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Research Paper

Lack of Improvement of Oral Absorption of ME3277 by Prodrug Formation Is Ascribed to the Intestinal Efflux Mediated by Breast Cancer Resistant Protein (BCRP/ABCG2)

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Purpose. ME3229, an ester-type prodrug of a hydrophilic glycoprotein IIb/IIIa antagonist (ME3277), failed to show improved oral absorption. Okudaira *et al.* (J. Pharmacol. Exp. Ther. 294. 580–587, 2000) provided a piece of evidence that this is ascribed to an efflux system, distinct from P-gp and MRP2, that extrudes ME3277 formed from ME3229 in the intestinal epithelial cells. The aim of the present study is to examine the involvement of breast cancer resistant protein (BCRP/ABCG2) as a cause of low oral absorption of ME3229.
Methods. The transport activity of ME3277 in the presence and absence of ATP was determined using a rapid filtration method with the membrane vesicles prepared from LLC-PK1 cells expressing BCRP. The plasma concentrations of ME3229 and its metabolites were compared between Bcrp1$^{-/-}$ mice and wild-type mice after a single-pass perfusion of small intestine with ME3229.
Results. The ATP-dependent uptake of ME3277 was greater in BCRP-expressing membrane vesicles than that in the control vesicles. Furthermore, it was found that after intestinal perfusion with ME3229 for 60 min, the plasma concentrations of ME3277 and PM-5, a metabolite of ME3229, increased 2-fold and 3-fold, respectively, in Bcrp1 knockout mice. It is possible that BCRP acts synergistically with intestinal carboxylesterases.
Conclusion. These results suggest that Bcrp1 plays an important role in the intestinal efflux of ME3277 and, probably, PM-10 and PM-11, metabolites of ME3229, and limits its BA after oral administration of ME3229.

KEY WORDS: absorption; BCRP/ABCG2; intestinal perfusion; ME3229; prodrug.

INTRODUCTION

The oral route is the most popular way of administering drugs. Orally administered compounds have to cross the intestinal enterocytes to reach the blood circulation and target tissue. In this process, enterocytes form a selective barrier to prevent the passage of drugs. ME3277, a newly developed glycoprotein IIb/IIIa receptor antagonist, has a poor oral bioavailability (BA), presumably because of its low lipophilicity (1). To improve the low BA, ME3229, an ester-type prodrug of ME3277, was designed (1). ME3229 is rapidly metabolized to ME3277 in epithelial cells via monoester compounds, PM-10 and PM-11, as shown in Fig. 1 (1). However, in spite of the increased log D value, the BA of ME3277 was still low (only 10%) after oral administration of ME3229 in rats (1). Further *in situ* studies revealed that ME3229 itself is taken up into enterocytes; however, ME3277 and its monoester compounds formed in the epithelial cells undergo active efflux into the gut lumen (1). Therefore, it has been suggested that the low absorption of ME3277 can be accounted for by rapid hydrolysis from its ester-prodrug ME3229 and subsequent active efflux in the intestine (1).

Efflux transporters, such as P-glycoprotein (P-gp) and multidrug resistance associated protein 2 (MRP2), that are expressed on the apical membrane of epithelial cells play an important role in this barrier function in the intestine. *In vitro* analysis has shown that the permeability of ME3277 across a monolayer of Caco-2 cells did not show any directional transepithelial transport and verapamil, an inhibitor of P-gp, had no effect on the permeability of ME3277 across rat intestinal tissue in an Ussing chamber study. Furthermore, in EHBR, the Eisai hyperbilirubinemic rat with a hereditary deficiency in MRP2, the translocation of ME3277 was not changed compared with that in normal rats (Sprague-Dawley rats). These results rule out the involvement of these two transporters (2).

The Breast Cancer Resistance Protein (BCRP/ABCG2) is another candidate transporter that plays an important role in drug absorption like P-gp and MRP2, and it is possible that BCRP may limit the absorption of ME3229 by extruding it

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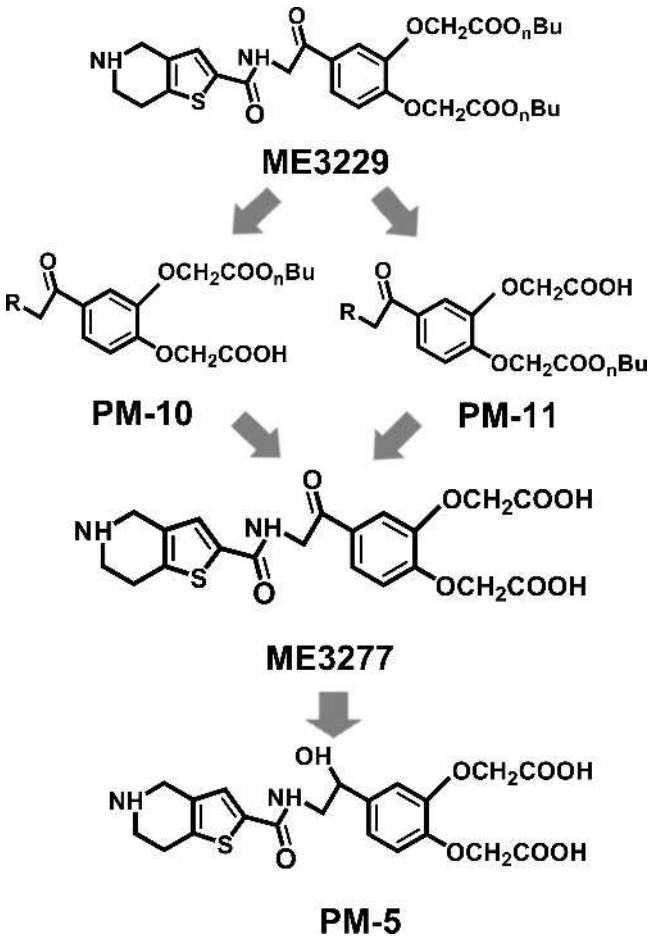

