$CL_{urine,plasma}$) gave the mean clearance values calculated by dividing the biliary and urinary excretion rates (V_{bile}, V_{urine}) by C_{ss}. $CL_{bile,liver}$ was the biliary clearance based on C_{liver}. The $K_{p,liver}$, $K_{p,kidney}$, and $K_{p,brain}$ represented the ratio of C_{liver}, C_{kidney}, and C_{brain} to C_{ss} ,respectively.

Liver uptake index (LUI) study.

Under anesthesia with pentobarbital sodium (Nembutal), the portal vein of male FVB and Mrp3 (-/-) mice (weighing 28 to 32 g), was cannulated with polyethylene tubing (PE-10). FEX dissolved in mouse plasma was rapidly injected into the portal vein. At 17 sec after the bolus administration of FEX (10 nmol/kg b.w.), which is long enough for the bolus to pass completely through the liver but short enough to prevent recirculation of the compound, the portal vein, hepatic artery, and bile duct were cut and the liver was excised. The tissue was weighed and stored at –80 °C until assay.

LC/MS analyses.

Sample pretreatment. The aliquots (50 µL) obtained from the transcellular transport study were precipitated with 200 µL methanol containing

10 nM midazolam as an internal standard. After centrifugation (15000 g, 10 min, 4 °C) of the mixture, 50 μL 0.05% formic acid was added to 50 μL supernatant. In the membrane vesicle studies, FEX retained on the filter was recovered in 1 mL methanol containing 1 nM midazolam as an internal standard by sonication for 15 min. After centrifugation, the supernatants (750 μL) were evaporated using a centrifugal concentrator (CC-105; TOMY, Tokyo, Japan), and dissolved in 100 μL mobile phase (see the section of "*LC/MS Instrumentation and Operating Conditions*"). Plasma (5 μL) obtained from the infusion study was mixed with 15 μL 0.05% vol. formic acid and precipitated with methanol (80 μL) containing midazolam (50 nM) as an internal standard. Bile (2 μL) obtained from the infusion study was mixed with 48 μL 0.05% vol. formic acid. Then, 90 μL 0.05% vol. formic acid was added to 10 μL of the mixed solution and precipitated with methanol (250 μL) containing the internal standard. Urine (10 μL) obtained from the infusion study was precipitated with methanol (500 μL) containing midazolam (internal standard). Liver, kidney, and brain obtained from the infusion study or the LUI study were added to a 3-fold volume of PBS and homogenized with a handy-type homogenizer (Multipro 395; Dremel Corp., Racine, WI). 50 μL homogenate obtained from the liver and kidney was

precipitated with methanol (750 μL) containing midazolam (internal standard) and then centrifuged, and the supernatant was diluted with an equal volume of 0.05% vol. formic acid. 50 μL homogenate obtained from the brain was precipitated with methanol (500 μL) containing 5 nM midazolam as an internal standard and centrifuged, then the supernatant (400 μL) was evaporated using a centrifugal concentrator (CC-105), and dissolved in 80 μL mobile phase. The obtained samples were subjected to the LC/MS analysis to determine the concentration of FEX.

LC/MS Instrumentation and Operating Conditions. An LC/MS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. The samples were separated on a CAPCELL PAK C18 MG column (3 μm, 4.6 mm ID, 75 mm, Shiseido, Tokyo, Japan) in binary gradient mode. The mobile phase consisted of 0.05% formic acid and methanol. The methanol concentration was initially 48%, then linearly increased up to 61.5% over 4.5 min. Finally, the column was re-equilibrated at a methanol concentration of 48% for 3 min. The total run time was 7.5 min. FEX and midazolam were eluted at 4.1 min and 2.8 min, respectively. In the mass analysis, FEX and midazolam were detected at a mass-to charge ratio of 502.3

and 326.1 under positive ionization conditions. The interface voltage was 3.5 kV, and the nebulizer gas (N_2) flow was 1.5 L/min. The heat block and curved desolvation line temperatures were 200 and 150 °C, respectively.

