In Vitro Biliary Clearance of Angiotensin II Receptor Blockers and 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Inhibitors in Sandwich-Cultured Rat Hepatocytes: Comparison with in Vivo Biliary Clearance

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ABSTRACT

Previous reports have indicated that in vitro biliary clearance (C_{\text{biliary}}) determined in sandwich-cultured hepatocytes correlates well with in vivo C_{\text{biliary}} for limited sets of compounds. This study was designed to estimate the in vitro C_{\text{biliary}} in sandwich-cultured rat hepatocytes (SCRHs) of angiotensin II receptor blockers and HMG-CoA reductase inhibitors that undergo limited metabolism, to compare the estimated C_{\text{biliary}} values with published in vivo C_{\text{biliary}} data in rats, and to characterize the mechanism(s) of basolateral uptake and canalicular excretion of these drugs in rats. The average biliary excretion index (BEI) and in vitro C_{\text{biliary}} values of olmesartan, valsartan, pravastatin, rosuvastatin, and pitavastatin were 15, 19, 43, 45, and 20%, respectively, and 1.7, 3.2, 4.4, 46.1, and 34.6 ml/min/kg, respectively. C_{\text{biliary}} predicted from SCRHs, accounting for plasma unbound fraction, correlated with reported in vivo C_{\text{biliary}} for these drugs. The rank order of C_{\text{biliary}} values predicted from SCRHs was consistent with in vivo C_{\text{biliary}} values. Bromosulfophthalein inhibited the uptake of all drugs. BEI and C_{\text{biliary}} values of olmesartan, valsartan, pravastatin, and rosuvastatin, known multidrug resistance-associated protein (Mrp) 2 substrates, were reduced in SCRHs from Mrp2-deficient (TR\_H11002) compared with wild-type (WT) rats. Although Mrp2 plays a minor role in pitavastatin biliary excretion, pitavastatin BEI and C_{\text{biliary}} were reduced in TR\_H11002 compared with WT SCRHs; Bcrp expression in SCRHs from TR\_H11002 rats was decreased. In conclusion, in vitro C_{\text{biliary}} determined in SCRHs can be used to estimate and compare in vivo C_{\text{biliary}} of compounds in rats and to characterize transport proteins responsible for their hepatic uptake and excretion.

Biliary excretion is a predominant route of elimination for bile acids and many xenobiotics (Trauner and Boyer, 2003; Shitara et al., 2006). ATP-dependent canalicular transport systems are responsible for the biliary excretion of xenobiotics (Chandra and Brouwer, 2004). One approach that drug development scientists have employed recently to decrease the lipophilicity of a drug candidate is to exchange the hydrophobic moiety for a more hydrophilic moiety, such as a carboxylic acid. Although this modification can enhance some drug-like properties of the molecule (e.g., increased solubility, decreased metabolism), it increases the possibility that the molecule will be recognized by hepatic transport systems. Accordingly, knowledge regarding the extent of biliary excretion of compounds in the early stages of drug development may be as important as absorption and metabolic properties when selecting drug candidates. From the point of view of drug-drug interactions and disease state alterations, elucidation of the biliary excretion properties of a drug candidate also is a critical issue.

Freshly isolated hepatocytes are widely accepted as a reliable model for characterizing drug uptake across the basolateral membrane (Hirano et al., 2004; Lam et al., 2006). However, hepatocytes lose cellular polarity rapidly after isolation (Groothuis et al., 1981; Talamini et al., 1997), and canalicular transport proteins are internalized or confined to junctions between adjacent cells (Bow et al., 2008). Therefore, suspended hepatocytes are not an appropriate system to
study canalicular excretion of drugs. Sandwich-cultured rat hepatocytes (SCRHs) rapidly regain polarity and maintain expression levels of uptake and efflux transport proteins for several days (Hoffmaster et al., 2004; Zhang et al., 2005). In vitro biliary clearance (Cl\text{biliary}) determined in sandwich-cultured rat and human hepatocytes correlates well with in vivo Cl\text{biliary} (Liu et al., 1999a; Ghibellini et al., 2007), suggesting that this system is useful for predicting the in vivo biliary excretion of drug candidates. However, there are few reports to systematically elucidate the prediction of biliary clearance and to assess the involvement of uptake or canalicular transport proteins in SCRHs using compounds that have similar chemical structures. Such data would be useful in the preliminary evaluation of the hepatobiliary disposition of drug candidates, predicting the rank order of in vivo Cl\text{biliary}, and assessing the potential for drug-drug interactions in hepatobiliary transport.