Fig. 1. Chemical structure of ME3229 and its metabolites.

and its metabolites from epithelial cells into the gut lumen. Recently, BCRP has been shown to be expressed at the apical membrane of small intestinal and colonic epithelia and mediate the secretory transport of substrate drugs from enterocytes to the gut lumen (3–5). Jonker *et al.* reported that BCRP had a marked effect on the oral bioavailability of its substrate (3–5). In Bcrp1 knockout mice, the oral availability of topotecan, a typical substrate of BCRP, was dramatically increased, indicating the importance of this transporter as far as drug absorption and disposition are concerned (4). By considering the substrate specificity of BCRP, it is possible that anionic compounds are also excreted into the intestinal lumen via BCRP (6,7). In this study, we investigated whether BCRP accepts ME3277 as a substrate and has an effect on the oral absorption of ME3229 by extruding ME3277 and other metabolites.

MATERIALS AND METHODS

Chemicals

ME3229 and its metabolite, ME3277, PM-5, PM-10, PM-11, and the internal standards for the assay of these compounds, EF5084 and EF5139, were synthesized by Meiji Seika Kaisha Ltd. (Yokohama, Japan). FITC-inulin was purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals were commercially available and of reagent grade. LLC-PK1 cells were cultured in Dulbecco's Modified Eagle's Medium (GIBCO BRL, Gaithersburg, MD, USA), after addition of 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Preparation of Membrane Vesicles

Wild-type BCRP cDNA was purchased from Invitrogen (Carlsbad, CA) (no. H24176). To prepare BCRP-expressing membrane vesicles, the recombinant adenovirus containing BCRP cDNA was constructed using the Adeno-X™ Expression System (BD Biosciences, Palo Alto, CA, USA) according to the user manual. LLC-PK1 cells were infected with recombinant adenoviruses containing BCRP cDNA at 100 multiplicity of infection (M.O.I.). As a negative control, cells were infected with virus containing GFP cDNA (pAd-GFP). Cells were harvested at 48 h after infection, and then the membrane vesicles were isolated using a standard method described previously in detail (8,9).

Vesicle Transport Assays

The uptake study of ME3277 was performed as reported previously (9). To measure the amount of ME3277 retained on the filter, the membrane filter was placed in 1 ml MeOH containing 10 μl H_2O and 10 μl internal standard (1 μg/ml EF5084 in H_2O), followed by sonication for 5 min. This aliquot was centrifuged for 5 min at 10,000 rpm and the supernatant and residue were separated. Then, 1 ml MeOH was added to the residue and the sample was centrifuged for 5 min at 10,000 rpm. The resulting supernatant was mixed with the earlier supernatant, and a 100 μl aliquot of propylene glycol / MeOH (10:90) was added. The sample was dried under N_2 at 40°C, the pellet was dissolved in 300 μl 1% HCOOH and passed through a 0.45-μm filter. The concentration of ME3277 in this sample was measured by liquid chromatography mass spectrometry (LC-MS/MS) using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) and an API 3000 MS/MS system (PE-Sciex). The separation was performed on a Xterra RP 18 column (2.1 mm ID × 50 mm, 3.5 mm; Waters), with 1 v/v % HCOOH/CH_3CN (90/10) as the mobile phase. The flow rate was 0.3 ml/min. Mass spectra were determined in the positive ion-detecting mode, and the monitored ion was 449.1 → 165.4 for ME3277, and 463.2 → 165.8 for EF5084, respectively. The amount of ligand taken up into vesicles was normalized in terms of the amount of membrane protein. The uptake activity was defined as the amount of ligand divided by the ligand concentration in the medium.

Animals

The animals used in this study were female Bcrp1$^{-/-}$ and wild-type mice, all of friend virus B-type susceptible (FVB) genetic background, produced as previously reported (4). They were between 28 and 32 weeks of age. Animals received food and water *ad libitum*. The animal works were performed according to the guidelines provided by the Institution Animal Care Committee (Graduate School of Pharmaceutical Science, The University of Tokyo).

