Culture Period-Dependent Changes in the Uptake of Transporter Substrates in Sandwich-Cultured Rat and Human Hepatocytes

Naoki Kotani, Kazuya Maeda, Takao Watanabe, Mariko Hiramatsu, Li-kun Gong, Yi-an Bi, Toshiaki Takezawa, Hiroyuki Kusuhara, and Yuichi Sugiyama

Graduate School of Pharmaceutical Sciences, the University of Tokyo, Tokyo, Japan (N.K., K.M., T.W., M.H., H.K., Y.S.); Center for Drug Safety and Evaluation Research, State Key Laboratory of New Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China (L.G.); Pfizer Global Research and Development, Groton, Connecticut (Y.B.); and Laboratory of Animal Cell Biology, National Institute of Agrobiological Sciences, Ibaraki, Japan (T.T.)

ABSTRACT:
Sandwich-cultured hepatocytes (SCH) are a useful tool for evaluating hepatobiliary drug transport in vitro. Some studies have investigated the in vitro-in vivo correlations of the biliary clearance of drugs using SCH. In most cases, the biliary clearance observed in vivo correlated well with the predicted clearance, but the predicted absolute values were underestimated when based on in vitro experiments with SCH. We hypothesized that the down-regulated function of uptake transporters is one of the causes of this underestimation. Therefore, the uptake of taurocholate, digoxin, pravastatin, and rosuvastatin was investigated in sandwich-cultured rat hepatocytes (SCRH) cultured for 5, 24, 48, and 96 h, and the predicted hepatic clearance from in vitro uptake clearance (CLH, vitro) was calculated with a dispersion model. In SCRH cultured for 96 h, the saturable uptake of taurocholate, digoxin, pravastatin, and rosuvastatin decreased to 7.5, 3.3, 64, and 23%, respectively, of their uptake in hepatocytes cultured for 5 h, and a better prediction of in vivo hepatic clearance (CLH, vivo) was achieved when based on CLH, vitro of 5-h-cultured hepatocytes. These results suggest that the uptake activity is considerably reduced in cell culture, even in a sandwich-culture format. In a similar study, we also examined taurocholate and rosuvastatin in sandwich-cultured human hepatocytes (SCHR). Unlike in SCRH, the saturable uptake of these compounds did not differ markedly in SCHR cultured for 5 or 96 h. Thus, the uptake activity in SCHR was maintained relatively well compared with that in SCRH.

Introduction
Together with their metabolism, many endogenous and exogenous compounds undergo hepatic uptake and biliary excretion mediated by transporters, which facilitate the efficient detoxification of xenobiotics. Several experimental tools have been established to evaluate hepatic uptake, such as cell lines stably expressing human uptake transporters and cryopreserved human hepatocytes. In contrast, it is fairly difficult to evaluate the biliary excretion of compounds from hepatocytes to bile in humans. Primary cultured hepatocytes are used to predict the hepatic transport and metabolism of drugs, but a previous report suggested that their polarity was lost, followed by the internalization of the efflux transporters a short time after the cells were isolated (Hoffmaster et al., 2004), with a rapid reduction in the expression and activities of the uptake transporters in long-term cultured hepatocytes (Liang et al., 1993; Ishigami et al., 1995; Rippin et al., 2001). This observation limits the utility of this experimental system for the assessment of the biliary excretion of drugs.

In sandwich-cultured hepatocytes (SCH), the hepatocytes are repolarized and the expression, cellular localization, and transport activities of the hepatic transporters are well maintained (Liu et al., 1998; Hoffmaster et al., 2004) and functional canalicular networks, called “bile pockets,” are also formed between these hepatocytes (Swift et al., 2010). The accumulation of compounds in the bile pockets can be measured by opening them in Ca<sup>2+</sup>-free buffer (Liu et al., 1999b; Bi et al., 2006). Therefore, SCH are the only in vitro experimental system in which the biliary excretion of drugs from the blood to the bile can be evaluated. SCH can be useful for several applications such as the prediction of overall biliary clearance of compounds whose clearance was not dominated only by uptake process, the determination of the contribution of each efflux transporter to the overall efflux of drugs, and the impact of the inhibition of efflux transporters by coadministered drugs on the change in the biliary transport of substrate drugs (Swift et al., 2010).

