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Improved Extrapolation of Hepatobiliary Clearance from *in Vitro* Sandwich Cultured Rat Hepatocytes through Absolute Quantification of Hepatobiliary Transporters

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Abstract: Previously we have reported that hepatobiliary transporter expressions in sandwich cultured hepatocytes (SCH) are altered 2- to 5-fold. This change could limit the model's predictive power for *in vivo* biliary clearance. The present study was designed to better establish *in vitro* to *in vivo* correlation (IVIVC) of biliary clearance. Eleven compounds representing the substrates of Mrp2/Abcc2, Bcrp/Abcg2 and Bsep/Abcb11 were tested in the sandwich cultured rat hepatocyte (SCRH) model. Simultaneously, the absolute difference of hepatobiliary transporters between rat livers and SCRH at day 5 post culture was determined by LC−MS/MS. This difference was integrated into the well-stirred hepatic prediction model. A correction factor named "g_factor" was mathematically defined to reflect the difference in hepatobiliary transporter expressions between the SCRH model and *in vivo models*, as well as the contribution of multiple transporters. When the g_factor correction was applied, the *in vivo* biliary clearance prediction was significantly improved. In addition, for those compounds which are poorly permeable and/or undergo transporter-dependent active uptake, the known intracellular concentrations of substrates were used to estimate intrinsic bile clearance. This led to further improvement in the prediction of *in vivo* bile secretion. While the rate-limiting processes of uptake transporters in the SCRH model remain to be further determined, we showed that integration of the absolute difference of hepatobiliary transporter proteins and transport contributions could improve the predictability of SCRH model. This integration is fundamental for increased confidence in the IVIVC of human biliary clearance.

Keywords: *In vitro/in vivo* correlation; biliary clearance; multidrug resistance protein 2 (Mrp2/Abcc2); breast cancer resistance protein (Bcrp/Abcg2); bile salt export pump (Bsep/Abcb11); sandwich cultured hepatocytes

Introduction

Accurate and more relevant prediction of human pharmacokinetics (PK) could efficiently accelerate the process of identifying new chemical entities as drug candidates. In past decades, *in vitro* models and preclinical species have been very useful for predicting drug disposition in humans, particularly in the early stages of drug discovery. However, confidence in human PK prediction is generally limited to drugs eliminated by hepatic enzymatic metabolism and/or urinary excretion.[1−4] Human PK prediction remains very challenging for those compounds that are mainly secreted into bile. Obtaining clinical bile samples is difficult in practice so predicting biliary secretion in humans is highly dependent on extrapolation from preclinical data or *in vitro* models. On the other hand, these extrapolations are

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complicated by the large inherent interspecies transporter expression differences and the lack of predictive *in vitro* models.

Biliary excretion is a dynamic, vectorial process mediated by uptake and efflux transporters located on the sinusoidal and canalicular membranes of liver hepatocytes. Several ATP binding cassette (ABC) efflux transporters, such as MRP2/ABCC2, BCRP/ABCG2 and BSEP/ABCB11, on the canalicular membrane of hepatocytes, are in charge of the biliary secretion of endogenous and exogenous substances.[5−9] During the past decade, numerous attempts have been made to find suitable *in vitro* models for predicting human biliary secretion. For instance, the transporter overexpressed membrane vesicles, terminal differentiated Caco-2 cells and transporter overexpressed cell lines have been widely employed to determine the transporters involved in biliary elimination of drugs and their metabolites. Among *in vitro* models, hepatocytes are a versatile model for studying drug metabolism and transporters. However, isolated hepatocytes lose cellular polarity immediately after isolation from the intact liver which limits their application for characterizing biliary secretion.[10,11] The SCH model has recently been demonstrated to be a useful model to evaluate bile clearance.[12,13] Sandwich culturing allows hepatocytes to repolarize and form bile canalicular networks providing a three-dimensional structure, proper localization of hepatobiliary transporters mimicking the *in vivo* hepatobiliary system. The polarized structure allows vectorial transport from blood to bile. Although it has been shown that the SCH predicted bile clearance is linearly correlated to *in vivo* bile clearance to a certain extent,[1,14,15] the predictive capability of the SCH model is still questionable. The proposed correction with protein unbound fraction could significantly underestimate bile clearance 10−100-fold, compared to *in vivo* observation. The major critiques of this model are that (1) it lacks dynamic bile flow, and (2) the protein expression of transporters is changed after culturing. The unknown expression level of hepatobiliary transporters between the SCH model and the liver might significantly affect the precision of prediction, as the transport kinetics of a given compound is characterized by K_m and V_{max} in which V_{max} is largely determined by the amount of transporter protein. Therefore, the poor correlation could be due to the alteration of the expression of biliary transporters that are not taken into account in the current extrapolation strategy. To fill the gap, in the present study, a series of compounds representing MRP2/ABCC2, BCRP/ABCG2 and BSEP/ABCB11 substrates were tested in the *in vitro* SC rat hepatocyte (SCRH) model and *in vivo* bile duct cannulated rats to obtain *in vitro* and *in vivo* bile clearance. By incorporating the differential expression of hepatobiliary transporters that are involved in biliary excretion of the selected compounds, the bile clearance prediction from *in vitro* SCRH model was significantly improved.

Materials and Methods

Chemicals and Reagents. Rosuvastatin, topotecan, valsartan and cefpiramide were purchased from AK Scientific, Inc. (Mountain View, CA). Pravastatin and perchloric acid were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile, water and methanol were purchased from Burdick & Jackson (Muskegon, MI) and EMD Chemicals, Inc. (Gibbstown, NJ), respectively. Hydroxychloride and dimethyl sulfoxide was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Phosphate buffered saline (PBS), Hanks

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balanced salt solution (HBSS) and customized Ca^{2+}/Mg^{2+} free HBSS were purchased from Gibco (Carlsbad, CA). Matrigel (phenol red free) and collagen I coated 24-well BioCoat plates were obtained from BD Biosciences (Bedford, MA). The hepatocyte plating medium (*InVitroGRO* CP medium), culture medium (*InVitroGRO* HI medium) and *Torpedo* Antibiotic Mix were purchased from Celsis IVT technologies (Baltimore, MD).

Hepatocyte Sandwich Culture. Hepatocytes freshly isolated from 8 weeks old male Sprague−Dawley rats were purchased from CellzDirect (Pittsboro, NC). The hepatocytes were cultured in a sandwich format based on the methods reported previously.[16] Briefly, the freshly isolated hepatocytes were precipitated by centrifuging at 500 rpm and resuspended in 5 mL of completed plating (CP) media. The cell viability was checked by the trypan blue exclusion method. The hepatocytes were seeded on 24-well BioCoat plates and allowed to attach for 2−4 h at 37 °C in a humidified incubator with 95%/5% of air/CO_2. On the second day after hepatocyte plating, the hepatocytes were washed once with warmed completed culture medium (HI medium) then overlaid with BD Matrigel at a concentration of 0.25 mg/mL in ice-cold completed HI incubation media. The culture media were refreshed every 24 h.

