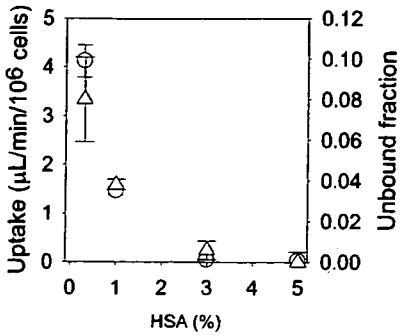

FIG. 1. Effect of various concentrations of human serum albumin on the uptake of tel-glu in isolated rat hepatocytes and the protein unbound fraction of tel-glu. △ and ○ represent the uptake of tel-glu into isolated rat hepatocytes (microliters per minute per 10^6 cells) and protein unbound fraction of tel-glu in the incubation media, respectively. Uptake of tel-glu was measured by incubating cells with 5 μM or 200 μM tel-glu, and the saturable uptake of tel-glu by isolated rat hepatocytes was determined by using eqs. 3 and 4. HSA concentrations used were 0.3, 1, 3, and 5%. Each point with vertical bar shows the mean ± S.E. of three separate determinations.

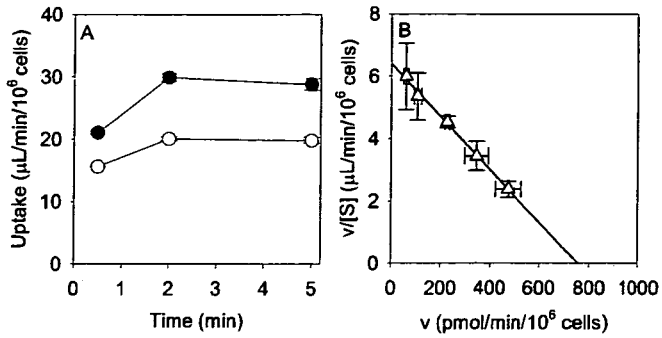

FIG. 2. Time profiles (A) and Eadie-Hofstee plot (B) of the uptake of tel-glu by isolated rat hepatocytes in the presence of 1% HSA. A, concentrations of tel-glu used were 2 (●) and 200 μM (○). B, the uptake of tel-glu in isolated rat hepatocytes as a function of a range of tel-glu concentrations (10–200 μM) was measured at a concentration between 10 and 200 μM tel-glu. The initial uptake rate of tel-glu in isolated rat hepatocytes was determined using (eq. 3). The solid line represents the fitted curve. Each point with bar represents the mean ± S.E. of three separate determinations.

inhibitors was obtained by examining their inhibitory effects on the uptake of CCK-8, $E_2$17βG, and tel-glu based on eq. 2:

$$CL_{+I} = CL \Big/ \left(1 + \frac{I}{IC_{50}}\right) \quad (2)$$

where CL and CL_{+I} represent the uptake clearance in the absence and presence of inhibitor, respectively, and I is the concentration of inhibitor. IC_{50} values were estimated by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981). To determine the saturable hepatic uptake clearance in human hepatocytes, we first determined the hepatic uptake clearance [$CL_{(2\ min-0.5\ min)}$] (microliters per minute per 10^6 cells) by calculating the slope of the uptake volume (V_d) (microliters per 10^6 cells) between 0.5 and 2 min (eq. 3). The saturable hepatic uptake clearance (CL_{hep}) was determined by subtracting $CL_{(2\ min-0.5\ min)}$ in the presence of an excess of cold substrate (excess) from that in the presence of tracer amount of substrate (tracer) (eq. 4).

$$CL_{(2\ min-0.5\ min)} = \frac{(V_{d,\,2\ min} - V_{d,\,0.5\ min})}{2-0.5} \quad (3)$$

$$CL_{hep} = CL_{(2\ min-0.5\ min),\,tracer} - CL_{(2\ min-0.5\ min),\,excess} \quad (4)$$

Results

Uptake of Tel-Glu into Isolated Rat Hepatocytes. First, we evaluated the involvement of transporters in the uptake of tel-glu by isolated rat hepatocytes. Because tel-glu is also anticipated to exhibit very high adsorption to the cell surface and/or culture materials as well as telmisartan (Ishiguro et al., 2006), we checked the effect of HSA on tel-glu uptake and measured the protein unbound fraction of tel-glu in incubation media in each condition before the uptake experiment with tel-glu. Tel-glu exhibited a very high apparent uptake, which may include nonspecific adsorption to cells, transporter-mediated uptake, and passive diffusion in the absence of HSA (tracer, 5 μM: 268 ± 5 μl/2 min/10^6 cells; excess, 200 μM: 200 ± 3 μl/2 min/10^6 cells). The apparent uptake was reduced with an increase in HSA concentration (Fig. 1). The protein unbound fractions of tel-glu in the incubation media with 0.3, 1, 3, and 5% HSA were reduced to 9.9, 3.5, 1.1, and 0.7%, respectively (Fig. 1). Both the apparent uptake into hepatocytes and the unbound fraction of tel-glu were reduced in parallel with increasing concentrations of HSA. This observation suggested that the saturable uptake of tel-glu followed the "free" hypothesis according to which only unbound ligand can be recognized by transporters (Fig. 1). Thus, the tel-glu uptake was assessed with at least 0.3% HSA in the incubation media to prevent the extensive adsorption of tel-glu to the cells and/or culture materials and to use the same experimental conditions as in the telmisartan study (Ishiguro et al., 2006). In the presence of 1% HSA, tel-glu was taken up into isolated rat hepatocytes in a linear fashion for up to 2 min. The uptake was reduced by the presence of an excess of tel-glu (200 μM), indicating that transporter(s) are involved in the tel-glu uptake by isolated rat hepatocytes (Fig. 2A). The concentration dependence of the uptake of tel-glu was studied over the concentration range of 10 to 200 μM in the presence of 1% HSA. An Eadie-Hofstee plot showed one saturable component (Fig. 2B), and the apparent K_m and V_{max} values for tel-glu uptake in the presence of 1% HSA were 118 ± 4 μM and 754 ± 12 pmol/min/10^6 cells, respectively. To evaluate the nonsaturable uptake of tel-glu, a tel-glu concentration of 200 μM was used, which is not high enough to saturate the transporter-mediated transport completely, but represents the maximum solubility of tel-glu in the incubation media. Depletion of Na^+ in the incubation media did not reduce the uptake of tel-glu (Fig. 3A). Although 10 μM pravastatin slightly stimulated the uptake of tel-glu, the uptake was inhibited by pravastatin, digoxin, and TCA, which are substrates and inhibitors of Oatp isoforms (Noe et al., 1997; Kouzuki et al., 1999; Tokui et al., 1999; Cattori et al., 2000; Sasaki et al., 2002, 2004), with IC_{50} values of 58.9 ± 26.7, 22.9 ± 9.8, and 7.12 ± 3.18 μM, respectively. An excess amount of TEA (1 mM), a typical substrate of organic cation transporters, caused at most a 30% reduction in the uptake of tel-glu (Fig. 3B). On the basis of these results, Oatp isoforms are considered to be involved in the tel-glu transport by isolated rat hepatocytes.

