Prediction of in vivo rat biliary drug clearance from an in vitro hepatocyte efflux model.

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Abbreviations: ACN, acetonitrile; AUC, Area Under the Concentration-time curve; BCRP, Breast cancer resistance protein; C_{blood}/C_{plasma}, blood-to-plasma concentration ratio; CL, clearance; CL_{int}, intrinsic clearance; CsA, Cyclosporine A; CYP, Cytochrome P450; DMSO, dimethyl-sulfoxide; Eq, equation; FTC, Fumitremorgin C; fu, fraction unbound, free fraction; IVIVE, In Vitro to In Vivo Extrapolation; KHL, Krebs-Henseleit buffer; LC-MS/MS, Liquid Chromatography tandem Mass Spectrometry; LOQ, Limit of Quantification; MRP2, Multidrug resistance associated protein 2; NTCP, Na+-taurocholate cotransporting polypeptide; OATP, Organic anion transporting polypeptide; P-gp, P-glycoprotein; PK, Pharmacokinetic; UGT, UDP-Glucuronosyltransferase; WE, William's E medium
Abstract

Well established techniques are available to predict in vivo hepatic uptake and metabolism from in vitro data, but predictive models for biliary clearance remain elusive. Several studies have verified the expression and activity of ATP-binding cassette (ABC) efflux transporters central to biliary clearance in freshly isolated rat hepatocytes, raising the possibility to predict biliary clearance from in vitro efflux measurements. In the present study short term plated rat hepatocytes were evaluated as a model to predict biliary clearance from in vitro efflux measurements, prior to major changes in transporter expression known to take place in long term hepatocyte cultures. The short term cultures were carefully characterized for their uptake and metabolic properties using a set of model compounds. In vitro efflux was studied using digoxin, fexofenadine, napsagatran, and rosuvastatin, representing compounds with over 100-fold differences in efflux rates in vitro and 60-fold difference in measured in vivo biliary clearance. The predicted biliary clearances from short term plated rat hepatocytes were within two-fold of measured in vivo values. As in vitro efflux includes both basolateral and canalicular effluxes, pronounced basolateral efflux may introduce errors in predictions for some compounds. In addition, in vitro rat hepatocyte uptake rates corrected for simultaneous efflux, predicted rat in vivo hepatic clearance of the biliary cleared compounds with less than two-fold error. Short term plated hepatocytes could thus be used to quantify hepatocyte uptake, metabolism, and efflux of compounds and considerably improve the prediction of hepatic clearance, especially for compounds with a large biliary clearance component.
Introduction

Transport processes such as hepatic uptake or biliary efflux are central to the clearance (CL) of many drug compounds (Giacomini et al., 2010). Hepatic drug metabolism is performed by enzymes commonly divided into phase I enzymes (mainly Cytochrome P450, CYP) and phase II enzymes (conjugating enzymes such as UDP-glucoronosyltransferase, UGT). A special challenge is to predict the in vivo pharmacokinetics (PK) of compounds that are subject to a combination of uptake, metabolism, and efflux (Li et al., 2010).

Today several techniques for investigation of hepatocyte uptake and metabolism of drug molecules are available, such as metabolic stability, or uptake measurements using suspended hepatocytes (Soars et al., 2007). However, since isolated hepatocytes lose their tissue geometry, active efflux and biliary secretion of compounds are properties difficult to study in vitro (De Bruyn et al., 2013; Li et al., 2010). Robust in vitro models that also reflect biliary efflux in addition to hepatic uptake and metabolism would be valuable tools to evaluate drug disposition, and for in vitro- in vivo extrapolation (IVIVE) of hepatic clearance. Compounds actively cleared by biliary or renal secretion belong in general to classes III and IV of the Biopharmaceutics Drug Disposition Classification System (BDDCS) proposed by Wu and Benet (2005). These classes are characterized by low metabolism - low permeability and large transporter effects. Classes I and II are characterized by high metabolism and permeability and small transporters effects.

Sandwich-cultured hepatocytes (SCH) are a convenient in vitro model to use for biliary clearance studies since the hepatocytes after 4-5 days of culture develop a rudimentary canalicular network and polarized membrane expression of many transporters. The excretion of compounds into the canalicular compartment is used
to calculate the biliary excretion (Liu et al., 1999; De Bruyn et al., 2013). This strategy has been successful in predicting bile efflux of several drugs, but as noted by Li and others, often under-predict bile excretion by 10- to 100-fold (De Bruyn et al., 2013; Li et al., 2010; Nakakariya et al., 2012). Partly this is likely due to the down-regulation of both drug uptake and efflux transporters that has been demonstrated to take place within the first days after plating of hepatocytes from both rat and man (De Bruyn et al., 2013; Kimoto et al., 2013; Kotani et al., 2011; Li et al., 2009; Liu et al., 1998; Tchaparian et al., 2011). Sinusoidal efflux transporters, such as rMRP3\(^1\) and rOST\(\alpha\)-OST\(\beta\)\(^1\) show low expression levels in normal livers but are up-regulated in cholestasis (Boyer et al., 2006; Luttringer et al., 2002; Rippin et al., 2001). rMRP4 and rMRP3 expression levels in rat liver, primary hepatocytes and short term cultures are low but they are extensively up regulated in longer term cultures (Luttringer et al., 2002; Rippin et al., 2001 Tchaparian et al., 2011). In addition the activity of many CYP-enzymes is considerably reduced in SCH (Boess et al., 2003; De Bruyn et al., 2013). To compensate for expression changes in SCH, proteomics-based correction factors have been used with some success, but these are difficult to implement if the transporters involved in the disposition of a compound are not known (Li et al., 2010).

The fate of canalicular ABC-efflux transporters central to biliary excretion such as P-glycoprotein (P-gp)\(^1\), breast cancer resistance protein (BCRP)\(^1\), and multidrug resistance protein-2 (MRP2)\(^1\) in isolated hepatocytes has been debated. Some reports suggest that isolated rat hepatocytes lose part of their ABC transporters (Bow et al., 2008). Others studies have demonstrated both expression and activity of rP-gp\(^1\), rBCRP\(^1\), and rMRP2\(^1\) in freshly isolated rat hepatocytes (Lam et al., 2006; Li et al., 2008, 2009a, 2009b). The retained activity of canalicular efflux transporters in primary rat hepatocytes demonstrated by these authors raises the possibility to predict biliary clearance from in vitro efflux measurements.
Short term cultures may comprise a reliable in vitro model for studies on drug disposition as changes in hepatocyte transporter and drug metabolizing enzyme expression and activity are minor after 4-6 hour culture (Beigel et al., 2008; Tchaparian et al., 2011; Jigorel et al., 2005).