Biliary Excretion Assay in SCRH. At day 5 post culture, the biliary secretion assay in SCRH was performed as described previously.[12] Briefly, SCRH cultures were rinsed twice with 0.5 mL of HBSS or Ca^{2+}/Mg^{2+}-free HBSS containing 1 mM EDTA. Disruption of the bile canalicular network was confirmed by phase contrast microscopy after preincubating with Ca^{2+}/Mg^{2+}-free HBSS for 10 min at 37 °C. Subsequently, the substrates dissolved in HBSS were added to initiate the vectorial transport of SCRH. At 2 min, 10 min, and 15 min postincubation, hepatocytes were washed three times with ice-cold HBSS and then lysed with 0.5 mL of methanol at room temperature for 20 min while shaking. The samples were transferred to a 96-deep-well microplate and evaporated on a SPE Dry 96 Dual Microplate Sample Concentrator Systems (Biotage, Uppsala, Sweden). The residue was reconstituted in 50/50 ACN/H_2O with 0.1% formic acid and subjected to analysis by LC−MS/MS. Parallel wells of hepatocytes were lysed with 1% Triton-X-100 for protein quantification by BCA kit (Pierce Biotechnology, Rockford, IL).

Metabolic Stability of Probe Substrates in Rat Liver Microsome and Hepatocytes. The metabolic stability of selected probe substrates was examined using pooled male Sprague−Dawley rat microsomes from Pfizer Global Supply (BD Biosciences, MA). The assays were performed in a total volume of 1 mL of incubation mixture containing 100 mM potassium phosphate (pH 7.4), 3.3 mM magnesium chloride, 0.5 mg/mL rat liver microsomal protein and 1.0 μM probes. Following 3 min preincubation at 37 °C, reaction was initiated by the addition of 2 mM NADPH. Rat cryopreserved hepatocytes from Pfizer's central hepatocyte stock (Celsis IVT, Baltimore, MD) were used for the hepatocyte stability assay. The cryopreserved hepatocytes were prepared according to the protocol provided by XenoTech, LLC (Lenexa, Kansas). The hepatocytes with viability greater than 80% were used in the study. The substrates (1 μM in William's E media) were incubated with hepatocytes in a CO_2 incubator with 95%/5% air/CO_2, 37 °C and 95% humidity. At designated time points, aliquots of the incubation mixture were taken out and the reaction was terminated by adding 10× volume of acetonitrile. The mixtures were then centrifuged at 5000 rpm for 10 min to precipitate protein. The supernatants were transferred into a 96-well assay plate to measure disappearance of parent compounds by LC−MS/MS.

Plasma Protein Binding. The pooled rat plasma was spiked with 2 μL of 2.5 mM stock solutions in DMSO (except topotecan−HCl in methanol) to reach the final concentration of 10 μM. Equilibrium dialysis was performed in a 96-well equilibrium dialyzer with MW cutoff of 5K (Harvard Apparatus, Holliston, MA), according to the protocol recommended by the manufacturer. Briefly, 200 μL of plasma samples or 1× PBS (pH 7.4) were added to the respective sides of the 96-well dialysis plate. The plate was then incubated at 37 °C while rotation for 6 h. 50 μL of samples were collected from buffer or plasma side and then mixed with 150 μL of the opposite matrix. After centrifugation at 5000 rpm for 10 min, the supernatant was transferred to a new 96-well microplates for analyte quantification on LC−MS/MS.

Biliary Excretion of Probe Substrates in Rats. Male Sprague−Dawley (SD) rats weighing 250−300 g were obtained from Charles River Laboratories (Wilmington, MA). The rats were surgically implanted with BASi vascular catheters (West Lafayette, IN) in carotid artery, jugular vein, common bile duct and duodenum before arrival. The bile duct and duodenal cannulae were exteriorized between the scapulae. Animals were acclimated in Culex cages (Bioanalytical Services, Inc., West Lafayette, IN) overnight prior to dosing. Food and water were provided *ad libitum*. Animals were dosed with rosuvastatin or topotecan through the jugular vein catheter. The dosing volume was controlled at about 300 μL (1 μL/g of body weight). Blood samples were automatically collected by the Culex at 0.083, 0.25, 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 16, and 24 h time points for the intravenous bolus administration. Bile samples were collected at 0−10 min, 10−20 min, 20−30 min, 30−40 min, 40−50 min, 50 min to 8 h and 8−24 h. Both plasma and bile samples were kept on ice during sampling and stored in −80 °C until analysis. All procedures were approved by the St. Louis Pfizer Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

High-Performance Liquid Chromatography (HPLC) Analysis for Topotecan. The chemical structure of topotecan is pH-dependent so quantification of the total amount of

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topotecan in the samples collected from *in vivo* rat experiments was accomplished by HPLC at an acidic pH. Briefly, 50 μL plasma samples were mixed with 50 μL of acetonitrile and 100 μL of 7% (v/w) perchloric acid. Bile samples were diluted in a ratio of 1 to 10 in PBS pH 3.0, then 50 μL of diluted bile sample was mixed with 100 μL of acetonitrile and 50 μL of 7% (v/w) perchloric acid. The mixture was vortex-mixed and centrifuged at 5000 rpm for 10 min. The supernatant was transferred to 96-well microplates, and then 100 μL was loaded on the HPLC system (Agilent) for quantification. The standard curve was prepared by topotecan in lactone form by diluting equal amount of stock solution in DMSO with PBS at pH 3.0. The standard curve ranged from 0.0024 μM to 5 μM. The liquid chromatographic system consisted of Aligent 1100 solvent delivery BinPump equipped with an auto injector system and degasser and G1321A fluorescence detector (Agilent). Chromatographic separation was achieved at ambient temperature using a SYNERGI column (4 μM, 250 mm $\times$ 4.6 mm) (Phenomenex) and a C18 guard column (10 μM, 20 mm $\times$ 3.9 mm) (Waters, MA) at isocratic flow rate of 1 mL/min. The mobile phase consisted of acetonitrile and PBS adjusted to pH3.6 with hydroxychloride at ratio of 1:3. The excitation and emission wavelengths were 380 and 527 nm respectively. The data was integrated and analyzed using ChemStation (Agilent).

