Investigation of the inhibitory effects of various drugs on the hepatic

uptake of fexofenadine in humans

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Abbreviations: D-MEM, Dulbecco's modified Eagle's medium; EG, estradiol-17β-D-glucuronide; FEX, fexofenadine; LC/MS, liquid chromatography/mass spectrometry; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; OATP organic anion transporting polypeptide

Abstract

Fexofenadine (FEX), an H_1-receptor antagonist, is mainly eliminated from the liver in an unchanged form. Our previous study suggested that organic anion transporting polypeptide (OATP) 1B3 mainly contributes to the hepatic uptake of FEX (Shimizu et al., 2005). On the other hand, a clinical report demonstrated that a T521C mutation of OATP1B1 increased its plasma AUC (Niemi et al., 2005). Several compounds are reported to have a drug interaction with FEX and some of this may be caused by the inhibition of its hepatic uptake. We checked which transporters are involved in the hepatobiliary transport of FEX by using double transfectants and examined whether clinically reported drug interactions with FEX could be explained by the inhibition of its hepatic uptake. Vectorial basal-to-apical transport of FEX was observed in double transfectants expressing OATP1B1/multidrug resistance-associated protein 2 (MRP2) and OATP1B3/MRP2, suggesting that OATP1B1 as well as OATP1B3 is involved in the hepatic uptake of FEX and that MRP2 can recognize FEX as a substrate. The inhibitory effects of compounds on FEX uptake in OATP1B3-expressing HEK293 cells were investigated and the maximum degree of increase in plasma AUC of FEX by drug interaction in

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clinical situations was estimated.   As a result, cyclosporin A and rifampicin were

found to have the potential to interact with OATP1B3-mediated uptake at clinical

concentrations.   From these results, most of the reported drug interaction

cannot be explained by the inhibition of hepatic uptake of FEX and different

mechanisms such as the inhibiton of intestinal efflux should be considered.

Introduction

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

The first step in the process of elimination from the liver is hepatic uptake across the sinusoidal membrane. Accumulated evidence has supported the idea that organic anion transporting polypeptide 1B1 (OATP1B1) and OATP1B3 play major roles in the hepatic uptake of a wide variety of compounds including endogenous compounds and clinically important drugs such as HMG-CoA reductase inhibitors (statins) (Hagenbuch and Meier, 2004). Shimizu et al. have demonstrated that OATP1B3 mainly contributes to the hepatic uptake of FEX using transporter-expressing HEK293 cells (Shimizu et al., 2005). On the

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other hand, a recent clinical report has shown that the genetic polymorphism of

OATP1B1 (T521C), which was reported to decrease the transport clearance,

increased the plasma AUC of FEX (Niemi et al., 2005).   These results

suggested that OATP1B1 as well as OATP1B3 is involved in the uptake of FEX

into human liver.

Currently, many clinical reports have indicated the drug interaction

between FEX and several drugs.   Among them, itraconazole (Shon et al., 2005;

Shimizu et al., 2006a; Shimizu et al., 2006b; Uno et al., 2006), ketoconazole

(Common Technical Document for the Registration of Pharmaceuticals for

Human Use), azithromycin (Gupta et al., 2001), erythromycin (Common

Technical Document), ritonavir, lopinavir (van Heeswijk et al., 2006), verapamil

and probenecid (Yasui-Furukori et al., 2005) increased the plasma AUC of FEX,

whereas rifampicin (Hamman et al., 2001) decreased it.   One of the possible

mechanisms for the increase of its AUC is the inhibition of multidrug resistance 1

(MDR1/ABCB1) in the small intestine by the concomitantly administered

compounds.   Vectorial transport was reported to be observed in

MDR1-expressing LLC-PK1 cells, but not in parent cells (Cvetkovic et al., 1999).

Furthermore, after oral administration of FEX, the plasma AUC in Mdr1a/1b (-/-)

mice was about 6 times greater than that in FVB mice, whereas after intravenous

administration of FEX, there was no difference in the pharmacokinetics of FEX

between FVB and Mdr1a/1b (-/-) mice, suggesting that Mdr1a/1b in the small

intestine limits the absorption of FEX (Tahara et al., 2005).   Since most of the

compounds, which clinically increase the AUC of FEX, have the ability to inhibit

MDR1 function, these are likely to increase the intestinal absorption of FEX by

the inhibition of MDR1 in the small intestine.

However, considering that the main elimination pathway of FEX is biliary

excretion of the unchanged form (Lippert et al., 1995), it is also possible that

drug interaction with FEX is caused by the inhibition of its hepatic uptake.