Angiotensin II receptor blockers (ARBs) and HMG-CoA reductase inhibitors (statins) are used widely in the treatment of cardiovascular disease (Brousil and Burke, 2003; Basile and Chrysant, 2006; Shitara and Sugiyama, 2006). Olmesartan and valsartan (ARBs) and pravastatin, rosuvastatin, and pitavastatin (statins) are all organic anions that have a carboxylic acid moiety and are known to be excreted extensively into bile as unchanged compound in rats (Yamazaki et al., 1997; Hirano et al., 2005; Kitamura et al., 2005; Nakagomi-Hagihara et al., 2006; Yamashiro et al., 2006) (Fig. 1). Data obtained from multidrug resistance-associated protein (Mrp) 2-deficient Eisai hyperbilirubinemic rats (EHBRs) suggested that Mrp2 is primarily involved in the biliary excretion of olmesartan, valsartan, and pravastatin, partially involved in the biliary excretion of rosuvastatin, but plays a minor role in the biliary excretion of pitavastatin in rats (Fig. 1).

The purpose of this study was to determine in SCRHs the in vitro Cl\text{biliary} of these ARBs and statins that undergo limited metabolism, to compare the predicted in vivo Cl\text{biliary} values based on the in vitro data with previously published in vivo Cl\text{biliary} data in rats, and to evaluate the involvement of basolateral uptake and canalicular efflux transporters in the hepatobiliary disposition of these drugs in rats.

Materials and Methods

**Chemicals.** Olmesartan was kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). Valsartan, pravastatin, rosvuastatin, pitavastatin, \( d \text{-olmesartan} \), \( d \text{-valsartan} \), and \( d \text{-rosuvastatin} \) were obtained from Toronto Research Chemicals Inc. (Ontario, Canada). Bromosulphophthalein (BSP) was obtained from Sigma-Aldrich (St. Louis, MO). \(^{3}H\text{-Taurocholate} \) (5 Ci/mmol; purity > 97%) and \( ^{3}H\text{-estradiol-17β-D-glucuronide} \) (E2-17β; 46.9 Ci/mmol; purity > 97%) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Collagenase (type I, class I) was purchased from Worthington Biochemicals (Freehold, NJ). Dulbecco’s modified Eagle’s medium (DMEM), MEM nonessential amino acids, and insulin were purchased from Invitrogen (Carlsbad, CA). Insulin/transferrin/selenium culture supplement, Matrigel, and BioCoat plates were purchased from BD Biosciences (San Jose, CA). Penicillin-streptomycin solution, fetal bovine serum, taurocholic acid, dexamethasone, and Triton X-100 were purchased from Sigma-Aldrich. All other chemicals and reagents were of analytical grade and readily available from commercial sources.

**Animals.** Male Wistar [wild-type (WT)] rats (177–318 g) from Charles River Laboratories, Inc. (Raleigh, NC) or male Mrp2-defi-
cient (TR) rats bred at the University of North Carolina (Chapel Hill, NC; 206–346 g; breeding stock obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were used for hepatocyte isolation. Animals had free access to water and food before surgery. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of North Carolina).

**Hepatocyte Isolation and Culture.** Hepatocytes were isolated from WT or TR−/− male rats by a modification of the two-step collagenase digestion method as described previously (Liu et al., 1999b). Hepatocyte viability was >85% as determined by trypan blue exclusion. Hepatocytes were seeded at a density of 1.75 million cells/well on six-well BioCoat plates in 1.5 ml of DMEM containing 5% fetal bovine serum, 10 mM dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units of penicillin G sodium, and 100 μg of streptomycin sulfate. Cells were incubated at 37°C in a humidified incubator with 95% O2/5% CO2 and allowed to attach for 2 h, at which time the medium was aspirated to remove unattached cells, and fresh medium was added. Twenty-four hours later, cells were overlaid with BD Matrigel basement membrane matrix at a concentration of 0.25 mg/ml in 2 ml of ice-cold DMEM containing 1% insulin/transferrin/selenium culture supplement, 0.1 μM dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units of penicillin G sodium, and 100 μg of streptomycin sulfate. Hepatocytes were cultured for 4 days, and medium was changed daily.

