

Quantitative Expression Profile of Hepatobiliary Transporters in Sandwich Cultured Rat and Human Hepatocytes

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Received February 5, 2009; Revised Manuscript Received May 17, 2009; Accepted June 22, 2009

Abstract: As sandwich cultured (SC) hepatocytes can repolarize to form bile canaliculi networks, allowing active excretion of compounds in a vectorial manner, the model has been widely used for assessing the transporter related complexity of ADME/tox issues. A lack of quantitative information on transporter expression during cell culture has made *in vitro* to *in vivo* extrapolation of hepatobiliary transport difficult. In the present study, using our newly developed LC–MS/MS absolute quantitative methods, we determined the quantitative expression profile of three biliary transporters in SC rat and human hepatocytes. A significant shift of hepatobiliary transporter proteins was observed both in human and rat sandwich cultures. A decrease of BSEP/Bsep protein and an increase of BCRP/Bcrp protein were detected in both rat and human hepatocytes over time in culture. Interestingly, Mrp2 in rat hepatocytes was significantly diminished, while MRP2 constantly increased in human hepatocytes during the cell culture. Consequently, the interspecies difference between rat and human in absolute amount of MRP2/Mrp2 was minimized over time in culture. Following the sandwich culture, the species difference of hepatobiliary transporter protein between human and rat at day 5 post SC was diminished (MRP2/Mrp2), identical (BSEP/Bsep) or reversed (BCRP/Bcrp), compared to the *in vivo* situation. In addition, the absolute protein amount of BCRP/Bcrp or MRP2/Mrp2 was proportionally correlated with the intrinsic biliary clearance estimated in various lots of SC rat and human hepatocytes. The results revealed that absolute protein amount is a key determinant for hepatobiliary clearance and could provide fundamental support on extrapolation of biliary secretion from *in vitro* to *in vivo*.

Keywords: LC–MS/MS; multidrug resistance protein 2 (MRP2/Mrp2); breast cancer resistance protein (BCRP/Bcrp); bile salt export pump (BSEP/Bsep); sandwich cultured hepatocytes

Introduction

The excretion of drugs/drug metabolites into bile by hepatocytes is one of the primary elimination routes for endogenous and exogenous compounds from blood circula-

tion.¹ Recently, along with the wide application of liver microsomal stability tests and advances in combinatorial chemistry for minimizing potential P450 interactions, an increasing number of drug candidates associated with biliary secretion are identified from the discovery chemical space. Therefore, understanding biliary excretion properties has been recognized as a critical consideration for candidate selection in drug discovery and development processes. Access to clinical bile samples is usually difficult; as a result, the extent

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(1) Arias, I. M.; Che, M.; Gatmaitan, Z.; Leveille, C.; Nishida, T.; St Pierre, M. The biology of the bile canaliculus, 1993. *Hepatology* **1993**, *17* (2), 318–29.

of biliary elimination of new drugs and/or their metabolites, or even marketed drugs, in humans remains unclear.² The information regarding human biliary secretion is generally obtained using *in vitro* model predictions and/or interspecies scaling from bile duct cannulated preclinical animals. However, the remarkable interspecies differences in biliary excretion of xenobiotics and drugs/metabolites^{3,4} might cause significant overestimation of biliary excretion in human as simply by an exponential allometric extrapolation approach.^{5–7} Although progress has been made in methodologies for absolute quantification of multiple species transmembrane hepatobiliary transporters,^{8,9} *in vitro* human cell models are still important components in drug discovery processes for understanding mechanism of human *in vivo* situation, particularly in hepatobiliary secretion. As such, a suitable *in vitro* model is desirable to assess the hepatic vectorial transport *in vivo*.

Hepatic uptake and efflux transporters, respectively located on sinusoidal or canalicular membrane, contribute to the vectorial transport of therapeutic reagents from systemic circulation to bile.^{10,11} Several ATP-binding cassette efflux

transporters are responsible for the hepatobiliary elimination of therapeutics and physiological substances.^{12–14} Pgp/ABCB1, MRP2/ABCC2, BCRP/ABCG2 and BSEP/ABCG11 on the canalicular membrane, have been identified and demonstrated to play active roles in biliary secretion^{15,16} (The use of uppercase letters in transporter nomenclature identifies the human protein, i.e., MRP2 and BCRP; lowercase letters indicate the transporter derives from a preclinical species, i.e., Mrp2 and Bcrp). MRP2/Mrp2 represents the primary transport proteins mediating excretion of organic anions including unmodified drugs as well as their metabolic conjugates. The function of Mrp2/Abcc2 has been extensively studied by comparing the hepatobiliary transport across the bile canalicular membrane via normal and Mrp2-deficient mutant animals or chemical knockout model (e.g., Mrp2 inhibitors).^{17,18} BSEP/Bsep transports monoanionic bile salts into bile canaliculi. BSEP/Bsep is also involved in the biliary excretion of pravastatin and vinblastine both in rats and human.^{19,20} BCRP/Bcrp recognizes molecules of either negative or positive charge including anticancer agents, dietary carcinogens and endogenous substrate^{21,22} and interacts with a growing number of drugs.²³