Hirano et al. performed detailed investigations to see whether the inhibitory

effects of various compounds on OATP1B1-mediated uptake of pitavastatin were

clinically relevant (Hirano et al., 2006).   However, inhibition potencies of several

compounds for OATP1B3-mediated uptake have not yet been clarified.

Therefore, the purpose of this study is to check which transporters are

involved in the hepatobiliary transport of FEX and explore which instances can

be explained by the inhibition of its hepatic uptake among clinically reported drug

interactions.   We identified the transporters that can transport FEX by using

double transfectants expressing OATP1B1/multidrug resistance-associated

protein (MRP) 2 and OATP1B3/MRP2 (Matsushima et al., 2005). It has often

been found that the transcellular transport assay using double transfectants is

more sensitive in detecting the transporter-mediated transport compared with

the uptake assay in single transporter-expressing cells. Then we checked the

inhibitory effects of several compounds that are reported to interact with FEX in

clinical situations on the FEX uptake in OATP1B3-expressing HEK293 cells and

compared the *in vitro* inhibition constant (K_i) for OATP1B3 with that for OATP1B1

obtained from a previous study (Hirano et al., 2006). Following that, to check

whether the inhibition of FEX uptake by several compounds is clinically relevant,

we also estimated the maximum degree of increase in plasma AUC of FEX by

considering the maximum unbound concentration of inhibitors at the inlet to the

liver estimated by an established method for this calculation (Ito et al., 1998).

Materials and Methods

Materials

[^{3}H]-estradiol-17β-D-glucuronide (EG) (1.6 TBq/mmol) was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, BA). FEX hydrochloride was purchased from Toronto Research Chemicals (North York, ON, Canada). All other chemicals and reagents were of analytical grade and commercially available.

Cell culture

MDCKII cells expressing OATP1B1/MRP2, OATP1B3/MRP2, OATP1B1, OATP1B3, MRP2 and vector-transfected control MDCKII cells have been constructed previously (Matsushima et al., 2005, Ishiguro et al., *submitted*). OATP1B3-expressing HEK293 cells and vector-transfected control cells were also constructed previously (Hirano et al., 2004). Transporter-expressing or vector-transfected MDCKII and HEK293 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (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

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95% humidity.

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 medium was replaced with D-MEM supplemented with 5 mM sodium butyrate 2 days before the transport study to induce the expression of exogenous transporter. In the transport assay, cells were first washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO$_3$, 4.83 mM KCl, 0.96 mM KH$_2$PO$_4$, 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 and preincubated with Krebs-Henseleit buffer for 10 min. Subsequently, substrates were added in Krebs-Henseleit buffer either to the apical compartment (250 μL) or to the basolateral compartment (1 mL). After a designated period, 50 μL or 100 μL of medium was taken from the opposite side to the added substrate. Using FEX as a substrate, 50 μL aliquots were used for LC/MS quantification as described below. 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 Lowry method with bovine serum

albumin as a standard.

Uptake study using OATP1B3-expressing HEK293 cells

Cells were seeded in 12-well plates coated with

poly-L-lysine/poly-L-ornithine (Sigma) at a density of 1.5×10^5 cells per well.

After 2 days, the cell culture medium was replaced with culture medium

supplemented with 5 mM sodium butyrate 24 hr before transport assay to induce

the expression of exogenous transporters.   The transport study was carried out

as described previously (Sugiyama et al., 2001).   Uptake was initiated by

adding Krebs-Henseleit buffer containing FEX.   All the procedures were

conducted at 37 °C.   The uptake was terminated at a designated time by adding

ice-cold Krebs-Henseleit buffer after removal of the incubation buffer.   Then,

cells were washed twice with 1 mL ice-cold buffer, solubilized in 500 μL 0.2 N

NaOH, and kept overnight at room temperature.   Using EG as a substrate,

aliquots (300 μL) were transferred to vials after adding 100 μL 1 N HCl.   Using

FEX as a substrate, aliquots (240 μL) were used for LC/MS quantification as described below.   The remaining 50 μL of the aliquots of cell lysate were used to determine the protein concentration by the Lowry method with bovine serum albumin as a standard.

Quantification of FEX by LC/MS

The aliquots (50 μL) obtained from the transcellular transport study were precipitated with 200 μL methanol containing 10 nM midazolam as an internal standard, while the aliquots (240 μL) obtained from the uptake study were precipitated with 480 μL methanol containing 50 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.   The obtained samples were subjected to LC/MS analysis to determine the concentration of FEX.   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.   For the mobile phase, 0.05% formic acid and methanol were used.   The methanol concentration was initially 48%, then linearly

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increased up to 61.5% over 4.5 min. Finally, the column was re-equilibrated in a methanol concentration of 48% for 3 min. The total run time was 7.5 min. Using this method, FEX was eluted at 4.1 min and midazolam at 2.8 min. 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.