**Accumulation Studies.** On day 4, hepatocytes were rinsed twice and then preincubated for 10 min at 37°C with 2 ml of warmed Hank’s balanced salt solution (HBSS) containing Ca2+ (standard; cells + bile) or Ca2+-free HBSS (cells) either to maintain or to disrupt the tight junctions sealing bile canalicular networks, respectively. Subsequently, hepatocytes were incubated with test compound (0.5–30 μM for ARB or statin; 1 μM for [3H]taurocholate or [3H]E2-17βG in standard HBSS for 5 to 20 min (ARB or statin) or 10 min ([3H]taurocholate or [3H]E2-17βG) at 37°C. For uptake inhibition studies, BSP (a known inhibitor of organic anion transporters and Na+ taurocholate-transporting polypeptide) was added simultaneously with test compound to the hepatocytes. After incubation, the dosing solution was aspirated from the cells, and uptake was stopped by washing the cells three times with ice-cold standard HBSS. For radiolabeled compounds, cells were lysed with 1 ml of 0.5% Triton X-100 in phosphate-buffered saline. For ARBs and statins, cells were lysed with 1 ml of 70% (v/v) methanol and sonicated for 20 s with a sonic dismembrator (model 100, Thermo Fisher Scientific, Waltham, MA) and stored at -70°C until analysis. The samples were analyzed for drug concentrations by liquid chromatography with tandem mass spectrometry. Substrate accumulation was corrected for nonspecific binding by using rat Matrigel-precoated dishes without cells. The total protein concentration in cell lysates was quantified by the BCA protein assay (Pierce Chemical, Rockford, IL) using bovine serum albumin as the reference standard, and accumulation was normalized to protein concentration. Because of incompatibility of the protein assay with methanol, the average protein concentration for standard HBSS or Ca2+-free HBSS incubations in the same liver preparation was used to normalize accumulation.

**Analysis of ARBs and Statins.** The cell lysate samples were centrifuged at 12,000g for 2 min at 4°C, and the supernatant was diluted 1:6 with water and methanol containing internal standard (d5-olmesartan for olmesartan; d7-valstatin for valsartan; d6-rosuvastatin for pravastatin, rosuvastatin, and pitavastatin). A Shimadzu solvent delivery system (Kyoto, Japan) and a Leap HTC Pal thermosated autosampler (Carrboro, NC) connected to an Applied Biosystems API 4000 triple quadrupole mass spectrometer with a TurboSpray ion source (Applied Biosystems, Foster City, CA) were used for analysis. Tuning, operation, integration, and data analyses were performed in positive mode using multiple reaction monitoring (Analyst software version 1.4.1; Applied Biosystems). Analysis required 10 μl of sample and a solvent flow of 0.75 ml/min. Reverse phase chromatography [aqueous phase, water with 0.1% formic acid (v/v) and organic phase, methanol with 0.1% formic acid, (v/v)] was used to elute the various compounds from an Aquasil C18, 50 × 2.1-mm column, with a 5-μm particle size (Thermo Electron Corporation, Waltham, MA). Initial gradient conditions (20% organic) were held for 0.75 min. From 0.75 to 1.39 min, the mobile phase composition increased linearly to 40% organic, and the eluent was directed to the mass spectrometer. At 3.3 min, the organic composition was increased to 90%. The flow was held at 90% organic until 4 min. At 4 min, the column was equilibrated with 20% organic. The total run time, including equilibration, was 5 min/injection. Eight point calibration curves (0.5–1000 nM for olmesartan; 2–1000 nM for pravastatin, rosuvastatin, and valsartan; 2–2000 nM for pitavastatin) were constructed based on peak area ratios of analyte and appropriate internal standard using the following transitions: olmesartan (447.5 → 207.2), valsartan (436.4 → 235.2), pravastatin (447.1 → 327.4), rosuvastatin (482.2 → 258.2), pitavastatin (422.1 → 290.3), d5-olmesartan (453.5 → 207.2), d7-valstatin (439.4 → 235.2), and d6-rosuvastatin (488.2 → 264.2). All points on the curves back-calculated to within ±15% of the nominal value.