- (2) Ghibellini, G.; Johnson, B. M.; Kowalsky, R. J.; Heizer, W. D.; Brouwer, K. L. A novel method for the determination of biliary clearance in humans. *AAPS J.* **2004**, *6* (4), e33.
- (3) Ishizuka, H.; Konno, K.; Shiina, T.; Naganuma, H.; Nishimura, K.; Ito, K.; Suzuki, H.; Sugiyama, Y. Species differences in the transport activity for organic anions across the bile canalicular membrane. *J. Pharmacol. Exp. Ther.* **1999**, *290* (3), 1324–30.
- (4) Shilling, A. D.; Azam, F.; Kao, J.; Leung, L. Use of canalicular membrane vesicles (CMVs) from rats, dogs, monkeys and humans to assess drug transport across the canalicular membrane. *J. Pharmacol. Toxicol. Methods* **2006**, *53* (3), 186–97.
- (5) Ayrton, A.; Morgan, P. Role of transport proteins in drug absorption, distribution and excretion. *Xenobiotica* **2001**, *31* (8–9), 469–97.
- (6) Lave, T.; Portmann, R.; Schenker, G.; Gianni, A.; Guenzi, A.; Girometta, M. A.; Schmitt, M. Interspecies pharmacokinetic comparisons and allometric scaling of napsagatran, a low molecular weight thrombin inhibitor. *J. Pharm. Pharmacol.* **1999**, *51* (1), 85–91.
- (7) Pahlman, I.; Andersson, S.; Gunnarsson, K.; Odell, M. L.; Wilen, M. Extensive biliary excretion of the sulfasalazine analogue, susalimod, but different concentrations in the bile duct in various animal species correlating to species-specific hepatobiliary toxicity. *Pharmacol. Toxicol.* **1999**, *85* (3), 123–9.
- (8) Li, N.; Palandra, J.; Nemirovskiy, O. V.; Lai, Y. LC-MS/MS Mediated Absolute Quantification and Comparison of Bile Salt Export Pump and Breast Cancer Resistance Protein in Livers and Hepatocytes across Species. *Anal. Chem.* **2009**, *81* (6), 2251–2259.
- (9) Li, M.; Yuan, H.; Li, N.; Song, G.; Zheng, Y.; Baratta, M.; Hua, F.; Thurston, A.; Wang, J.; Lai, Y. Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human. *Eur. J. Pharm. Sci.* **2008**, *35* (1–2), 114–26.
- (10) Kullak-Ublick, G. A.; Beuers, U.; Paumgartner, G. Hepatobiliary transport. *J. Hepatol.* **2000**, *32* (1 Suppl.), 3–18.
- (11) Meier, P. J.; Eckhardt, U.; Schroeder, A.; Hagenbuch, B.; Stieger, B. Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* **1997**, *26* (6), 1667–77.
- (12) Keppler, D.; Arias, I. M. Hepatic canalicular membrane. Introduction: transport across the hepatocyte canalicular membrane. *FASEB J.* **1997**, *11* (1), 15–8.
- (13) Muller, M.; Jansen, P. L. Molecular aspects of hepatobiliary transport. *Am. J. Physiol.* **1997**, *272* (6 Pt 1), G1285–303.
- (14) Suzuki, H.; Sugiyama, Y. Transporters for bile acids and organic anions. *Pharm. Biotechnol.* **1999**, *12*, 387–439.
- (15) Chandra, P.; Brouwer, K. L. The complexities of hepatic drug transport: current knowledge and emerging concepts. *Pharm. Res.* **2004**, *21* (5), 719–35.
- (16) Ishikawa, T.; Akimaru, K.; Kuo, M. T.; Priebe, W.; Suzuki, M. How does the MRP/GS-X pump export doxorubicin. *J. Natl. Cancer Inst.* **1995**, *87* (21), 1639–40.
- (17) Keppler, D.; Kamisako, T.; Leier, I.; Cui, Y.; Nies, A. T.; Tsujii, H.; Konig, J. Localization, substrate specificity, and drug resistance conferred by conjugate export pumps of the MRP family. *Adv. Enzyme Regul.* **2000**, *40*, 339–49.
- (18) Suzuki, H.; Sugiyama, Y. Excretion of GSSG and glutathione conjugates mediated by MRP1 and cMOAT/MRP2. *Semin. Liver Dis.* **1998**, *18* (4), 359–76.
- (19) Hirano, M.; Maeda, K.; Hayashi, H.; Kusuha, H.; Sugiyama, Y. Bile salt export pump (BSEP/ABCB11) can transport a nonbile acid substrate, pravastatin. *J. Pharmacol. Exp. Ther.* **2005**, *314* (2), 876–82.
- (20) Lecœur, V.; Sun, D.; Hargrove, P.; Schuetz, E. G.; Kim, R. B.; Lan, L. B.; Schuetz, J. D. Cloning and expression of murine sister of P-glycoprotein reveals a more discriminating transporter than MDR1/P-glycoprotein. *Mol. Pharmacol.* **2000**, *57* (1), 24–35.
- (21) Merino, G.; Jonker, J. W.; Wagenaar, E.; van Herwaarden, A. E.; Schinkel, A. H. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. *Mol. Pharmacol.* **2005**, *67* (5), 1758–64.
- (22) Jonker, J. W.; Musters, S.; Vlaming, M. L.; Plosch, T.; Gooijert, K. E.; Hillebrand, M. J.; Rosing, H.; Beijnen, J. H.; Verkade, H. J.; Schinkel, A. H. Breast cancer resistance protein (Bcrp1/Abcg2) is expressed in the Harderian gland and mediates transport of conjugated protoporphyrin IX. *Am. J. Physiol.* **2007**, *292* (6), C2204–12.

Isolated hepatocytes are a versatile *in vitro* model for the study of drug metabolism and transporters. However, the cellular polarity that allows vectorial transport *in vivo* is disrupted rapidly when cells are isolated from the intact organ.^{24,25} Therefore, the restoration of the bile canalicular network is desired for investigating the vectorial transport of drug candidates. Culturing hepatocytes in a sandwich configuration results in repolarization and the development of intact bile canaliculi, thereby provides a three-dimensional orientation and proper localization of hepatobiliary transporters, including MRP2/Mrp2, BSEP/Bsep and BCRP/Bcrp on the canalicular membrane. The use of sandwich-cultured (SC) hepatocytes has become a valuable tool to mimic the *in vivo* situation, such as vectorial transport processes and hepatobiliary disposition of drugs.^{26,27} Although the impact of culture conditions on transporter expression in SC hepatocytes has been thoroughly investigated,^{28,29} the quantitative expression profiles of hepatobiliary transporters in SC hepatocytes still remain unknown. The change of transporter expression over time in culture can be critical for the *in vitro*/*in vivo* extrapolation of biliary secretion. In the present study, we determined the time-dependent expression profiles of hepatobiliary transporters in SC human and rat hepatocytes by using an absolute LC–MS/MS quantitative method. The correlative relationship between the absolute amount of biliary efflux transporters and the corresponding biliary efflux activity as assessed by selected substrate probes suggests the expression profile of transporters in SC hepatocytes could advance the *in vitro*/*in vivo* extrapolation and further promote the progression of pharmaceutical practice in drug discovery.

Table 1. The Characterization of Single Donor Hepatocyte Lots Used for Sandwich Hepatocyte Culture

	lots	species	sex	age	race or rat strain
cryopreserved	FEP	human	F	59 y	African American
	NRL	human	M	13 y	Caucasian
fresh	Rat468	rat	M	8 w	Sprague–Dawley
	Rs485	rat	F	9 w	Sprague–Dawley
	Rs481	rat	M	8 w	Sprague–Dawley
	Hu0824	human	M	49 y	Caucasian
	Hu0856	human	M	66 y	Caucasian
	hu0859	human	F	39 y	Caucasian
	Hu0824	human	M	49 y	Caucasian
	Hu0839	human	F	71 y	Caucasian