Until now, several studies have tried to demonstrate an in vitro-in vivo correlation for the biliary clearance of drugs using SCH. In most cases, a good correlation between the biliary clearance predicted from in vitro SCH experiments (CLbile, vitro) and the biliary clearance

ABBREVIATIONS: SCH, sandwich-cultured hepatocyte(s); SCRH, sandwich-cultured rat hepatocytes; SCHH, sandwich-cultured human hepatocytes; BSA, bovine serum albumin; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; OATP/Oatp, organic anion transporting polypeptide; NTCP/Ntcp, Na<sup>+</sup>-taurocholate cotransporting polypeptide.
observed in vivo (CL\textsubscript{bile, vitro}) has been reported, but the absolute values for CL\textsubscript{bile, vitro} are often underestimated. For example, Fukuda et al. (2008) showed that the absolute CL\textsubscript{bile, vitro} values for some angiotensin II receptor blockers, HMG-CoA reductase inhibitors (statins), and antibiotics were approximately 20- to 200-fold lower than the CL\textsubscript{bile, vitro} values, although good correlations between CL\textsubscript{bile, vitro} and CL\textsubscript{bile, vivo} were observed (Fukuda et al., 2008). Abe et al. (2008, 2009) compared the CL\textsubscript{bile, vitro} of some angiotensin II receptor blockers and statins with their CL\textsubscript{bile, vivo} in both sandwich-cultured rat hepatocytes (SCRH) and sandwich-cultured human hepatocytes (SCHH), and these CL\textsubscript{bile, vitro} values were also 20- to 200-fold lower in the SCRH and approximately 20-fold lower in the SCHH than the CL\textsubscript{bile, vivo} values (Abe et al., 2008, 2009). Li et al. (2010) tried to improve the in vitro-in vivo correlation of the biliary clearance of drugs using SCRH by introducing an appropriate scaling factor, because CL\textsubscript{bile, vitro} was approximately 6-fold lower than CL\textsubscript{bile, vivo} based on a conventional calculation method (Li et al., 2010). All of these results indicated that the transport activity was reduced in SCRH, but the exact reasons for its underestimation were still unknown.

CL\textsubscript{bile, vitro} is usually calculated on the basis of the drug concentration in the medium of SCH and corresponds to the biliary clearance based on the plasma unbound concentration in vivo. On the basis of the pharmacokinetic theory, the uptake process is the first step in the hepatic elimination of drugs, and changes in uptake activity directly affect the overall hepatic clearance. Therefore, we hypothesized that the down-regulation of uptake transporters is one of the major causes of the underestimation of CL\textsubscript{bile, vitro} in the SCH system.

In the present study, changes in transporter activity in SCRH were investigated using several transporter substrates (taurocholate, digoxin, pravastatin, and rosuvastatin), as were these changes in SCHH (taurocholate and rosuvastatin). The predicted hepatic clearance (CL\textsubscript{H, vitro}) values were calculated on the basis of a dispersion model, under the assumption that the uptake process is the rate-determining process in hepatic clearance, and were also compared with CL\textsubscript{H, vivo} values in hepatocytes cultured for either 5 or 96 h.