**Data Calculation.** The accumulation (picomoles per milligram of protein), biliary excretion index (BEI; percentage), and in vitro intrinsic Clbiliary (milliliters per minute per kilogram) were calculated in hepatocytes using B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC) based on the following equations (Liu et al., 1999a):

\[
\text{BEI} = \frac{\text{Accumulation}_{\text{cells}} - \text{Accumulation}_{\text{bile}}}{\text{Accumulation}_{\text{cells}}} \times 100 \quad (1)
\]

**Intrinsic Clbiliary** was calculated according to the equations below based on the well stirred model of hepatic disposition, assuming that red blood cell partitioning of test compounds was minimal.

\[
\text{Predicted Cl}_{\text{biliary}} = \frac{Q_p \times \text{intrinsic Cl}_{\text{biliary}}}{Q_p + \text{intrinsic Cl}_{\text{biliary}}} \quad (3)
\]

\[
\text{Predicted Cl}_{\text{biliary}} = \frac{Q_p \times f_u \times \text{intrinsic Cl}_{\text{biliary}}}{Q_p + f_u \times \text{intrinsic Cl}_{\text{biliary}}} \quad (4)
\]

Where Qp represents the hepatic plasma flow rate (40 ml/min/kg).

In eq. 3, plasma unbound fraction (fu,p) was assumed to be unity. Taking into consideration the unbound fraction, the observed in vivo Clbiliary values were obtained from references.

**Immunoblot.** Freshly isolated rat hepatocytes on day 0 and SCRhs on day 4 of culture were washed once with HBSS and then lysed with 400 μl of lysis buffer (1 mM EDTA, 1% SDS, pH 8, with Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics, Mannheim, Germany)). Lysates were stored at −80°C until immunoblot analysis. The whole cell lysates were thawed, and protein concentrations were determined using the BCA protein assay. Protein samples (30 μg/well) were resolved on NuPAGE 4 to 20% Bis-
Tris gel (Invitrogen) and electrotransferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). Blots were blocked with Tris-buffered saline with 0.1% Tween 20 containing 5% nonfat dry milk for 1 h at room temperature. Subsequently, the membrane was incubated with appropriate primary antibodies for 2 h at room temperature or overnight at 4°C and then rinsed three times at 10-min intervals with Tris-buffered saline with 0.1% Tween 20. Mrp2, breast cancer resistance protein (Bcrp), and β-actin were detected using monoclonal antibodies M2III-6 (1:1000 dilution; Alexis Laboratories, San Diego, CA), BXP-53 (1:1000 dilution; Alexis Laboratories), and C4 (1:5000 dilution; Millipore Bioscience Research Reagents, Temecula, CA), respectively. After incubation with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Chalfont St. Giles, UK), immunoreactive protein bands were detected by chemiluminescent substrate SuperSignal West Dura (Pierce Chemical) using a Bio-Rad VersaDoc imaging system (Bio-Rad, Hercules, CA).

**Results**

**Cumulative Uptake in SCRHs.** Substrate accumulation in SCRHs was concentration-dependent and seemed to be in the linear range of uptake at concentrations between 2 and 5 μM for all drugs incubated for 10 min (Fig. 2). Accumulation was also time-dependent; BEI (eq. 1), equivalent to the percentage of retained substrate in the canalicular networks, was measurable for all drugs at each incubation time (Fig. 3). For a given drug, the BEI determined after 10- and 20-min incubations was comparable. Rosuvastatin and pravastatin have high BEI values. Both rosuvastatin and pitavastatin exhibited strikingly higher accumulation in SCRHs compared with the other drugs, suggesting higher uptake activity (Fig. 3). The BEI of taurocholate ranged from 82.1 to 90.4% in these experiments, demonstrating functional excretion of substrates into the canalicular network in the SCRH system.

**In Vitro-in Vivo Correlation of Clbiliary of ARBs and Statins in Rats.** The in vitro intrinsic Clbiliary values were higher for both rosuvastatin and pitavastatin compared with the other drugs (Table 1). The relationship between predicted Clbiliary values from SCRHs using eq. 4 and observed in vivo Clbiliary values for these drugs was excellent, although the compounds selected clustered into one of two categories, either low or high Clbiliary (Fig. 4). Even though the predicted Clbiliary values were 10- to 100-fold lower than the in vivo Clbiliary values, the rank order of the predicted Clbiliary values was consistent with the in vivo Clbiliary values for these drugs. Both the predicted [based on eq. 4] and the in vivo Clbiliary values of pravastatin were the highest, followed by rosuvastatin, pitavastatin, valsartan, and olmesartan (Fig. 4; Table 1). As shown in Fig. 4, there was no discernible relationship between predicted Clbiliary based on eq. 3 and observed in vivo Clbiliary values for these drugs.

