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 ($CL_{bile,plasma}$,

$CL_{urine,plasma}$) 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