Materials and Methods

Chemicals and Reagents. \textsuperscript{[3]H}Pravastatin (45.5 Ci/mmol) and unlabeled pravastatin were kindly donated by Daichi Sankyo Co. Ltd (Tokyo, Japan). \textsuperscript{[3]H}Rosuvastatin (79 Ci/mmol) and unlabeled rosuvastatin were donated by AstraZeneca (Macclesfield, UK). \textsuperscript{[3]H}Taurocholate (4.6 Ci/mmol) and \textsuperscript{[3]H}digoxin (40 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), Triton X-100, EGTA, and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO), t-Glutamine, bovine serum albumin (BSA), and collagenase (type I) were obtained from Wako Pure Chemicals (Tokyo, Japan). Williams’ medium E (WME), human recombinant insulin, and standard (containing Ca\textsuperscript{2+}/Mg\textsuperscript{2+}) and Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free Hanks’ balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). BioCoat 24-well plates, Percoll, BD Matrigel, and ITS premix, 0.1 mg/ml insulin, and 2 mM t-glutamine was added to each well (this procedure was not applied to the 5-h-cultured hepatocytes). Within 18 to 24 h of seeding, the cells were overlain with ice-cold 0.25 mg/ml BD Matrigel in WME supplemented with 5% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1 µM dexamethasone, 4 µg/ml insulin, and 2 mM t-glutamine. Cell viability was determined by trypan blue exclusion. Only those hepatocytes with greater than 90% viability were used for further experiments. The hepatocytes were seeded onto BioCoat 24-well plates at a density of 2.0 × 10\textsuperscript{5} viable cells/well in 0.5 ml of supplemented WME. The hepatocytes were allowed to attach for 5 h at 37°C in a humidified chamber under a 95% air/5% CO\textsubscript{2} atmosphere. The plates were then swirled for 5 min at 37°C in a humidified atmosphere. Only those hepatocytes with greater than 90% viability were used for further experiments. The hepatocytes were seeded onto BioCoat 24-well plates at a density of 2.0 × 10\textsuperscript{5} viable cells/well in 0.5 ml of supplemented WME. The hepatocytes were allowed to attach for 5 h at 37°C in a humidified chamber under a 95% air/5% CO\textsubscript{2} atmosphere. The plates were then swirled gently, and the medium containing the unattached cells was aspirated. WME supplemented with 5% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1 µM dexamethasone, 4 µg/ml insulin, and 2 mM t-glutamine was added to each well (this procedure was not applied to the 5-h-cultured hepatocytes). Western Blot Analysis of Uptake Transporters in SCRH. Crude membrane was prepared from SCRH cultured for designated time as reported previously (Sasaki et al., 2002). After the crude membrane fraction was suspended in phosphate-buffered saline, it was immediately frozen in liquid N\textsubscript{2} and stored at −80°C until used. The protein concentration of the crude membrane prepared from SCRH was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The crude membrane fraction was dissolved in EzApply containing diithiothreitol (ATTO, Tokyo, Japan) and loaded onto an SDS-polyacrylamide gel electrophoresis mini 4 to 12% gel (TEFCO, Tokyo, Japan). The molecular weight was determined using a Precision Plus Western C protein standard (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred electrophoretically to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories) using a blotter (Trans-Blot; Bio-Rad Laboratories) at 100 V for 1 h. The membrane was blocked with 10-fold diluted 1× EzBlock (ATTO) by Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 h at room temperature. After washing with TTBS, the membrane was incubated overnight at 4°C in 1× EzBlock with 1000-fold diluted anti-Oatp1a1 (antisemur that we had produced previously in rabbit) (Aoki et al., 2008), 120-fold diluted anti-Oatp1a4 (Alpha Diagnostic Intl. Inc., San Antonio, TX), 100-fold diluted anti-Oatp1b2 (N-17 antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-actin clone C4 (Millipore Corporation, Billerica, MA). For the detection of Oatp1a1 and 1a4, the membrane was placed in contact with 5000-fold diluted goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories); for the detection of Oatp1b2, the membrane was placed in contact with 1000-fold diluted donkey anti-goat IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Inc.); and for the detection of β-actin, the membrane was placed in contact with 1000-fold diluted goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories) for 1 h in 1× EzBlock. The immunoreactive band was detected using an AP Conjugate Substrate Kit (Bio-Rad Laboratories).

Culture of Cryopreserved Human Hepatocytes. Three batches of cryopreserved human hepatocytes were used in this study: lot KQG was purchased from Celsis In Vitro Technologies; lot HH190 was purchased from BD Gentest (Woburn, MA); and lot Hu930 was purchased from CellzDirect (Durham, NC). The cryopreserved human hepatocytes were prepared according to the
protocol suggested by Celsis In Vitro Technologies. Torpedo Antibiotic Mix (1.1 ml) was mixed into 50 ml of InVitroGRO HT medium, InVitroGRO CP medium, and InVitroGRO HI medium, to produce complete thawing medium, plating medium, and incubation medium, respectively. Before these media were used, they were prewarmed in a water bath at 37°C. The cryopreserved human hepatocytes were incubated for approximately 1 min in the water bath were used, they were prewarmed in a water bath to 37°C. The cryopreserved plating medium, and incubation medium, respectively. Before these media were overlain with ice-cold 0.25 mg/ml BD Matrigel in 0.5 ml/well incubation medium. BD Matrigel was only applied to the 96-h-cultured hepatocytes. The cultures were maintained in the incubation medium, which was changed every 24 h. Hepatocytes cultured for 5 or 96 h were used for the uptake study.

Transport Assay with SCH. The uptake studies were performed according to the method described previously (Liu et al., 1999a). In brief, rat hepatocytes cultured in a collagen-sandwich configuration (cultured for 96 h) were rinsed twice and preincubated at 37°C for 10 min with 0.5 ml of standard HBSS to maintain their tight junctions and bile canaliculi or Ca2+/Mg2+/H+ ions causes the disruption of the tight junctions that seal the bile pockets. The hepatocytes were then rinsed three times with 1 ml of ice-cold HBSS and lysed with 0.5% Triton X-100 in phosphate-buffered saline (0.5 ml/well) with vigorous shaking for 30 min at room temperature. The radioactivity associated with the cell lysate (500 μl), and the incubation buffer (100 μl) was analyzed with a liquid scintillation counter (Tri-Carb 3100TR; PerkinElmer Life and Analytical Sciences) after the addition of 3 ml of scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan) to the scintillation vials. The remaining 25 μl of cell lysate was used to determine the protein concentration with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA), with BSA as the standard. All data were corrected for nonspecific binding by subtracting the radioactivity in hepatocyte-free wells from that in hepatocyte-containing wells. The biliary excretion index (BEI), which is defined as the percentage of the amount of substrates accumulated in the bile pockets in the total accumulation in SCH after incubation for 10 min, was calculated with eq. 1 (Liu et al., 1999a):  