Materials and Methods

Chemicals and Reagents. Rosuvastatin, topotecan, and cefpiramide were purchased from AK Scientific, Inc. (Mountain View, CA). 7-Ethyl-10-hydroxycamptothecin (SN38) was purchased from Ivy Fine Chemicals (Cherry Hill, NJ). Pravastatin sodium and antihuman antibodies (MRP2, BCRP and BSEP) produced in rabbit were purchased from Sigma-Aldrich (St. Louis, MO). ProteoExtract Native Membrane Protein Extraction Kit was purchased from Calbiochem International, Inc. (Temecula, CA). HPLC grade acetonitrile, water and methanol were purchased from Burdick & Jackson (Muskegon, MI) and EMD Chemicals, Inc. (Gibbstown, NJ), respectively. 5-Chloromethylfluorescein diacetate (CMFDA) and Hanks balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). The protein quantification BCA kit and the in-solution digestion kit were purchased from Pierce Biotechnology (Rockford, IL). Trypsin was purchased from Promega (Madison, WI). Matrigel (phenol red free) and collagen I coated 24 well plates were obtained from BD Biosciences (Franklin Lakes, NJ). 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. Freshly isolated rat and human hepatocytes were purchased from CellzDirect (Pittsboro, NC). The plateable cryopreserved human hepatocytes were purchased from Celsis IVT Technologies (Baltimore, MD). The lot numbers of fresh/cryopreserved hepatocytes are listed in Table 1. The completed plating media or culture media were prepared by mixing 1 mL of Torpedo Antibiotic Mix with 45 mL of InVitroGRO CP medium or InVitroGRO HI medium, respectively. The media were prewarmed before usage. Upon arrival, the fresh hepatocytes were centrifuged at 500 rpm at 4 °C for 4 min. The supernatant was removed and the cell pellet was resuspended with 5 mL of completed plating medium to check the cell viability with the trypan blue exclusion method. The cryopreserved human hepatocytes were prepared following the protocol suggested by Celsis IVT Technologies. After incubating 40–50 s in 37 °C water bath, the hepatocytes were immediately diluted into completed CP media and then spun down at 500 rpm for 4 min. The fresh hepatocytes and cryopreserved hepatocytes

- (23) Burger, H.; Nooter, K. Pharmacokinetic resistance to imatinib mesylate: role of the ABC drug pumps ABCG2 (BCRP) and ABCB1 (MDR1) in the oral bioavailability of imatinib. *Cell Cycle* **2004**, 3 (12), 1502–5.
- (24) Bow, D. A.; Perry, J. L.; Miller, D. S.; Pritchard, J. B.; Brouwer, K. L. Localization of P-gp (Abcb1) and Mrp2 (Abcc2) in freshly isolated rat hepatocytes. *Drug Metab. Dispos.* **2008**, 36 (1), 198–202.
- (25) Roelofsens, H.; Bakker, C. T.; Schoemaker, B.; Heijn, M.; Jansen, P. L.; Elferink, R. P. Redistribution of canalicular organic anion transport activity in isolated and cultured rat hepatocytes. *Hepatology* **1995**, 21 (6), 1649–57.
- (26) Bi, Y. A.; Kazolias, D.; Duignan, D. B. Use of cryopreserved human hepatocytes in sandwich culture to measure hepatobiliary transport. *Drug Metab. Dispos.* **2006**, 34 (9), 1658–65.
- (27) Ghibellini, G.; Vasist, L. S.; Leslie, E. M.; Heizer, W. D.; Kowalsky, R. J.; Calvo, B. F.; Brouwer, K. L. In vitro-in vivo correlation of hepatobiliary drug clearance in humans. *Clin. Pharmacol. Ther.* **2007**, 81 (3), 406–13.
- (28) Chandra, P.; Lecluyse, E. L.; Brouwer, K. L. Optimization of culture conditions for determining hepatobiliary disposition of taurocholate in sandwich-cultured rat hepatocytes. *In Vitro Cell Dev. Biol. Anim.* **2001**, 37 (6), 380–5.
- (29) Turncliff, R. Z.; Tian, X.; Brouwer, K. L. Effect of culture conditions on the expression and function of Bsep, Mrp2, and Mdr1a/b in sandwich-cultured rat hepatocytes. *Biochem. Pharmacol.* **2006**, 71 (10), 1520–9.

were further diluted to 0.7 million per mL with completed CP media. Hepatocytes suspension of 0.5 mL were then seeded on 24-well BioCoat collagen I plates and allowed to attach for 2–4 h at 37 °C in a humidified incubator with 95%/5% of air/CO₂. The unattached hepatocytes were removed and the media replaced with fresh CP medium. On day 2, the hepatocytes were washed once with warmed completed HI culture media, and then overlaid with BD Matrigel at a concentration of 0.25 mg/mL in ice-cold completed HI incubation media.

Confirmation of Canalicular Structure and Biliary Excretion Function in Sandwich Cultures. To confirm the formation of bile canaliculi networks, SC hepatocytes were washed with standard HBSS and incubated with 5-chloromethylfluorescein diacetate (CMFDA, 2 μ M) for 15 min. After rinsing three times with HBSS, hepatocyte morphology and accumulated glutathione–methylfluorescein (GS–MF) in bile canaliculi were photographed under phase-contrast mode or fluorescent excitation mode using Nikon TE-300 (Nikon, Melville, NY). Biliary secretion assays in SC hepatocytes were conducted at day 5 as described previously.²⁶ Briefly, on day 5, SC cultures were rinsed twice with 0.5 mL of HBSS or Ca²⁺/Mg²⁺-free HBSS containing 1 mM EDTA. Disruption of the bile canalicular network was checked by phase contrast microscopy after preincubating with HBSS or Ca²⁺/Mg²⁺-free HBSS for 10 min at 37 °C. Subsequently, the substrates dissolved in HBSS were added to initiate the vectorial transport of SC hepatocytes. At 2 min, 10 min, and 15 min post incubation, 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/H₂O 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 total protein quantification by BCA kit.

Immunohistochemistry. To confirm the expression of efflux transporters, SC hepatocytes were fixed in 4% paraformaldehyde at 4 °C for 2 h at day 5 post culture, and cryoprotected in 30% sucrose at 4 °C overnight. The fixed hepatocytes were treated with 3% hydrogen peroxide for 5 min at room temperature. After rinsing with PBS containing 0.02% Tween 20, the hepatocytes were incubated respectively with rabbit antihuman BSEP, MRP2 and BCRP antibodies (1:15, Sigma-Aldrich) diluted in PBS containing 1% bovine serum albumin/0.3% Triton X-100 for 1 h at room temperature. Reactivity was visualized by using an Extravidin peroxidase staining kit (Sigma), following the procedures recommended by supplier.

LC–MS/MS Analysis of Probe Substrates. LC–MS/MS analysis of probe substrates was conducted with an API 3000 triple quadrupole 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 autosam-

pler (Carrboro, NC). Reverse phase chromatography (mobile phase A, 0.1% formic acid in H₂O; 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 μ m, Fortis Technologies Ltd.). Injections of 10 μ L were analyzed using a flow rate of 0.4 mL/min. The following transitions were monitored: 422.3 *m/z* to 377.2 *m/z* for topotecan, 482.2 *m/z* to 258 for rosuvastatin, 393.2–349.4 for SN38, 447.1–327.4 for pravastatin, 613.3–257.2 for cefpiramide and 237.4–194 for carbamazepine (internal standard). The instrument settings of the API 3000 TQMS were as follows: ion spray voltage, 4 kV; temperature, 400 °C; and collision energy was set at 29 eV for topotecan, 49 eV for rosuvastatin, 37 eV for SN38, 25 eV for pravastatin, 40 eV for cefpiramide and 27 eV for IS.