In the present study short term cultures were demonstrated to be a robust model to predict biliary clearance of a set of compounds with a large range of biliary clearances in vivo. Predictions were within two-fold of measured biliary clearances, which is a considerable improvement compared with published methods. In addition uptake and metabolic clearances were evaluated using short term plated hepatocytes and results indicate that the model provides the possibility to assess uptake, metabolic and efflux clearances.
Material and methods

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Animals

Male Sprague-Dawley rats were acquired from Harlan Laboratories B.V. (Venray, The Netherlands). Rats were housed up to 3 per cage with free access to food and water. All animals were acclimatized for 2 weeks prior to studies. All animal experiments were approved by the local animal ethics committee (Stockholms Södra djurförsöksnämnd) and were conducted in compliance with national guidelines for the care and use of laboratory animals.

Preparation of rat hepatocytes

Rat hepatocytes were isolated according to a modified collagenase perfusion method previously described (Bissell and Guzelian, 1980) from 7-8 weeks old male Sprague-Dawley rats, anesthetized with Isoflurane (Abbott Scandinavia AB, Kista, Sweden). Shortly, the liver was perfused in situ, first with 37 °C calcium-free William’s E medium containing 0.5 M EGTA, followed by William’s E medium, 10 mM HEPES (Gibco, Life Technologies, Paisley, UK), pH 7.4 (WE), and supplemented with 0.16 mg/ml Collagenase type XI. The suspension of released hepatocytes was filtered through gauze, repeatedly centrifuged at 50 x g for 2 minutes, followed by aspiration of supernatant and resuspension of the pellet in WE. Cell number and viability were determined using the Trypan blue exclusion method. Viability typically exceeded 90%. Cell preparations with viability of <80% were discarded.
Uptake and metabolism measurements of test compound with plated rat hepatocytes and suspended cells

0.75 million cells, from a suspension of 10^6 cells/ml in culture medium (WE supplemented with 10% fetal calf serum, 2 mM Glutamine, and 0.01% Insulin-transferrin-selenium A solution (Gibco)) were seeded into each well of collagen I coated 12-well plates (Biocoat, BD Biosciences, Franklin Lakes, NJ, USA). Cells were incubated at RT for 10 min and were then left to attach to the plates for 90 min at 37°C, and 5% CO₂. The plates were washed three times in 37°C Krebs-Henseleit buffer, with 10 mM HEPES (Gibco), pH 7.4 (KHL). Cells were pre-incubated in KHL, 37°C, for 10 minutes before each experiment.

To start the uptake KHL was removed and 300 µl of 1 µM of test compound diluted in KHL, containing 0.1% DMSO, was added to each well. Each time point was measured in triplicate. To stop the uptake reaction 200 µl buffer from each well was transferred to a 96 well plate containing 300 µl stop solution of acetonitrile (ACN) with 200 nM Warfarin (internal standard). Remaining buffer was removed and the wells were washed twice in ice-cold KHL. The cell layer in washed wells was lysed with 300 µl lysis solution, 2 parts of stop solution, 1 part KHL with 200 nM warfarin. Lysates were incubated on ice for at least 10 minutes, mixed and centrifuged at 1200 x g for 10 minutes. Before analysis each fraction was diluted in KHL to contain 25 % ACN. All samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Binding was measured in empty plates and subtracted from uptake measurements.

The activity of drug phase I and II metabolizing enzymes in rat hepatocytes in suspension and attached to plates was compared using a cocktail containing substrates for several CYP and phase II enzymes; 7-hydroxycoumarin (UGT), bufuralol (CYP2D2), diazepam (CYP2C11), diclofenac (CYP2C6), midazolam...
(CYP3A) and phenacetin (CYP1A2), each at 2 μM final concentration (isoform specificities from Kobayashi et al., 2002). Hepatocytes in suspension (10^6/ml) in KHL were incubated with the cocktail for 0 to 90 minutes. Samples were precipitated with 2 volumes of stop solution. Cells attached to plates were processed as described for uptake experiments.

For comparative purposes in IVIVE, metabolic stability in rat liver microsomes was determined as described (Sohlenius-Sternbeck et al., 2010).

To test uptake activity in suspended and plated rat hepatocytes, five well characterized uptake transporter substrates (atorvastatin, bosentan, estrone-3-sulfate, fexofenadine, and montelukast) and two passively transported substrates atenolol and metoprolol (AstraZeneca) were tested. Experiments in plated cells were performed as described above. Uptake measurements in suspended cells were performed using the media loss technique described by Soars et al., (2007).

To further investigate active transport in plated cells atorvastatin uptake measurements were done in the absence and presence of 2 μM of the organic anion transporting polypeptide (OATP) inhibitor Cyclosporine A (CsA). The activity of Na^+-taurocholate cotransporting polypeptide (NTCP) activity in plated cells were studied by comparing uptake of taurocholate in sodium-containing buffer and sodium-free buffer, substituted with equimolar choline. The influence of temperature on passive uptake in plated rat hepatocytes was investigated using the passively uptaken substrate atenolol at 37ºC and 4ºC.

**In vitro efflux measurements**

Four compounds with complete human in vivo pharmacokinetics were identified (digoxin, napsagatran, fexofenadine, and rosuvastatin). Napsagatran was a kind gift
from Hoffman-La Roche, Basel, Switzerland. Inclusion criteria were plasma clearance and biliary clearance measured after intravenous administration of compound and published values on human plasma protein binding and blood-plasma concentration ratio (Cblood/Cplasma).

Hepatocytes in 12 well plates were preloaded until a maximal intracellular concentration was reached as described for uptake experiments. Measuring compound efflux at maximal intracellular concentration gives the efflux at steady state. Preload times and compound concentrations were decided from measured uptake data in pilot experiments (digoxin, 10 µM for 15 min; fexofenadine, 1 µM for 30 min; napsagatran, 10 µM for 30 min; and rosvastatin, 1 µM for 15 min). After preloading, the wells were washed three times in 37°C KHL to ensure that the wells were free from remaining compound. Efflux experiments were started by adding 300 µl KHL (37°C) to each well. Cells were also lysed immediately after washing to determine initial intracellular concentrations at the start of the experiment. The contribution of ABC-efflux transporter activity to the measured efflux was assayed using 0.5 µM of the inhibitors elacridar (GF120918, P-gp and BCRP inhibitor) and Fumitremorgin C (FTC, BCRP inhibitor).