Uptake of Tel-Glu in Transporter-Expressing HEK293 Cells. To identify which transporters are important for the hepatic uptake of tel-glu in humans, uptake assays were performed using OATP1B1-, OATP1B3- and OATP2B1-expressing HEK293 cells. For these studies the HSA concentration was reduced from 1 to 0.3% in the incubation media. This was done because only minimal transport activity of $E_2$17βG, which is a typical ligand for OATP1B1, was detected in OATP1B1-expressing cells in the presence of 1% HSA owing to the significant reduction in its unbound concentration by binding to HSA (data not shown). Tel-glu was transported by OATP1B3 and OATP2B1 but not by OATP1B1 (Fig. 4A). The concentration dependence of the initial uptake of tel-glu by OATP1B3 and OATP2B1 was studied over the concentration ranges of 0.2 to 20 μM and 0.2 to 50 μM, respectively (Fig. 4B). The K_m and V_{max} values of OATP1B3- and OATP2B1-mediated tel-glu uptake were 3.40 ±

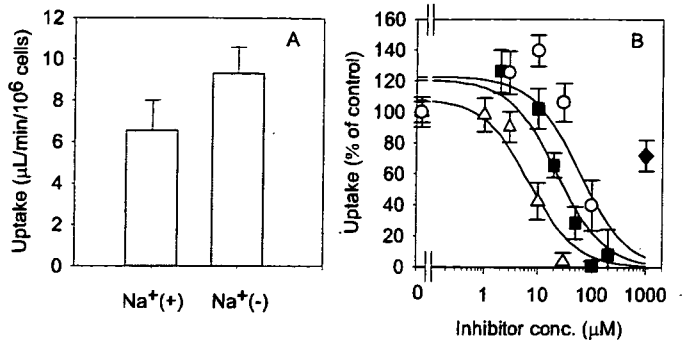

FIG. 3. Effect of Na^+ ion (A) and various compounds (B) on the uptake of tel-glu in isolated rat hepatocytes in the presence of 1% HSA. The concentration of tel-glu used was 10 μM. Saturable uptake of tel-glu by isolated rat hepatocytes was determined using eqs. 3 and 4. B, data are shown as the percentage of the saturable uptake of tel-glu in the absence of inhibitors. ■, △, ○, and ◆ represent the uptake of tel-glu in the presence of digoxin, taurocholate, pravastatin, and TEA, respectively. Solid lines represent the fitted curves obtained by nonlinear regression analysis. Each bar and vertical bar represents the mean ± S.E. of three separate determinations.

0.16 μM and 124 ± 4 pmol/min/mg of protein and 1.09 ± 0.10 μM and 22.3 ± 1.0 pmol/min/mg of protein, respectively.

Inhibitory Effect of E-sul on OATP1B3- and OATP2B1-Mediated Uptake of Tel-Glu in Transporter Expression Systems. We previously reported that E-sul is a selective inhibitor against OATP1B1 and that 30 μM E-sul completely inhibited OATP1B1-mediated $E_2$17βG uptake but did not inhibit OATP1B3-mediated CCK-8 uptake in transporter-expressing HEK293 cells (Ishiguro et al., 2006). In this study, we investigated the effects of E-sul on OATP1B3- and OATP2B1-mediated tel-glu uptake using OATP1B3- and OATP2B1-expressing HEK293 cells. E-sul hardly inhibited the OATP1B3- and OATP2B1-mediated tel-glu uptake with high IC_{50} values of 216 ± 28 and 223 ± 37 μM, respectively (Fig. 5).

Estimation of Relative Contributions of OATP1B1 and OATP1B3 to the Hepatic Uptake of Tel-Glu in Cryopreserved Human Hepatocytes. To confirm the minor contribution of OATP1B1 to overall hepatic tel-glu transport, three batches of cryopreserved human hepatocytes (Lots HH-OCF, HH-094, and HH-TDH) were used for the inhibition study. The uptake of 1 μM $E_2$17βG and 2 μM tel-glu by three different batches of cryopreserved human hepatocytes in the presence of 0.3% HSA was increased from 0.5 to 2 min [uptake of $E_2$17βG and tel-glu by cryopreserved human hepatocytes (HH-TDH): 3.40 ± 0.49 and 15.4 ± 2.5 μl/min/10^6 cells, respectively]. The uptake was reduced in the presence of an excess of unlabeled $E_2$17βG (200 μM) and tel-glu (50 μM) to 1.33 ± 0.30 and 4.05 ± 0.47 μl/min/10^6 cells, respectively (HH-TDH). The uptake of $E_2$17βG into human hepatocytes was inhibited by more than 50% by an E-sul concentration of 30 μM, whereas the uptake of tel-glu was hardly inhibited by 30 μM E-sul (Table. 1).

Expression of Human OATP1B3, MRP2, MDR1, and BCRP in MDCKII Cells. The expression of OATP1B3, MRP2, MDR1, and BCRP in the double-transfected MDCKII cell lines was confirmed by Western blot analysis (Fig. 6). In the parental MDCKII cells, no expression of OATP1B3, MRP2, MDR1, and BCRP was observed. The major band, which appeared at approximately 120 kDa, was detected in all kinds of OATP1B3-transfected cells (Fig. 6A), as

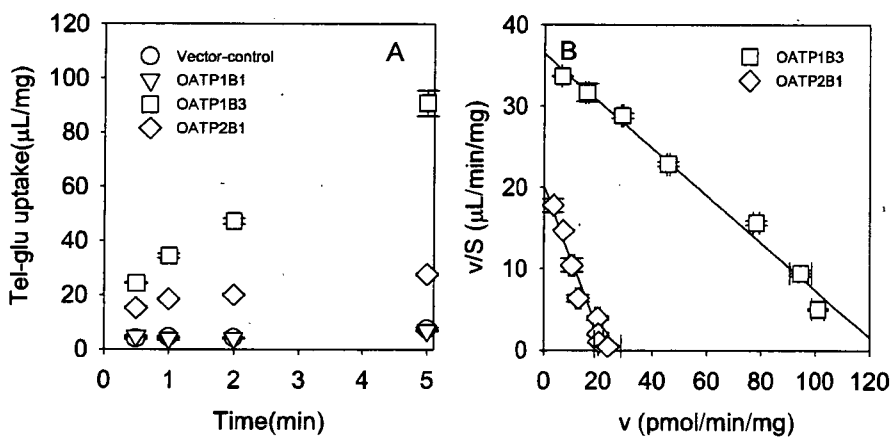

FIG. 4. Time profiles (A) and Eadie-Hofstee plots (B) of the uptake of tel-glu in transporter-expressing cells in the presence of 0.3% HSA. A, the concentration of tel-glu used was 2 μM. ▽, □, ◇, and ○ indicate the uptake of tel-glu by OATP1B1-, OATP1B3-, and OATP2B1-expressing cells and vector-transfected cells, respectively. B, uptake of tel-glu by OATP1B3-expressing cells (□) and OATP2B1-expressing cells (◇) was measured at a concentration between 0.2 and 20 μM for OATP1B3 and between 0.2 and 50 μM for OATP2B1. The transporter-mediated uptake was expressed by the difference in the uptake clearance between transporter-expressing cells and vector-transfected cells. Solid lines represent the fitted curves. Each point with bar represents the mean ± S.E. of three separate determinations.