Extraction of Membrane Protein and Proteomic Digestion. At the indicated day post culture (from day 1 to day 8 for human hepatocytes, from day 1 to day 5 for rat hepatocytes), the SC hepatocytes were harvested and washed with HBSS. The membrane protein fraction of hepatocytes was extracted as described previously.³⁰ Briefly, hepatocyte pellets were homogenized in extraction buffer I containing protease inhibitor cocktail followed by incubation at 4 °C for 10 min with gently rocking. The homogenate was centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant containing cytosolic protein was discarded, and the pellets were resuspended in the extraction buffer II containing protease inhibitor cocktail. After 30 min incubation at 4 °C with gentle rocking, the suspension was centrifuged at 14,000 rpm for 15 min at 4 °C. Protein concentrations of extracted membrane fraction were determined by BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL).

The samples were then subjected to tryptic digestion in the presence of stable isotope labeled (SIL) internal standard overnight, followed by LC–MS/MS quantitative analysis as described previously.³⁰ Briefly, 30 μ g of membrane fraction protein was reduced with 10 mM DTT and alkylated with iodoacetamide (IAA) in 50 mM ammonium bicarbonate digestion buffer and then digested by trypsin, with 50 fmol of SIL peptide serving as internal standard.³⁰ At the end of digestion, samples were acidified with equal amounts of 0.2% formic acid in H₂O (BSEP/Bsep and BCRP/Bcrp) or 1:1 acetonitrile/H₂O (MRP2/Mrp2), then centrifuged at 5000 rpm for 20 min prior to LC–MS/MS analysis. The synthetic peptides corresponding to MRP2/Mrp2, BCRP/Bcrp and BSEP/Bsep tryptic fragments (Celtek Bioscience, Nashville, TN) and the corresponding SIL peptides (Sigma-Aldrich) were synthesized (Table 2) for LC–MS/MS protein quantification. Data were processed by integrating the appropriate peak areas generated from the reconstructed ion chromato-

(30) Li, N.; Nemirovskiy, O. V.; Zhang, Y.; Yuan, H.; Mo, J.; Ji, C.; Zhang, B.; Brayman, T. G.; Lepsey, C.; Heath, T. G.; Lai, Y. Absolute quantification of multidrug resistance-associated protein 2 (MRP2/ABCC2) using liquid chromatography tandem mass spectrometry. *Anal. Biochem.* **2008**, *380* (2), 211–22.

Table 2. Proteomic Peptides for Absolute Quantification of Hepatobiliary Transporters

transporter	AQUA peptide	parent ion	SRM/MRM
MRP2/Mrp2	LTIIPQDPILFSGSLR	885.9	1330.6
	LTIIPQDPILFSGSL*R ^a	889.4	1337.6
BSEP/Bsep	STALQLIQR	515.4	529.5
			657.8
	STALQL*IQR	518.9	536.6
BCRP/Bcrp	ENLQFSAALR	575.1	517.6
			664.8
	ENLQFSAAL*R	578.8	524.5
			671.7

^a *: isotope labeled.

grams for the analyte peptides and the SIL internal standard peptides by Analyst 1.4.1 (Applied Biosystems, Foster City, CA).

LC–MS/MS Quantitative Measurement of MRP2/Mrp2, BSEP/Bsep and BCRP/Bcrp Protein. To quantify the absolute amount of hepatocyte transporters in *in vivo/in vitro* models, we recently developed a novel LC–MS/MS AQUA method. Proteotypic peptides and SIL peptides of MRP2/Mrp2, BSEP/Bsep and BCRP/Bcrp were selected and synthesized as surrogate analytes for the corresponding protein quantification by LC–MS/MS.^{30,31} The calibration curve was prepared at a range of concentrations of the synthetic proteotypic peptide with a fixed concentration of SIL peptide as internal standard. Sample quantification was conducted by coupling a triple quadrupole mass spectrometer (TQ-MS, API4000, Applied Biosystem, Foster City, CA) to a Shimadzu LC (SLC-10A) system (WoolDale, IL) and HTS PAL Leap autosampler (Carrboro, NC). For MRP2/Mrp2, a Vydac EVEREST C18 column (2.1x 100 mm, 5 μm 300 Å) was used and, for BCRP/Bcrp and BSEP/Bsep, an Agilent Eclipse XDB C18 column (2.1 × 150 mm 5 μm 80 Å) was used. A linear gradient elution program was conducted to achieve chromatographic separation with mobile phase A (0.1% formic acid in HPLC grade water), and mobile phase B (0.1% formic acid in acetonitrile). MRP2/Mrp2 gradient conditions were 5% B to 35% B over a period of 30 min, while BCRP/Bcrp and BSEP/Bsep gradient conditions were 5% B to 25% B over a period of 20 min. A sample volume of 20 μL was injected onto the LC column at a flow rate of 0.4 mL/min. The parent-to-product transitions for the proteotypic peptide represent the doubly charged parent ion to the single charged product y ions for each transporter (Table 2). The instrument settings of the API4000 TQMS were as follows: ion spray voltage, 4 kV; temperature, 400 °C;

declustering potential (DP), collision energy (CE), entrance potential (EP), and collision cell exit potential (CXP) were followed as reported previously.^{30,31}

Data Analysis. The apparent *in vitro* intrinsic biliary clearance (CL_{bile,int})³⁴ was determined by the equations shown below:

$$CL_{bile,int} = \frac{\text{accumulation}_{(std,HBSS)} - \text{accumulation}_{(Ca2+/Mg2+,free)}}{\text{incubation_time} * \text{concentration}_{(medium)}}$$

The transporter quantification data are conducted with the SC hepatocytes from different donors, at least three donors from day 1 and day 5 and at least three donors from other days as indicated. The changes of the expression of three major hepatobiliary transporters were statistically analyzed using one-way ANOVA. A *p* value less than 0.05 was considered as statistically significant.

Results

Bile Canalicular Structure and Vectorial Transport in SC Hepatocytes. SC hepatocytes are cultured between two layers of either gelled collagen/gelled collagen or biocoat/matrigel in a sandwich configuration.^{32–35} During the time in sandwich culture, the hepatocytes structurally and functionally repolarize to form a bile canalicular network, allowing for the vectorial transport of xenobiotics.³⁶ To examine the conformation of bile canalicular structure and the vectorial transport, 5-chloromethylfluorescein diacetate (CMFDA) was incubated with the SC cells. CMFDA is nonfluorescent compound which readily diffuses through the hepatocyte sinusoidal membrane and is rapidly metabolized to form glutathione–methylfluorescein (GS–MF) in the hepatocyte cytoplasm,³⁷ which is a membrane impermeable fluorescent dye. The dye then is actively effluxed to bile canaliculi of SC hepatocytes by MRP2/Mrp2 transporter. As shown in Figure 1A of SC hepatocytes at day 5 post culture, under the fluorescent microscope, GS–MF was found distributing around the bile canalicular network, suggesting the conformation of polarized hepatocytes and vectorial transport of SC hepatocytes. The polarized structure was obtained at day 3 in both rat and human SC hepatocytes,

- (31) Li, N.; Palandra, J.; Nemirovskiy, O. V.; Lai, Y. LC–MS/MS Mediated Absolute Quantification and Comparison of Bile Salt Export Pump and Breast Cancer Resistance Protein in Livers and Hepatocytes across Species. *Anal. Chem.* **2009**, 2251–9.
- (32) Liu, X.; Brouwer, K. L.; Gan, L. S.; Brouwer, K. R.; Stieger, B.; Meier, P. J.; Audus, K. L.; LeCluyse, E. L. Partial maintenance of taurocholate uptake by adult rat hepatocytes cultured in a collagen sandwich configuration. *Pharm. Res.* **1998**, 15 (10), 1533–9.