\[
\text{BEI} = \frac{\text{Accumulation}_{(\text{Ca}^{2+}/\text{Mg}^{2+}/\text{H}^+})} {\text{Accumulation}_{(\text{Ca}^{2+}/\text{Mg}^{2+}/\text{free})}} \times 100  
\]

where Accumulation_{Ca^{2+}/Mg^{2+} and Accumulation_{Ca^{2+}/Mg^{2+}/free}} indicate the cumulative uptake amount of compounds for 10 min in SCH preincubated with buffer in the presence or absence of Ca2+/Mg2+ ions, respectively. By using the B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC) (Liu et al., 1999a), the amount of substrate secreted into the bile pockets can be estimated as the difference in the cumulative uptake of the compound into the SCH in the presence and absence of Ca2+/Mg2+ ions, because these ions are required in the medium to maintain the tight junctions that seal the bile pockets tightly. The absence of Ca2+/Mg2+ ions causes the disruption of the tight junctions and the subsequent leakage of the contents from the bile pockets into the medium.

Determination of the Uptake Clearance of the Compounds Using SCH. To evaluate the uptake clearance of each compound in SCH, uptake studies were performed as described above, using only standard HBSS. The hepatocytes cultured for the designated time periods were incubated in standard HBSS containing radiolabeled compounds and unlabeled substrates for designated times. The uptake studies were performed using two different concentrations of each compound to estimate the contribution of transporter-mediated saturable uptake and passive diffusion to the overall uptake clearance. Saturable uptake clearance (CL_{uptake, satu}) was estimated as the difference between the in vitro uptake clearance of a tracer concentration (CL_{uptake, tracer} in microliters per minute per milligram of protein) and an excess concentration (CL_{uptake, excess}) (eq. 2):  

\[
\text{CL}_{\text{uptake, satu}} = \text{CL}_{\text{uptake, tracer}} - \text{CL}_{\text{uptake, excess}}  
\]

All data were corrected for nonspecific binding by subtracting the radioactivity in hepatocyte-free wells from that in hepatocyte-containing wells.

Calculation Method Used to Predict the In Vivo Biliary Clearance from the In Vitro Data. CL_{uptake} was determined by calculating the slope of the distribution volume (V_d) (microliters per milligram of protein), given as the amount of radioactivity associated with the cells (disintegrations per minute per milligram of protein) divided by its concentration in the incubation medium (disintegrations per minute per microliter), between 0.5 and 1 min (5 min for taurocholate, pravastatin, and rosuvastatin; 2 min for digoxin) (eq. 3). In these ranges, the linearity of the time-dependent uptake was confirmed for each compound:

\[
\text{CL}_{\text{uptake}} = \frac{V_{d,0.5\text{min}} - V_{d,0.5\text{min}}}{x - 0.5}  
\]

The hepatic clearance (CL_{H, inVitro}) predicted from the in vitro uptake clearance was calculated with eqs. 4 to 7 based on a dispersion model (Roberts and Rowland, 1986), under the assumption that the uptake process is a rate-determining process in the overall biliary clearance and uptake clearance is thought to be equal to the hepatic intrinsic clearance (CL_{intrinsic, B} as CL_{uptake, satu}).

\[
\text{CL}_{\text{H, inVitro}} = \frac{Q_h (1 - f_h)}{4a}  
\]

\[
F_h = (1 + a)^{-1} \cdot \exp((1 - a)/2D_h) - (1 - a) \cdot \exp(1 - a/2D_h)  
\]

\[
a = (1 + 4R_S \cdot D_h)^{1/2}  
\]

\[
R_S = \frac{f_h \cdot \text{CL}_{\text{intrinsic, B}}}{Q_h}  
\]

where Q_h represents the hepatic blood flow, which is 67 ml/min/kg in rats (Nies et al., 1976) and 20.7 ml/min·kg⁻¹ in humans (Davies and Morris, 1993), and f_h represents the blood/normal concentration ratio (R_h), and the in vivo hepatic clearance are cited from other reports. Dispersion number (D_h) was defined as an indicator for the degree of spreading of an injected solute on transit through an organ in the dispersion model and set at 0.17 according to the previous reports (Roberts and Rowland, 1986; Iwatsubo et al., 1996). Physiological scaling factors of 40 g of liver/kg, 10⁶ cells/g liver were used for in vivo scaling up (Davies and Morris, 1993; Bayliss et al., 1999). In humans, physiological scaling factors of 25.7 g of liver/kg body weight, 1.5 × 10⁶ cells/g liver, and 107 × 10⁶ cells/g liver were used for in vivo scaling up (Davies and Morris, 1993; Gibellini et al., 2007).