Kinetic analyses

Ligand uptake in transporter-expressing cells was expressed as the uptake volume (μL/mg protein), given as the amount associated with the cells divided by its initial concentration in the incubation medium. Transporter-specific uptake was obtained by subtracting the uptake into vector-transfected cells from that into transporter-expressing cells. Inhibition constants (K_i) of a series of compounds could be calculated by the following equation, if the substrate concentration was low enough compared with its K_m value:

$$CL(+I) = \frac{CL}{1 + I/K_i} \qquad \text{(Eq. 1)}$$

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where CL represents the uptake clearance in the absence of inhibitor, CL($+I$) represents the uptake clearance in the presence of inhibitor, and I represents the inhibitor concentration. When fitting the data to determine the K_i value, the input data were weighted as the reciprocal of the observed values. The Damping Gauss-Newton method algorithm was used with a MULTI software program (Yamaoka et al., 1981) to fit the data.

Prediction of clinical drug-drug interactions between FEX and various compounds

The degree of inhibition of uptake via OATP1B1 and OATP1B3 in humans was estimated by calculating the following R value, which represents the ratio of the uptake clearance in the absence of inhibitor to that in its presence:

$$R = 1 + \frac{f_u * I_{in,max}}{K_i} \qquad \text{(Eq. 2)}$$

where f_u represents the protein unbound fraction of the inhibitor in blood, $I_{in,max}$ represents the estimated maximum blood concentration of the inhibitor at the inlet to the liver. K_i value for OATP1B3 was obtained from the present *in vitro* study using OATP1B3-expressing HEK293 cells described above and K_i value

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for OATP1B1 is quoted from the previous reports in which pitavastatin is used as

a substrate of OATP1B1 (Hirano et al., 2006). For estimation of the R value,

$I_{in,max}$ was calculated by the method of Ito et al. (1998) as shown below:

$$I_{in,max} = I_{max} + \frac{F_a * Dose * k_a}{Q_h} \qquad \text{(Eq. 3)}$$

where I_{max} represents the reported value for the maximum blood concentration of

the inhibitor in the systemic circulation in clinical situations, F_a represents the

fraction absorbed from intestine of the inhibitor, k_a is the absorption rate constant

in the intestine and Q_h represents the hepatic blood flow rate in humans (1610

mL/min) (Ito et al., 1998). To estimate the maximum $I_{in,max}$, F_a was set at 1, k_a

was set at 0.1 mL^{-1} [minimum gastric emptying time (10 min)], and the

blood-to-plasma concentration ratio was assumed to be 1.

Results

Transcelluar Transport of FEX and EG across the MDCKII cell monolayer

The transcellular transport of 5 μM FEX and 0.1 μM EG across the

MDCKII monolayer was determined.   The basal-to-apical transport of FEX was

approximately 3.6 times higher than that in the opposite direction in

OATP1B1/MRP2 double transfectant (Figure 1 D), whereas no difference in

basal-to-apical transcellular transport of FEX could be observed in

vector-transfected control cells and single transfectants expressing OATP1B1 or

MRP2 (Figure 1 A-C).   On the other hand, the basal-to-apical transport of EG

was approximately 22 times higher than that in the opposite direction in

OATP1B1/MRP2 double transfectants (Figure 1 H), whereas we could not see

any basal-to-apical transcellular transport of EG in vector-transfected control

cells and single transfectants expressing OATP1B1 or MRP2 (Figure 1 E-G).

The basal-to-apical transport of FEX was approximately 2.9 times higher than

that in the opposite direction in OATP1B3/MRP2 double transfectant (Figure 2 D),

whereas the difference in each directional transport of EG was less than 2 times

in vector-transfected control cells and single transfectants expressing OATP1B3

or MRP2 (Figure 2 A-C).   As a positive control, the basal-to-apical transport of

EG was approximately 3.0 times higher than that in the opposite direction in

OATP1B3/MRP2 double transfectant (Figure 2 H), whereas no significant

difference in basal-to-apical transcellular transport of EG was less than 2 times

that in vector-transfected control cells and single transfectants expressing

OATP1B3 or MRP2 (Figure 2 E-G).