LC−MS/MS Analysis. Fifty microliter plasma or bile samples (1 to 10 diluted) were mixed with 150 μL of acetonitrile containing 0.2 μM carbamazepine as an internal standard for protein precipitation. After centrifugation at 5000 rpm for 10 min, the supernatant was transferred and subjected to LC−MS/MS analysis. LC−MS/MS analysis was conducted with an API 3000 triple quadruple mass spectrometer (PE Sciex, Ontario, ON, Canada) coupled with a turbo ion spray interface in positive ion mode, and connected with a Shimadzu LC (SLC-10A) system (WoolDale, IL) and HTS PAL Leap autosampler (Carrboro, NC). Reverse phase chromatography (mobile phase A, 0.1% formic acid in H_2O; mobile phase B, 0.1% formic acid in acetonitrile; linear gradient from 5% to 95% over 3 min) was used to elute and separate the different substrates with a Fortis phenyl C6 column (2.1 $\times$ 50 mm, 5 μm, Fortis Technologies Ltd.). Injections of 10 μL were analyzed using a flow rate of 0.4 mL/min. The following transitions were monitored: *m/z* 422.3−377.2 for topotecan, 482.2−258 for rosuvastatin, 393.2−349.4 for SN38, 436.4−235.1 for valsartan, 613.3−257.2 for cefpiramide, and 237.4− 194 for internal standard (carbamazepine). The instrument settings of the API 3000 were as follows: ion spray voltage, 4 kV; temperature, 400 °C; and collision energy was set at 29 eV for topotecan, 49 eV for resovastatin, 29 eV for valsartan, 25 eV for pravastatin, 40 eV for cefpiramide and 27 eV for IS. Estradiol-17β-glucuronide from *in vitro* assays and rosuvastatin from the *in vivo* assay were analyzed at negative mode and positive mode, respectively, on an API-4000 triple quadruple mass spectrometer (PE Sciex, Ontario, ON, Canada). The analytes were separated with GEMINI 5 μm C6 phenyl column (2.1 $\times$ 50 mm, 5 μm, Phenomenex). The transitions monitored for estradiol-17β-glucuronide and the internal standard tolbutamide were *m/z* 447.2−271.1 and *m/z* 269.1−169.8 with collision energy of −48 eV and −13 eV, respectively. The peak areas of all the analytes and internal standard were integrated and quantified using Analyst 1.4.1 (MDS Sciex).

Data Analysis. The biliary excretion index (BEI) was determined according to eq 1.[17] The apparent uptake rate ($CL_{uptake,int}$) and apparent *in vitro* intrinsic biliary clearance ($CL_{bile,int}$) were determined by the eq 2 and 3. Equation 1 and 3 use B-Clear Technology, which is patented by Qualyst, Inc.

$$BEI = \frac{accumulation_{(std,HBSS)} - accumulation_{(Ca^{2+}/Mg^{2+},free)}}{accumulation_{(std,HBSS)}} \quad (1)$$

$$CL_{uptake,int} = \frac{\Delta accumulation_{(std,HBSS,10-2min)}}{incubation\_time \times concentration_{(medium)}} \quad (2)$$

where Δaccumulation represents the cumulative amount of compound from 2 to 10 min (8 min in incubation), the $concentration_{(medium)}$ represents the starting concentration at time 0 with the assumption that the tested compounds quickly equilibrate between the intracellular compartment and culture media.

$$CL_{bile,int} = \frac{accumulation_{(std,HBSS)} - accumulation_{(Ca^{2+}/Mg^{2+},free)}}{incubation\_time \times concentration_{(medium)}} \quad (3)$$

where $accumulation_{(std,HBSS)}$ and $accumulation_{(Ca^{2+}/Mg^{2+},free)}$ represent the cumulative amount of compound in SCRH in the presence or absence of Ca^{2+}/Mg^{2+}. The $CL_{bile,int}$ and BEI were determined at 10 min based on eq 3 and eq 1. The unit of calculated $CL_{bile,int}$ was μL/min/mg protein, and further transformed to the unit of mL/min/kg based upon the assumption of 200 mg of protein/g of rat liver tissue and 40 g of liver/kg of body weight.

The predicted bile clearance ($CL_{pred,bile}$), the predicted bile clearance based on plasma unbound fraction ($CL_{pred,bile,fu,p}$) and the predicted bile clearance corrected by g_factor ($CL_{pred,bile,g}$) were determined by eq 4, eq 5 and eq 6 respectively, where Q_p represents rat hepatic plasma flow rate (40 mL/min/kg), $f_{u,p}$ represents the plasma unbound fraction of test compounds and g is the correction factor defined by eq 10. In these equations, $CL_{pred,bile}$ and $CL_{pred,bile,fu,p}$ represent the predicted bile clearance based on the total plasma concentration and plasma unbound concentration, respectively.

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$$CL_{pred,bile} = \frac{Q_p \times CL_{bile,int}}{Q_p + CL_{bile,int}} \quad (4)$$

$$CL_{pred,bile,fu,p} = \frac{Q_P \times f_{u,p} \times CL_{bile,int}}{Q_p + f_{u,p} \times CL_{bile,int}} \quad (5)$$

$$CL_{pred,bile,g} = \frac{Q_P \times g \times CL_{bile,int}}{Q_p + g \times CL_{bile,int}} \quad (6)$$

The pharmacokinetic parameter of *in vivo* studies, AUC (area under the plasma concentration−time curve), was estimated based on the noncompartmental model using WinNonlin Professional (version 5.2; Pharsight, Mountain View, CA). The percentage dose excreted into bile and CL_{bile} (mL/min/kg) were calculated according to eqs 7 and 8:

$$\%\ \text{dose in bile} = \frac{\text{accumulation in bile}_{(0-T)}}{\text{dose}} \times 100 \quad (7)$$

$$CL_{bile} = \frac{\text{dose}}{AUC_{(0-T)}} \times \%\ \text{dose in bile} \quad (8)$$

Data represent the mean ± SEM of n animals ($n = 3$ or 5).

Mathematical and Statistical Analysis. MATLAB software version 2007b (The Mathworks, Inc., Natick, MA) was used for all the mathematical analysis related to g_factor calculations and for generating the three-dimensional surface plot and corresponding contour diagrams. Additionally, the curve fitting toolbox within the 2007b package was utilized for the linear least-squares fitting of the data and estimating the correlation coefficient (R^2) and the confidence intervals.

Results

Selection of Probe Substrates for Investigating IVIVC of Biliary Excretion in Rat. Hepatic drug clearance consists of enzymatic metabolic pathways as phase I and phase II metabolism, and/or hepatobiliary elimination *via* transporters. Toward to the goal of investigating IVIVC of biliary secretions, 11 compounds that are predominantly secreted into bile were selected for testing in the SCRH model. The corresponding pharmacokinetic parameters were obtained from bile duct cannulated rats. Additionally, the metabolic stability of these selected compounds were determined in rat liver microsome and rat hepatocyte incubation in order to rule out the possibility of involvement of phase I and phase II enzymes in hepatic clearance (Table 1). Other compounds that were not listed in Table 1 have been reported to be stable or only undergo minor metabolism with hepatic enzymes (Table 1). Among the compounds selected, estradiol-17β-glucuronide, cefpiramide, valsartan, olmesartan, cefoperazone, cefmetazole, pravastatin, rosuvastatin are Mrp2/Abcc2 substrates; rosuvastatin, topotecan and pitavastatin are the substrates of Bcrp/Abcg2; taurocholate and pravastatin are the substrate of Bsep/Abcb11.