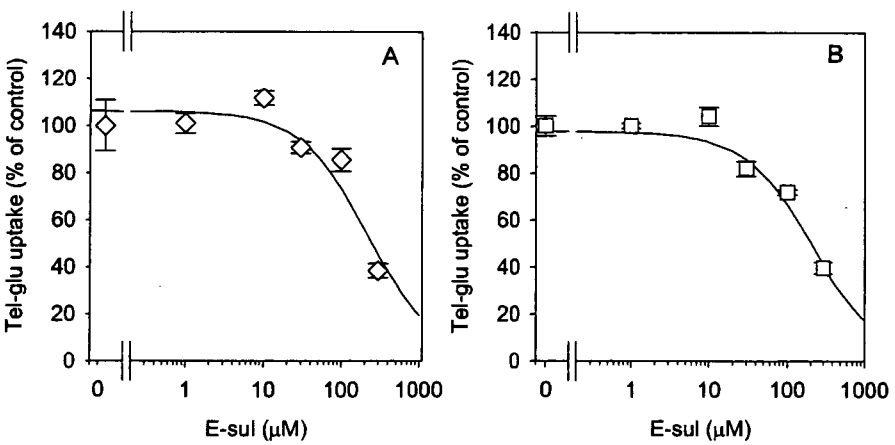

FIG. 5. The inhibitory effect of E-sul on OATP2B1 (A)- and OATP1B3 (B)-mediated tel-glu uptake in the presence of 0.3% HSA. The concentration of tel-glu used was 2 μM. The OATP2B1- and OATP1B3-mediated transport was obtained by subtracting the uptake in vector-transfected cells from that in OATP2B1- or OATP1B3-expressing cells for 2 min. The data are shown as the percentage of the OATP2B1- and OATP1B3-mediated tel-glu uptake in the absence of E-sul. The solid lines represent the fitted curves obtained by nonlinear regression analysis. Each point with bar represents the mean ± S.E. of three separate determinations.

TABLE 1

Effect of E-sul on the uptake of tel-glu and $E_2$17βG by cryopreserved human hepatocytes in the presence of 0.3% HSA

The substrate concentration used was 2 and 50 μM for tel-glu and 1 and 200 μM for $E_2$17βG. The saturable uptake of tel-glu and $E_2$17βG into cryopreserved human hepatocytes was determined after the subtraction of nonsaturable uptake (evaluated as the uptake clearance in the presence of 50 μM tel-glu or 200 μM $E_2$17βG). The term "% control" represents the ratio of the uptake clearance in the presence of 30 μM E-sul to that in its absence.

	HH-OCF		HH-094		HH-TDH	
	$E_2$17βG	Tel-Glu	$E_2$17βG	Tel-Glu	$E_2$17βG	Tel-Glu
			μl/min/10⁶ cells			
E-sul						
0 μM	5.66 ± 1.05[a]	12.8 ± 0.7	1.85 ± 0.72[a]	15.7 ± 0.7	2.08 ± 0.5	11.4 ± 2.5
30 μM	1.50 ± 0.75[a]	9.84	1.03 ± 0.76[a]	14.1 ± 1.0	0.0472 ± 0.1155	10.6 ± 0.4
% control	26.6%	76.9%	55.8%	89.9%	2.3%	93.0%

[a] Values from Ishiguro et al. (2006).

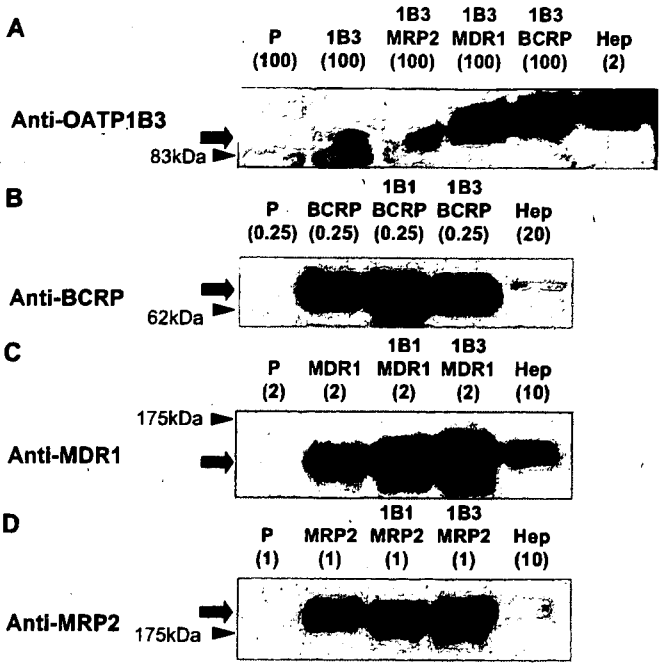

FIG. 6. Western blot analysis of OATP1B3 (A), BCRP (B), MDR1 (C), and MRP2 (D) in crude membrane vesicles obtained from MDCKII transfectants. Crude membrane prepared from MDCKII transfectants was separated by SDS-polyacrylamide gel electrophoresis, and each transporter was detected using antiserum or monoclonal antibody against respective transporter. The figures in parentheses represent the amount of protein applied to each lane (unit: micrograms). Arrows represent the specific bands for each transporter. P, parental MDCKII cells; Hep, hepatocytes (lot HH-OCF).