Triplicate wells from uptake and efflux experiments were lysed with 200 µl 1% SDS and the protein concentration was determined with the BCA protein assay kit (Pierce Biotechnology, Thermo Fischer Scientific Inc., Rockford, IL, USA). Hepatocyte number per well was calculated using published data (Sohlenius-Sternbeck, 2006).

**Determination of plasma protein binding**

Plasma protein binding was determined by equilibrium dialysis. Rat plasma was mixed with the compound at a concentration of 10 µM (in 0.1% DMSO). A volume of
180 µl 0.122 M phosphate buffer, pH 7.4, 75 mM NaCl was added to each well on one side of a dialysis plate, and 180 µl of the plasma/compound mix to the opposite side, separated by a dialysis membrane (Spectra/Por MWCO 6-8000, Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA). After incubation on an orbital shaker at 37°C for 18 h, the samples were analyzed by LC-MS/MS. All compounds were stable during the 18 h incubation.

**Determination of blood-plasma concentration ratio (C\text{blood}/C\text{plasma})**

$C_{\text{blood}}/C_{\text{plasma}}$ was determined in fresh rat whole blood for digoxin, fexofenadine, napsagatran, rosuvastatin and midazolam (positive control). Two blood samples were spiked with 0.2 µM test compound and incubated on a waddling mixer at 37°C for 1 h. The blood was centrifuged and the resulting plasma was analyzed by LC-MS/MS.

**Plasma clearance of test compound**

Blood samples were drawn from male Sprague-Dawley rats, at an age of approximately 8 weeks, just before and at 1 min; 5 min; 20 min; 40 min; 1 h; 1.5 h; 3 h; 6 h and at 24 h after intravenous bolus administration of 3 µmol/kg of test compound (in 0.3 M N-methyl-D-glucamine). Standards and samples were diluted in blank plasma and analyzed by LC-MS/MS.

**Bile clearance of test compound**

Male Sprague-Daley rats, at an age of approximately 8 weeks, were anaesthetized with Isoflurane (Abbott, Scandinavia AB, Kista, Sweden) by use of a vaporiser, and then surgically prepared with a bile-pancreatic catheter (constructed by polyethene catheter Physiocath and P90). A small incision was made in the duodenum and the catheter was inserted for recycling of bile. A skin incision was made in the scapula
region. A loop of the catheter was subcutaneously tunnelled from the abdominal incision to the scapula region and then secured with a draconic button. After 3 days recovery the animals were connected to a swivel system (Instech Laboratories, Inc. Plymouth Meeting, PA, USA) allowing freedom of movement during the experiment and administered a bolus dose of 3 μmol/kg of compound via a lateral caudal vein. Fractions of bile were collected every 10 minutes for the first hour and every hour until 6 hours after dosing. Plasma was drawn at 6 hours after dosing. The animals were finally sacrificed with an overdose of the barbiturate Pentobarbital, veterinary grade (Apoteket Produktion & Laboratorier AB, Sweden). Standards and samples were diluted in a 1:10 dilution of blank bile and analyzed by LC-MS/MS.

**LC-MS/MS analysis**

Analysis of parent compounds and metabolites was performed by LC-MS/MS using a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, UK). The software MassLynx was used to control the LC-system and mass spectrometer (Waters Corporation, Milford, MA, USA). A High Purity C\textsubscript{18} 5 μm 30 x 2.1 mm (Thermo Electron Corporation, Waltham, USA) analytical column was used. A standard curve was included for each compound analysed spanning a concentration range from twice the initial experimental concentration to below the limit of quantification. LOQ, signal to noise ratio for detection, was set to 5 times.
Pharmacokinetics and IVIVE

Determination of in vitro intrinsic clearance

The elimination rate constant in metabolic stability and uptake experiments was calculated from the first order elimination equation:

\[ [S] = [S_0] \cdot e^{-kt} \]  \hspace{1cm} (1)

where \([S]\) is concentration of substrate at a given time point, \(t\), and \([S_0]\) is the initial concentration of substrate. \(k\) is the elimination rate constant and was determined by non-linear regression within a time point interval representing the initial elimination rate (Soars et al., 2007a). For in vitro efflux \(k\) is given by the rate of compound effluxed into the medium.

In vitro intrinsic clearances (\(CL_{\text{int, met}}, CL_{\text{int, uptake}}, CL_{\text{int, efflux}}\)) for the compounds could then be calculated from:

\[ CL_{\text{int}} = k \cdot V \]  \hspace{1cm} (2)

where \(V\) represents the volume of incubation. \(CL_{\text{int, efflux}}\) is calculated using the volume and concentration of the preloading medium. This is the rate of efflux back into the medium when uptake experiments have reached steady state.

Intrinsic hepatic clearance according to the extended clearance concept incorporating both transport and metabolism is given by:
\[ CL_{\text{int,all}} = CL_{\text{int, uptake}} \times \frac{(CL_{\text{int, efflux}} + CL_{\text{int, met}})}{(CL_{\text{int, efflux}} + CL_{\text{int, met}} + CL_{\text{int, back}})} \]  

(Maeda et al., 2010). \( CL_{\text{int, uptake}} \) is the uptake \( CL_{\text{int}} \), \( CL_{\text{int, efflux}} \) is the efflux of compound from hepatocytes to bile, \( CL_{\text{int, met}} \) is the metabolic \( CL_{\text{int}} \), and \( CL_{\text{int, back}} \) is the basolateral efflux from hepatocytes back to the circulation. Our in vitro efflux experiments cannot distinguish \( CL_{\text{int, efflux}} \) and \( CL_{\text{int, back}} \), and \( CL_{\text{int, back}} \) was therefore arbitrarily set to zero for these experiments to allow calculations (see Discussion for further details).