***In Vitro* $CL_{bile,int}$ Estimated in SCRH Model and *in Vivo* CL_{bile} in Rat.** The in vitro $CL_{bile,int}$ for estradiol-17β-glucuronide, cefpiramide, valsartan, rosuvastatin, pravastatin, topotecan were obtained using the SCRH model. The others were acquired from literature reports (valsartan, olmesartan, cefoperazone, cefmetazole, pitavastatin and taurocholate).[1,15] Similarly, *in vivo* CL_{bile} was obtained by in-house pharmacokinetic studies using bile duct cannulated rats for rosuvastatin and topotecan or cited from the literature reports (Table 2). The BEI, representing the extent of compound secretion into the bile canaliculi formed in SCRH, was determined by use of eq 1. The apparent $CL_{uptake,int}$ and apparent $CL_{bile,int}$ were determined by eqs 2 and 3.[14] Table 2 summarizes both *in vitro* and *in vivo* kinetic parameters for the 11 selected compounds that were used for establishing IVIVC. It was noticed that estradiol-17β-glucuronide, rosuvastatin, pitavastatin and taurocholate exhibited high level uptake activity (nearly 18 μL/min/mg protein), while cefoperazone and cefmetazole showed the least hepatic uptake (<0.2 μL/min/mg protein). In general, the medium concentration of tested compounds was applied in the mathematic equation to determine the apparent $CL_{bile,int}$, as shown in eq 3. Conversely, the intracellular concentration of tested compounds was used to estimate the *in vitro* clearance when hepatic uptake is the rate-limiting process in hepatobiliary efflux, e.g. pravastatin, cefoperzone and cefmetazole.

Table 1. Enzyme Metabolic Stability of Selected Substrates[a]

compound name	RLM % at 60 min	RCH % at 60 min
estradiol-17β-glucuronide	96.9 ± 3.4	109.1 ±15.6
rosuvastatin	104.7 ± 3.3	109.1 ±15.6
pravastatin	101.8 ±12.2	96.2 ± 1.5
valsartan	96.9 ±13.5	94.2 ± 7.8
cefpiramide	102.3 ± 5.6	102.6 ± 1.0
topotecan	77.9 ± 4.3	81.8 ±12.7

[a] To evaluate the hepatic metabolic stabilities, 1 μM substrates were incubated with either rat liver microsomes (phase I metabolism) or cryopreserved rat hepatocytes (phase I and phase II metabolism), respectively. The percentage of parent compounds remaining after 60 min incubation is summarized. Other compounds that are not listed in Table 1 have been reported to be metabolically stable or undergo minor metabolism: olmesartan;[41] cefoperazone;[42] cefmetazole;[43] pitavastatin.[44] Data represent the mean ± SEM ($n = 3$). RLM is rat liver microsome; RCH stands for rat cryopreserved hepatocytes.

Absolute Difference in Hepatobiliary Transporter Proteins between SCRH and Rat Liver. The absolute amount of hepatobiliary transporters Mrp2/Abcc2, Bsep/Abcb11 and Bcrp/Abcg2 in rat livers and SCRH at day 5 was determined by LC−MS/MS.[18,19] The absolute amount of transporters in SCRH at day 5 post SC (fmol/μg membrane protein) was converted to pmol/g of liver weight by using the physiological parameters of rat liver (107×10^6 hepatocytes per g of rat liver with known hepatocyte number in each well)[20] and the total membrane protein recovery from rat liver (26 mg membrane protein/g liver).[18,19] Figure 1 shows the differences in absolute amount of transporter proteins between SCRH and

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Table 2. *In Vitro* Bile Clearance Obtained from SCRH Model and *in Vivo* Rat Biliary Clearance[a]

	in vitro SCRH				rat in *vivo*	
	$CL_{uptake,int}$ (mL/min/mg protein)	$CL_{bile,int}$ (mL/min/mg protein)	BEI (%)	f_u	CL_{bile} (mL/min/kg)	excretion ratio (% of dose)
estradiol-17β-glucuronide	18.9 ± 1.1	5.7 ± 1.0	15.9 ± 2.6	0.069	32.1[41]	77[41]
pravastatin	0.82 ± 0.1	0.51 ± 0.09	34.0 ± 5.9	0.572	27.9 ± 5.8[45]	62.7 ± 9.0[45]
cefpiramide	0.59 ± 0.06	0.59 ± 0.09	36.7 ± 2.4	0.099	5.47 ± 1.48[46]	57.5 ± 15.5[46]
valsartan[1]	1.6	0.405 ± 0.25	18.9 ± 2.6	0.006	3.5 ± 0.6[45]	83.2 ± 4.8[45]
olmesartan[1]	1.3	0.215 ± 0.031	14.7 ± 0.5	0.01	2.22 ± 0.47[15]	83 ± 28[15]
cefoperazone[15]	0.17	0.07 ± 0.01	30.8 ± 5.7	0.429	11.0 ± 4.0[45]	75.0 ± 14.3[45]
cefmetazole[15]	0.14	0.005 ± 0.019	0.1 ± 4.5	0.738	6.5 ± 1.0[45]	64.7 ± 5.1[45]
rosuvastatin	18.3 ± 1.0	15.2 ± 0.6	54.8 ± 2.8	0.115	27.4 ± 2.8	67.4 ± 10.2
topotecan	1.35 ± 0.32	1.67 ± 0.34	39.5 ± 6.8	0.647	5.3 ± 1.0	13.2 ± 1.2
pitavastatin[1]	17.6	4.32 ± 0.39	19.9 ± 1.4	0.008	4.55 ± 0.13[47]	72.7[47]
taurocholate[1]	ND	6.37 ± 0.81	76.8 ± 2.3	0.15	29.8[48]	100[48]

[a] Intrinsic uptake clearance ($CL_{uptake,int}$), intrinsic bile clearance ($CL_{bile,int}$) and biliary excretion index (BEI) were calculated based on eq 1, eq 2, and eq 3. The kinetic parameters are determined at 10 min incubation at indicated concentration. All compounds were tested at 2 μM, except for pravastain and cefpiramide at 10 μM. The $CL_{bile,int}$ of the tested compounds determined in the SCRH model was converted to mL/min/kg by determining total protein in each well and using the physiological parameters of the rat (40 g liver/kg body weight and 200 mg total protein/g of liver).[20] Data represent mean ± SEM (n = 3 or 5). Superscripts represent the citations for the in vitro SCRH data or *in vivo* bile clearance data of compounds.[1,15,41,45–48] ND: Not determined.

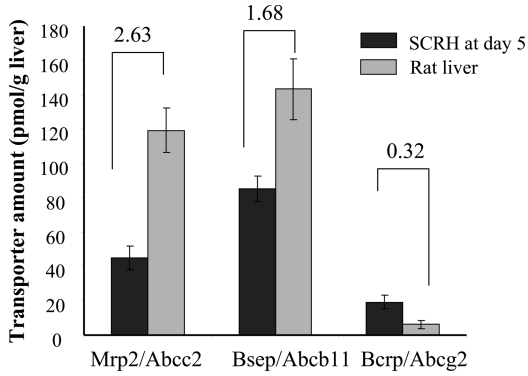

Figure 1. Comparison of absolute amount of major hepatobiliary transporters in SCRH and rat livers. Mrp2/Abcc2, Bsep/Abcb11 and Bcrp/Abcg2 protein levels were determined in SCRH at day 5 post SC and rat liver tissues by LC−MS/MS mediated quantification. The absolute amount of transporters in SCRH at day 5 post SC (fmol/μg membrane protein) was converted to pmol/g of liver weight by using the physiological parameters of rat liver[20] (107×10^6 hepatocytes per g of rat liver with known hepatocyte numbers in each well) and the total membrane protein recovery from rat liver (26 mg membrane protein/g liver).[18,19] Numbers above the bar are the fold difference between rat liver and SCRH at day 5. Data represent mean ± SEM (n = 3 for SCRH or 5 for rat livers).

rat liver tissues. This finding of distinct protein levels of hepatobiliary efflux transporters in *in vitro* SCRH models vs *in vivo* rat liver was subsequently utilized to improve the mathematical model for predicting *in vivo* biliary secretion as described in the following sections.