Statistics. Statistical differences were analyzed with Student’s t test to identify the significant differences between two sets of data and by one-way analysis of variance with Dunnett’s test for multiple pairwise comparisons. Differences were considered statistically significant at P < 0.05.

Results

Confirmation of Hepatic Transport Function in SCH. To confirm that sandwich culture was successfully performed and that the bile pockets were reestablished in this study, we performed an accumulation study of taurocholate using standard and Ca2+/Mg2+/-free HBSS. A significantly higher accumulation of taurocholate was observed in the hepatocytes incubated with standard HBSS than in hepatocytes incubated with Ca2+/Mg2+/-free HBSS in both SCRH (Fig. 1) and SCHH (Fig. 2). The BEI values for taurocholate, calculated with eq. 1, in SCRH and SCHH were 63.1 ± 5.8 and 51.0 ± 17.3%, respectively.
In contrast, the absence of Ca\(^{2+}\) in the incubation buffer had no effect on the accumulation of salicylic acid in the SCRH or SCHH.

Changes in Uptake Activities in SCRH. The amount of each test compound taken up into the rat hepatocytes decreased in a culture period-dependent manner (Fig. 3). The same accumulation study was performed three times, and reproducible results were obtained. Typical data are shown in Fig. 3 because the absolute values of the uptake amount into hepatocytes varied among experiments. Uptake was period-dependent manner (Fig. 3). The same accumulation study was also performed in SCHH to check whether the down-

Alteration of Expression Levels of Uptake Transporters in SCRH. The expression levels of Oatp1a1/1a4/1b2 in SCRH over time in culture were evaluated by Western blot analysis (Fig. 5). The expression levels of these transporters hardly changed between 5- and 24-h-cultured hepatocytes. Oatp1a1 expression was maintained even in 96-h-cultured hepatocytes. However, the expression levels of Oatp1a4 and 1b2 drastically decreased in 96-h-cultured hepatocytes.

Comparison of CL\(_{\text{H,vivo}}\) and CL\(_{\text{H, in vitro}}\) in SCRH. To investigate the time-dependent changes in the uptake activities in SCRH, we calculated the predicted CL\(_{\text{H, in vitro}}\) on the basis of CL\(_{\text{H, in vitro}}\) for the 5- or 96-h-cultured hepatocytes using a dispersion model (eqs. 4–7). CL\(_{\text{H,vivo}}\) of taurocholate, digoxin, pravastatin, and rosuvastatin, calculated with eq. 2, decreased from 23.5 ± 1.1 to 1.79 ± 0.70, from 2.73 ± 0.86 to 0.016 ± 0.327, from 6.40 ± 0.65 to 4.10 ± 0.99, and from 46.7 ± 4.5 to 10.9 ± 1.4 µl · min\(^{-1}\) · mg protein\(^{-1}\), respectively.

Changes in Uptake Activities in SCHH. A similar type of uptake study was also performed in SCHH to check whether the down-

Changes in Uptake Activities in SCRH. The amount of each test compound taken up into the rat hepatocytes decreased in a culture period-dependent manner (Fig. 3). The same accumulation study was performed three times, and reproducible results were obtained. Typical data are shown in Fig. 3 because the absolute values of the uptake amount into hepatocytes varied among experiments. Uptake was period-dependent manner (Fig. 3). The same accumulation study was performed three times, and reproducible results were obtained. This figure shows typical data, which are means ± S.E., from single preparations (n = 3). The same accumulation study was performed three times and reproducible results were obtained. Where vertical bars are not shown, the S.E. values are within the limits of the symbols.

cited in other reports (Table 1). The predicted CL\(_{\text{H,vivo}}\) values for these compounds, calculated from the data for the 5-h-cultured hepatocytes, were 1- to 3.6-fold lower than the CL\(_{\text{H,vivo}}\) values, whereas those calculated from the data for the 96-h-cultured hepatocytes were very low.