Inhibitory effects of various drugs on OATP1B3-mediated uptake of FEX

The inhibitory effects of various drugs on the uptake of FEX were

examined using OATP1B3-expressing HEK293 cells (Figure 3). Some of the

drugs we tested were reported to cause drug-drug interactions with FEX in

clinical situations. Most of the compounds could inhibit OATP1B3-mediated

FEX uptake. On the other hand, even 100 μM fluconazole, 30 μM itraconazole

and 100 μM cimetidine did not significantly inhibit the FEX uptake (Figure 3 C, D,

N). We also obtained the protein unbound fraction in blood (f_u) from the

literature and calculated the estimated maximum concentration at the inlet to the

liver ($I_{in,max}$) of the inhibitors. Inhibition constants (K_i) of various compounds for

OATP1B3 obtained in the present study and the ratio of the uptake clearance in

the absence of inhibitor to that in its presence (R value) are summarized in Table

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1.   Among several drugs we tested, only the R values of cyclosporin A and rifampicin for OATP1B3-mediated uptake exceeded 2.0.   The K_i values of various compounds for OATP1B1 obtained in the previous study and the respective R values are also shown in Table 1 (Hirano et al., 2006).   The K_i values of several compounds in the uptake mediated by OATP1B1 and OATP1B3 were not so different.   However, the K_i values of clarithromycin and ritonavir for OATP1B1-mediated uptake of pitavastatin were more than 5-fold less compared with that for OATP1B3-mediated uptake of FEX.

Discussion

To check which transporters are involved in the hepatobiliary transport of

FEX, we investigated the transcellular transport of FEX using OATP1B1/MRP2

and OATP1B3/MRP2 double transfected cells. Furthermore, to investigate

whether the inhibition of FEX hepatic uptake by several drugs is clinically

relevant, the inhibition constants (K_i) of several drugs for OATP1B3-mediated

FEX uptake obtained from *in vitro* analyses were determined and the maximum

degrees of increase in plasma AUC through drug interaction were calculated

using estimated maximum protein unbound concentrations of inhibitors at the

inlet to the liver.

In the transcellular transport study using double transfected cells, we

observed the basal-to-apical vectorial transport of FEX not only in

OATP1B3/MRP2- but also in OATP1B1/MRP2-double transfected cells (Figure 1

and 2). Our previous report indicated that OATP1B3 mainly contributes to the

hepatic uptake of FEX in humans and that OATP1B1-mediated uptake was not

statistically significant though the uptake in OATP1B1-expressing cells was

slightly larger compared with that in control cells (Shimizu et al., 2005). The

involvement of OATP1B1 in FEX uptake in humans was also supported by the

recent clinical report demonstrating that the genetic polymorphism of OATP1B1

(T521C) increased the plasma concentration of FEX (Niemi et al., 2005).   The

apparently conflicting results obtained from the present transcellular transport

study and the previous uptake study may be caused by the difference in the

sensitivity for the detection of the transport.   We found that transcellular

transport assay using a double transfectant is more sensitive in detecting the

transporter-mediated transport compared with an uptake assay in single

transporter-expressing cells (Sasaki et al., 2002; Matsushima et al., 2005).   For

example, the ratio of the basal-to-apical transport of pravastatin to that in the

opposite direction was 3.3, while it barely estimates the kinetics of pravastatin

transport in OATP1B1-expressing HEK293 cells because of its small

OATP1B1-mediated uptake (Matsushima et al., 2005).   Therefore, these results

suggest a significant contribution of OATP1B1 as well as OATP1B3 to the

hepatic uptake of FEX.   Further evaluation is required for the determination of

the precise relative contribution of OATP1B1 and OATP1B3 to the hepatic

uptake of FEX in humans.

The finding presented here is the first demonstration that human MRP2

can recognize FEX as a substrate.   MRP2 is expressed in the apical membrane

of the liver, kidney, and intestine. An *in vivo* infusion study using Eisai hyperbilirubinemic rats (EHBR), Mrp2-deficient rats, revealed that Mrp2 is not important for the biliary excretion of FEX (Tahara et al., 2005). However, it is still possible that MRP2 will play an important role in the disposition of FEX in humans. This situation is very similar to that reported for pitavastatin (Hirano et al., 2005). The transcellular transport study using OATP1B1/MRP2 double transfected cells indicated pitavastatin could be transported by MRP2. However, the biliary excretion of pitavastatin in EHBR was not changed compared with control rats. Moreover, there are some reports that show species differences in the expression and function of MRP2 (Ishizuka et al., 1999; Ninomiya et al., 2005; Ninomiya et al., 2006; Takekuma et al., 2007). These may also indicate the species difference in the relative contribution of efflux transporters to the biliary excretion of compounds. The methodology to determine the contribution of efflux transporters in human liver needs to be established by checking the effect of transporter-specific inhibitors on the efflux of compounds in membrane vesicles prepared from human liver or sandwich-cultured human hepatocytes.