IVIVC of Rat Biliary Secretion Based on Conventional Approaches. The $CL_{bile,int}$ of the tested compounds determined in the SCRH model was converted to mL/min/kg by determining total protein in each well and using the physiological parameters of the rat (40 g liver/kg body weight and 200 mg total protein/g of liver).[20] First, the *in vivo* biliary excretion ratio (% of dose) was plotted against BEI estimated from SCRH. However, there was not an apparent correlation observed for these parameters between *in vitro* and *in vivo* (Figure 2A). Subsequently, a well-stirred hepatic model was applied to predict the *in vivo* CL_{bile} from *in vitro* $CL_{bile,int}$ as determined in SCRH by eq 3. The predicted CL_{bile} values for estradiol-17β-glucuronide, rosuvastatin, taurocholate, cefpiramide, valsartan and olmesartan were fairly close to the *in vivo* CL_{bile} observed in rat (Figure 2B). However, apparent under- (cefoperazone, cefmetazole and pravastatin) or overestimation (pitavastatin and topotecan) in the prediction of CL_{bile} was also exhibited (Figure 2B). When the predicted CL_{bile} was normalized by plasma protein unbound fraction ($f_{u,p}$) to predict $CL_{pred,bile,fu,p}$,[15] the linearity of correlation was still poor for the 11 selected compounds (R^2 = 0.32) (Figure 2C). In addition, the predicted $CL_{pred,\ bile,\ fu,p}$ was significantly underestimated by about 10−100-fold, compared to *in vivo* CL_{bile}.

Integration of Absolute Difference of Hepatobiliary Transporters into Extrapolation of Biliary Excretion. Based on the current understanding of transporter kinetics, transporter-mediated drug clearance is determined by maximum transport velocity (V_{max}) and the Michaelis−Menten constant (K_m) of the transporter assuming linear kinetics when the concentration of substrates is much lower than K_m. As indicated by the marked differences in the protein levels of Mrp2/Abcc2, Bcrp/ABcg2 and Bsep/Abcb11 between SCRH

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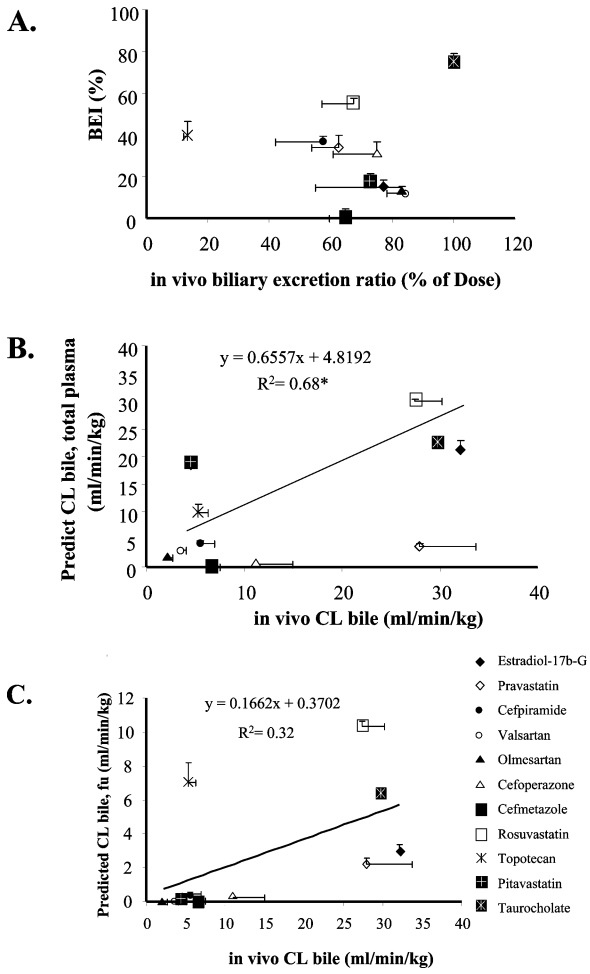

Figure 2. Comparison of *in vitro* biliary secretion and in vivo biliary clearance. (A) Correlation of BEI estimated in SCRH with % of dose in bile *in vivo*. (B) IVIVC of CL_{bile} predicted from *in vitro* SCRH based on total plasma concentration with *in vivo* observed CL_{bile} based on eq 4. The R^2 was 0.68, when cefmetazole, cefoperazone and pravastatin were excluded. (C) IVIVC of CL_{bile} predicted from *in vitro* SCRH based on plasma unbound fraction using eq 5. The R^2 of the correlation was 0.32.

and rat liver (Figure 1), the *in vitro* value of V_{max} could significantly differ from that in rat liver since it is a function of the absolute amount of transporter proteins. Therefore, accounting for the protein expression levels *in vitro* and *in vivo* becomes critical for the precise prediction of *in vivo* biliary secretion. Toward that end, a new correction factor named g_factor was introduced to take into account two important considerations: (1) the correction of *in vitro* $CL_{bile,int}$ by incorporating the absolute difference of protein expressions of various transporters between rat liver and SCRH at day 5 post culture (the day when the biliary excretion assay was usually performed) and (2) the relative contribution of multiple transporters in biliary excretion.

The g_factor was mathematically defined as the ratio of the sum of the intrinsic clearance from each transporter involved. Assuming that the Mrp2/Abcc2, Bcrp/Abcg2 and Bsep/Abcb11 mediated transport is the rate-limiting process that is mainly responsible for the biliary excretion, the g_factor was derived by eq 9:

$$\text{g\_factor} = \frac{\left[\frac{V_{max\_MRP2}}{K_{m\_MRP2}} + \frac{V_{max\_BSEP}}{K_{m\_BSEP}} + \frac{V_{max\_BCRP}}{K_{m\_BCRP}}\right]^{\text{rat liver}}}{\left[\frac{V_{max\_MRP2}}{K_{m\_MRP2}} + \frac{V_{max\_BSEP}}{K_{m\_BSEP}} + \frac{V_{max\_BCRP}}{K_{m\_BCRP}}\right]^{\text{SCRH at day 5}}} \quad (9)$$

where V_{max} and K_m are the Michaelis−Menten parameters for the corresponding transporters of a certain substrate. Being an intrinsic property of a given compound, the K_m is assumed to remain the same in both *in vitro* and *in vivo* models, whereas V_{max} is determined by amount of protein and intrinsic activity (K_{cat}) for a given substrate following the equation of V_{max} = [protein Amt] × K_{cat}. Since V_{max}/K_m = (amt × K_{cat})/K_m = amt/K_m/K_{cat} = amt/K_m', where K_m' is a apparent equilibrium constant for a given drug, eq 9 can be rearranged to eq 10. Plugging in the actual amounts listed in Figure 1 in the above equation with some rearrangement results in eq 11.