When \( CL_{\text{int, back}} = 0 \) we have a compound with no sinusoidal active efflux and little passive permeability (i.e. a BDDCS class III or IV compound without sinusoidal active efflux (Benet et al., 2011). All biliary probe compounds in this study are BDDCS Class III compounds with unknown sinusoidal active efflux.) \( CL_{\text{int, all}} \) is in this case determined by the hepatocyte uptake:

\[ CL_{\text{int, all}} = CL_{\text{int, uptake}} \]  

However, in our in vitro experiments efflux is active and the measured uptake is an apparent uptake diminished by the active efflux of compound back into the medium. To compensate for this the total uptake \( CL_{\text{int}} \) is calculated.

\[ CL_{\text{int, all}} = CL_{\text{int, uptake, total}} = CL_{\text{int, uptake}} + CL_{\text{int, efflux}} \]  

This equation gives the uptake rate that would be seen after complete inhibition of active efflux.
Fraction unbound in the incubations

The fraction unbound in the hepatocyte incubation was predicted according to Kilford et al. (2008):

\[
\frac{f_{u_{inc,hep}}}{1+125 \times V_R \times 10^{0.072 \times \log P - 0.067 \times \log D + 1.126}}
\]  

(6)

where \(V_R\) is the ratio between the cell volume and the incubation volume with a value of 0.005 at the cell concentration \(10^6\) cells/ml. \(\log P\) is used for bases, \(\log D\) for other ion classes. Predicted \(\log P\) and \(\log D\) values were generated using a commercial package from Advanced Chemistry Development (Toronto, Canada).

Determination of in vivo clearance

In vivo blood clearance was calculated according to

\[
CL_{blood} = \frac{\text{Intravenous Dose}}{AUC_{blood}}
\]  

(7)

where \(AUC_{blood} = \frac{AUC_{plasma} \times C_{blood}}{C_{plasma}}\)  

(8)

Blood to bile CL was calculated with the equation:

\[
CL_{bile} = \frac{X_{t_1-t_2}}{AUC_{blood,t_1-t_2}}
\]  

(9)

where \(X_{t_1-t_2}\) denotes the amount secreted in bile between the two time points and \(AUC_{blood,t_1-t_2}\) is the blood AUC for the same time interval (Fukuda et al., 2008). In this
study the time interval spans the first hour after intravenous administration of compound.

In vivo hepatic clearance, $CL_{hepatic}$, is calculated according to:

$$CL_{hepatic} = CL_{blood} \times f_{hepatic}$$  \hspace{1cm} (10)$$

where the hepatic fraction, $f_{hepatic}$, for the biliary cleared test compounds is given in the literature (Kamath et al., 2005, Lave et al., 1999, Yang et al., 2009). $CL_{hepatic}$ comprises both metabolic and biliary efflux clearances.

**Calculation of fraction unbound in the blood**

The fraction unbound in the blood ($f_{u_{blood}}$) was calculated using the following equation:

$$f_{u_{blood}} = \frac{f_{u_{plasma}}}{\left(\frac{C_{blood}}{C_{plasma}}\right)}$$  \hspace{1cm} (11)$$

**Prediction of in vivo $CL_{int}$**

The predicted $CL_{int}$ in vivo for the liver was calculated as follows:

$$Predicted\ CL_{int\ in\ vivo} = \frac{CL_{int\ in\ vitro} \times \text{physiological scaling factors} \times f_{u_{blood}}}{f_{u_{inc\ hep}}}$$  \hspace{1cm} (12)$$

The physiological scaling factors for rat hepatocytes were: $(120 \times 10^6 \text{ cells} / \text{g liver}) \times (10 \text{ g liver} / 250 \text{ g body weight})$
(Bayliss et al., 1990; Sohlenius-Sternbeck 2006). CL\textsubscript{int, in vitro} can be either CL\textsubscript{int, uptake}, CL\textsubscript{int, efflux}, CL\textsubscript{int, met} (all from equation 2), or CL\textsubscript{int, uptake, total} (eq. 3-5) which are then used to predict CL\textsubscript{hepatic} or CL\textsubscript{bile} using eq. 13.

**Prediction of in vivo CL from in vitro data**

Predictions were based on the well stirred model:

$$\text{Predicted CL}_{\text{hepatic or bile}} = \frac{Q_{h,b} \times CL_{\text{int,in vivo}}}{Q_{h,b} + CL_{\text{int,in vivo}}} \quad (13)$$

Hepatic blood flow, $Q_{h,b}$, was set to 72 ml/min/kg for rat (Brown, et al., 1997). CL\textsubscript{bile} was predicted from CL\textsubscript{int, efflux} and CL\textsubscript{hepatic} was for comparative purposes predicted from CL\textsubscript{int, met}, CL\textsubscript{int, uptake} and CL\textsubscript{int, uptake, total} (using eqs. 12 and 13).

**Statistical analysis**

Data were analyzed using Graphpad Prism 5 software (Graphpad Software, Inc, La Jolla, CA, USA). Data are given as mean ± SD. Statistical significance was tested using the two-tailed Student’s t-test. Probability values (P-values) are symbolized by: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. P-values < 0.05 were considered statistically significant.
Results

Plating of hepatocytes

After plating the hepatocytes formed a loosely confluent layer exhibiting a cobblestone appearance (data not shown). Experiments started within 4 h of liver collagenase perfusion, including 90 min plating.

Metabolism and uptake in plated cells

A range of highly permeable BDDCS class I and II compounds (Benet et al., 2011), known to be metabolized by CYPs and UGTs, were used to assess drug metabolic capacity in plated and suspended rat hepatocytes. The results presented in Table 1 indicates that short term plated hepatocytes retain similar metabolic capacity as freshly isolated cells.

Uptake rates for a number of well characterized uptake substrates from high and low permeable BDDCS classes were compared in plated and suspended hepatocytes (Table 2). The $\text{CL}_{\text{int. uptake}}$ of atorvastatin, bosentan, and montelukast, which are predominantly dependent on transporters to be taken up by cells, was similar in plated and suspended hepatocytes. The uptake rates of estrone-3-sulfate and fexofenadine, well known transporter substrates, were however higher in suspended than in plated cells. Atenolol, metoprolol and terfenadine, substances that are not known to be dependent on uptake transporters also showed higher $\text{CL}_{\text{int. uptake}}$ in suspensions than in plated hepatocytes, as a larger membrane area is exposed in the suspended cells. CsA, a well-characterized OATP-inhibitor, efficiently inhibited atorvastatin uptake in plated hepatocytes (Supplemental Fig. 1A). In the absence of Na⁺ a significant decrease of taurocholate uptake was observed, indicating NTCP activity in the plated hepatocytes (Supplemental Fig. 1B).
Temperature had a significant effect on uptake of atenolol, a drug which is passively taken up by the cell, reducing uptake 5-fold at 4°C as compared to 37°C (Supplemental Fig. 1C). As a consequence, low temperature uptake was not used as a negative control for active uptake in this study.