Changes in Uptake Activities in SCHH. A similar type of uptake study was also performed in SCHH to check whether the down-
regulation of these uptake activities also occurs in human hepatocytes. Unlike the results for SCRH, the uptake of the test compounds into three different batches of human hepatocytes did not differ much between the 5- and 96-h-cultured hepatocytes (Fig. 6). The contribution of passive diffusion to the overall uptake of taurocholate and rosuvastatin was also very small (Fig. 7). When the uptake activities of the 5-h-cultured hepatocytes and the 96-h-cultured SCHH were compared, the saturable uptake calculated with eq. 2, in each batch of human hepatocytes (KQG, HH190, and Hu0930) changed from 18.2 ± 0.5, 15.6 ± 0.4, and 22.9 ± 0.4 μl · min⁻¹ · mg protein⁻¹, respectively, to 15.5 ± 0.5, 20.6 ± 0.6, and 11.9 ± 0.3 μl · min⁻¹ · mg protein⁻¹, respectively, for taurocholate, and from 7.47 ± 0.36, 8.71 ± 0.43, and 7.99 ± 0.28 μl · min⁻¹ · mg protein⁻¹, respectively, to 7.58 ± 0.26, 6.79 ± 0.22, and 4.52 ± 0.19 μl · min⁻¹ · mg protein⁻¹, respectively, for rosuvastatin.

Comparison of CLHT,vitro and CLHT,vivo in SCHH. The CLHT,vitro values were calculated on the basis of CLuptake,satu in 5-h-cultured hepatocytes from each batch of human hepatocytes, using the same method we used for SCRH, and were compared with CLHT,vivo (Table 2). The CLHT,vitro values for taurocholate and rosuvastatin were 9.68 ± 0.69 and 2.96 ± 0.17 ml · min⁻¹ · kg⁻¹, respectively (means ± S.E. of three batches), and both of these values were 1.9- to 3.0-fold lower than the CLHT,vivo values. CLHT,vitro for taurocholate in the 96-h-cultured hepatocytes was 87% (KQG), 132% (HH190), or 54% (Hu0930) of the CLHT,vitro observed in the 5-h-cultured hepatocytes, and the CLHT,vitro for rosuvastatin was 104% (KQG), 90% (HH190), or 59% (Hu0930) of the CLHT,vitro observed in the 5-h-cultured hepatocytes (Fig. 7), so the CLHT,vitro values did not differ as much in the 96-h culture in SCHH as they did for SCRH.

**TABLE 1**

| Pharmacokinetic parameters for taurocholate, digoxin, pravastatin, and rosuvastatin in rats obtained from the literature and a comparison of rat hepatic clearance in vivo (CLH,vivo) with the hepatic clearance predicted from the uptake clearance in SCH (CLH,vitro) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | CLH,vitro        | CLH,vivo        | fH               | CLH,vitro (5 h)  | CLH,vitro (96 h) |
|                 | ml · min⁻¹ · kg⁻¹ | ml · min⁻¹ · kg⁻¹ |                | ml · min⁻¹ · kg⁻¹ (CLH,vitro/CLH,vivo ratio) | ml · min⁻¹ · kg⁻¹ |
| Tyrocholate     | 31.4 (0.6)       | 52.3            | 0.4 (0.1)        | 32.1 (0.615)     | 5.65 (0.108)     |
| Digoxin         | 13.3 (0.4)       | 12.7            | 0.615 (0.1)      | 12.7 (1.00)      | 7.96 (0.627)     |
| Pravastatin     | 62 (0.7)         | 0.3 (0.1)       | 2.94 (0.475)     | 22.5 (0.362)     |
| Rosuvastatin    | 37.6 (0.5)       | 59.6            | 0.083 (0.03)     | 16.7 (0.281)     | 5.21 (0.0874)    |

CLH,vitro, CLH,vivo: hepatic clearance in vivo and in vitro, respectively. CLH,vitro/CLH,vivo ratio: predicted hepatic clearance for each compound. Rb, ratio of the blood concentration to the plasma concentration of each compound; fH, unbound fraction of each compound in the total blood.

* Data from Hayashi and Sugiyama (2007).
* Blood-to-plasma concentration ratio of taurocholate in rats is assumed to be 0.6.
* Data from Inoue et al. (1985).
* Data from Harrison and Gibaldi (1976).
* Data from Liu et al. (2005).
* Data from Watanabe et al. (2010).
* Data from Kitamura et al. (2008).
* Data from Watanabe et al. (2009).
Western blot analysis (Fig. 5). Although the expression level of Oatp1a1 was maintained relatively well in 96-h-cultured SCRH compared with that in 5-h-cultured hepatocytes, that of Oatp1a4 decreased drastically in 96-h-cultured SCRH. These results are comparable with the previous report (Hoffmaster et al., 2004) and support our results in which the saturable uptake of digoxin, an Oatp1a4-selective substrate, was drastically decreased in 96-h-cultured SCRH. Oatp1b2 expression was also largely decreased after long-term culture, whereas uptake activity of pravastatin was well preserved, which suggests that pravastatin uptake into hepatocytes is dominated not only by Oatp1b2 but also by Oatp1a1 (Hsiang et al., 1999) and Oatp1a4 (Tokui et al., 1999).