$$\text{g\_factor} = \frac{\left[\frac{\text{amt}_{\_MRP2}}{K_{m}'_{\_MRP2}} + \frac{\text{amt}_{\_BSEP}}{K_{m}'_{\_BSEP}} + \frac{\text{amt}_{\_BCRP}}{K_{m}'_{\_BCRP}}\right]^{\text{rat liver}}}{\left[\frac{\text{amt}_{\_MRP2}}{K_{m}'_{\_MRP2}} + \frac{\text{amt}_{\_BSEP}}{K_{m}'_{\_BSEP}} + \frac{\text{amt}_{\_BCRP}}{K_{m}'_{\_BCRP}}\right]^{\text{SCRH at day 5}}} \quad (10)$$

$$\text{g\_factor} = \frac{\left[119.18\frac{K_{m}'_{\_BSEP}}{K_{m}'_{\_MRP2}} + 6.11\frac{K_{m}'_{\_BSEP}}{K_{m}'_{\_BCRP}} + 143.37\right]^{\text{rat liver}}}{\left[45.25\frac{K_{m}'_{\_BSEP}}{K_{m}'_{\_MRP2}} + 19.29\frac{K_{m}'_{\_BSEP}}{K_{m}'_{\_BCRP}} + 85.27\right]^{\text{SCRH at day 5}}} \quad (11)$$

From the above equation, it follows that the absolute magnitude of correction factor that is needed depends upon the K_m' values of a given compound being excreted. In order to explore the range within which the g_factor is expected to vary as a function of these K_m' values, we performed a mathematical analysis of eq 11. Figure 3A depicts the 3-dimensional surface plot of the g_factor as a function of two variables: the ratio of K_m' values for Bsep vs Mrp2; and the ratio of K_m' values for Bsep vs Bcrp. The mathematical analysis revealed that at its extremities the g_factor converges to 2.63, 1.68 and 0.32, which corresponds to the ratio of transporter amount in rat liver to SCRH model when the Mrp2/Abcc2, Bsep/Abcb11 and Bcrp/Abcg2 are the respective dominant transporters (Figure 1). In cases where more than one transporter is involved, as indicated by the comparable K_m' values, the g_factor is expected to adopt intermediate values that will depend upon the exact magnitude of K_m' values for all three transporters. This statement is supported by the analysis presented *via* the contour diagrams in Figure 3B−D. When Mrp2/Abcc2 is the most dominant

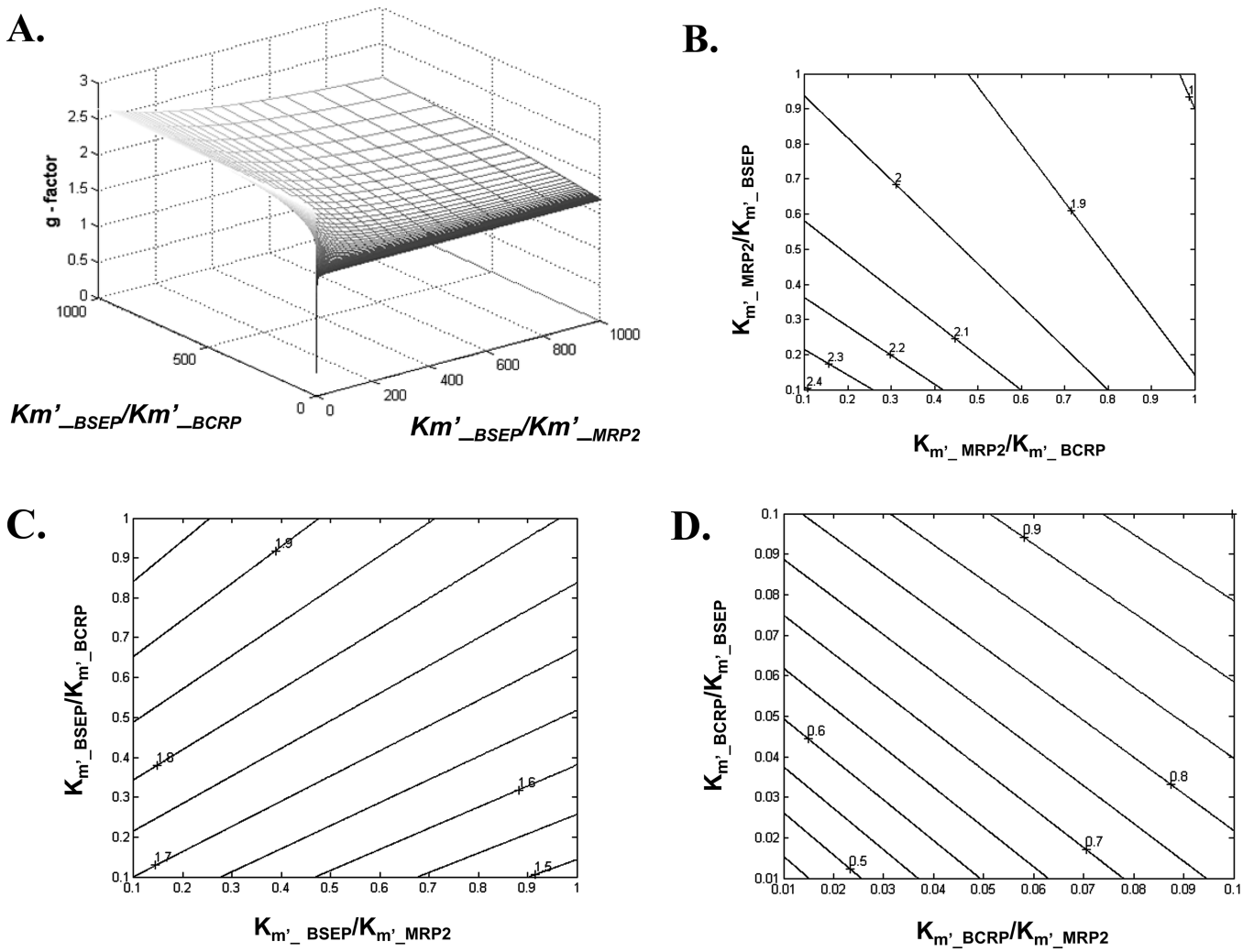

Figure 3. g_factor dynamic range. (A) 3-Dimensional surface plot depicting g_factor (eq 11) as a function of two variables: the ratio of K_m' values for Bsep vs Mrp2; and the ratio of K_m' values for Bsep vs Bcrp. This plot was generated using mesh function within MATLAB using a range of (0.001, 1000) for both variables. (B−D) The 2-dimensional contour lines depicting the range within which the g_factor varies as the K_m' ratios of respective enzymes (as labeled on the individual axis) are changed within the 10-fold range. The g_factor value as shown on the plots remains constant on a particular contour line. These contour plots were generated using contour function within MATLAB.

transporter (Figure 3B), i.e. has the lowest K_m' value, the g_factor is assumed to range in the vicinity of 1.9−2.0 when the ratios are below but close to unity. Ultimately, the g_factor converges to the maximum value of 2.63 when the K_m' ratios become lower than 0.1. In the case of Bsep/Abcb11 being the dominant transporter in terms of K_m' values (Figure 3C), the g_factor lies within a close range (1.5−1.9), finally converging to 1.68 when corresponding K_m' ratios became smaller than 0.2. In the case of Bcrp/Abcg2 being the dominant transporter, the convergence of the g_factor to a value of 0.32 was only achieved when the K_m' ratios became smaller than 0.01 (Figure 3D). In contrast to other scenarios, when Bcrp/Abcg2 is the dominant transporter, the g_factor was 3-fold higher than the ultimate value for Mrp2/Abcc2 when the K_m' ratios were in the range of 0.1. This can be explained by the relatively low levels of BCRP expression when compared to the activity of other transporters.