Properties of the selected test set of biliary cleared compounds
The selected biliary cleared compounds exhibited diverse clearance pathways and drug transporter involvement (Table 3). The compounds showed low to moderate plasma protein binding, diverse lipophilicities and limited passive permeability (BDDCS class III (Benet et al., 2011)).

In vitro pharmacokinetic properties of the biliary cleared compounds
The compounds showed limited metabolic CL_{int} (Table 4). All compounds in the test set were taken up by plated rat hepatocytes (Table 4). Efflux measurements for all compounds were performed after preloading when the compound had reached a maximal intracellular concentration.

The decreasing intracellular amount and efflux of compound from the cells into the medium was measured as illustrated in Fig. 1A and 1B for rosuvastatin. CL_{int, efflux} for the tested compounds are given in Table 4. The measured CL_{int, efflux} values were 0.13 ± 0.06, 3.2 ± 0.6, 10.1 ± 2.3, and 110 ± 2.8 for digoxin, fexofenadine, napsagatran, and rosuvastatin, respectively, which thus represents drugs with a > 800-fold range of efflux rates. As efflux in these experiments counteracts and diminishes uptake measurements, CL_{int, uptake, total} was calculated according to Eq. 5.

Efflux from plated rat hepatocytes could be inhibited by ABC-transporter inhibitors. In rat hepatocytes efflux of fexofenadine was reduced in the presence of 0.5 μM of the
P-gp and BCRP inhibitor elacridar (Fig. 1C). Efflux of rosuvastatin could be inhibited with 0.5 μM fumitremorgin C (FTC), an inhibitor of BCRP (Fig. 1D).

**Rat in vivo pharmacokinetics**

Rat in vivo blood and biliary CL were determined in three animals for each of the four biliary cleared compounds (Table 4). All compounds were rapidly excreted into bile. The rates of biliary clearance varied 60-fold between digoxin and rosuvastatin (Table 4). For all compounds the concentration in bile peaked 20 min after intravenous administration of compound. Peak bile concentrations varied from 4 μM for digoxin to 3000 μM for rosuvastatin. The fraction of the given dose recovered in the bile during 3 h varied from 10 % for digoxin over napsagatran (20 %) and fexofenadine (29 %) to 85 % for rosuvastatin.

Bile flow was similar between animals and over time during the collection period (28 ± 5 μl/min, Mean ± SD, n = 8). There was no correlation between bile flow and secretion rate or between bile flow and amount of compound excreted in bile.

Biliary CL (CL\(_{\text{bile}}\)) was calculated according to Eq. 9 (Fukuda et al., 2008) and is presented in Table 4. Biliary CL constituted a significant portion of the clearance of all tested compounds. In vitro efflux and in vivo CL\(_{\text{bile}}\) showed the same rank order of rosuvastatin > napsagatran > fexofenadine > digoxin (Table 4).

**In vitro-in vivo extrapolation**

When CL\(_{\text{hepatic}}\) was predicted from hepatocyte or microsomal metabolic CL\(_{\text{int,met}}\) using the well-stirred model (Table 4, Eqs. 12 and 13) an absolute average fold error of more than 10-fold was obtained and the slope of the best fit line was not significant (data not shown).
CL\textsubscript{bile} of digoxin, fexofenadine, napsagatran, and rosuvastatin was well predicted from CL\textsubscript{int, efflux} using the well-stirred model (Table 4, Eqs. 12 and 13). Extrapolations showed an even distribution of over- and under-predictions and an absolute average fold error of 1.7. Digoxin, fexofenadine and napsagatran CL\textsubscript{bile} were predicted within 2-fold of measured in vivo values, while the prediction for rosuvastatin showed an error of 2.2-fold (Table 4). The best fit line of the IVIVE data showed $r^2 = 0.89$ and its slope was significantly different from 0 (Fig. 2A).

The results from the present study indicated that scaling CL\textsubscript{hepatic} of digoxin, fexofenadine, napsagatran and rosuvastatin from plated rat hepatocyte CL\textsubscript{int, uptake} (Table 4, Eqs. 12 and 13) consistently under-predicted in vivo observations with errors ranging from 1.6- to 10-fold. The best fit line was not significantly different from 0 (Table 4, Fig. 2B).

Since active efflux in the plated hepatocytes will affect uptake measurements CL\textsubscript{int, uptake, total} was calculated according to equation 5 (Table 4). This compensates for reductions in measured uptake due to active efflux from the hepatocytes. CL\textsubscript{hepatic} was again predicted, now from CL\textsubscript{int, uptake, total} (Table 4, Eqs. 12 and 13). Predictions were significantly improved. Three out of four compounds were slightly under-predicted. Predictions of CL\textsubscript{hepatic} for digoxin, fexofenadine, and napsagatran were within 2-fold of observed in vivo values, whereas the prediction for rosuvastatin showed an error of 2.1-fold. The absolute average fold error of the predictions was 1.7 (Table 4). The best fit line of the IVIVE data showed an $r^2 = 0.84$ and its slope was significantly different from 0 (Fig. 2C).
Discussion

Short term plated hepatocytes have previously been used for kinetic characterization and modeling of hepatic drug uptake and metabolism but little attention has been paid to the impact of active efflux in the system (Menochet et al., 2012a, b). The active efflux component may have a major influence on cellular kinetics and thus merits further investigation. As demonstrated in this study active efflux of digoxin, fexofenadine, napsagatran, and rosuvastatin in short term plated rat hepatocytes predicts biliary clearance within 2-fold and inclusion of the efflux component in IVIVE of hepatic clearance from in vitro uptake data improved predictions considerably.