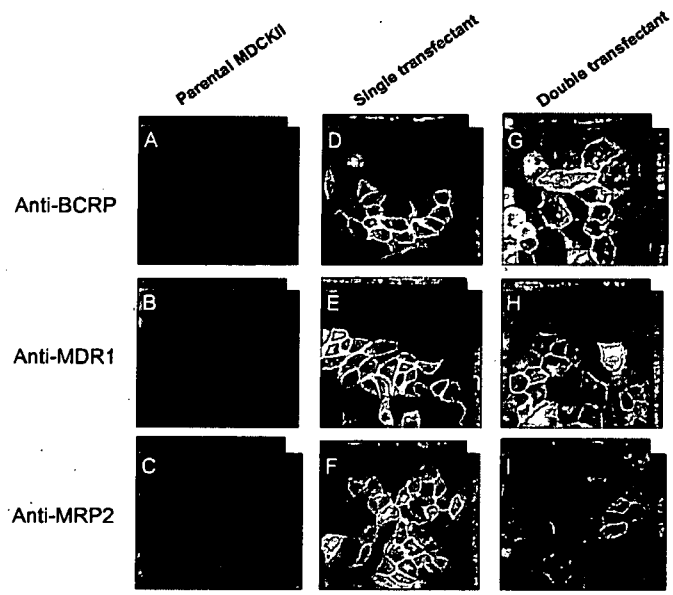

FIG. 7. Immunolocalization of recombinant OATP1B3, BCRP, MDR1, and MRP2 in MDCKII cells. MDCKII cells transfected with empty vector (A, B, and C), OATP1B3 (D, E, and F), both OATP1B3 and BCRP (G), both OATP1B3 and MDR1 (H), and both OATP1B3 and MRP2 (I) were stained with polyclonal antiserum against human OATP1B3 (green fluorescence, A–I), monoclonal antibody against human MRP2 (red fluorescence, C, F, and I), human MDR1 (red fluorescence, B, E, and H), and human BCRP (red fluorescence, A, D, and G). Nuclei were stained with TO-PRO-3 (blue fluorescence). Pictures are single optical sections (*x*,*y*) (center) with *xz* (top) and *yz* (right) projections, respectively.

shown previously (Hirano et al., 2004). The expression level of OATP1B3 in OATP1B3-transfected MDCKII cells per unit of protein was lower than that in cryopreserved human hepatocytes (Lot HH-OCF). We easily detected human MRP2, MDR1, and BCRP with apparent molecular masses of approximately 190, 170, and 70 kDa, respectively (Fig. 6, B–D). The MRP2, MDR1, and BCRP expression levels in double transfectants per unit of protein were almost comparable with those in single transporter-expressing cells and were much higher than those in cryopreserved human hepatocytes (Lot HH-OCF).

Localization of Recombinant Human OATP1B3, MRP2, MDR1, and BCRP. The cellular localization of the recombinant transporters in each transfectant was confirmed by confocal laser scanning microscopy (Fig. 7). OATP1B3 was correctly localized in the basolateral membrane of each transfectant expressing OATP1B3 (Fig. 7, D–I), whereas MDR1 and MRP2 were localized in the apical membrane (Fig. 7, H and I). BCRP was detected mainly in the apical membrane, but some fractions were also detected in the basolateral membrane (Fig. 7G).

Transcellular Transport of $E_2$17βG, CCK-8, and Tel-Glu across the MDCKII Monolayer Expressing Uptake and Efflux Transporters. To characterize the double-transfected MDCKII cells coexpressing OATP1B3 and MRP2, MDR1, and BCRP, we evaluated the transcellular transport of $E_2$17βG (OATP1B1/OATP1B3 bisubstrates) and CCK-8 and tel-glu (specific substrates of OATP1B3) across the MDCKII monolayer expressing uptake and efflux transporters (Figs. 8, 9, and 10). Significantly higher basal-to-apical transport of $E_2$17βG was observed in OATP1B1/MRP2, OATP1B1/MDR1, OATP1B1/BCRP, and OATP1B3/MRP2 double transfectants. However, such transport was not observed in parental MDCKII cells, single transfectants (OATP1B1, OATP1B3, MRP2, MDR1, and BCRP) and OATP1B3/MDR1 and OATP1B3/BCRP double transfectants (Fig. 8). Transcellular transport of the OATP1B3-selective substrate of CCK-8

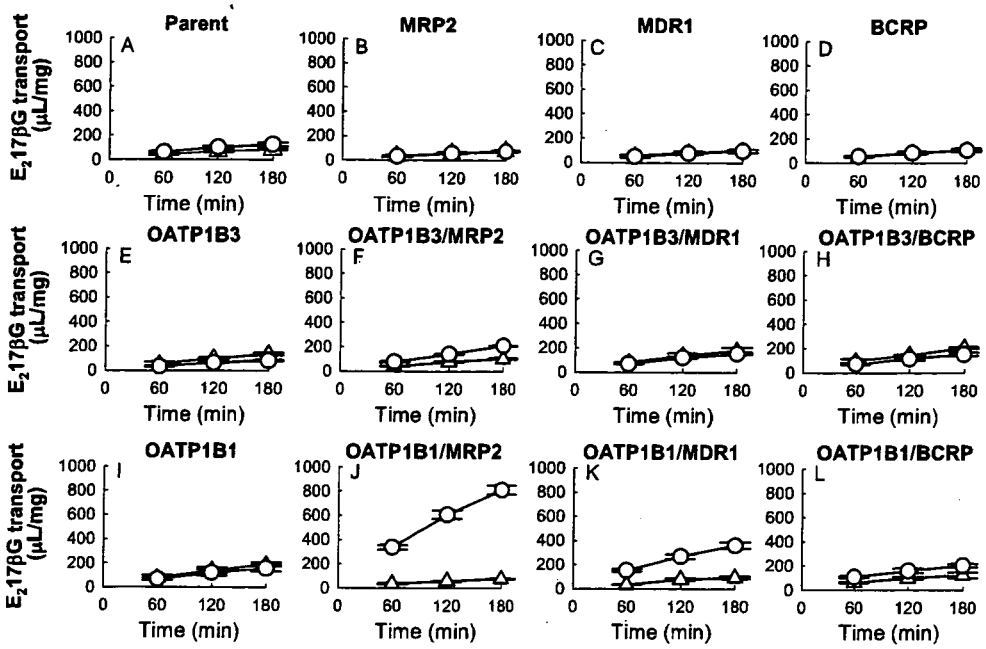

FIG. 8. Time profiles for the transcellular transport of $E_2$17βG across MDCKII monolayers. Transcellular transport of $E_2$17βG (0.1 μM) across MDCKII monolayers expressing MRP2 (B), MDR1 (C), BCRP (D), OATP1B3 (E), both OATP1B3 and MRP2 (F), both OATP1B3 and MDR1 (G), both OATP1B3 and BCRP (H), OATP1B1 (I), both OATP1B1 and MRP2 (J), both OATP1B1 and MDR1 (K), and both OATP1B1 and BCRP (L) was compared with that across the parental MDCKII monolayer (A). ○, transcellular transport in the basal-to-apical direction; △, transcellular transport in the apical-to-basal direction. Each point with vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

and tel-glu was also determined in the transporter-expressing MDCKII cells. The basal-to-apical vectorial transport of CCK-8 was significantly higher in the OATP1B3/MRP2 double transfectant than in the OATP1B3 single transfectant. However, no significant difference in vectorial transport was seen between OATP1B3-expressing cells and double transfectants expressing OATP1B3/MDR1 and OATP1B3/BCRP. In cell lines expressing OATP1B1 as an uptake transporter, no transcellular transport of CCK-8 was observed (Fig. 9). Before the transcellular transport study using tel-glu, the stability of tel-glu during the incubation was examined, and the decomposition from tel-glu into tel was negligible up to 3 h in the presence of 0.3% HSA. A higher basal-to-apical transport of tel-glu was found in all three cell lines expressing OATP1B3 and in the OATP1B1/MRP2 double transfectant. The vectorial transport in the double transfectants expressing OATP1B3 was higher than that in OATP1B3-expressing cells (Fig. 10).