**Effect of BSP on Accumulation of ARBs and Statins in SCRHs.** BSP (30 and 100 μM) inhibited the uptake of each drug tested (5 μM) in day 4 sandwich-cultured hepatocytes in a concentration-dependent manner (Fig. 5).

**BEI of ARBs and Statins in SCRHs from WT and TR−Rats.** BEI values and in vitro intrinsic Clbiliary values (Table 2) of E2-17β, an Mrp2 substrate (Morikawa et al., 2000), olmesartan, valsartan, pravastatin, and rosuvastatin in TR−SCRHs were reduced compared with WT SCRH. This was consistent with decreased biliary excretion observed in vivo in EHB rats compared with WT rats, as mentioned and referenced in Fig. 1. Because Mrp2 plays only a minor role in pitavastatin biliary excretion in rats (Hirano et al., 2005), it was surprising to find that both the BEI and in vitro intrinsic Clbiliary of pitavastatin were reduced in TR−SCRHs compared with WT SCRHs. There were marginal differences in accumulation (cell + bile) between WT and TR−SCRHs for all of these compounds (data not shown), suggesting no differences in uptake and hepatocyte concentrations.

**Expression Levels of Mrp2 and Bcrp in Hepatocytes from WT and TR−Male Rats.** Protein levels of Mrp2 and Bcrp in freshly isolated hepatocytes on day 0 and SCRHs on day 4 of culture were determined by immunoblot analysis and compared between WT and TR−male rats (Fig. 6). The absence of Mrp2 protein in hepatocytes from TR−rats in both day 0 freshly isolated hepatocytes and day 4 SCRHs was confirmed. Interestingly, Bcrp protein levels in hepatocytes from TR−rats were much lower than in hepatocytes from WT rats in freshly isolated hepatocytes and day 4 SCRHs. The mol. wt. of both Mrp2 and Bcrp protein in the hepatocytes from WT rats on day 4 of culture was greater than their counterpart in freshly isolated hepatocytes.

**Discussion**

The present study was undertaken to examine the relationship between in vitro and in vivo biliary excretion of ARBs and statins and to investigate the usefulness of SCRHs as an in vitro model to identify transport proteins involved in the basolateral uptake and canalicular excretion of these drugs in rats. As shown in Table 1 and Fig. 4, the predicted Clbiliary values from SCRHs based on eq. 4 correlated well with the in vivo Clbiliary values for these drugs compared with predicted Clbiliary values based on eq. 3. Among statins, the predicted Clbiliary values assuming fup was unity (eq. 3) did not reflect the rank order of the observed in vivo Clbiliary values. However, the predicted Clbiliary values for pravastatin, rosuvastatin, and pitavastatin, accounting for plasma...
reported that accounting for plasma protein binding was necessary to predict in vivo values. Fukuda et al. (2008) also showed that the unbound fraction as shown in eq. 4 would be necessary to traverse the hepatic basolateral membrane. Thus, accounting for the unbound fraction (eq. 4), were consistent with the rank order observed for the in vivo Cl_biliary values. The rank order of predicted Cl_biliary values for ARBs was in good agreement with in vivo results, even though both ARBs have low in vivo Cl_biliary values. Based on the assumptions of the well stirred model of hepatic disposition, only unbound concentrations are able to traverse the hepatic basolateral membrane. Thus, accounting for the unbound fraction as shown in eq. 4 would be necessary to predict in vivo values. Fukuda et al. (2008) also reported that accounting for plasma protein binding was necessary to obtain a good in vivo prediction based on SCRH data using valsartan, pravastatin, rosuvastatin, and two other antibiotics. In contrast, Liu et al. (1999a) reported that predicted Cl_biliary values determined in SCRHs assuming fu,p was unity correlated well with reported in vivo Cl_biliary using structurally different compounds (inulin, salicylate, methotrexate, [+]pen2,5]enkcephalin, and taurocholate). According to the present data, when in vivo Cl_biliary values are predicted based on SCRH data between structurally similar compounds, the unbound fraction should be included as shown in eq. 4.