Several reports regarding drug-drug interaction with FEX have been

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published.   It is generally believed that one of the major mechanisms of the

reported drug-drug interactions between FEX and concomitantly administered

drugs is the inhibition of MDR1-mediated efflux in the small intestine, which

plays an important role in limiting the entry of FEX into circulating blood (Tahara

et al., 2005).   However, some of the reported cases are thought to be caused

by mechanisms other than the inhibition of MDR1.   A regional perfusion study

showed that ketoconazole and verapamil did not have a significant effect on the

in vivo intestinal absorption of FEX when co-administered or given as a

pretreatment in spite of increasing the plasma AUC of FEX (Tannergren et al.,

2003).   Accordingly, the involvement of other mechanisms in addition to the

inhibition of MDR1 has been supposed.   One of the candidate mechanisms is

considered to reduce the hepatic uptake clearance because the major route of

FEX elimination is biliary excretion of the unchanged form.   Since FEX is barely

metabolized, the apparent intrinsic hepatic clearance is described as follows:

$$CL_{int,h} = CL_{uptake} * \frac{CL_{excretion}}{CL_{efflux} + CL_{excretion}} \qquad \text{(Eq. 4)}$$

where $CL_{int,h}$, represents the apparent intrinsic hepatic clearance, CL_{uptake}, the

hepatic uptake clearance, $CL_{excretion}$, the biliary excretion clearance, and CL_{efflux},

the backflux clearance from liver to blood.   According to Eq. 4, the change in

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the hepatic uptake clearance always directly affects the overall intrinsic hepatic

clearance.   The present study indicates that both OATP1B1 and OATP1B3

contribute to the hepatic uptake of FEX.   We previously reported the inhibitory

effects of various drugs on the OATP1B1-mediated uptake and their clinical

relevance of drug-drug interaction (Hirano et al., 2006).   However, this kind of

systematic investigation for OATP1B3 has not been conducted.   Therefore, the

inhibitory effects of various drugs on the OATP1B3-mediated uptake were

determined.   Among several compounds we tested, the R values of cyclosporin

A and rifampicin for OATP1B3 as well as OATP1B1 exceeded 2.0 (Table 1).   To

date, we have not been able to find a published report regarding a drug-drug

interaction between FEX and cyclosporin A.   Many clinical reports have been

indicated that cyclosporin A increases the AUC of a variety of substrates of

OATP transporters, particularly HMG-CoA reductase inhibitors (Shitara et al.,

2005).   Although cyclosporin A is known as a clinically relevant potent

OATP1B1 inhibitor (Shitara et al., 2003), we showed that cyclosporin A can also

potently inhibit OATP1B3-mediated uptake.   Accordingly, it is necessary to pay

attention not only to the OATP1B1- but also the OATP1B3-mediated drug-drug

interaction between FEX and cyclosporin A in clinical situations.   Repetitive

administration of rifampicin reduced the plasma AUC of FEX in a previous clinical study (Hamman et al., 2001).   This report apparently conflicts with the present results in which rifampicin inhibited the OATP1B3-mediated uptake. However, rifampicin is a well-known PXR-mediated inducer and increases the expression level of MDR1 in the small intestine (Schuetz et al., 1996). Therefore, in this case, repeated dosing of rifampicin increased the expression level of MDR1 in the small intestine, which masked its inhibitory effects on the OATP1B3-mediated uptake of FEX.   This concept is supported by the recent report from Lam et al. (2006) indicating that drugs should be administered 1 day after final dosing of rifampicin to minimize potential inhibitory effects of OATP transporters in the induction study (Lam et al., 2006).

When comparing the Ki values for OATP1B1 with those for OATP1B3, except clarithromycin and ritonavir, Ki values for OATP1B1 and OATP1B3 were within the range of 5-fold difference, suggesting that the inhibitory potency of compounds for OATP1B1-mediated transport can be considered similar to that for OATP1B3-mediated transport.   A specific inhibitor for each individual transporter is very useful for determining the contribution of each transporter to the overall membrane transport.   Although EG and estrone-3-sulfate are

recognized as selective inhibitors for OATP1B1/OATP1B3 and OATP1B1/OATP2B1, respectively (Hirano et al., 2006), unfortunately, specific inhibitors for OATP1B3 have not yet been identified. Because of the high homology and overlapped substrate specificities between OATP1B1 and OATP1B3, the aid of *in silico* screening using a ligand-based drug design approach may be necessary to search for the selective inhibitors for OATP1B3 (Hirono et al., 2004).