Discussion

Although OATP1B1 and MRP2 are thought to play a major role in the hepatic transport of several organic anions (Evers et al., 1998; Cui et al., 1999; König et al., 1999; Vavricka et al., 2002; Hirano et al., 2004), the importance of OATP1B3, BCRP, and MDR1 has also recently been indicated. Previously we showed that double transfectants coexpressing OATP1B1 and an efflux transporter are a useful system to identify efflux transporters of organic anions in human liver (Matsushima et al., 2005). However, we found that some compounds such as fexofenadine and telmisartan are taken up into human hepatocytes mainly via OATP1B3 (Shimizu et al., 2005; Ishiguro et al., 2006). Telmisartan is excreted into bile predominantly as tel-glu (Wienen et al., 2000). To understand the pharmacokinetics of telmisartan, it is important to clarify the transport mechanisms of tel-glu as well as that of telmisartan. In the present study, we constructed a novel set of double-transfected cells expressing OATP1B3 and MDR1, MRP2, or BCRP and examined the transport mechanisms of tel-glu using rat and human hepatocytes, OATP transporter-expressing HEK293 cells, and double transfectants.

Previously, there were substantial difficulties to overcome in evaluating the transport of lipophilic telmisartan because of its extensive adsorption to cells (Ishiguro et al., 2006). Therefore, we examined whether tel-glu also exhibits this unfavorable property as does telmisartan. In the absence of HSA, the apparent uptake of tel-glu by isolated rat hepatocytes was very high and almost the same as that of telmisartan. To avoid the extensive adsorption of tel-glu to cells by HSA, we decided to use the same experimental conditions as for telmisartan, 1 and 0.3% HSA in the incubation media, for the further evaluation of tel-glu uptake by rat and human hepatocytes, respectively.

Because the involvement of Oatp isoforms on the hepatic uptake of tel-glu into isolated rat hepatocytes was anticipated by uptake experiments using isolated rat hepatocytes, we assessed which OATP transporters were involved in the human hepatic uptake of tel-glu by using cryopreserved human hepatocytes and transporter expression systems. Tel-glu was taken up by OATP1B3 and OATP2B1 but not by OATP1B1 (Fig. 4A). To confirm the minor contribution of OATP1B1 to the hepatic uptake of tel-glu, we performed an inhibition study using three batches of cryopreserved human hepatocytes and an OATP1B1-selective inhibitor, E-sul. Previously we reported that 30 μM E-sul can selectively inhibit the OATP1B1-mediated uptake in the presence of 0.3% HSA compared with OATP1B3 (Ishiguro et al., 2006). As a result, tel-glu was taken up into cryopreserved human hepatocytes in a saturable manner, and 30 μM E-sul did not significantly inhibit the uptake of tel-glu into all batches of cryopreserved human hepatocytes that we tested and OATP1B3- and OATP2B1-expressing HEK293 cells (Table 1; Fig. 5, A and B). These results confirmed the minor contribution of OATP1B1 to tel-glu uptake in cryopreserved human hepatocytes. The transport clearance (V_{max}/K_m) of tel-glu by OATP1B3-expressing cells was approximately 2-fold higher than that by OATP2B1-expressing cells. Hirano et al. (2004, 2006) reported that the ratio of the protein expression level of OATP2B1 in human hepatocytes to that in our expression system was less than 0.2, whereas for OATP1B3, that ratio was almost 1 by Western blot analysis. Our results suggested that the contribution of OATP2B1 to the hepatic uptake of tel-glu into human hepatocytes was at most one-tenth that of OATP1B3. Therefore, we concluded that tel-glu

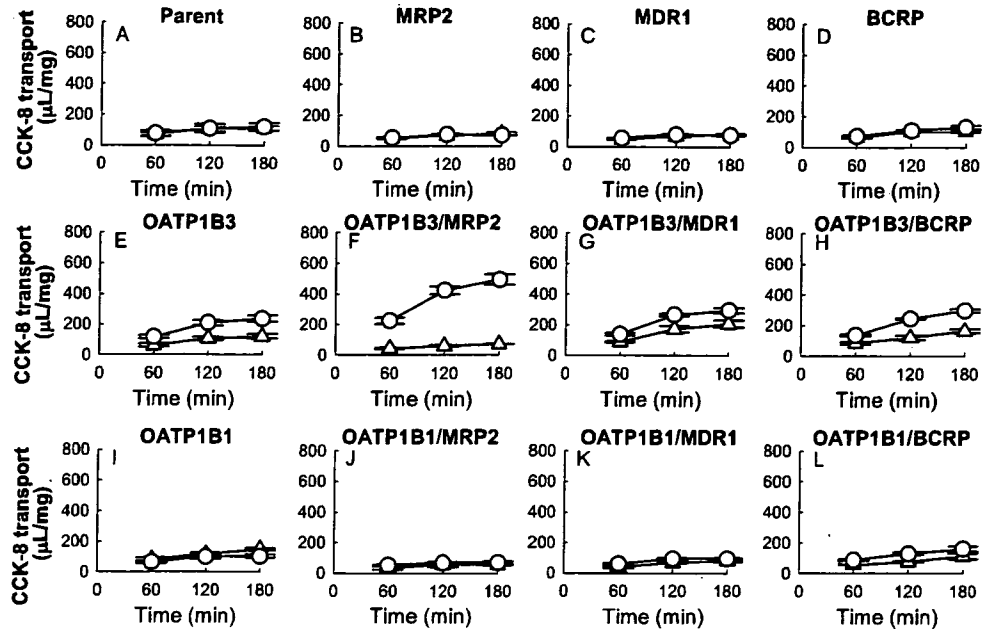

FIG. 9. Time profiles for the transcellular transport of CCK-8 across MDCKII monolayers. Transcellular transport of CCK-8 (0.1 μM) across MDCKII monolayers expressing MRP2 (B), MDR1 (C), BCRP (D), OATP1B3 (E), both OATP1B3 and MRP2 (F), both OATP1B3 and MDR1 (G), both OATP1B3 and BCRP (H), OATP1B1 (I), both OATP1B1 and MRP2 (J), both OATP1B1 and MDR1 (K), and both OATP1B1 and BCRP (L) was compared with that across the parental MDCKII monolayer (A). ○, transcellular transport in the basal-to-apical direction; △, transcellular transport in the apical-to-basal direction. Each point with vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