Quantification of mRNA and protein expression levels of the hepatic transporters in mice.

Total RNA was isolated from the livers of three wild-type FVB mice and Mrp3 (-/-) mice using ISOGEN (Nippon Gene, Tokyo, Japan) and converted to cDNA using a random primer. Real-time quantitative PCR was performed using a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) and LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' instructions. The primers used in the quantification are listed in Table 1. G3pdh was used as a housekeeping gene for the internal standard. An external standard curve was generated by dilution of the target PCR product which was purified by agarose gel electrophoresis. The absolute concentration of external standard was measured by PicoGreen dsDNA Quantification Reagent (Molecular Probes, Eugene, OR). To confirm the amplification specificity, PCR products were subjected to a melting curve analysis and gel

electrophoresis. All gene expressions in each reaction were normalized by the expression of G3pdh in the same sample.

For Western blot analyses, crude membrane was prepared from the livers of five wild-type FVB and Mrp3 (-/-) mice according to the method used in the previous report (Niinuma et al., 1999). After the crude membrane was suspended in PBS, it was frozen in liquid N_2 and stored at –80 °C until used. The protein concentration in the crude membrane vesicles was determined by the method of Lowry with bovine serum albumin as a standard. The membrane fraction was dissolved in 3 x SDS sample buffer (New England Biolabs, Beverly, MA) and loaded on to a 7% or 12.5% SDS-polyacrylamide electrophoresis gel with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England Biolabs). Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-lad, Hercules, CA) at 15 V for 1 hr. The membrane was blocked with PBS containing 5% skimmed milk overnight at 4 °C. After washing with Tris-buffered saline with 0.05% Tween 20 (TTBS), the membrane was incubated at room temperature in PBS containing 5% skimmed milk with 125-fold diluted anti-Mrp2 monoclonal antibody (M_2III-6; Alexis,

Gruenberg, Germany) for 2 hr, 100-fold diluted anti-Mdr1 monoclonal antibody (C219; Signet, Dedham, MA) for 1 hr, 200-fold diluted anti-Bcrp monoclonal antibody (BXP-53; Signet) for 2 hr, 500-fold diluted anti-Bsep polyclonal antibody (Santa Cruz, California, CA) for 3 hr, 1000-fold diluted anti-Mrp3 polyclonal antibody for 2 hr (Akita et al., 2002), 100-fold diluted anti-Mrp4 monoclonal antibody (M_4I-10; Abcam, Cambridge, UK) for 1 hr, or 1000-fold diluted anti-mouse β-actin monoclonal antibody (Millipore) for 2 hr. For the detection of mMrp2, mMdr1, mBcrp, mMrp4, and mβ-actin, the membrane was placed in contact with 1000-fold diluted Alexa Flour 680 goat anti-mouse IgG (Molecular Probes) for 1 hr. For the detection of mMrp3, the membrane was placed in contact with 1000-fold diluted Alexa Flour 680 goat anti-rabbit IgG (Molecular Probes). For the detection of mBsep, the membrane was placed in contact with 1000-fold diluted Alexa Flour 680 donkey anti-goat IgG (Molecular Probes). The fluorescence was assessed in a densitometer (Odessey, ALOKA, Tokyo, Japan).

Measurement of the concentration of total bile acids and GSH in the liver and bile in FVB mice and Mrp3 (-/-) mice.

Mice of both strains, weighing approximately 30g, were used throughout the experiments. Under anesthesia with pentobarbital sodium (Nembutal, Dainippon Pharmaceutical), the bile duct was cannulated with a teflon tube (UT-3) for bile collection. Bile was collected in pre-weighed test tubes for a designated time. For the measurement of GSH, bile was collected in tubes filled by 5% metaphosphoric acid (MPA)-dissolved solution. After collecting bile, the mice were sacrificed and the entire liver was excised immediately. The liver was weighed and a part of it was placed in MPA solution (for the measurement of GSH) and the remainder was placed in PBS. Each tissue was homogenized using a handy-type homogenizer (Multipro 395). The concentrations of total bile acids and GSH in bile and liver homogenate were measured using assay kits (total bile acids; Wako, Osaka, Japan: GSH; Oxis, Portland, OR).