Next, we tried to predict the hepatic clearance of drugs in vivo (CL_{H, vivo}) from the in vitro uptake clearance in 5- and 96-h-cultured hepatocytes under the assumption that the uptake process is a rate-determining process in the overall biliary clearance (Table 1). Because the hepatic clearance of some of the test compounds was very close to the hepatic blood flow rate, a dispersion model (D_{N} = 0.17) was used to test this prediction (Roberts and Rowland, 1986). Our results indicate that the predicted hepatic clearance (CL_{H, vitro}) decreased together with the reduced uptake function in the 96-h-cultured SCRH compared with that in the 5-h-cultured hepatocytes. In the sandwich-culture format, the in vitro clearance from the medium to the bile pockets is defined as the accumulation rate of the compound in the bile pockets divided by the medium concentration and corresponds to the in vivo biliary clearance from the blood to the bile. Considering eq. 8, the CL_{H, vitro} predicted from uptake clearance in vitro is the upper limit of the clearance calculated from SCRH. Therefore, the CL_{H, vitro} predicted from uptake clearance in SCRH could be further underestimated relative to CL_{H, vivo}. These results suggest that we must consider the reduced function of uptake transporters in SCRH when biliary clearance in vivo is predicted using SCRH.

To understand the predictability of SC in humans, the same type of study was performed in SC. The uptake of taurocholate and rosuvastatin did not decrease as much in the 96-h-cultured SC compared to that in the 5-h-cultured human hepatocytes (Figs. 6 and 7), so the function of uptake transporters was maintained relatively well.

We also evaluated the expression levels of Oatps in SC by Western blot analysis (Fig. 5). Although the expression level of Oatp1a1 was maintained relatively well in 96-h-cultured SCRH compared with that in 5-h-cultured hepatocytes, that of Oatp1a4 decreased drastically in 96-h-cultured SCRH. These results are comparable with the previous report (Hoffmaster et al., 2004) and support our results in which the saturable uptake of digoxin, an Oatp1a4-selective substrate, was drastically decreased in 96-h-cultured SCRH. Oatp1b2 expression was also largely decreased after long-term culture, whereas uptake activity of pravastatin was well preserved, which suggests that pravastatin uptake into hepatocytes is dominated not only by Oatp1b2 but also by Oatp1a1 (Hsiang et al., 1999) and Oatp1a4 (Tokui et al., 1999).

Next, we tried to predict the hepatic clearance of drugs in vivo (CL_{H, vivo}) from the in vitro uptake clearance in 5- and 96-h-cultured hepatocytes under the assumption that the uptake process is a rate-determining process in the overall biliary clearance (Table 1). Because the hepatic clearance of some of the test compounds was very close to the hepatic blood flow rate, a dispersion model (D_{N} = 0.17) was used to test this prediction (Roberts and Rowland, 1986). Our results indicate that the predicted hepatic clearance (CL_{H, vitro}) decreased together with the reduced uptake function in the 96-h-cultured SCRH compared with that in the 5-h-cultured hepatocytes. In the sandwich-culture format, the in vitro clearance from the medium to the bile pockets is defined as the accumulation rate of the compound in the bile pockets divided by the medium concentration and corresponds to the in vivo biliary clearance from the blood to the bile. Considering eq. 8, the CL_{H, vitro} predicted from uptake clearance in vitro is the upper limit of the clearance calculated from SCRH. Therefore, the CL_{H, vitro} predicted from uptake clearance in SCRH could be further underestimated relative to CL_{H, vivo}. These results suggest that we must consider the reduced function of uptake transporters in SCRH when biliary clearance in vivo is predicted using SCRH.