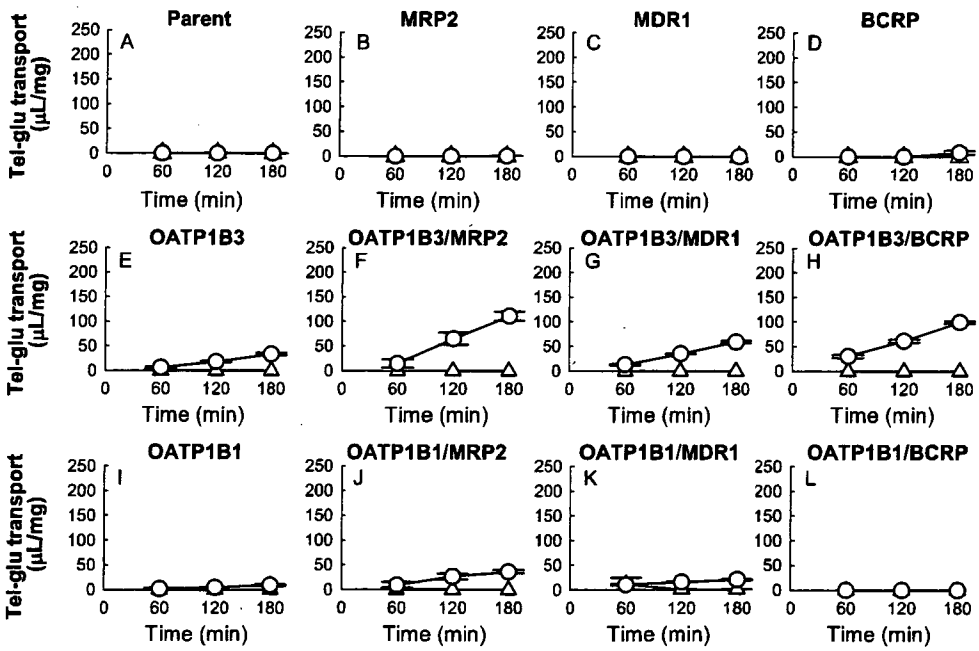

FIG. 10. Time profiles for the transcellular transport of tel-glu across MDCKII monolayers in the presence of 0.3% HSA. Transcellular transport of tel-glu (5 μM) across MDCKII monolayers expressing MRP2 (B), MDR1 (C), BCRP (D), OATP1B3 (E), both OATP1B3 and MRP2 (F), both OATP1B3 and MDR1 (G), both OATP1B3 and BCRP (H), OATP1B1 (I), both OATP1B1 and MRP2 (J), both OATP1B1 and MDR1 (K), and both OATP1B1 and BCRP (L) was compared with that across the parental MDCKII monolayer (A). ○, transcellular transport in the basal-to-apical direction; △, transcellular transport in the apical-to-basal direction. Each point with vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

is taken up into human hepatocytes predominantly by OATP1B3 as telmisartan.

Regarding the biliary excretion of tel-glu, we previously showed that tel-glu is transported by both MRP2 and another transporter(s) that are also expressed in Eisai hyperbilirubinemic rats (Nishino et al., 2000). Because of its extensive adsorption of tel-glu, it was not possible to assess its transport into canalicular membrane vesicles. CCK-8 is a recognized substrate of OATP1B3 and MRP2 (Ismair et al., 2001; Letschert et al., 2004, 2005), but the involvement of BCRP and MDR1 in its efflux remains to be investigated. To identify the efflux transporters of OATP1B3-selective substrates, CCK-8 and tel-glu, we established a set of novel double-transfected MDCKII cells coexpressing OATP1B3 and MDR1, MRP2, or BCRP and characterized their transcellular transports using several kinds of double transfectants.

Western blot and immunocytochemical analyses revealed that OATP1B3, MRP2, and MDR1 were expressed in MDCKII cells and localized correctly on the basolateral (OATP1B3) and apical membrane (MRP2 and MDR1), respectively. However, BCRP was localized mainly on the apical membrane and partially on the basolateral membrane (Figs. 6 and 7). This phenomenon was also observed in OATP1B1/BCRP double transfectants, but basal-to-apical transcellular transport of several compounds could be observed in these cell lines. This finding suggested that a minor distribution of BCRP on the basolateral side may not become a major concern to characterize efflux transport processes (Matsushima et al., 2005).

$E_2$17βG is a bisubstrate of OATP1B1 and OATP1B3, and its efflux is mediated by MRP2, MDR1, and BCRP (Hirano et al., 2004; Matsushima et al., 2005). No vectorial transport of $E_2$17βG was observed in MDCKII cells expressing only efflux transporter, whereas higher basal-to-apical transport was seen in double transfectants expressing OATP1B1 (Fig. 8) as had been reported previously (Matsushima et al., 2005). This result was most likely due to the limited access of $E_2$17βG inside the cells without the action of a suitable uptake transporter such as OATP1B1. Our findings showed that the transcellular transport of $E_2$17βG was significantly enhanced only in OATP1B3/MRP2 double transfectants among the three kinds of double transfectants expressing OATP1B3. This result can be explained by the previous findings that the transport clearance of $E_2$17βG by OATP1B3 is 7-fold lower than that by OATP1B1 (Hirano et al., 2004) and that $E_2$17βG is a good substrate of MRP2 compared with BCRP and MDR1 (Matsushima et al., 2005). In the case of CCK-8, a higher vectorial transport compared with the OATP1B3 single transfectant was observed only in OATP1B3/MRP2 double transfectants (Fig. 9). This finding indicated that CCK-8 was preferably transported by MRP2 rather than by MDR1 and BCRP, which was also in line with a previous report (Letschert et al., 2005).

In a following set of experiments, the vectorial basal-to-apical transcellular transport of tel-glu was observed in all kinds of double transfectants expressing OATP1B3, indicating that tel-glu was a substrate of MRP2, MDR1, and BCRP (Fig. 10). This result implies that other transporter(s), as was predicted by the previous study using Eisai hyperbilirubinemic rats (Nishino et al., 2000), might be MDR1 and/or BCRP. The vectorial transport of tel-glu was also seen in OATP1B1/MRP2 double transfectants (Fig. 10J) but not in MRP2-expressing cells (Fig. 10B), which was apparently inconsistent with the result that tel-glu was a specific substrate of OATP1B3. In our experiment, only a very small uptake of tel-glu by OATP1B1-expressing cells was observed, compared with that by vector-transfected cells in the absence of HSA (vector: 21.5 $\pm$ 0.9 μl/0.5 min/mg; OATP1B3: 193 $\pm$ 8 μl/0.5 min/mg; and OATP1B1: 36.2 $\pm$ 0.9 μl/0.5 min/mg). Because of the high sensitivity of the detection of transport in double-transfected cell lines, this observation may explain the apparent discrepancy.

Taken together, the results of this study using transporter expression systems and human hepatocytes suggested that tel-glu is taken up into human liver mainly via OATP1B3. For the more general purpose of the identification of efflux transporters of OATP1B3-selective substrates, we constructed a novel set of double transfectants coexpressing OATP1B3 and MDR1, MRP2, or BCRP. Telmisartan 1-*O*-acylglucuronide was excreted via MDR1, MRP2, and BCRP, although the relative quantitative contribution of each transporter has not been determined yet.