Statistical Analyses

Statistical differences were analyzed by using Student's *t* test to identify significant differences between two sets of data. Significant differences were

considered to be present at $p<0.05$.

Results

Transcellular transport of FEX and pitavastatin across the MDCKII cell monolayer.

In order to examine whether FEX is a substrate of hBSEP and hBCRP, transcellular transport of 5 μM FEX across the MDCKII monolayer was determined in hOATP1B3/hBSEP- and hOATP1B3/hBCRP-double transfectants. The basal-to-apical transport of FEX was approximately 2.6 times greater than that in the opposite direction in hOATP1B3/hBSEP double transfectants (Figure 1 D), whereas the difference in each direction of transport of FEX was no more than 2-fold in control cells, and hOATP1B3- and hBSEP-single transfectants (Figure 1 A-C). The difference in each direction of transport of FEX was no more than 2-fold in control cells, and hOATP1B3-, hBCRP-, and hOATP1B3/hBCRP-transfectants (Figure 1 E-H). On the other hand, the basal-to-apical transport of 0.1 μM pitavastatin, a bisubstrate of hOATP1B3 and hBCRP (Hirano et al., 2005b), was approximately 2.6 and 2.9 times greater than that in the opposite direction in transfectants expressing hBCRP and hOATP1B3/hBCRP (Figure 1 K, L), respectively, whereas the difference in each direction of transport of pitavastatin was no more than 2-fold in control cells and

hOATP1B3 transfectants (Figure 1 I, J).

ATP-dependent transport of FEX by hBSEP and rBsep.

In order to examine the substrate specificity of hBSEP and rBsep for FEX, membrane vesicles were prepared from HEK293 cells either infected with recombinant adenovirus harboring hBSEP, rBsep, or GFP cDNA. The uptake clearance of 0.1 μM TC as a positive control by hBSEP and rBsep after a 2-min incubation at 37 °C in the presence of ATP or AMP was 788 and 43.6 μL/mg protein (hBSEP; mean, n=2), 378 and 43.3 μL/mg protein (rBsep; mean, n=2), respectively. The time-dependent uptake of 10 μM FEX by hBSEP- and rBsep-enriched membrane vesicles is shown in Figure 2 A and B. The uptake of FEX was significantly stimulated by ATP in membrane vesicles prepared from hBSEP- and rBsep-enriched cells but not in those from HEK293 cells infected with GFP cDNA-harboring recombinant adenovirus. The concentration-dependent uptake of FEX is shown in Figure 2 C and D. The uptake clearance in the presence of ATP was saturated in hBSEP- and rBsep-enriched membrane vesicles. However, the Michaelis constant (K_m) could not be evaluated because we could not measure the uptake clearance at

less than 3 μM because this was below the detection limit.

Steady-state pharmacokinetics of FEX in wild type FVB and Mrp2 (-/-) mice.

Although the previous study suggests that rMrp2 is not involved in the biliary excretion of FEX, there may be a species difference in the mechanism of FEX biliary excretion between rats and mice. Therefore, intravenous constant infusion into wild type FVB and Mrp2 (-/-) mice was performed. The plasma concentration, biliary excretion, and urinary excretion of FEX at steady state in wild type FVB and Mrp2 (-/-) mice are shown in Figure 3. The pharmacokinetic parameters are summarized in Table 2. The plasma concentrations of FEX reached steady-state within 120 min during the constant infusion to both strains of mice (Figure 3 A). The C_{ss} in Mrp2 (-/-) mice significantly increased and the $CL_{tot,plasma}$ significantly decreased compared with the values for FVB mice ($p<0.05$). The $CL_{bile,plasma}$ and $K_{p,liver}$ in Mrp2 (-/-) mice significantly decreased compared with that for FVB mice ($p<0.01$), whereas the $CL_{bile,liver}$ in Mrp2 (-/-) mice slightly decreased by 20% in comparison with FVB mice, although the difference was not statistically significant. There were no statistically significant differences in the other parameters.

