Oatp1a1, Oatp1a4, and Oatp1b2 in the Mrp3 (-/-) mice were almost the same as, or slightly lower than, those in the wild-type mice (Table 5). The extraction ratio of FEX in Mrp3 (-/-) mice was not different from that in wild-type mice in LUI experiment. However, because FEX was highly extracted into the liver in both strains and the hepatic uptake clearance was much larger than the blood flow rate, so the change of uptake clearance doesn't affect its extraction ratio. Therefore, unfortunately we cannot conclude that the uptake clearance of FEX was not different between the wild-type and Mrp3 (-/-) mice from this experiment. However, we have not obtained any evidence indicating that the uptake clearance of FEX increased in the Mrp3 (-/-) mice. We currently think that the increase in the $K_{p,liver}$ was mainly caused by the decrease in the sinusoidal efflux by the absence of Mrp3 expression rather than the enhanced uptake in the Mrp3 (-/-) mice. Moreover, it can be considered that the increase in the sinusoidal efflux clearance in the Mrp2 (-/-) mice was mainly due to an increase in the Mrp3 expression on the sinusoidal membrane.

Surprisingly, $CL_{bile,liver}$ increased in Mrp3(-/-) mice. It is difficult to explain why the efflux via the canalicular membrane was affected by Mrp3 on sinusoidal membrane. A significant increase in the bile flow rate was observed

in Mrp3 (-/-) mice in comparison with wild-type mice (Tables 3, 5). It is generally accepted that the bile flow rate depends on the biliary excretion of GSH and bile acids, which are mainly excreted by Mrp2 and Bsep, respectively (Elferink and Groen, 2002), so it is possible that the functions of Mrp2 and Bsep were changed in the Mrp3 (-/-) mice. Therefore, the mRNA and protein expression levels of Mrp2, Bsep, and the other efflux transporters expressed in the canalicular membrane were compared between the wild-type and Mrp3 (-/-) mice. Unexpectedly, the difference in the expression levels of all the transporters was no more than 2-fold (Table 5, Figure 7). In addition, in order to investigate whether the function of Mrp2 and Bsep was changed, the biliary excretion clearance based on the intrahepatic concentration of GSH and total bile acids were calculated. However, no significant difference in the clearance of both GSH and bile acids was observed (Table 5). On the other hand, the excretion rate and hepatic concentration of GSH in Mrp3 (-/-) mice were slightly higher than those in wild-type mice (Table 5). Manautou et al., showed that hepatic GSH content in untreated Mrp3 (-/-) mice was slightly higher than that in wild type mice (Manautou et al., 2005). Accordingly, the increase of hepatic GSH synthesis in Mrp3 (-/-) mice might lead to an increase in the bile flow rate,

following the increase of the excretion rate of GSH. Thus, mMrp2 and mBsep are not likely to contribute to the increase of $CL_{bile,liver}$ of FEX in Mrp3 (-/-) mice and unidentified transporter(s) may be involved in the excretion of FEX in mice. It is also possible that the increase in the excretion of FEX might result in an increase in the secretion of FEX since GSH is known to stimulate transport of substrates via Mrp2 (Van Aubel et al., 1999). Multiplicity of canalicular transporters has been proposed for the excretion of some compounds. For example, the excretion of bilirubin glucuronide across the canalicular membrane contains ATP-independent transport system which is stimulated by bicarbonate ion in addition to Mrp2 (Adachi et al., 1991). Further studies are required to clarify the multiple canalicular transport systems for xenobiotics.

The results obtained in the present and previous studies are summarized in Figure 8. The *in vitro* studies clarified that FEX is a substrate of hBSEP/rBsep and hMRP3. In addition, the *in vivo* studies show that mMrp3 plays an important role in the sinusoidal efflux of FEX and consequently its pharmacokinetics, whereas mMrp2 plays a minor role in the canalicular excretion of FEX.

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Footnotes

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Legends for Figures

Figure 1 Time profiles for the transcellular transport of FEX and pitavastatin across hOATP1B3-, hBSEP- and hBCRP-expressing MDCKII cell monolayers.