In consideration of the fact that OATP1B1 and OATP1B3 are responsible for the hepatic uptake of organic anions in human liver, a set of double transfectants expressing OATP1B3 combined with double transfectants expressing OATP1B1 can be used as a powerful tool for the rapid identification of efflux transporters of many organic anions that are substrates of OATP1B1 or OATP1B3.

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Involvement of multiple efflux transporters in hepatic disposition of fexofenadine

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Abbreviations: BCRP; breast cancer resistance protein; BSEP, bile salt export pump; CL, clearance; EG, estradiol-17β-D-glucuronide; EHBR, Eisai hyperbilirubinemic rat; FEX, fexofenadine; GFR, glomerular filtration rate; LUI, liver uptake index; Mate, multidrug and toxin compound extrusion; MDR, multidrug resistance; MPA, metaphosphoric acid; MRP, multidrug resistance-associated protein; OATP, organic anion transporting polypeptide; P-gp, P-glycoportein; TC, taurocholate

Abstract

Fexofenadine (FEX) is mainly eliminated from the liver into bile in unchanged form. We previously demonstrated that organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 are involved in the hepatic uptake of FEX. However, little is known about the mechanisms controlling the hepatic efflux of FEX from the liver to bile and blood. In the present study, the involvement of hepatic efflux transporters in the pharmacokinetics of FEX was investigated in both *in vitro* and *in vivo* studies. Vectorial transport of FEX was observed in OATP1B3/human bile salt export pump (hBSEP)-double transfectants, but not in OATP1B3/human breast cancer resistance protein (hBCRP)-double transfectants, which indicates the possible contribution of hBSEP to the biliary excretion of FEX in humans. In multidrug resistance associated protein 2 (Mrp2) (-/-) mice, the biliary excretion clearance based on the plasma concentration and the liver-to-plasma concentration ratio significantly decreased, whereas the biliary excretion clearance based on the liver concentration decreased only with 20%, suggesting the minimum contribution of Mrp2 to its biliary excretion. ATP-dependent transport of FEX was observed in hMRP3-enriched membrane vesicles, but not hMRP4. In Mrp3 (-/-) mice, the biliary excretion clearance based on both the plasma and liver concentration, and the liver-to-plasma concentration ratio increased, suggesting the significant contribution of Mrp3 to its sinusoidal efflux and the up-regulation of its biliary excretion in Mrp3 (-/-) mice. On the other hand, pharmacokinetics of FEX remained unchanged in Mrp4 (-/-) mice. This information provides a novel insight into the transporters important for FEX disposition.

Introduction

Fexofenadine (FEX) is an orally active nonsedating histamine H_1-receptor antagonist that is prescribed for oral treatment of allergic rhinitis and chronic idiopathic urticaria. After oral administration of [^{14}C]FEX to healthy volunteers, 80% of the dose was recovered in feces and 12% in urine, in unchanged form (Lippert et al., 1995). Since the absolute oral bioavailability of FEX is reported to be 33% (product information, Hoechst Marion, Roussel, Laval, Quebec, Canada), it follows that two-thirds of the bioavailable FEX is excreted into bile. Accordingly, hepatic transport of FEX is one of the determinants for its systemic clearance.

Previously, we demonstrated that human organic anion transporting polypeptide 1B1 (hOATP1B1/SLCO1B1) and hOATP1B3 (SLCO1B3) contribute to the hepatic uptake of FEX in humans (Shimizu et al., 2005; Matsushima et al., *submitted*). On the other hand, the transporters involved in its biliary excretion have not been clarified yet. In the canalicular membrane, several ATP-binding cassette transporters such as multidrug resistance-associated protein 2 (MRP2/ABCC2), P-glycoprotein/multidrug resistance 1 (P-gp/MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and bile salt export pump

(BSEP/ABCB11), are involved in the excretion of several compounds. MRP2 is responsible for the biliary excretion of a wide variety of organic anions including glutathione- and glucuronide-conjugates and drugs such as pravastatin (Suzuki and Sugiyama, 1998). MDR1 preferentially accepts hydrophobic cationic and neutral compounds (Hoffmann and Kroemer, 2004), while BCRP accepts various kinds of organic anions (Hirano et al., 2005b; Merino et al., 2005; Suzuki et al., 2003; van Herwaarden et al., 2003). Though BSEP was thought to accept only bile salts (Byrne et al., 2002), recent studies indicate that BSEP transports some drugs such as vinblastine and pravastatin (Hirano et al., 2005a; Lecureur et al., 2000).

It has been shown that FEX is a substrate of P-gp and hMRP2 (Cvetkovic et al., 1999; Matsushima et al., *submitted*), whereas nobody has checked whether FEX is a substrate of hBCRP and hBSEP. Tahara et al. (2005) investigated biliary excretion of FEX using Eisai hyperbilirubinemic rats (EHBRs/Mrp2-deficient rats), and Mdr1a/1b (-/-) and Bcrp1 (-/-) mice. Surprisingly, these transporters didn't have an effect on FEX biliary excretion clearance based on the liver concentration (Tahara et al., 2005). These results suggest that the biliary excretion of FEX is mediated by unknown transporters

distinct from rat Mrp2 (rMrp2), mouse Mdr1a/1b (mMdr1a/1b) and mBcrp1. However, there may be a species difference in the mechanisms of FEX biliary excretion between rats and mice. Recently, Mrp2 (-/-) mice have been established and the impact of mMrp2 on the pharmacokinetics of some drugs and toxins has been characterized (Chu et al., 2006; Vlaming et al., 2006). Therefore, to clarify the biliary excretion mechanisms of FEX in greater detail, we investigated whether FEX is accepted by hBCRP and hBSEP/rBsep in *in vitro* studies and demonstrated the importance of mMrp2 in its biliary excretion using Mrp2 (-/-) mice.

On the other hand, it has become clear that MRP3 (ABCC3) and MRP4 (ABCC4) are important transporters in sinusoidal efflux (Borst et al., 2007). MRP3 can transport a wide variety of organic anions, such as glucuronides, glutathione-conjugates, bile acids and methotrexate (Hirohashi et al., 1999; Hirohashi et al., 2000; Kool et al., 1999; Zelcer et al., 2001; Zeng et al., 2001). Because rMrp3 is expressed at low levels in normal rat liver and its expression markedly increases in EHBRs (Hirohashi et al., 1998), the physiological role of rMrp3 has been thought to be the protection of hepatocytes from intrahepatic toxins such as bile acids only under pathological conditions (*e.g.* cholestatis).