Rat CL\textsubscript{bile} rates of digoxin, fexofenadine, napsagatran, and rosuvastatin as predicted from in vitro efflux data from plated rat hepatocytes were not related to a general under prediction of the studied compounds as often experienced using other in vitro systems. Earlier attempts to scale rat in vivo biliary CL from rat SCH efflux, incorporating \textit{fu}\textsubscript{blood}, for the biliary cleared compound set resulted in 1.6- (digoxin), 3.5- (fexofenadine), and 15- to 17-fold (rosuvastatin) under predictions (Annaert et al., 2001; Fukuda et al., 2008; Nakakariya et al., 2012; Turncliff et al., 2006). Nakakariya and coworkers have recently shown that biliary clearance predictions from rat SCH can be considerably improved by predicting biliary intrinsic clearance using liver tissue drug concentrations and free intracellular concentrations in rat SCH (Nakakariya et al., 2012). Predicting rat biliary CL using rat SCH efflux in the traditional manner, yielded under predictions ranging from 7- to 300-fold. Predicting intrinsic biliary efflux using intracellular SCH and liver concentrations improved results giving under predictions with an average fold error of 4.5, representing the most successful in vitro method for prediction of biliary clearance to this date (Nakakriya et al., 2012).
While a few previous studies suggest that isolated rat hepatocytes lose part of their ABC transporters (Bow et al., 2008), others have demonstrated both expression and activity of efflux transporters in freshly isolated rat hepatocytes (Lam et al., 2006; Li et al., 2008, 2009a, 2009b, 2009c). In a recent study we have demonstrated retained protein levels, membrane localization and activity for rBCRP, rP-gp, and rMRP2 in freshly isolated rat hepatocytes as well as cryopreserved human hepatocytes (Lundquist et al., unpublished). In the present study efflux from preloaded rat hepatocytes could be inhibited with well characterized ABC efflux transporter inhibitors, indicating that rBCRP and rP-gp are active in short term cultured hepatocytes.

Despite being cleared by a wide range of mechanisms and being substrates of many different transporters as listed in Table 3, prediction errors were similar for all compounds tested. Investigation of bile clearance using the in vitro model proposed in the present study will best predict in vivo biliary clearance for compounds exhibiting limited sinusoidal efflux, low passive permeability and low lipophilicity/low unspecific cellular binding. These are characteristics shared by many compounds with pronounced biliary clearance (Yang et al., 2009). Short term cultures of human cryopreserved hepatocytes should be investigated in future studies to help improve predictions of human biliary clearance. The pronounced down regulation of OATP transporters recently demonstrated in human cryopreserved hepatocytes might however present a limitation (Kimoto et al., 2013).

Scaling $\text{CL}_{\text{hepatic}}$ from plated rat hepatocyte uptake data, in vivo values were consistently under-predicted in the present study. Similar results were obtained by Gardiner and Paine (2011) who noted a 6-fold under-prediction from rat hepatocyte uptake data. Menochet et al., (2012b) also presented under-predictions of in vivo hepatic uptake from in vitro uptake measurements using rat hepatocytes. As can be
seen in Table 4 and Figure 2B the under-prediction of $CL_{hepatic}$ from plated hepatocyte uptake data became larger for compounds with higher efflux rates. In the study by Menochet et al., (2012b) rosuvastatin needed the largest correction factor and is also the compound with highest $CL_{bile}$ and in vitro efflux. Due to active efflux from the hepatocytes the true cellular uptake is probably underestimated. We recently showed that the apparent fexofenadine uptake in rat hepatocytes increased more than 4-fold when inhibiting P-gp during the uptake experiment (Lundquist et al., unpublished), supporting this hypothesis. As clearly shown in the present study compensating for active efflux in the plated hepatocytes by calculating $CL_{int, uptake, total}$ (eq. 5) significantly improved IVIVE of $CL_{hepatic}$.

The loss of polarity of the isolated hepatocytes used in cell suspension or short term cultures results in both basolateral (sinusoidal, facing the circulation) and apical (canalicular, facing the bile) efflux transporters exporting compound into the same medium compartment. The presence of sinusoidal efflux could therefore lead to over-prediction of biliary CL for certain compounds since the measured $CL_{int, efflux}$ will be overestimated and contain both canalicular and sinusoidal efflux. As can be inferred from Eq. 3 biliary efflux will be over-predicted by a factor of 2 if sinusoidal and canalicular efflux are of the same magnitude ($CL_{int, efflux} = CL_{int, back}$). Little is known of basolateral efflux for the study compounds. Digoxin is an OSTα-OSTβ substrate (Seward et al., 2003), but nothing is known of the in vivo significance. Fexofenadine is a MRP3 substrate in mouse liver where sinusoidal and basolateral fexofenadine effluxes are of similar magnitude (Matsushima et al., 2008; Tian et al., 2008). Mice have been shown to express high levels of MRP3 compared to rat where basolateral efflux thus might be less pronounced (Chu et al., 2006). Likewise, rosuvastatin has recently been shown to exhibit similar basolateral and canalicular efflux in rat SCH, after the large up regulation of rMRP3 and rMRP4 taking place in rat SCH (Pfeiffer et
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al., 2013). The error introduced by basolateral efflux in short term plated rat hepatocytes is thus likely to be small for these compounds.

In the liver most sinusoidal efflux transporters show low expression compared to the canalicular ones, MRP3 being barely detectable in rat liver and short term hepatocyte cultures while MRP2 shows a very high expression (Luttringer et al., 2002; Rippin et al., 2001). The expression of rMRP2 in rat liver is more than twice the combined levels of rMRP3, and rMRP4 (Luttringer et al., 2002; Jigorel et al., 2005; Rippin et al., 2001). It is known that for some drug metabolites, particularly sulfate conjugates, sinusoidal efflux may be very high, but data on parent compounds are scarce and this area would merit further studies (Zamek-Gliszczynski et al., 2006). In the present study CL bile was not over-predicted when scaling from in vitro CL int, efflux and the rank orders for in vitro efflux and CL bile were identical, suggesting that basolateral efflux was not a major component in the efflux of the tested compounds.

Compounds with high passive permeability may also present a problem in the experimental model as this component may be difficult to determine experimentally. The biliary cleared compound set used consists of low permeable BDDCS class III compounds (Benet et al., 2011; Lavé et al., 1999). For rosuvastatin and fexofenadine the passive uptake component has been estimated to be less than 10% of total hepatocyte uptake and is thus unlikely to have a major impact on measurements (Menochet et al., 2012a).