ATP-dependent transport of FEX by hMRP3 and hMRP4.

In order to examine whether sinusoidal efflux transporters, hMRP3 and hMRP4, can accept FEX as a substrate, membrane vesicles were prepared from HEK293 cells infected with recombinant adenovirus harboring hMRP3, hMRP4, or GFP cDNA. As a positive control, the uptake of 0.1 μM EG by hMRP3 and hMRP4 after a 2-min incubation at 37 °C in the presence of ATP or AMP was 298 and 3.26 μL/mg protein (hMRP3; mean, n=2), 170 and 5.17 μL/mg protein (hMRP4; mean, n=2), respectively. The time-dependent uptake of 10 μM FEX by hMRP3- and hMRP4-enriched membrane vesicles is shown in Figure 4 A and B. The uptake of FEX was significantly stimulated by ATP in membrane vesicles prepared from only hMRP3-expressing cells, but not in those from hMRP4- and GFP-expressing cells. The concentration-dependent uptake of FEX is shown in Figure 4 C. The uptake clearance in the presence of ATP was saturated in hMRP3-enriched membrane vesicles. However, the Michaelis constant (K_m) could not be evaluated because we could not measure the uptake clearance at less than 3 μM because this was below the detection limit.

Steady-state pharmacokinetics of FEX in wild type FVB mice and Mrp3 (-/-) mice.

In order to investigate the effect of mMrp3 on the pharmacokinetics of FEX in *in vivo*, intravenous constant infusion into Mrp3 (-/-) mice was performed. The plasma concentration, biliary excretion, and urinary excretion of FEX at steady state in wild type FVB and Mrp3 (-/-) mice are shown in Figure 5 and the pharmacokinetic parameters are summarized in Table 3. The plasma concentrations of FEX reached steady-state within 120 min during the constant infusion to both strains of mice (Figure 5 A). The C_{ss} in Mrp3 (-/-) mice significantly decreased and the $CL_{tot,plasma}$ significantly increased compared with that in FVB mice ($p<0.01$). The $CL_{bile,plasma}$ and $K_{p,liver}$ in Mrp3 (-/-) mice significantly increased compared with that in FVB mice ($CL_{bile,plasma}$, $p<0.01$; $K_{p,liver}$, $p<0.05$) and the $CL_{bile,liver}$ in Mrp3 (-/-) mice significantly increased ($p<0.01$). There were no statistically significant differences in the other parameters.

Steady-state pharmacokinetics of FEX in wild type C57BL/6 mice and Mrp4 (-/-) mice.

In order to investigate the effect of mMrp4 on the pharmacokinetics of FEX in *in vivo*, intravenous constant infusion into Mrp4 (-/-) mice was performed. The plasma concentration, biliary excretion, and urinary excretion of FEX at steady-state in wild type C57BL/6 and Mrp4 (-/-) mice are shown in Figure 6 and the pharmacokinetic parameters are summarized in Table 4. The plasma concentrations of FEX reached steady-state within 120 min during the constant infusion to both strains of mice (Figure 6 A). There were no statistically significant differences in any of the evaluated parameters.

LUI study in wild type FVB mice and Mrp3 (-/-) mice.