- (33) Liu, X.; Chism, J. P.; LeCluyse, E. L.; Brouwer, K. R.; Brouwer, K. L. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. *Drug Metab. Dispos.* **1999**, 27 (6), 637–44.
- (34) Liu, X.; LeCluyse, E. L.; Brouwer, K. R.; Gan, L. S.; Lemasters, J. J.; Stieger, B.; Meier, P. J.; Brouwer, K. L. Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am. J. Physiol.* **1999**, 277 (1 Part 1), G12–21.
- (35) Liu, X.; LeCluyse, E. L.; Brouwer, K. R.; Lightfoot, R. M.; Lee, J. I.; Brouwer, K. L. Use of Ca2+ modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. *J. Pharmacol. Exp. Ther.* **1999**, 289 (3), 1592–9.
- (36) LeCluyse, E. L.; Bullock, P. L.; Parkinson, A.; Hochman, J. H. Cultured rat hepatocytes. *Pharm. Biotechnol.* **1996**, 8, 121–59.
- (37) Gutmann, H.; Fricker, G.; Torok, M.; Michael, S.; Beglinger, C.; Drewe, J. Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake. *Pharm. Res.* **1999**, 16 (3), 402–7.

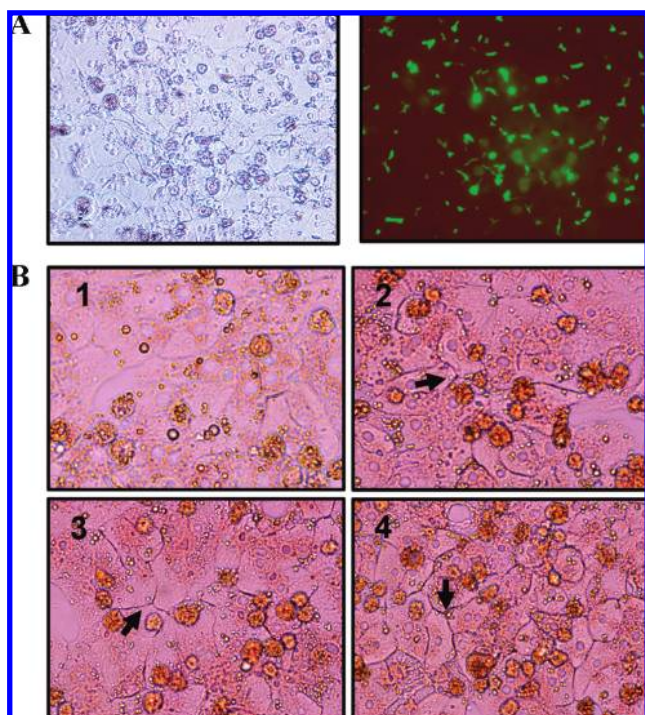


Figure 1. Confirmation of bile canicular network and transporter expression in SC hepatocytes. On day 5 post culture, 2 μ M CMFDA was added into the sandwich cultures and incubated for 15 min. The cell morphology (A, left panel) was photographed in phase-contrast microscopy, and the formed glutathione conjugate (GS–MF) of CMFDA was visualized by fluorescent microscopy (A, right panel). The expression of MRP2, BCRP and BSEP in SC human hepatocyte was detected by immunohistochemistry (B). The reactivity with antibodies was visualized by an Extravidin peroxidase staining kit. 1: Negative control. 2: BSEP. 3: BCRP. 4: MRP2. Arrows indicates the canalicular expression of BSEP, BCRP and MRP2, respectively.

and disrupted post 8 days (human) or 5 days (rat) in SC hepatocytes (data not shown). Furthermore, the canalicular expressions of BCRP, MRP2 and BSEP were confirmed by immunostaining at day 5 post culture (Figure 1B).

Expression Time Profiles of MRP2/Mrp2 in SC Hepatocytes. One of the major hepatobiliary efflux transporters, MRP2/Mrp2 is localized on the canalicular membrane of hepatocyte. To verify the effect of sandwich culture on MRP2/Mrp2 expression, the time profile of MRP2/Mrp2 protein in membrane fraction of SC hepatocytes was quantified by our newly developed LC–MS/MS absolutely quantitative method.³⁰ At day 1, Mrp2 protein in rat hepatocytes was $\sim$ 7-fold higher than that in human (Figure 2), which was comparable to those in freshly isolated or cryopreserved suspension hepatocytes found previously.³⁸ Surprisingly, a

5–6-fold increase of MRP2 protein was detected in SC human cryopreserved and freshly isolated hepatocytes at 5 days post culture ($P = 0.003$ by ANOVA) (Figure 2). MRP2 levels in cryopreserved human SC hepatocytes were lower than freshly isolated hepatocytes; however no statistical testing could be conducted for the difference, as only two lots of plateable human cryopreserved hepatocytes are commercially available. In contrast, the amount of Mrp2 protein in 5-day SC rat hepatocytes significantly declined to about half of the initial amount in freshly isolated rat hepatocytes. There was no significant difference in absolute amount of MRP2/Mrp2 protein between human and rat SC hepatocytes at day 4 or day 5 post cell culture ($P > 0.05$), when the biliary excretion assay is usually performed.

Expression Time Profile of BSEP/Bsep in SC Hepatocytes. As shown in Figure 3, at day 1 of SC hepatocytes, the amount of Bsep in membrane fraction of rat SC hepatocytes is about 2-fold higher than that in human, which was comparable to the level found in liver tissues.³¹ BSEP/Bsep proteins significantly decreased both in rat and human SC hepatocytes over time in culture ($p < 0.005$ by ANOVA) (Figure 3). Compared to the initial level of BSEP/Bsep (at day 1), only 60% remained in the membrane fraction of SC hepatocytes at day 5 post culture. Although the amount of BSEP/Bsep protein in SC hepatocytes significant decreased, the fold difference between the two species at 5 days post culture observed for *in vitro* SC hepatocytes was similar to that found in liver tissues and freshly isolated hepatocytes.³¹

Expression Time Profile of BCRP/Bcrp in SC Hepatocytes. At day 1, absolute amount of Bcrp protein in membrane fraction of rat SC hepatocytes was similar to that in human SC hepatocytes (Figure 4), which was consistent with that found in liver tissues. BCRP/Bcrp proteins in human and rat SC hepatocytes significantly increased over time post culture (Figure 4). About 2-fold and 5-fold increases of BCRP/Bcrp were found in human and rat SC hepatocytes at day 5, respectively. The Bcrp protein in rat SC hepatocytes was about 2-fold higher (1.0 *vs* 0.5 fmol/mg protein) than that in human at day 5 post culture (Figure 4).