**Fig. 3.** Accumulation [cells + bile (solid bars) and cells (white bars)] and BEI of olmesartan (A), valsartan (B), pravastatin (C), rosuvastatin (D), and pitavastatin (E) in sandwich-cultured rat hepatocytes. Dosing concentration, 5 μM. Data represent mean ± S.E.M. (n = 3 livers).

**TABLE 1**

In vitro intrinsic Cl_biliary and predicted Cl_biliary, calculated based on eqs. 3 and 4 from data generated in sandwich-cultured rat hepatocytes, compared with observed in vivo Cl_biliary for selected angiotensin II receptor blockers and HMG-CoA reductase inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>In Vitro Intrinsic Cl_biliary</th>
<th>fu,p</th>
<th>Predicted Cl_biliary</th>
<th>Observed in Vivo Cl_biliary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/min/kg</td>
<td></td>
<td>From Eq. 3</td>
<td>From Eq. 4</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>1.72 ± 0.25</td>
<td>0.010[^b]</td>
<td>1.65 ± 0.23</td>
<td>0.0172 ± 0.0025</td>
</tr>
<tr>
<td>Valsartan</td>
<td>3.24 ± 0.51</td>
<td>0.006[^c]</td>
<td>2.99 ± 0.45</td>
<td>0.0194 ± 0.0031</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>4.43 ± 0.75</td>
<td>0.472[^c]</td>
<td>3.97 ± 0.60</td>
<td>1.98 ± 0.32</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>46.1 ± 0.4</td>
<td>0.044[^c]</td>
<td>21.4 ± 0.1</td>
<td>1.93 ± 0.02</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>34.8 ± 3.1</td>
<td>0.008[^d]</td>
<td>18.5 ± 0.9</td>
<td>0.275 ± 0.024</td>
</tr>
</tbody>
</table>

[^a] In vitro intrinsic Cl_biliary values were calculated according to eq. 2 based on a 10-min incubation at 5 μM substrate concentration.
[^b] Olmesartan regulatory documentation.
[^c] Valsartan regulatory documentation.
[^d] Pravastatin regulatory documentation.
[^e] Hirano et al. (2005).
to be in the linear range at concentrations between 2 and 5 μM, which was reasonable based on the reported $K_m$ values for pravastatin, rosuvastatin, and pitavastatin. Thus, 5 μM was selected as the substrate concentration for the present study. Rosuvastatin exhibited approximately 10-fold higher accumulation in SCRHs compared with pravastatin. This finding is consistent with the intrinsic clearance for the hepatic uptake of rosuvastatin, which was reported to be approximately 10-fold higher than pravastatin in rats (Nezasa et al., 2003), even though accumulation in SCRHs was not determined as the initial uptake velocity.

BSP is a known inhibitor of Oatps, organic anion transporters, and Na$^+$-taurocholate-cotransporting polypeptide (Hagenbuch et al., 1991; Sekine et al., 1998; Tokui et al., 1999). Inhibition of ARB and statin uptake in SCRHs by BSP in a concentration-dependent manner (Fig. 5) suggests that one or more of these transport proteins is/are involved in the carrier-mediated uptake of these compounds from sinusoidal blood into hepatocytes. Inhibition of the hepatic uptake of pitavastatin by BSP was relatively less sensitive compared with the other compounds tested. This finding may be related to the higher contribution of passive diffusion to pitavastatin uptake because of the hydrophobic structural features of pitavastatin compared with pravastatin and rosuvastatin (Shitara and Sugiyama, 2006).