Hirano et al. (2006) have indicated that cyclosporin A, rifampicin, clarithromycin and ritonavir (R value for OATP1B1 > 2.0) have a potential to interact with OATP1B1-mediated transport of pitavastatin in clinical situations (Hirano et al., 2006). Though the Ki values for OATP1B1-mediated uptake were determined by using pitavastatin as a substrate because of no significant uptake of FEX into OATP1B1-expressing HEK293 cells, considering the possible contribution of OATP1B1 as well as OATP1B3 to the hepatic uptake of FEX, these drugs may also affect the hepatic clearance of FEX. In order to avoid false-negative predictions of drug-drug interactions, the maximum plasma unbound concentration of inhibitors at the inlet to the liver was calculated using Eq. 3, which can overestimate these concentrations (Ito et al., 1998).

Therefore, in most cases, drug-drug interaction caused by inhibition of hepatic uptake of FEX might not occur in clinical situations.

In conclusion, both OATP1B1 as well as OATP1B3 are involved in the hepatic uptake of FEX and MRP2 can recognize FEX as a substrate. Among compounds we tested, cyclosporin A and rifampicin have the potential to inhibit the OATP1B1- and OATP1B3-mediated hepatic uptake of FEX at clinically relevant concentrations. However, most of the reported clinical drug-drug interactions cannot be simply explained by the inhibition of hepatic uptake of FEX and other mechanisms should be taken into account (e.g, inhibition of MDR1-mediated efflux in small intestine).

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Footnotes

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Legends for Figures

Figure 1.   Time profiles for the transcellular transport of FEX and EG

across MDCKII cell monolayers expressing OATP1B1 and/or MRP2

The transcellular transport of 5 μM FEX (A-D) and 0.1 μM EG (E-H)

across MDCKII cell monolayers expressing OATP1B1 (B, F), MRP2 (C, G), both

OATP1B1 and MRP2 (D, H) was compared with that across the

vector-transfected control MDCKII cell monolayer (A, E). Open and closed

circles represent the transcellular transport in the apical-to-basal and

basal-to-apical directions, respectively. These data were obtained from three

independent experiments and each experiment was performed in triplicate.

Each point and vertical bar represents the mean and standard deviation.

Where a vertical bar is not shown, the standard deviation was contained within

the limits of the symbol.

Figure 2.   Time profiles for the transcellular transport of FEX and EG

across MDCKII cell monolayers expressing OATP1B3 and/or MRP2.

The transcellular transport of 5 μM FEX (A-D) and 0.1 μM EG (E-H)

across MDCKII cell monolayers expressing OATP1B3 (B, F), MRP2 (C, G), both

OATP1B3 and MRP2 (D, H) was compared with that across the control MDCKII

cell monolayer (A, E).   Open and closed circles represent the transcellular

transport in the apical-to-basal and basal-to-apical directions, respectively.

These data were obtained from three independent experiments and each

experiment was performed in triplicate.   Each point and vertical bar represents

the mean and standard deviation.   Where a vertical bar is not shown, the

standard deviation was contained within the limits of the symbol.

Figure 3. **Inhibitory** **effects** **of** **various** **drugs** **on** **the**

OATP1B3-mediated uptake of FEX

The OATP1B3-mediated uptake of FEX (10 μM) was determined in the

absence or presence of inhibitors; (A) cyclosporin A, (B) rifampicin, (C)

fluconazole, (D) itraconazole, (E) ketoconazole, (F) clarithromycin, (G)

erythromycin, (H) azithromycin, (I) ritonavir, (J) lopinavir, (K) diltiazem, (L)

verapamil, (M) omeprazole, and (N) cimetidine using OATP1B3-expressing

HEK293 cells.   These data were obtained from three independent experiments

and each experiment was performed in triplicate.   Each point and vertical bar

represents the mean and standard deviation.   Where a vertical bar is not shown,

the standard deviation was contained within the limits of the symbol.   Each solid

line represents the fitting curve obtained by non-linear regression analysis.

Table 1. **Comparison of K_i values of inhibitors for OATP1B1-mediated uptake of pitavastatin and OATP1B3-mediated uptake of FEX**