Transcellular transport of 5 μM FEX (A-H) and 0.1 μM pitavastatin (I-L) across MDCKII cell monolayers expressing hOATP1B3 (B, F, J), hBSEP (C), hBCRP (G, K), both hOATP1B3 and hBSEP (D), and both hOATP1B3 and hBCRP (H, L) was compared with that across the control MDCKII cell monolayer (A, E, I). Open and closed circles represent the transcellular transport in the apical-to-basal and basal-to-apical direction, respectively. Each point and vertical bar represents the mean ± S.E. of three determinations. Where no vertical bar is shown, the S.E. was contained within the limits of the symbol.

Figure 2 The uptake of FEX in the membrane vesicles prepared from hBSEP- (A, C) and rBsep- (B, D) expressing HEK293 cells

(A, B) The uptake of 10μM FEX by hBSEP (A) and rBsep (B) for 5 min was examined at 37 °C in the buffer containing 5 mM ATP (closed symbols) or AMP (open symbols). Circles and squares represent the uptake in hBSEP- (A)

or rBsep- (B) and GFP-enriched membrane vesicles, respectively.

(C, D) The concentration-dependent uptake of FEX by hBSEP (C) and rBsep (D) was examined at 37 °C in the medium containing 5 mM ATP (closed columns) or AMP (open columns). Each point and vertical bar represents the mean ± S.E. (n=3). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols. *: $p<0.05$, **: $p<0.01$

Figure 3. Plasma concentration, biliary excretion rate and urinary excretion rate of FEX during constant intravenous infusion into FVB mice and Mrp2 (-/-) mice.

The plasma concentration (A), biliary excretion rate (B) and urinary excretion rate (C) of FEX were determined during constant intravenous infusion into FVB mice (open circles) and Mrp2 (-/-) mice (closed circles). Each point and vertical bar represents the mean ± S.E. (FVB mice, n=5; Mrp2 (-/-) mice, n=6). *: $p<0.05$, **: $p<0.01$

Figure 4. The uptake of FEX in the membrane vesicles prepared from hMRP3- (A, C) and hMRP4- (B) expressing HEK293 cells.

(A, B) The uptake of 10 μM FEX by hMRP3 (A) and hMRP4 (B) was examined at 37 °C in the medium containing 5 mM ATP (closed symbols) or AMP (open symbols). Circles and squares represent the uptake in hMRP3- (A) or hMRP4- (B) and GFP-enriched membrane vesicles, respectively.

(C) The concentration-dependent uptake of FEX by hMRP3 was examined at 37 °C in the medium containing 5 mM ATP (closed columns) or AMP (open columns). Each point and vertical bar represents the mean ± S.E. (n=3). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols. **: $p<0.01$

Figure 5 Plasma concentration, biliary excretion rate and urinary excretion rate of FEX during constant intravenous infusion into FVB mice and Mrp3 (-/-) mice.

The plasma concentration (A), biliary excretion rate (B) and urinary excretion rate (C) of FEX were determined during constant intravenous infusion into FVB mice (open circles) and Mrp3 (-/-) mice (closed circles). Each point and vertical bar represents the mean ± S.E. (FVB mice, n=7; Mrp3 (-/-) mice, n=6). Where no vertical bars are shown, the S.E. values were contained within

the limits of symbols. *: $p<0.05$, **: $p<0.01$

Figure 6 Plasma concentration, biliary excretion rate and urinary excretion rate of FEX during constant intravenous infusion into C57BL/6 mice and Mrp4 (-/-) mice.

The plasma concentration (A), biliary excretion rate (B) and urinary excretion rate (C) of FEX were determined during constant intravenous infusion into C57BL/6 mice (open circles) and Mrp4 (-/-) mice (closed circles). Each point and vertical bar represents the mean ± S.E. (C57BL/6 mice, n=4; Mrp4 (-/-) mice, n=3). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols.