The Correlation of Absolute Amount of Transporter Protein and Bile Intrinsic Clearance in SC Hepatocytes. To investigate whether the alteration of absolute amount of transporter proteins in SC hepatocytes affects the functional transport of the models, the *in vitro* intrinsic biliary clearance of known BCRP/Bcrp substrates (SN38, topotecan and rosuvastatin) and MRP2/Mrp2 substrates (pravastatin and cefpiramide) was measured in various lots of rat and human SC hepatocytes. Simultaneously, the absolute amount of BCRP/Bcrp or MRP2/Mrp2 protein in these SC hepatocytes was quantified on day 5, when the functional assay was performed. The time dependent accumulation of these compounds is shown in Figure 5. For all five tested compound, the maximum hepatic accumulation was achieved at 10 min; therefore, the intrinsic clearance calculated at 10 min was used to perform the following correlation study.

(38) Li, N.; Zhang, Y.; Hua, F.; Lai, Y. Absolute Difference of Hepatobiliary Transporter MRP2/Mrp2 in Liver Tissues and Isolated Hepatocytes from Rat, Dog, Monkey and Human. *Drug Metab. Dispos.* **2009**, *37*, 66–73.

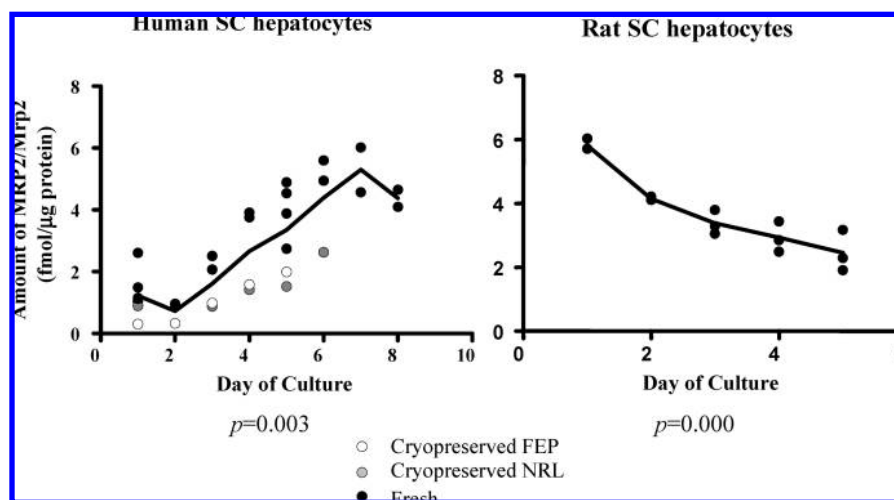


Figure 2. MRP2/Mrp2 expression in SC human and rat hepatocytes over time in culture. Left panel: MRP2 protein in SC human fresh or cryopreserved hepatocytes over time in culture. Right panel: Mrp2 protein in SC rat fresh hepatocytes over time in culture. Each point represents the amount of transporter protein for each individual donor at indicated time point. Solid circle represents fresh isolated human or rat hepatocytes; open circle represents cryopreserved human hepatocyte lot FEP; gray circle represents cryopreserved human hepatocyte lot NRL. One way ANOVA statistic analysis showed the significance of the alteration in BCRP/Bcrp protein in both SC rat and human hepatocytes over the time of culture.

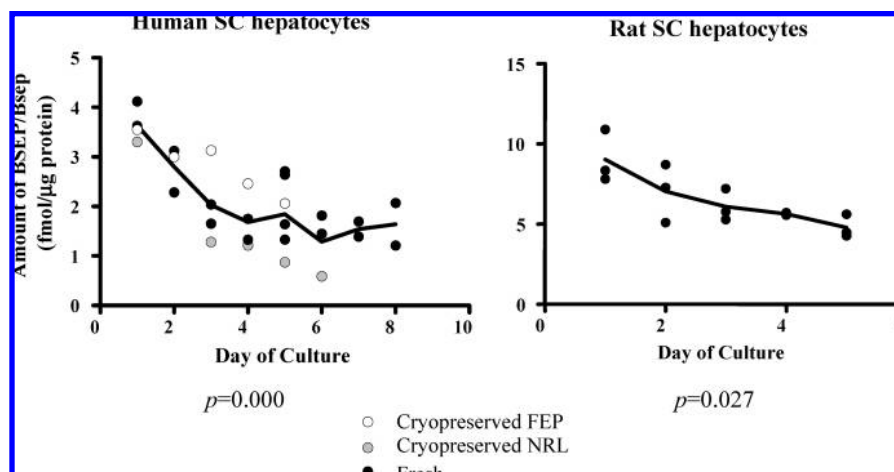


Figure 3. BSEP/Bsep expression in SC human and rat hepatocytes over time in culture. Left panel: BSEP protein in SC human fresh or cryopreserved hepatocytes over the time of culture. Right panel: Bsep protein in SC rat fresh hepatocytes over the time of culture. Each point represents the amount of transporter protein for each individual donor at indicated time point. Solid circle represents fresh isolated human or rat hepatocytes; open circle represents cryopreserved human hepatocyte lot FEP; gray circle represents cryopreserved human hepatocyte lot NRL. One way ANOVA statistic analysis indicated the alteration in BSEP/Bsep protein in both SC rat and human hepatocytes over the time of culture was significant.

As shown in Figure 6A, the intrinsic bile clearances of SN38, topotecan and rosuvastatin were proportionally correlated to the absolute amount of BCRP/Bcrp protein in the SC hepatocytes of corresponding lots. The R^2 values were 0.77, 0.98, and 0.89 for SN38, topotecan and rosuvastatin in various lots of human and rat hepatocytes ($P < 0.05$), respectively. Similarly, the absolute amount of MRP2/Mrp2 protein was proportionally correlated with the intrinsic biliary clearance estimated from SC rat and human hepatocytes of pravastatin and cefpiramide, the known MRP2/Mrp2 substrates (Figure 6B). The R^2 values were 0.95 and 0.93 for pravastatin and cefpiramide ($P < 0.05$), respectively. In contrast, no correlation was found between the intrinsic bile

clearance and the absolute amount of hepatobiliary transporters that are unrelated to the transport of selected compounds, e.g. BSEP/Bsep (data not shown).