In conclusion, we have developed an experimental technique based on short term plated hepatocytes that allows the measurement of uptake, metabolism, and efflux of drug compounds. Basolateral efflux is however not considered and may introduce errors into the predictions. In vitro efflux measurements using this model predicted biliary clearance within, or close to, two-fold of measured in vivo values for four
compounds, digoxin, fexofenadine, napsagatran, and rosuvastatin that exhibit a wide range of in vivo biliary clearances. Efflux corrected in vitro uptake measurements were used to improve the IVIVE of hepatic CL. The short term hepatocyte culture model described thus provides a simple way to study uptake, metabolic, and efflux clearance pathways in the liver of new chemical entities and considerably improves the prediction of hepatic clearance, especially for compounds with a large biliary clearance component.
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Performed data analysis: Lundquist, Lööf, Fagerholm

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assessment of uptake and metabolism in rat hepatocytes: a comprehensive

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significantly to rosvastatin disposition: characterization of basolateral versus


Footnotes

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¹Transporter nomenclature; as recommended by Hagenbuch and Stieger (2013) this article follows the gene and protein nomenclature guidelines of the HUGO Gene Nomenclature Committee (Gray et al., 2013) and the International Committee for Standardized Genetic Nomenclature in Mice (2009). Protein names for both human and rodent proteins are written in capital letters (e.g. BCRP). In the interest of clarity human protein names are prefixed with h while rat proteins are prefixed r (e.g. hBCRP, rBCRP). Protein names without prefix refer to the protein in general, not belonging to any particular species (e.g. a BCRP-inhibitor).
Figure legends

Fig. 1
Efflux of rosuvastatin and fexofenadine from plated rat hepatocytes. **A:** Rosuvastatin remaining in the cell layer. **B:** Accumulation of rosuvastatin in efflux media. **C:** Efflux of rosuvastatin could be significantly reduced by addition of the BCRP inhibitor FTC. **D:** Efflux of fexofenadine from plated rat hepatocytes is diminished in the presence of the P-gp and BCRP inhibitor elacridar. CTL: control experiment, standard conditions. (Mean ± SD, n = 3, * = p < 0.05)

Fig. 2
In vitro – in vivo extrapolation of rat pharmacokinetics from Table 4. **A:** Prediction of in vivo CL_{bile} from plated hepatocyte CL_{int, efflux}. **B:** Prediction of in vivo CL_{hepatic} from plated hepatocyte CL_{int, uptake}. **C:** Prediction of in vivo CL_{hepatic} from plated hepatocyte CL_{int, uptake, total}. The line of unity between extrapolation and observation as well as lines representing 2-fold errors are shown by dotted lines. Best fit line from linear regression is represented by a solid line with an r^2 = 0.89 and a significant slope (p < 0.05) for 6A. The best fit line in 6C shows an r^2 = 0.84 and a significant slope (p < 0.05). D, digoxin; F, fexofenadine; N, napsagatran; R, rosuvastatin. (Mean ± SD, n = 3).
Table 1. A comparison of metabolic CL$_{int}$ for several phase I and II metabolic substrates in plated rat hepatocytes and suspended cells.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>BDDCS class$^a$</th>
<th>Main metabolic enzyme$^b$</th>
<th>Plated cells</th>
<th>Suspended cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bufuralol</td>
<td>I</td>
<td>CYP2D2</td>
<td>64 ± 12</td>
<td>68 ± 9</td>
</tr>
<tr>
<td>Diazepam</td>
<td>I</td>
<td>CYP2C11</td>
<td>24 ± 6</td>
<td>34 ± 8</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>I</td>
<td>CYP2C6</td>
<td>23 ± 5</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>7-Hydroxycoumarin</td>
<td>I</td>
<td>UGT</td>
<td>38 ± 24</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>Midazolam</td>
<td>I</td>
<td>CYP3A</td>
<td>40 ± 9</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>II</td>
<td>CYP1A2</td>
<td>29 ± 4*</td>
<td>39 ± 11</td>
</tr>
</tbody>
</table>

$^a$BDDCS classes as described by Benet et al. (2011).

$^b$Rat CYP isoform specificities were from Kobayashi et al. (2002).

$^c$Mean ± SD is shown, n=3 for plated cells, > 100 for suspended cells (long term lab average). Statistical significance was tested with the two-tailed Student’s t-test. *, significantly lower than in suspended hepatocytes, p < 0.05.
### Table 2. Comparison of uptake rates (CL\textsubscript{int, uptake}) for plated rat hepatocytes and suspended cells.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>BDDCS class\textsuperscript{a}</th>
<th>Uptake transporter\textsuperscript{b}</th>
<th>Plated cells (\mu l/min \times 10^6) cells)</th>
<th>Suspended cells (\mu l/min \times 10^6) cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol\textsuperscript{c}</td>
<td>III</td>
<td>None</td>
<td>1.2 ± 0.2 ***</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Metoprolol\textsuperscript{c}</td>
<td>I</td>
<td>None</td>
<td>10 ± 2 **</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>II</td>
<td>OATPs</td>
<td>30 ± 3 ***</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Bosentan</td>
<td>II</td>
<td>OATPs</td>
<td>11.5 ± 3 NS</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Estrone-3-sulfate\textsuperscript{d}</td>
<td>III</td>
<td>NTCP/OATs/OATPs</td>
<td>75 ± 9 **</td>
<td>210 ± 32</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>III</td>
<td>OATPs</td>
<td>3 ± 0.5 **</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Terfenadine\textsuperscript{e}</td>
<td>II</td>
<td>None</td>
<td>153 ± 19 **</td>
<td>324 ± 53</td>
</tr>
<tr>
<td>Montelukast</td>
<td>II</td>
<td>OATPs</td>
<td>145 ± 20 NS</td>
<td>143 ± 14</td>
</tr>
</tbody>
</table>

\textsuperscript{a}BDDCS class as described by Benet et al. (2011).

\textsuperscript{b}Transporter data are from the University of Tokyo and UCSF-FDA online transporter databases (Ozawa et al., 2004; Morrissey et al., 2012).

\textsuperscript{c}Compounds with predominantly passive uptake.

\textsuperscript{d}As no dose value can be assigned to Estrone-3-sulfate no true BDDCS class assignment can be given, except to note that it is a highly soluble compound with little passive permeability.

\textsuperscript{e}Mean ± SD is shown, n=3. Statistical significance was tested with the two-tailed Student’s t-test. *** = \(p < 0.001\), ** = \(p < 0.01\), * = \(p < 0.05\). NS = not significant.
Table 3. In vivo and in vitro properties of the biliary cleared test set.