Figure 7 Comparison of the protein expression levels of various transporters expressed in the crude membrane of mouse liver between FVB mice and Mrp3 (-/-) mice using Western blot analyses.

The expression levels of the efflux transporters expressed in liver canalicular membrane (A) and sinusoidal membrane (B) in the hepatic crude membrane fraction prepared from five FVB mice and Mrp3 (-/-) mice were

determined by Western blot analyses. β-actin was used for the normalization of the expression level of each transporter.

Figure 8. Schematic diagrams of the proposed transport mechanisms of FEX in wild-type, Mrp2(-/-) and Mrp3(-/-) mice

FEX is a substrate of hOATPs, hMRP2, hMRP3, hBSEP, and P-gp in humans. In this figure, it is assumed that there is no difference in the substrate specificity of each transporter for FEX between humans and mice. In the Mrp2 (-/-) mice, the expression levels of Mrp3 and Mrp4 are increased compared with those in the wild-type mice. In the Mrp3 (-/-) mice, the unidentified transporter(s) may be increased compared with the wild-type mice.

Table 1. Nucleotide sequences of the primers used in real-time quantitative PCR.

Transporter	Forward primer	Reverse primer
Oatp1a1	cagataaatggatttgccag	gtcaacaaatagttacagag
Oatp1a4	atagcttcaggcgcatttac	ttctccatcattctgcatcg
Oatp1b2	ttcaccacaacaatggccta	ttttccccacagacaggttc
Mrp2	tctctggtttgcctgtta	gcagaagacaatcaggttt
Mrp3	gctctcacaaggtggtacaa	caggttgaaacaggcactca
Mrp4	gatcgcctacgtttctcagc	ccggtctcctataaccgtca
Mdr1a	tcattgcgatagctggag	caaacttctgctcccgagtc
Mdr1b	acctgctgttggcgtatttg	ttcctccagactgctgttgc
Bcrp	aaatggagcacctcaacctg	cccatcacaacgtcatcttg
Bsep	aaatcggatggtttgactgc	tgacagcgagaatcaccaag
Mate1	aacaccatctcccagtttgc	gccaaggataccactcagga
G3pdh	tgcgacttcaacagcaactc	cttgctcagtgtccttgctg

Table 2. Pharmacokinetic parameters of FEX during constant intravenous infusion into FVB mice (n=5) and Mrp2 (-/-) mice (n=6).

Parameters	FVB mice (n=5)	Mrp2 (-/-) mice (n=6)
C_{ss} (nM) [1)]	414 ± 54	597 ± 49*
$CL_{tot,plasma}$ (mL/min/kg b.w.)	29.8 ± 3.9	20.1 ± 1.4*
$CL_{bile,plasma}$ (mL/min/kg b.w.)	9.48 ± 0.82	3.38 ± 0.38**
$CL_{bile,liver}$ (mL/min/kg b.w.)	0.250 ± 0.053	0.198 ± 0.022
V_{bile} (nmol/min/kg b.w.)	3.71 ± 0.32	1.93 ± 0.14**
Bile flow rate (μL/min/kg b.w.)	64.0 ± 7.1	53.1 ± 4.6
$K_{p,liver}$	40.7 ± 6.8	16.2 ± 2.0*
$CL_{urine,p}$ (mL/min/kg b.w.)	16.9 ± 2.3	15.2 ± 3.2
V_{urine} (nmol/min/kg b.w.)	6.59 ± 0.91	8.66 ± 1.79
GFR (mL/min/kg b.w.) [2)]	17.3 ± 0.9	17.7 ± 2.5
$K_{p,kidney}$	22.7 ± 4.4	21.8 ± 3.1
$K_{p,brain}$	0.0183 ± 0.0028	0.0190 ± 0.0043

Data represent the mean ± S.E. (n=5 or 6). The meanings of these parameters are explained in the "**Materials & Methods**" section.

1) Corrected steady-state plasma concentration at the infusion rate of 700 nmol/hr/kg

2) GFR represents the glomerular filtration rate.

*: $p<0.05$, **: $p<0.01$