Experiments in TR$^-$ SCRHs demonstrated that Mrp2 was involved in the biliary excretion of olmesartan, valsartan, pravastatin, and rosuvastatin in rats, consistent with previously published in vivo data (see Fig. 1). It is interesting to note that the TR$^-$ SCRH data (Table 2) also suggested that pitavastatin was an Mrp2 substrate. However, as previously reported, the in vivo biliary excretion of pitavastatin was similar in EHBRs and WT rats, suggesting that pitavastatin was not an Mrp2 substrate. Pitavastatin is hypothesized to be a Bcrp substrate (Hirano et al., 2005). This apparent discrepancy may be because of a novel finding based on immunoblots showing that Bcrp expression levels were much lower in SCRHs from TR$^-$ rats used in this study compared with WT rats. Expression levels of Bcrp in TR$^-$ rats based on earlier studies using a different Bcrp antibody (Hoffmaster et al., 2005; Johnson et al., 2006) were similar or decreased compared with WT rats. The decreased BEI for pitavastatin in TR$^-$ SCRHs may be because of reduced Bcrp protein levels in these Mrp2-deficient TR$^-$ rats. Accordingly, reduced Bcrp levels in TR$^-$ SCRHs could affect the evaluation of the other compounds tested. For instance, rosuvastatin has been shown to be a substrate of BCRP in humans (Huang et al., 2006). However, Mrp2 is known to be primarily involved in the biliary excretion of olmesartan, valsartan, and pravastatin in vivo, so the reduction of BEI values in TR$^-$ SCRHs for these compounds may be attributed, in large part, to the absence of Mrp2. These data confirm that reduced transport protein expression in SCRHs leads to decreased BEI and in vitro intrinsic $C_{\text{biliary}}$, further demonstrating the usefulness of the SCRH system to evaluate transport protein function and the involvement of specific transport proteins in substrate excretion.

Immunoblot analysis demonstrated an increase in molecular mass of Mrp2 on day 4 compared with day 0, which has been attributed previously in SCRHs to glycosylation of Mrp2 during the culture time (Zhang et al., 2005). It is interesting to note the $C_{\text{biliary}}$ values predicted based on eq. 4 were 10- to 100-fold lower than the observed in vivo $C_{\text{biliary}}$ values for all compounds tested. These findings may be because of less extensive canalicular network formation in culture compared with liver tissue in vivo. Consistent underestimation of in vivo $C_{\text{biliary}}$ values also may be because of several factors including, but not limited to, decreased activity of transport proteins in culture or leakage from the biliary compartment over time in SCRHs as discussed previously (Liu et al., 1999c; Hoffmaster et al., 2005).

Multiple organic anion-transporting polypeptide (Oatp) isoforms are involved in the hepatic uptake of pravastatin (Yamazaki et al., 1993; Tokui et al., 1999), rosuvastatin (Nezasa et al., 2003), and pitavastatin (Fujino et al., 2005) in rats. Transport proteins responsible for the hepatic uptake of olmesartan and valsartan in rats were unknown, but these ARBs are substrates for multiple OATP isoforms in humans (Nakagomi-Hagihara et al., 2006; Yamashiro et al., 2006). Nezasa et al. (2003) reported that $K_m$ values for the hepatic uptake of pravastatin and rosuvastatin were 16.5 and 9.17 μM in rat hepatocytes, respectively; $K_m$ values for pitavastatin uptake in oocytes expressing rat Oatp1 and Oatp4 were 7.9 and 4.8 μM, respectively (Fujino et al., 2005). In the present study, uptake in SCRHs for all drugs tested seemed
that immunoblot analysis also revealed an increase in Bcrp molecular mass at day 4 in culture compared with day 0.

Turncliff et al. (2006) demonstrated the usefulness of the SCRH model to evaluate the hepatobiliary disposition of generated metabolites. Although biliary excretion of pitavastatin is the main elimination pathway in rats, pitavastatin also is metabolized to pitavastatin lactone by UDP-glucuronosyl transferase as a minor component and excreted into bile in rats (Kojima et al., 1999; Fujino et al., 2003). Simultaneous determination of the amount of pitavastatin and the lactone form in SCRHs revealed pitavastatin lactone in both the cell and bile compartments, consistent with biliary excretion of pitavastatin lactone in vivo (data not shown). Studies currently are underway to examine the metabolic disposition of several drugs in SCRHs.

In conclusion, in vitro Cl\text{biliary} determined in SCRHs can be used to estimate and compare in vivo Cl\text{biliary} and to ascertain the involvement of transport proteins in the basolateral uptake and canalicular excretion of ARBs and statins that undergo limited metabolism. Knowledge regarding the contribution of hepatic uptake and efflux transporters to the pharmacokinetics of a drug is essential to predict potential alterations in systemic and hepatobiliary disposition when the expression or function of hepatic transport proteins is altered by disease states, genetic variability, and/or transporter-mediated drug-drug interactions.

### References


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