<table>
<thead>
<tr>
<th></th>
<th>BDDCS class(^a)</th>
<th>Clearance mechanism(^b)</th>
<th>Uptake transporters(^c)</th>
<th>Efflux transporters(^c)</th>
<th>Rat plasma protein binding ((fu,\text{plasma}))</th>
<th>(C_{\text{biod}}/C_{\text{plasma}})</th>
<th>Chemical class(^e)</th>
<th>(c\text{logD}_{7.4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>III</td>
<td>Renal &gt; biliary</td>
<td>OATP1B1</td>
<td>P-gp</td>
<td>0.84</td>
<td>1.2</td>
<td>N/Z/A/B</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OATP1B3</td>
<td>P-gp</td>
<td>MDR3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown(^d)</td>
<td>P-gp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>III</td>
<td>Biliary &gt; renal</td>
<td>OATP1A2</td>
<td>P-gp</td>
<td>0.27</td>
<td>1</td>
<td>Z</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OATP1B1</td>
<td>P-gp</td>
<td>MRP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OATP2B1</td>
<td>P-gp</td>
<td>MRP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OAT3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Napsagatran</td>
<td>III</td>
<td>Biliary &gt; renal</td>
<td>?</td>
<td>?</td>
<td>0.87</td>
<td>0.6</td>
<td>Z</td>
<td>-2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>?</td>
<td>?</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>?</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>III</td>
<td>Biliary &gt; renal &gt; metabolic</td>
<td>NTCP</td>
<td>BCRP</td>
<td>0.1</td>
<td>0.6</td>
<td>A</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OATP1B1</td>
<td>BCRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OATP1B3</td>
<td>MRP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OATP2B1</td>
<td>MRP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OAT3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)BDDCS classes as described by Benet et al. (2011).

\(^b\)Clearance mechanisms are given in: Kamath et al., 2005; Lave et al., 1999; Poirier et al., 2009; Yang et al., 2009.

\(^c\)Transporter data are from the University of Tokyo and UCSF-FDA online transporter databases (Ozawa et al., 2004; Morrissey et al., 2012).

\(^d\)The involvement of OATPs in digoxin uptake has recently come under debate, and an alternative, as yet unidentified, sodium-dependent transporter has been proposed (Taub et al., 2011).

\(^e\)Chemical class: N, Neutral; Z, Zwitterion; A, Acid; B, Base.
Table 4. Rat in vitro and in vivo pharmacokinetics and in vitro- in vivo extrapolation of rat CL for the biliary cleared compound set.\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat in vitro PK</th>
<th>Rat in vivo PK</th>
<th>Rat IVIVE Predicted CL(^b) from CL(^i), uptake</th>
<th>Fold error(^b)</th>
<th>Predicted CL(^i) from CL(^i), uptake, total</th>
<th>Fold error(^b)</th>
<th>Predicted CL(^i) from CL(^i), efflux</th>
<th>Fold error(^b)</th>
<th>In vitro efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL(^{int, net}) (^b) Microsome</td>
<td>CL(^{int, net}) (^b) Hepatocyte</td>
<td>CL(^{int, uptake})</td>
<td>CL(^{int, efflux})</td>
<td>CL(^{int, uptake, total})</td>
<td>CL(^{hepatic})</td>
<td>CL(^{hepatic})</td>
<td>CL(^{hepatic}) Predicted CL(^{hepatic}) from CL(^{int, uptake, total})</td>
<td>Fold error(^b)</td>
</tr>
<tr>
<td>Digoxin</td>
<td>10 ± 2.8</td>
<td>7 ± 1.5</td>
<td>3 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td>3.6 ± 0.9</td>
<td>0.13 ± 0.06</td>
<td>73.6 ± 5</td>
<td>84.6 ± 7.4</td>
<td>110 ± 28</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>6 ± 1.1</td>
<td>&lt; 5</td>
<td>3 ± 0.6</td>
<td>3.2 ± 0.4</td>
<td>6.2 ± 1.3</td>
<td>3.2 ± 0.6</td>
<td>4.8 ± 0.8</td>
<td>7 ± 1.6</td>
<td>10.1 ± 2.3</td>
</tr>
<tr>
<td>Napsagatran</td>
<td>14 ± 5.2</td>
<td>5 ± 0.8</td>
<td>2.2 ± 0.4</td>
<td>4.8 ± 0.8</td>
<td>7 ± 1.6</td>
<td>14.6 ± 2</td>
<td>24.8 ± 1</td>
<td>48 ± 10.8</td>
<td>100.6 ± 33</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>6 ± 1.8</td>
<td>&lt; 5</td>
<td>11 ± 1.5</td>
<td>73.6 ± 5</td>
<td>84.6 ± 7.4</td>
<td>110 ± 28</td>
<td>21.8 ± 1</td>
<td>23.6 ± 1.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SD is shown, n=3.

\(^b\)LOQ for intrinsic metabolism experiments were 5 µl/min/mg protein or 5 µl/min/10^6 hepatocytes.

\(^c\)CL\(^{int, uptake, total}\) = CL\(^{int, uptake}\) + CL\(^{int, efflux}\) (Eq. 5).

\(^d\)CL\(^{blood}\) was calculated according to Eqs. 7 and 8.

\(^e\)CL\(^{hepatic}\) was calculated using equation 10. f\(^{hepatic}\) data was taken from the literature (Kamath et al., 2005, Lave et al., 1999, Yang et al., 2009).
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\( ^{f} \text{CL}_{\text{bile}} \) denotes blood to bile CL (Eq. 9).

\( ^{g} \text{In vitro efflux measurements} \) (\( \text{CL}_{\text{int, efflux}} \), Table 5) were extrapolated to in vivo \( \text{CL}_{\text{bile}} \) (Eqs. 12 and 13).

\( ^{h} \text{Absolute fold error and average absolute fold error are given for predictions.} \)

\( ^{i} \text{CL}_{\text{hepatic}} \) was predicted from \( \text{CL}_{\text{int, uptake}} \) and \( \text{CL}_{\text{int, uptake, total}} \) (data from Table 5, Eqs. 12 and 13).
Figure 2

A

Predicted CL_{bile} (ml/min/kg) vs. In vivo CL_{bile} (ml/min/kg) with a correlation coefficient of r^2=0.89.

B

Predicted CL_{hepatic} (ml/min/kg) vs. In vivo CL_{hepatic} (ml/min/kg).

C

Predicted CL_{hepatic} (ml/min/kg) vs. In vivo CL_{hepatic} (ml/min/kg) with a correlation coefficient of r^2=0.84.
Supplemental Fig 1. Effects on drug uptake kinetics in rat hepatocytes of transporter inhibitors and temperature. A: Uptake of atorvastatin in plated rat hepatocytes with or without the OATP inhibitor CsA. B: Effect of sodium on taurocholate uptake. C: Effect of temperature on atenolol uptake in plated rat hepatocytes.