The major efflux transporter responsible for biliary elimination of estradiol-17β-glucuronide, cefpiramide, valsartan, olmesartan, cefoperazone and cefmetazole is Mrp2/Abcc2, therefore, a g_factor of 2.63 was applied to normalize the $CL_{bile,int}$ obtained from the SCRH model. Bcrp/Abcg2 is the major transporter responsible for biliary elimination of topotecan and pitavastatin; therefore, a g_factor of 0.32 was applied for extrapolation of these two substrates. Similarly, as taurocholate is mostly eliminated through Bsep/Abcb11, a g_factor of 1.68 was applied. The K_m values for rosuvastatin's interaction with Mrp2/Abcc2 and Bcrp/Abcg2 are 2.02 μM and 29.9 μM,[21] respectively. The g_factor for rosuvastatin estimated *via* eq 11 was 0.63. Similarly, the K_m values for pravastatin interaction with Mrp2/Abcc2 and Bsep/Abcb11 are 48.3 μM[22] and 124 μM,[23] respectively. The g_factor for

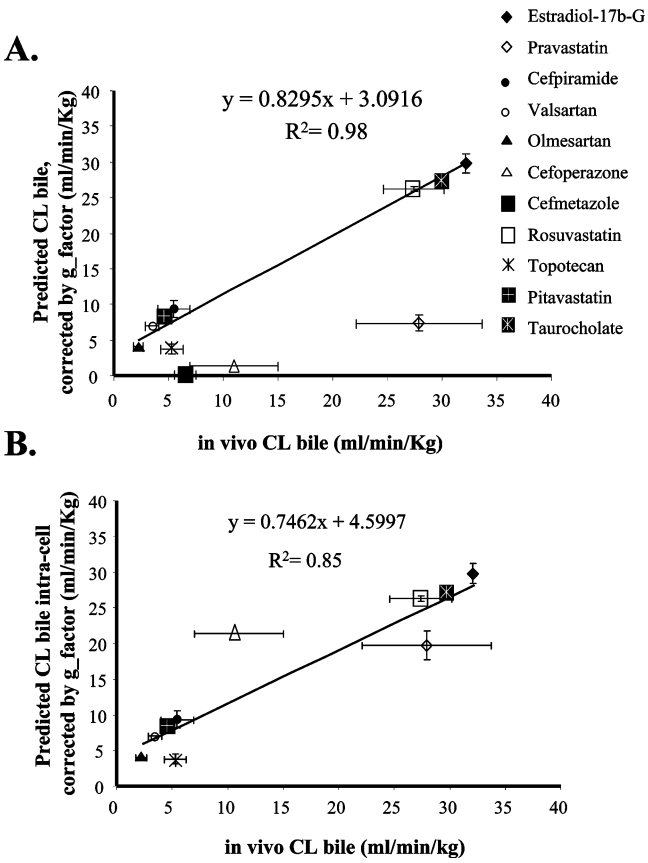

Figure 4. IVIVC of biliary excretion based on absolute difference of efflux transporters. (A) IVIVC of predicted CL_{bile} from *in vitro* SCRH with correction of the transporter protein amounts between *in vivo* and *in vitro* based on eq 6. Linear regression analysis of 8 out 11 compounds indicated the R^2 of the correlation was 0.98. The outliers were pravastatin and cefoperazone and cefmetazole. (B) $CL_{bile,int}$ was estimated based on the intracellular concentration in SCRH, for pravastatin and cefoperazone. Linear regression analysis of 10 out 11 compounds indicated the R^2 of the correlation was 0.85.

pravastatin *via* eq 11 was 2.23. Caution is needed, to be aware that the g_factor values for rosuvastatin (0.63) and pravastatin (2.23) are only an approximation given the fact that actual $K_m' = K_m$ is an approximation in the absence of experimental data on intrinsic V_{max} values. The transporter(s) that are not involved in handling a substrate, e.g. Bsep/Abcb11 for rosuvastatin, were mathematically eliminated from the effect of the scaling factor by applying infinity for the K_m. The observed rat CL_{bile} was plotted against the predicted $CL_{pred,bile,g}$ obtained from eq 6 (Figure 4A). Linear regression (R^2) indicated that the correction with g_factor significantly improved the accuracy of the predicted CL_{bile}, compared to the *in vivo* observations. However, as shown in Figure 4A, the CL_{bile} of pravastatin, cefoperazone and cefmetazole remained significantly underestimated. Considering that the apparent hepatic uptake indexes of these three compounds were obvious outliers (Table 1), the biliary secretion in the SCRH model was hypothesized to be the rate-limiting uptake process for these compounds. Subsequently, the intracellular concentration, which was determined by the total amount of compounds accumulated at 10 min in Ca^{2+}/Mg^{2+} free condition and the volume of rat hepatocytes ($6.2 \pm 0.11 \times 10^{-12}$ L/cell),[24] was applied to predict the $CL_{bile,int}$, instead of using the concentration in extra cellular matrix (concentration in media), where it is assumed that all intracellular drugs freely access the drug transporters. As shown in Figure 4B, the prediction of CL_{bile} for cefoperazone and pravastatin was apparently improved, while cefmetazole was ruled out from the calculation due to the absence of any hepatic uptake in a 10 min incubation in SCRH. In contrast, either excluding the outliers cefmetazole, cefoperazone and pravastatin ($R^2 = 0.68$) or applying intracellular contraction of cefoperazone and pravastatin ($R^2 = 0.56$, data not shown) did not improve the IVIVC of CL_{bile} predicted from *in vitro* SCRH without applying g_factor corrections. This indicated the fact that the linearity of the correlation of *in vitro* and *in vivo* CL_{bile} for 10 selected (except cefmetazole) compounds was obtained ($R^2 = 0.85$) (Figure 4B) by g_factor corrections.