Discussion

Despite being a popular tool to assess biliary secretion, a common criticism of the sandwich culture model is the potential for alteration of transporter expressions and for static bile accumulation, because pulsatile draining does not occur in the bile canalicular space as it does *in vivo*. In addition, extracellular matrix is reported to affect hepatocyte morphology and subse-

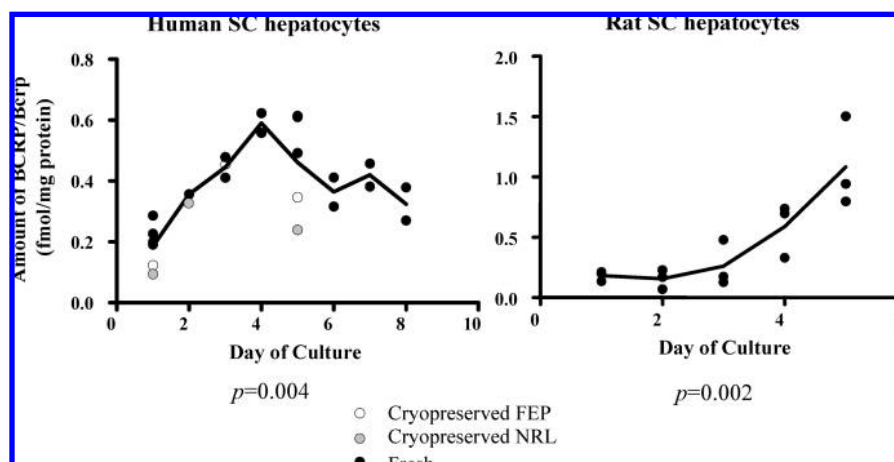


Figure 4. BCRP/Bcrp expression in SC human and rat hepatocytes over time in culture. Left panel: BCRP protein in SC human fresh or cryopreserved hepatocytes over time in culture. Right panel: Bcrp protein in SC rat fresh hepatocytes over time in culture. Each point represents the amount of transporter protein for each individual donor at indicated time point. Solid circle represents fresh isolated human or rat hepatocytes; open circle represents cryopreserved human hepatocyte lot FEP; gray circle represents cryopreserved human hepatocyte lot NRL. One way ANOVA statistic analysis showed the significance of the alteration in BCRP/Bcrp protein in both SC rat and human hepatocytes over the time of culture.

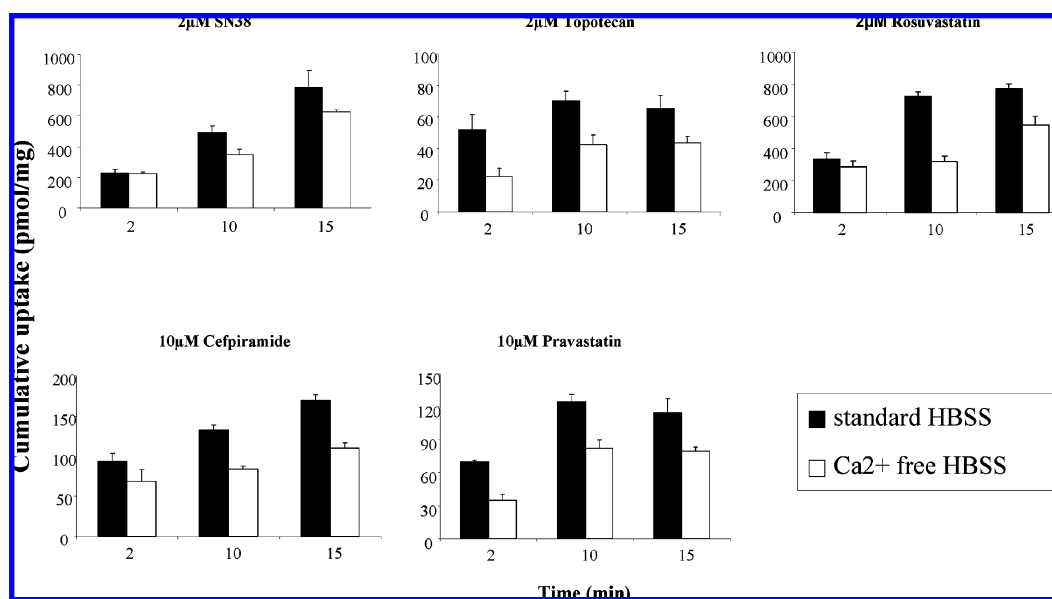


Figure 5. Cumulative uptake of selected transporter substrates in SC rat hepatocytes. Each compound was incubated in HBSS buffer with (black bars) or without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (white bars). Data represents the mean $\pm$ SEM ($n = 3$ independent experiments). Dose concentration for each compound is shown above each figure.

quently influence the formation of the canalicular network.³⁹ Consequently, accumulative bile salts or other physiological substances in bile canalicular space could further regulate the expression of canalicular transporters.⁴⁰ In fact, Turncliff et al.²⁹ have reported the effect of culture conditions on the expressions

of Bsep, Mrp2, and Mdr1a/b proteins in SC rat hepatocytes. The modification of the proteins might lead to the functional alteration, as transporter-mediated drug clearance is determined by maximum transport rate ($V_{\max}$) and Michaelis–Menten constant (K_m) of the transporter (While the K_m is a unique property of a particular substrate and usually is a fixed parameter, $V_{\max}$ is determined by protein level of the transporter). Therefore, modification of protein expression could significantly affect the estimates of biliary clearance from SC hepatocyte model, and consequently lead to an inaccurate extrapolation from *in vitro* to *in vivo*, or inaccurate interspecies scaling.

Recently, LC–MS/MS AQUA methods have been successfully adapted for determining the absolute amount of

- (39) Dunn, J. C.; Yarmush, M. L.; Koebe, H. G.; Tompkins, R. G. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J.* **1989**, *3* (2), 174–7.
- (40) Keitel, V.; Burdelski, M.; Warskulat, U.; Kuhlkamp, T.; Keppler, D.; Haussinger, D.; Kubitz, R. Expression and localization of hepatobiliary transport proteins in progressive familial intrahepatic cholestasis. *Hepatology* **2005**, *41* (5), 1160–72.

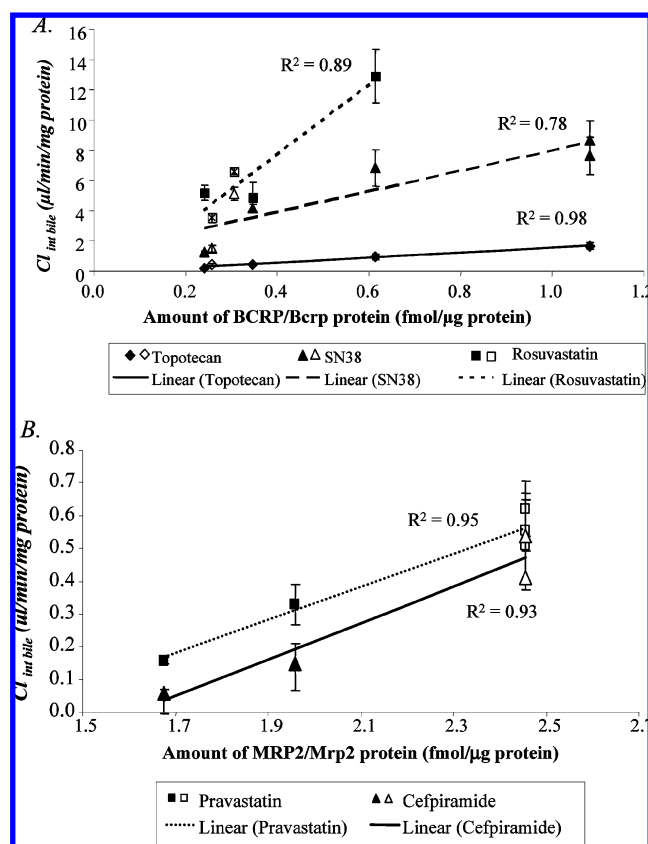


Figure 6. Correlation of protein amount and intrinsic biliary secretion in SC hepatocytes. (A) The intrinsic biliary secretion of SN38, topotecan and rosuvastatin was plotted against the protein level of BCRP/Bcrp in difference lots of human or rat hepatocytes. Solid symbols represent freshly isolated human or rat hepatocytes, while the open symbols represent cryopreserved human hepatocytes. (B) The intrinsic biliary clearance of cefpiramide and pravastatin was plotted against to the amount of MRP2/Mrp2 protein in the corresponding lot of SC hepatocytes. Solid symbols represent cryopreserved human hepatocytes, while open symbols represent fresh rat hepatocytes.