Recent *in vivo* studies using Mrp3 (-/-) mice suggest that mMrp3 contributes to sinusoidal efflux of various glucuronide conjugates (Borst et al., 2007). The substrate specificity of MRP4 overlaps with that of MRP3, but it is somewhat distinguished from MRP3 by its ability to transport nucleotide analogues (Reid et al., 2003; van Aubel et al., 2002). The physiological function of MRP4 in hepatocytes is considered to protect hepatocytes from bile acids under cholestatic conditions as if it is upregulated during cholestasis (Keitel et al., 2005; Mennone et al., 2006). Since both hMRP3 and hMRP4 are expressed in human liver under physiological conditions (Konig et al., 1999; Rius et al., 2003), these transporters may also be involved in the hepatic distribution of drugs. Accordingly, we investigated the role of MRP3 and MRP4 in the sinusoidal efflux of FEX using hMRP3- and hMRP4-enriched membrane vesicles and Mrp3 (-/-) and Mrp4 (-/-) mice.

Materials and Methods

Materials.

[^{3}H]estradiol-17β-D-glucuronide (EG; 45 Ci/mmol) and [^{3}H]taurocholate (TC; 3.5 Ci/mmol) were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). [^{3}H]pitavastatin (44.6 Ci/mmol) was donated by Kowa Co. Ltd. (Tokyo, Japan). FEX hydrochloride was purchased from Toronto Research Chemicals (North York, ON, Canada). All other chemicals and reagents were of analytical grade and commercially available.

Animals.

Male FVB mice (wild type) and Mrp2 (-/-) mice were described previously (Vlaming et al., 2006). Male FVB mice (wild type) and Mrp3 (-/-) mice were kindly donated by Dr. P. Borst (Division of Molecular Biology and Cancer of Biomedical Genetics, The Netherlands Cancer Institute) (Zelcer et al., 2006). Male C57BL/6 mice (wild type) and Mrp4 (-/-) mice were kindly donated by Dr. J. D. Schuetz (Department of Pharmaceutical Sciences, St Jude Children's Research Hospital, Memphis, Tennessee, USA) (Leggas et al., 2004). All animals were maintained under standard conditions with a reverse dark-light

cycle and were treated humanely. Food and water were available ad libitum. All the animal studies performed in this paper were approved by the Institutional Animal Care Committee and carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

Cell culture.

hOATP1B3-expressing MDCKII cells and vector-transfected control cells used in this study were constructed previously (Ishiguro et al., *submitted*). Transporter-expressing, vector-transfected MDCKII, or parent HEK293 cells were grown in Dulbecco's modified Eagle's medium (low glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich) at 37 °C under 5% CO_2 and 95% humidity.

Construction of hBSEP- and hBCRP-expressing cells.

To construct MDCKII cells expressing hBSEP and hBCRP, MDCKII cells were infected with recombinant adenovirus containing hBSEP and hBCRP cDNA

at a multiplicity of infection of 150, 48 hr prior to all experiments. The virus titer was determined as described previously (Hayashi et al., 2005).

Transcellular transport study.

The transcellular transport study was performed as reported previously (Matsushima et al., 2005). Briefly, MDCKII cells were grown on Transwell membrane inserts (6.5 mm diameter, 0.4 μm pore size; Corning Coster, Bodenheim, Germany) at confluence for 7 days, and the expression level of transporters was induced by the replacement of culture medium with that supplemented with 5 mM sodium butyrate 48 hr before the transport study. Cells were first washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4) at 37 °C. Subsequently, Krebs-Henseleit buffer containing substrates was added either to the apical compartments (250 μL) or the basolateral compartments (1 mL). After a designated period, 50 μL of medium was taken from the opposite side to the added substrate. When using FEX as a substrate, 50 μL aliquots were used for LC/MS quantification as described below. When using [^{3}H]pitavastatin

as a substrate, the radioactivity in the sample was measured in a liquid scintillation counter (LS 6000SE; Beckman Coulter, Fullerton, CA). At the end of the experiments, cells were washed with ice-cold Krebs-Henseleit buffer and solubilized in 500 μL 0.2 N NaOH. After addition of 100 μL 1 N HCl, 50 μL aliquots were used to determine protein concentrations by the method of Lowry with bovine serum albumin as a standard.

Transport studies with membrane vesicles.

Membrane vesicles were prepared from human BSEP-, rat Bsep-, human MRP3- and human MRP4-transfected HEK293 cells according to the method described previously (Hayashi et al., 2005; Hirouchi et al., *submitted*). The transport studies were performed using a rapid filtration technique. Briefly, 15 μL transport medium (10 mM Tris-HCl, 250 mM sucrose, and 10 mM $MgCl_2$, pH 7.4) containing FEX, EG, or TC was preincubated at 37 °C for 3 min and then rapidly mixed with 5 μL membrane vesicle suspension (10 μg, time course study or 15 μg, saturation study of protein). The reaction mixture contained 5 mM ATP or AMP, along with the ATP-regenerating system (10 mM creatinine phosphate and 100 μg/μL creatinine phosphokinase). The transport reaction

was terminated by the addition of 1 mL ice-cold stop solution (containing 10 mM Tris-HCl, 250 mM sucrose, and 0.1 N NaCl, pH 7.4). The reaction mixture was passed through a 0.45-μm HA filter (Millipore Corporation, Billerica, MA) and then washed twice with 5 mL stop solution. FEX retained on the filter was then quantified by LC/MS as described below. In the case of [^{3}H]EG and [^{3}H]TC, filters with trapped membrane vesicles were mixed with scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan), and the radioactivity retained on the filter was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter Inc., Fullerton, CA).

Intravenous constant infusion studies in mice

Mice weighing approximately 24 to 32 g were used throughout the experiments. Under anesthesia with pentobarbital sodium (Nembutal, Dainippon Pharmaceutical, Osaka, Japan), the jugular vein was cannulated with a polyethylene catheter (PE-10; Becton Dickinson, Sparks, MD) for the injection of FEX. The bile duct was cannulated with a teflon tube (UT-3; Unique Medical, Tokyo, Japan) for bile collection and the urinary bladder was cannulated with a teflon tube (industrial use) for urine collection. The mice received a constant

infusion of FEX at a dose of 623 to 804 nmol/hr/kg b.w. for 180 min (Harvard Apparatus syringe infusion pump; Harvard Apparatus Inc., Holliston, MA). Since mice were anesthetized throughout the experiment, they were kept warm with a hot plate for experimental animals (Natsume Seisakusyo, Tokyo, Japan). Bile and urine were collected in pre-weighed test tubes at 20-min intervals throughout the experiment. Blood samples (approximately 30 μL) were collected from the jugular vein at 120, 140, 160, and 180 min after starting the infusion. Plasma was prepared by centrifuging the blood samples (3000 g). The mice were sacrificed after 180 min, and the entire liver, kidney, and brain were excised immediately. The tissues were weighed and stored at –80 °C until the assay.

Kinetic analyses in the infusion study.

The steady-state plasma concentration (C_{ss}) was assessed as the mean plasma concentration at 120, 140, 160, and 180 min, whereas the steady-state liver (C_{liver}), kidney (C_{kidney}), and brain (C_{brain}) concentrations were determined at 180 min. The total plasma clearance ($CL_{tot,plasma}$) was obtained by dividing the infusion rate by C_{ss}. The biliary and urinary clearances ($CL_{bile,plasma}$,