Discussion

Biliary excretion is one of the primary elimination routes of xenobiotics and their conjugate metabolites from the body, along with metabolic elimination, renal secretion or a combination thereof.[25] Human bile samples are difficult to obtain in the clinic,[26] so the extent of biliary secretion of drugs and/or their metabolites in humans remains unclear. In most cases, the mechanisms of human bile clearance are estimated from *in vitro* or preclinical models. Encouraged by the success in extrapolating *in vivo* P450 mediated

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clearance from *in vitro* data during the past decade,[4,27] various *in vitro* models have been investigated for human biliary secretion prediction capability in a similar manner. However, the extrapolation of *in vivo* biliary secretion from *in vitro* SC hepatocyte models is not straightforward due to the lack of expression level of hepatobiliary transporters in the model. Knowing the expression level of P450 enzymes has been considered the key component of success in establishing IVIVC for P450 mediated metabolism.[28] Recently, because of significant progress in developing methodologies for absolute quantification of hepatobiliary transporters,[18,29] absolute protein levels of hepatic transporters for both *in vitro* and *in vivo* models have become attainable. The information might potentially facilitate the precise extrapolation of biliary secretion from *in vitro* data. Kipp and Arias have demonstrated that ABC transporter traffic from Golgi to the canalicular membrane is regulated by physiological factors.[30] Since isolating "pure" plasma membrane protein from SCRH and rat liver tissue is technically unfeasible, the protein mistrafficking can potentially affect the extrapolation from *in vitro* to in *in vivo*. Considering that less than 8% of total membrane protein is from organelles,[31] however, their contribution should be minimal in the overall predication. Furthermore, establishing IVIVC in preclinical species could increase confidence in prediction of human bile secretion using an *in vitro* SC human hepatocyte model. Toward to that goal, 11 compounds that are the substrates of Mrp2/Abcc2, Bcrp/Abcg2 or Bsep/Abcb11 were intentionally selected based on three criteria: (1) substrates of hepatobiliary efflux transporters; (2) metabolically stable with phase I and phase II enzymes; (3) high biliary excretion *in vivo*.

In the present study, several approaches reported in the literature were tested for IVIVC of biliary secretion. Within our data set, we did not observe a correlation between BEI obtained from the SCRH model and *in vivo* bile excretion ratio of the selected compounds. The result was consistent as previously reported.[1] BEI represents the translocation of compound from the intracellular compartment to the canalicular network; in *vivo* bile excretion is a dynamic process, including blood flow, hepatic uptake and bile flow, etc. The lack of dynamic bile flow and the limited space of bile canaliculi in the SCRH model could result in quick saturation of biliary secretion. Subsequently, the well-stirred hepatic model was applied for prediction of *in vivo* CL_{bile} from *in vitro* $CL_{bile,int}$, with or without plasma protein binding normalization. In agreement with the previous reports, the predicted CL_{bile} based on *in vitro* $CL_{bile,int}$ was fairly consistent with rat *in vivo* CL_{bile}, and the correction with plasma unbound fraction did slightly improve the correlation (Figure 2B,C).[1,15] However, the predictability of the models was still poor (Figure 2C) as the predicted bile clearances were underestimated by about 10−100-fold for the majority of compounds, except rosuvastatin, topotecan and taurocholate. It is true that only protein-unbound compounds are able to be metabolized or transported.[32−34] On the other hand, the dynamic equilibrium processes by which the drugs are released into the unbound fraction pool might partially explain the frequent underestimation of *in vivo* bile clearance with the incorporation of the plasma protein binding factor. Nevertheless, the poor IVIVC (regardless of plasma protein correction) could be caused by factors other than access to the transporters.

The kinetic parameters estimated using *in vitro* models have been successfully applied to estimate the enzymatic metabolism kinetics *in vivo* when the absolute content of each P450 enzymes expressed in human liver was taken into account.[4,35] Similarly, transporter expression levels could also be the determining factor for the prediction, when transporter mediated hepatobiliary secretion is the predominant route for drug clearance. In this case, the unknown expression level of transporter proteins could lead to poor predictive capability. Particularly, significant alteration (2−5-fold) of transporter expression has been recognized previously in hepatocytes post sandwich culture.[16] In addition, difference in transporter protein levels has been observed between *in vitro* models and *in vivo* systems.[36,37] To overcome this obstacle, in the present study, a g_factor was introduced to capture the ratio of the amount of transporter in rat liver to that in sandwich cultured hepatocytes at day 5, when the intrinsic

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bile clearance is usually determined. In addition, the g_factor also takes into consideration of the complexities of the transporter overlapping in substrate specificity. As shown in eq 6, the g_factor could be estimated when the major transporters responsible for biliary efflux are determined for a certain substrate.[38] The g_factor could converge at a range when a certain transporter is the major player in the biliary excretion mechanism, which is determined by the K_m' value of the specific transporter. By applying the g_factor, *in vivo* CL_{bile} of 8 out of 11 compounds were closely predicted from *in vitro* $CL_{bile,int}$, while the predicted biliary excretion remained underestimated for 3 of 11 compounds. The 3 compounds were characterized by extremely low hepatic uptake indexes in the SCRH model (Table 2). Insufficient hepatic uptake in the SCRH model might prevent these compounds from accessing the efflux transporters on the canalicular membrane. The $CL_{bile,int}$ represents the exact translocation across the canalicular membrane; therefore, eqs 2 and 3 were derived based on the assumption that the equilibrium of tested compounds between the intracellular compartment and the extracellular media is reached quickly. Nevertheless, the hepatic uptake process was the rate-limiting step for the hepatobiliary secretion for these 3 compounds. In that case, eq 2 might not be suitable for compounds with low passive permeability. However, protein expression of hepatic uptake transporters could also be significantly modified during cell culturing in the sandwich configuration.[17,39] Due to limited information on the protein expression of hepatic uptake transporters in SCRH models, alternatively, a modification of eq 2 was applied by using the intracellular concentration, instead of the concentration in extra cellular media. By determining the intracellular concentration of pravastatin and cefoperazone based on the total cellular concentration of compound and the cellular volume, the prediction of biliary excretion by the SCRH model was significantly improved. This implies that it is critical to determine the rate limiting step for hepatic clearance for a particular compound, allowing the proper *in vitro* to *in vivo* extrapolation of biliary excretion. Due to the extremely insufficient uptake in SCRH model, the BEI of cefmetazole is nearly zero. Therefore, the SCRH model was not able to provide any predictive information of *in vivo* bile secretion for such kind of compounds. A previous study suggested that uptake transporter PEPT2 was involved in cellular uptake of cefmetazole.[40] Therefore, the expression of PEPT2 and/or other uptake transporters in SCRH should be further determined in order to better understand the predictability of SCRH for biliary excretion of cefmetazole or other compounds that are dependent on hepatic uptake transporter(s) to enter into hepatocytes.

In conclusion, a well-stirred hepatic model was used to estimate the bile clearance from the SCRH model. By integrating the g_factor, which was introduced to reflect expression level of hepatobiliary transporters between *in vitro* SCRH and *in vivo*, and the respective contribution when multiple transporters are involved, the prediction of *in vivo* biliary secretion could be improved greatly, as determined by linear regression analysis. On the other hand, the caution was raised that the well-stirred hepatic model is dependent upon the accessibility of efflux transporters to compounds on canalicular membranes and it is critical to determine the rate limiting step of the hepatic clearance for a particular compound to allow the proper *in vitro* to *in vivo* extrapolation of biliary excretion. The IVIVC results in rat could be expanded to build up the confidence for IVIVC of human biliary secretion using SC human hepatocyte model.

Abbreviations Used

ADME, absorption, disposition, metabolism and elimination; IVIVC, *in vitro* to *in vivo* correlation; LC−MS/MS,

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liquid chromatography tandem mass spectrometry; Mrp2/Abcc2, multidrug resistance protein 2; Bcrp/Abcg2, breast cancer resistance protein; Bsep/Abcb11, bile salt transporter protein; SCRH, sandwich cultured rat hepatocytes; BEI, biliary excretion index.

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