To understand the predictability of SC in humans, the same type of study was performed in SC. The uptake of taurocholate and rosuvastatin did not decrease as much in the 96-h-cultured SC compared to that in the 5-h-cultured human hepatocytes (Figs. 6 and 7), so the function of uptake transporters was maintained relatively well.
in SCHH compared with that in SCRH. NTCP also makes a large contribution to the uptake of taurocholate in human hepatocytes (Hagenbuch and Dawson, 2004), whereas OATP1B1 rather than OATP1B3 plays a major role in the uptake of rosuvastatin (Kitamura et al., 2008). These results are consistent with a previous study demonstrating that the expression levels of OATP1B1 and OATP1B3 in SCHH were almost the same on days 1 and 6 (Hoffmaster et al., 2004). Why the expression and function of uptake transporters are better maintained in SCHH than in SCRH is unknown, but this tendency was also observed in primary cultured hepatocytes (Jigorel et al., 2005). The predicted CLH_vitro values were 1.9- to 3.0-fold lower than the CL_H_vivo for taurocholate and rosuvastatin in both the 5-h-cultured hepatocytes and the 96-h-cultured SCHH (Table 2). Watanabe et al. (2009) reported that a scaling factor (×1.7–4.3) was required to fit the calculated value to the CLH_vivo value when they assessed the CL_H_vitro value for four kinds of statins using cryopreserved human hepatocytes in suspension, which is comparable to our situations.

In the previous report, there was 20-fold difference at least between in vitro biliary clearance predicted by SCHH and human in vivo biliary clearance (Abe et al., 2009), but in our results, the difference between the predicted hepatic clearance from in vitro uptake assay and in vivo hepatic clearance was only approximately 2.5-fold. This discrepancy may be due to the change in the backflux or biliary efflux intrinsic clearance, the large interbatch difference in the uptake clearance of substrates, and partial saturation of OATP transporters because the substrate concentration they used was almost similar to the K_M values for OATP transporters.

In conclusion, we have investigated the changes in the uptake of transporter substrates over several days in culture in both SCHH and SCRH. In SCHH, the uptake activity was dramatically down-regulated throughout the culture period, and the reduced uptake activity led to the underestimation of CL_H_vitro from the in vitro data for SCHH. One of the strategies for the prediction of in vivo biliary clearance may be available by taking correlation between in vitro and in vivo biliary clearance of many compounds with some scaling factors. Conversely, the uptake activity was maintained relatively well in SCHH. Thus, our results suggest that we can correctly estimate the hepatobiliary transport clearance of transporter substrates from in vitro transport assay with SCHH. More data regarding the prediction of biliary clearance in vivo from experiments in SCHH in vitro are required to confirm the usefulness of SCHH.

Acknowledgments
We sincerely thank AstraZeneca and Daiichi Sankyo Co., Ltd. for kindly providing us with radiolabeled and unlabeled rosuvastatin and pravastatin, respectively.

Authorship Contributions
Participated in research design: Kotani, Maeda, Kusuhara, and Sugiyama. Conducted experiments: Kotani, Maeda, Watanabe, Hiramatsu, Gong, and Sugiyama. Contributed new reagents or analytic tools: Bi and Takezawa. Performed data analysis: Kotani, Maeda, Watanabe, Hiramatsu, Gong, Kusuhara, and Sugiyama. Wrote or contributed to the writing of the manuscript: Kotani, Maeda, and Sugiyama.

References

TABLE 2
Pharmacokinetic parameters for taurocholate and rosuvastatin in humans and a comparison of human nonrenal (hepatic) clearance (CLH_vivo) with the biliary clearance in vitro predicted from the uptake clearance in SCHH (CL_H_vitro)

<table>
<thead>
<tr>
<th></th>
<th>CLH_P (ml·min⁻¹·kg⁻¹)</th>
<th>R_B (ml·min⁻¹·kg⁻¹)</th>
<th>CLH_vitro (5 h) (ml·min⁻¹·kg⁻¹)</th>
<th>CLH_vitro (96 h) (ml·min⁻¹·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurocholate</td>
<td>0.6⁷⁺</td>
<td>20.7⁷⁺</td>
<td>0.4⁺</td>
<td>9.50 (0.459)</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>4.83⁷⁺</td>
<td>0.6⁷⁺</td>
<td>8.06⁷⁺</td>
<td>0.18⁺</td>
</tr>
</tbody>
</table>

CL_H_vitro the hepatic clearance in vivo based on the plasma concentration was assessed as the difference between the total plasma clearance and the renal plasma clearance for each compound; R_B, ratio of the blood concentration to the plasma concentration of each compound; f_B, unbound fraction of each compound in the total blood.

* Footnotes:
1. Bloodplasma concentration ratio of these compounds in human was assumed to be 0.6.
2. Biliary clearance of taurocholate was assumed to be the same as the hepatic blood flow rate.
3. These are data for the blood unbound fraction of taurocholate in humans, so we used the value for rats (Inoue et al., 1985).
4. These data were obtained from the interview form of CRESTOR.


Address correspondence to: Dr. Yuichi Sugiyama, Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033 Japan. E-mail: sugiyama@mol.f.u-tokyo.ac.jp