hepatobiliary transporters.³⁸ The method has been demonstrated as a highly sensitive and selective quantification approach with great accuracy and precision.³⁰ Technically, the AQUA methods consist of three major steps:³⁰ (i) membrane protein extraction; (ii) tryptic digestion; and (iii) LS/MS/MS. As the isolation and identification of subcellular membrane proteins have historically posed problems for scientists, it should be noted that the extracted membrane protein represents total membrane proteins of SC hepatocytes contaminated by endoplasmic reticulum/Golgi complex.⁴¹ For our particular interest, however, the effect of ER/Golgi retaining protein on our overall conclusion was considered as minimal, as (1) contamination of ER/Golgi portion

is limited, as less than 8% of the total membrane protein is from organelles;⁴² and (2) the contamination of ER/Golgi is contributed equally to each species. Transporter proteins detected in total membrane fraction of SC hepatocytes should mainly reflect the functional expressions on plasma membrane, and therefore are of value to fill the gaps in the translational aspect of functional extrapolation.

Conventionally, the biliary clearance estimated from SC hepatocytes model is normalized by total protein of SC hepatocytes. However, the data obtained from the model are not able to explain the significant variations from date-to-date experiments or from different lots/resources of hepatocytes (our in house unpublished data). Indeed, about 3-fold differences of biliary intrinsic clearance for MRP2 or BCRP were detected among various lots of hepatocytes (Figures 6A, 6B). In addition, as MRP2/Mrp2 has been demonstrated to significantly contribute to interspecies difference in hepatobiliary secretion of drugs/drug metabolites,^{3,43,44} the capability of using current *in vitro* model for extrapolating species difference remains unknown. In the present studies, a significant increase of human MRP2 and decrease of rat Mrp2 in SC hepatocytes was detected. Consequently, the interspecies differences between rat and human in MRP2/Mrp2 protein were minimized over time in culture, compared to that observed *in vivo*.³⁸ Similarly, a significant increase of BCRP/Bcrp protein in SC human and rat hepatocytes was also observed over time in culture. Even though a significant decrease of BSEP/Bsep protein was detected in both rat and human hepatocytes following sandwich culture (Figure 3), interestingly, the fold differences at day 4 or 5 post culture (functional assay end point) between human and rat remained identical (2.5-fold higher in rat) to the difference observed on day 1 or to that detected *in vivo* or in isolated hepatocytes.³¹ The correlation of intrinsic clearance for MRP2/Mrp2 or BCRP/Bcrp substrates with absolute amount of corresponding transporter in various lots of hepatocytes (Figure 5) demonstrated the fact that transporter protein level is the key determinant in biliary clearance in SC hepatocyte model. Therefore, caution should be raised when interpreting the intrinsic biliary clearance estimated from SC hepatocytes for MRP2/Mrp2 substrates, particularly when the model is applied for scaling up interspecies differences in biliary clearance. Conversely, the correlation between absolute protein amount and functional measurement could be used to estimate the affinity of substrate to corresponding trans-

(41) Schirmer, E. C.; Gerace, L. Organellar proteomics: the prizes and pitfalls of opening the nuclear envelope. *Genome Biol.* **2002**, *3* (4), REVIEWS1008.

(42) Wilfong, R. F.; Neville, D. M., Jr. The isolation of a brush border membrane fraction from rat kidney. *J. Biol. Chem.* **1970**, *245* (22), 6106–12.

(43) Shitara, Y.; Sato, H.; Sugiyama, Y. Evaluation of drug-drug interaction in the hepatobiliary and renal transport of drugs. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 689–723.

(44) Niinuma, K.; Takenaka, O.; Horie, T.; Kobayashi, K.; Kato, Y.; Suzuki, H.; Sugiyama, Y. Kinetic analysis of the primary active transport of conjugated metabolites across the bile canalicular membrane: comparative study of S-(2,4-dinitrophenyl)-glutathione and 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole glucuronide. *J. Pharmacol. Exp. Ther.* **1997**, *282* (2), 866–72.

porters. For instance, as shown in Figure 6A, the increase in BCRP/Bcrp protein level is about 5-fold, while the increase in intrinsic biliary clearance of topotecan is much less. Similarly, as shown in Figure 6B, the MRP2/Mrp2 protein increased about 2-fold, but the corresponding intrinsic biliary clearance increased about 8-fold. The difference in slope suggested the different affinity (K_m) for each substrate.

OATP/Oatps on sinusoidal membrane of hepatocytes are considered to be involved in the uptake of rosuvastatin and pravastatin. Therefore, the hepatic uptake could also be a rate-limiting process for hepatobiliary secretion. To minimize the effect of rate-limiting uptake process on the readouts of hepatobiliary transport, in the present study, low concentrations of tested substrates for incubation ($2\ \mu\text{M}$) were used. In addition, the cumulative uptakes of substrates in SC rat hepatocytes (Figure 5) were also monitored to further confirm that hepatobiliary efflux, but not the hepatocyte uptake, was the determination for biliary clearance in SC hepatocyte models. The point of maximum hepatic accumulation (10 min post incubation) was used to calculate the intrinsic biliary clearance. Practically, the identification of rate-limiting processes should be critical for establishing correlations between intrinsic bile clearances and the absolute amount of corresponding transporter, when information of hepatic uptake transporters remains missing.

In conclusion, by utilizing the LC–MS/MS AQUA method, we quantitatively measured the expression profiles of hepatobiliary efflux transporter MRP2/Mrp2, BCRP/Bcrp and BSEP/Bsep in SC rat and human hepatocytes. The sandwich culture exhibited significant impacts on the expression of hepatobiliary transporters. Following sandwich culture, the species difference of hepatobiliary transporter protein between human and rat was diminished (MRP2/Mrp2), identical (BSEP/Bsep) or reversed (BCRP/Bcrp), compared to the *in vivo* situation. The absolute amount of transporter detected correlated with the intrinsic clearance in SC hepatocytes, which could explain the variations of biliary clearance measurement on date-to-date experiments or various lots of hepatocytes. These results provide supportive information for SC hepatocyte models in extrapolating *in vitro* biliary clearance to *in vivo*.

Acknowledgment. We would like to thank Drs. Joseph C. Fleishaker, Matthew D. Troutman and Timothy G. Heath for their helpful comments and suggestions on our manuscript. We would also thank Timothy G. Brayman for proofreading of the manuscript.

MP900044X