The $CL_{bile,plasma}$ and $K_{p,liver}$ in Mrp3 (-/-) mice significantly increased compared with that in FVB mice as mentioned previously. One of the possible reasons is the increase in the uptake clearance of FEX in Mrp3 (-/-) mice. Therefore, we measured the initial uptake clearance of FEX in Mrp3 (-/-) mice and FVB mice by using LUI experiment. After FEX (10 nmol/kg b.w.) was injected into the portal vein of wild type FVB mice and Mrp3 (-/-) mice, the

hepatic extraction ratio was calculated. There was no significant difference in the extraction ratio between the FVB mice (0.885 ± 0.014; mean ± S.E., n=3) and Mrp3 (-/-) mice (0.885 ± 0.022; mean ± S.E., n=3).

Relative expression of Oatps, Mrps, Bcrp, Bsep and multidrug and toxin compound extrusion 1 (Mate1) in the liver, bile flow rate, and biliary excretion of total bile acids and GSH in wild type FVB and Mrp3 (-/-) mice.

The various kinetic parameters of FEX and the bile flow rate were changed in the Mrp3 (-/-) mice. It is possible that the expression levels of hepatic transporters are different between Mrp3 (-/-) and FVB mice. Accordingly, the hepatic mRNA and protein expression levels of the transporters involved in drug transport were compared between FVB and Mrp3 (-/-) mice using real-time quantitative PCR and Western blot analyses (Table 5, Figure 7). The no expression of Mrp3 mRNA and protein was confirmed in Mrp3 (-/-) mice. The mRNA levels of mOatp1b2 and mMate1 significantly decreased in Mrp3 (-/-) mice ($p<0.05$), whereas mBcrp significantly increased ($p<0.05$; Table 5). However, these differences were no more than 2-fold. There were no statistically significant differences in the mRNA levels of the other transporters.

The protein expression levels of the transporters in the crude membrane fraction normalized by the expression level of β-actin were also evaluated. There were less than 2-fold differences in the protein levels of mMrp2, mMrp4, mBsep, mMdr1, and mBcrp (Figure 7). Since it is generally accepted that the bile flow rate depends on the biliary excretion of GSH and bile acids, the bile flow rate and biliary excretion of total bile acids and GSH were examined in wild type FVB mice and Mrp3 (-/-) mice and summarized in Table 5. The bile flow rate significantly increased in Mrp3 (-/-) mice compared with FVB mice. Although the excretion rate of GSH in Mrp3 (-/-) mice was about 1.3 times higher than that in FVB mice, the efflux clearance based on liver concentration in Mrp3 (-/-) mice was not significantly different from that in FVB mice. And there is no statistically significant difference in the biliary excretion of total bile acids between FVB mice and Mrp3 (-/-) mice.

Discussion

In the present study, we examined which hepatic efflux transporters can recognize FEX as a substrate by using transporter-expressing cells and membrane vesicles. We also investigated the importance of Mrp2, Mrp3, and Mrp4 in the *in vivo* pharmacokinetics of FEX by using the corresponding knockout mice.

The basal-to-apical transport of FEX was larger than the apical-to-basal transport in the hOATP1B3/hBSEP double transfectant, but not in the hOATP1B3/hBCRP double transfectant (Figure 1). Moreover, ATP-dependent uptake of FEX was observed in hBSEP- and rBsep-enriched membrane vesicles (Figure 2). These results indicate that FEX is a substrate of hBSEP and rBsep, but not hBCRP. This result is consistent with a previous *in vivo* result demonstrating that the absence of mBcrp1 didn't change its biliary excretion. We previously hypothesized that efflux transporters other than rMrp2, mMdr1, and mBcrp1 contribute to the biliary excretion of FEX (Tahara et al., 2005). Interestingly, BSEP could be a potential candidate transporter for its biliary excretion. BSEP is generally recognized as an efflux transporter for bile acids. However, recent studies have revealed that BSEP can also transport non-bile

acids such as vinblastine and pravastatin (Hirano et al., 2005a; Lecureur et al., 2000). Further investigations to clarify the contribution of BSEP to the biliary excretion of drugs will be of interest.