Inhibitor	Dosage (mg)	I_{max} (µM)	$I_{in,max}$ (µM)	f_u	OATP1B3 K_i (µM)	R	OATP1B1 K_i (µM) [5]	R
cyclosporin A	100	0.596	5.76	0.10[1]	0.573 ± 0.172	2.01	0.242 ± 0.029	3.55
rifampicin	600[2]	7.90[2]	53.2	0.11[2]	1.45 ± 0.28	5.03	0.477 ± 0.030	13.3
fluconazole	100	8.62	28.9	0.89[2]	>100	<1.26	>100	<1.26
itraconazole	100	0.0792	8.88	0.002[2]	>30	1.00	>100	1.00
ketoconazole	200[2]	3.20[2]	26.6	0.01[2]	18.5 ± 3.0	1.01	19.2 ± 3.9	1.01
clarithromycin	400	1.86	35.1	0.54[2]	53.6 ± 15.9	1.42	8.26 ± 0.54	3.29
erythromycin	200	1.12	18.0	0.16[2]	38.3 ± 7.7	1.08	11.4 ± 2.1	1.25
azithromycin	1200	0.881	100	0.84	161 ± 7.7	1.52	n.d.[6]	
ritonavir	800	28.5	97.4	0.02[2]	5.64 ± 1.39	1.35	0.781 ± 0.048	3.49
lopinavir	400	15.2	54.8	0.015	18.4 ± 7.2	1.04	n.d.[6]	
probenecid	2000[3]	52.0[3]	487	0.10[2]	130 ± 40[4]	1.37	76.2 ± 7.1	1.64
diltiazem	100	0.0536	15.0	0.22[2]	193 ± 112	1.03	>100	<1.03
verapamil	80	0.190	11.1	0.10[2]	89.5 ± 52.9	1.01	51.6 ± 15.9	1.02
omeprazole	20	1.18	4.77	0.03	53.9 ± 14.3	1.00	n.d.[6]	
cimetidine	200	2.75	52.0	0.81[2]	>100	<1.42	>300	<1.14

The K_i values are expressed as the mean ± computer-calculated S.D. The inhibitors, which are shown by boldface, increased the AUC of the plasma AUC in the previous clinical studies. Data for dosages, I_{max}, and f_u are from

product information of each drug except the data with footnotes. R and $I_{in,max}$ values are calculated according to (Eq. 2) and (Eq.3), respectively (see **Materials and Methods**).

*1 Data for protein unbound fraction of cyclosporin A was from a previous report (Lemaire and Tillement, 1982).

*2 These data are from Goodman & Gilman's The Pharmacological Basis of Therapeutics, 10th Edition.

*3 Data for dosages, I_{max} of probenecid from a previous report (Selen et al., 1982).

*4 K_i values of probenecid were previously determined (Tahara et al., 2006).

