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

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.

Acknowledgements

We are deeply grateful to Dr. Piet Borst and Dr. Koen van de Wetering (The Netherlands Cancer Institute, Amsterdam, The Netherlands) for donating male Mrp3 (-/-) mice and FVB mice and providing their fruitful comments. We are deeply grateful to Dr. Junko Iida and Futoshi Kurotobi (Shimadzu Corporation, Kyoto, Japan) for the technical support of the LC/MS system. We are deeply grateful to Atsushi Ose for providing valuable comments about the LC/MS system. We appreciate Kowa Co. Ltd. (Tokyo, Japan) for providing radiolabeled and unlabeled pitavastatin.

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Footnotes

This work was supported by Grant-in-Aid for Scientific Research (A)(KAKENHI 17209005) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and Health and Labor Sciences Research Grantsfrom the Ministry of Health, Labor, and Welfare for the Research on Toxicogenomics.

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)