Though previous results indicated a minor role of rMrp2 in the biliary excretion of FEX in rats (Tahara et al., 2005; Tian et al., 2008), species difference in the contribution of Mrp2 might exist between rats and mice. Therefore, to clarify the contribution of mMrp2 to FEX excretion, an *in vivo* infusion study was carried out using wild-type mice and Mrp2 (-/-) mice (Figure 3 and Table 2). The $CL_{bile,plasma}$ in the Mrp2 (-/-) mice was approximately one-third of that in the FVB mice, whereas the $CL_{bile,liver}$ in the Mrp2 (-/-) mice was only 20% lower than that in the FVB mice. The $K_{p,liver}$ in the Mrp2 (-/-) mice was much lower than that in the FVB mice. These results indicate that mMrp2 plays a limited role in the biliary excretion of FEX in mice and unknown transporter(s) other than mMdr1, mMrp2 and mBcrp1 is/are involved in FEX transport across the canalicular membrane. The possible reason of the great decrease in the $CL_{bile,plasma}$ in the Mrp2 (-/-) mice was a fall in the hepatic uptake clearance and/or a rise in the sinusoidal efflux clearance from the liver to blood. The expression levels of mMrp3 and mMrp4 in the Mrp2 (-/-) mice are increased

compared with wild-type mice, whereas no change in the expression levels of mOatp transporters in the liver was observed (Chu et al., 2006; Vlaming et al., 2006). Therefore, the increase in the sinusoidal efflux of FEX was probably caused by the increase in the expression of mMrp3 and/or mMrp4. While this manuscript was under review, Tian et al. (2008) published the interesting findings in which biliary excretion clearance of FEX based on the unbound hepatic concentration decreased by more than 50% and its hepatic concentration also considerably decreased in Mrp2 (-/-) mice (Tian et al., 2008). Though the reason for the difference in the quantitative contribution of mMrp2 between previous study (*in situ* perfusion) and current study (*in vivo* pharmacokinetics) remains unclear, these results suggest that mMrp2 may be partly involved in its biliary excretion and its sinusoidal efflux considerably increases in Mrp2 (-/-) mice.

To examine the involvement of MRP3 and MRP4 in the sinusoidal efflux of FEX, *in vitro* uptake studies using hMRP3- and hMRP4-enriched membrane vesicles and *in vivo* infusion studies using Mrp3 (-/-) and Mrp4 (-/-) mice were performed. ATP-dependent uptake of FEX was only observed in hMRP3-enriched vesicles, but not hMRP4- and GFP-enriched vesicles,

indicating that FEX is a substrate of hMRP3, but not hMRP4 (Figure 4). Up to now, methotrexate and etoposide were the only unconjugated drugs reported to be transported by hMRP3 (Zelcer et al., 2001; Zeng et al., 2001). The identification of FEX as an MRP3 substrate suggests that it is worthwhile to check whether other drugs are substrates of MRP3. In *in vivo* kinetic analyses, the $CL_{bile,plasma}$, $CL_{bile,liver}$, and $K_{p,liver}$ of FEX in the Mrp3 (-/-) mice were greater than those in the wild-type mice (Figure 5 and Table 3), while there was no difference in the pharmacokinetic parameters between the wild-type and Mrp4 (-/-) mice (Figure 6 and Table 4). This result is difficult to explain because if the increase in the $CL_{bile,plasma}$ was simply caused by the increase in the $CL_{bile,liver}$, the $K_{p,liver}$ should be reduced in the Mrp3 (-/-) mice, which is opposite to our results. To resolve this discrepancy, the increase in the uptake clearance and/or the decrease in the sinusoidal efflux clearance in Mrp3 (-/-) mice should be considered.

In order to examine whether the uptake clearance of FEX was increased in the Mrp3 (-/-) mice, the expression levels of the Oatp transporters in the liver and the extraction ratio of FEX estimated by the LUI experiment were compared between the wild-type mice and the Mrp3 (-/-) mice. The expression levels of