*5 K_i values of inhibitors for the OATP1B1-mediated uptake of pitavastatin were previously determined (Hirano et al., 2006).
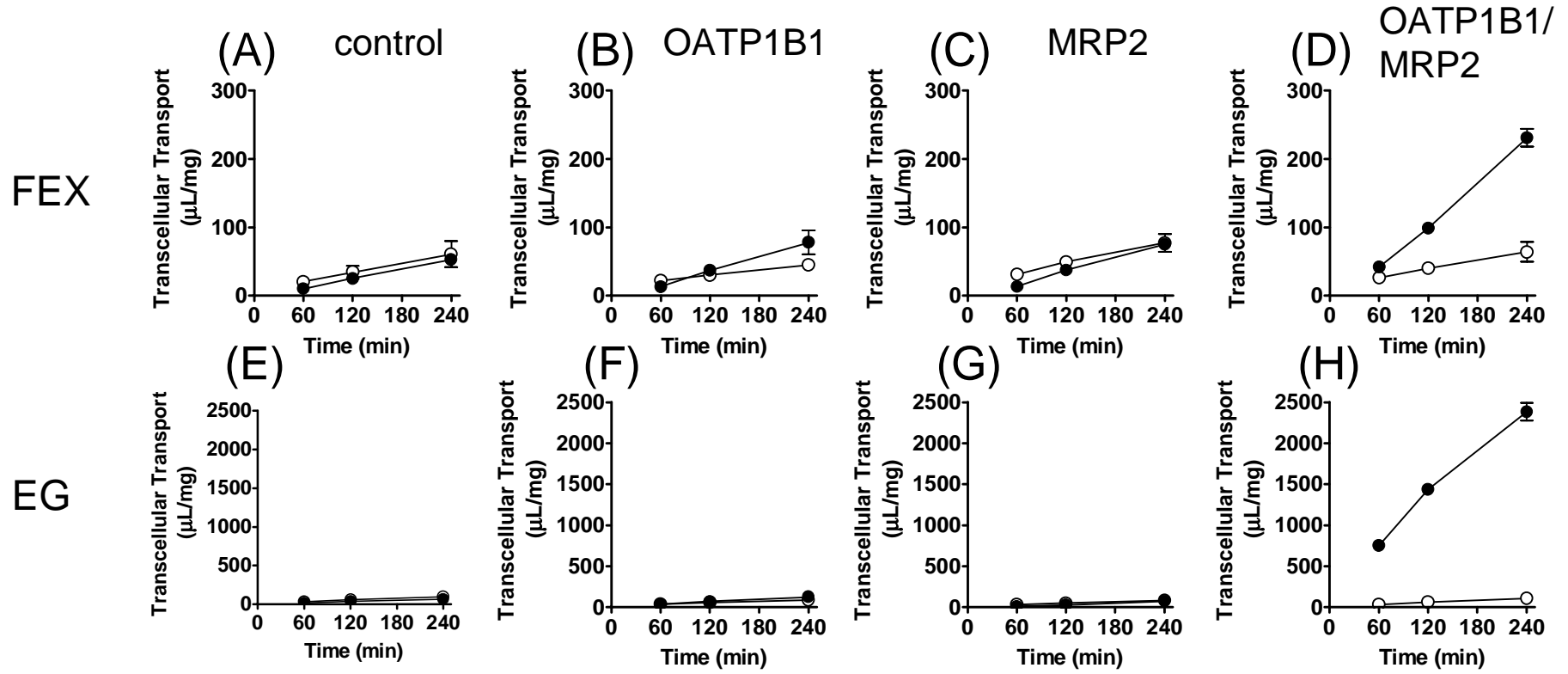
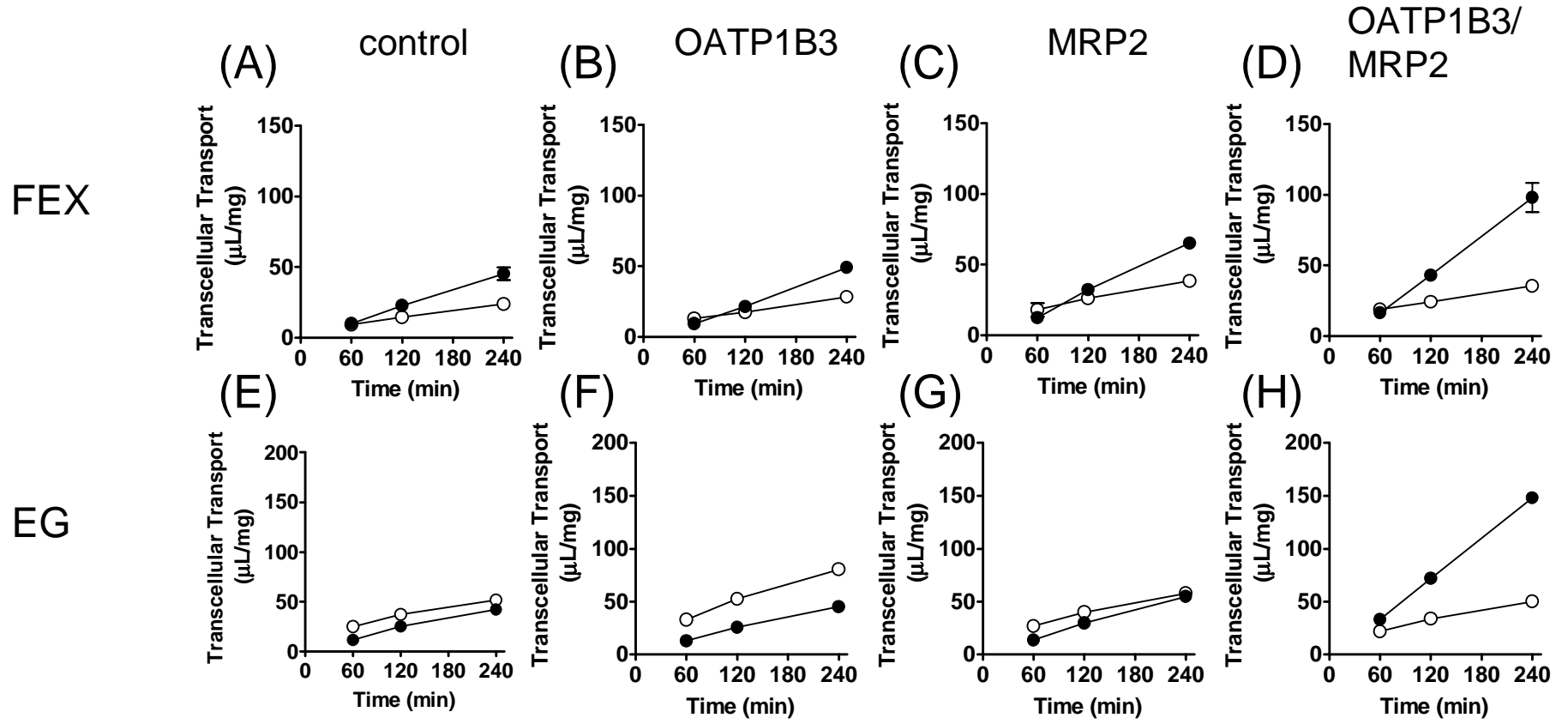*6 Not determined.

Figure 1

Figure 2

Figure 3