Single-Pass Perfusion of Mouse Small Intestinal Segments

Single-pass perfusion of mouse small intestine was performed using a method described previously (10–12). Before the experiments, the mice were fasted overnight, but water was available *ad libitum*. Mice were then anesthetized with

Nembutal, and after opening the abdominal cavity, a 7-cm jejunal segment was prepared. The top side of the segment was cannulated with silicon tubing (OD 1.2 mm ID 0.8 mm) connected to a perfusion pump, and the intestinal contents were flushed out with 3 ml perfusion buffer at 37°C (20.1 mM Na_2HPO_4, 47 mM KH_2PO_4, 101 mM NaCl), followed by 3 ml air. The outflow from the segment was led into the sampling tube via polyethylene tubing. The segment was perfused with perfusion buffer containing 112 μg/ml ME3229 as a test compound and 40 μM FITC-inulin as a nonabsorbable marker. The dimethyl sulfoxide (DMSO) used to dissolve ME3229 did not exceed 0.5% of the perfusate volume. The perfusion flow rate was 0.1 ml/min. The outflow from the intestinal segment was collected for 2 min at 10 min intervals, followed by deproteinization with an equivalent volume of CH_3CN. Blood samples were collected from the jugular vein at 35, 45, and 60 min, and thereafter samples were centrifuged to prepare plasma samples. All samples were frozen in liquid nitrogen and stored at –20°C until required.

Determination of ME3229 and ME3277 and Their Metabolites (PM-5, PM-10, and PM-11) in the Samples from the Perfusion Study

Outflow samples were diluted 10-fold with 20% CH_3OH to measure the concentration of ME3277 and PM-5 and diluted 10-fold with 50% CH_3CN for ME3229, PM-10, and PM-11. Each concentration was determined by HPLC-UV. Compounds were separated on an Inertsil ODS-2 Column (4.0 mm ID × 10 mm + 4.6 mm ID × 250 mm) using gradient elution with 0.5 v/v % CH_3COOH / CH_3CN as the mobile phase. The detection wavelength was 280 nm. To determine the concentration of each compound in the plasma specimens, 1 ml HCOOH (v/v %) and 50 μl internal standard solution (EF5084 for ME3277 and PM-5, EF5139 for ME3229, PM-10, and PM-11) were added to 25 μl plasma samples and then mixed. The samples were centrifuged and the supernatant was transferred to an Empore Disk Cartridge C18 (4 mm/1 ml) or Empore Disk Cartridge SDB-XC (4 mm/1 ml) to extract ME3277 and PM-5, or ME3229, PM-10, and PM-11, respectively. The concentration of each compound in the elute solution was determined by LC-MS/MS, using an Agilent 1100 HPLC system (Agilent Technologies) and an API 3000 MS/MS system (PE-Sciex). Separation of ME3229, PM-10, and PM-11 was conducted using an Atlantis dC18 column (2.1 mm ID × 50 mm, 3 mm; Waters), and gradient elution with 1 v/v % HCOOH/CH_3CN as the mobile phase. The flow rate was 0.35 ml/min. ME3277 and PM-5 were separated on an Atlantis dC18 column (2.1 mm ID × 50 mm, 3 mm; Waters), with 1 v/v % HCOOH/CH_3CN (90/10) as the mobile phase. The flow rate was 0.3 ml/min. Mass spectra were determined in the positive ion-detecting mode. The monitored ion was 561.2 → 165.7 for ME3229, 505.1 → 165.7 for PM-10, 505.2 → 165.7 for PM-11, 575.2 → 165.5 for EF5139, 449.1 → 165.4 for ME3277, 450.9 → 165.8 for PM-5, and 463.2 → 165.8 for EF5084, respectively.

FITC-labeled inulin was determined using a Hitachi fluorescence spectrophotometer F-3000 (Hitachi, Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. The concentration of the outflow samples was corrected using the concentration of FITC-inulin.

RESULTS AND DISCUSSION

Vesicle Transport Assays

ME3229 is metabolized to ME3277 in epithelial cells via monoester compounds, PM-10 and PM-11. The structures of these compounds are shown in Fig. 1. In the human jejunum, BCRP showed quite high levels of mRNA together with MRP2, and compared with other ABC transporters, including P-gp (13). To determine whether BCRP accepts ME3277 as a substrate, ATP-dependent uptake of ME3277 was examined in membrane vesicles prepared from BCRP-expressing LLC-PK1 cells. As shown in Fig. 2, ATP-dependent uptake of ME3277 was observed by membrane vesicles from BCRP-expressing LLC-PK1 cells. A little uptake was observed by membrane vesicles from control cells, presumably due to the endogenous transporter. However, the uptake by BCRP-expressing cells was significantly greater than that of control cells. This result shows that BCRP can accept ME3277 as a substrate. It is possible that PM-10 and PM-11 may be substrates of BCRP as well. However, the detection range is limited and, therefore, we focused on ME3277 in the current study.

Single-Pass Perfusion of Mouse Small Intestinal Segments

To estimate the contribution of BCRP to the absorption of ME3229, we used Bcrp1$^{-/-}$ mice and performed single-pass perfusion experiments on mouse intestinal segments. Figure 3 shows the concentration of ME3229, ME3277, PM-10, and PM-11 in the outflow samples. Steady-state was assumed for the perfusate between 10 and 60 min, and the composition of

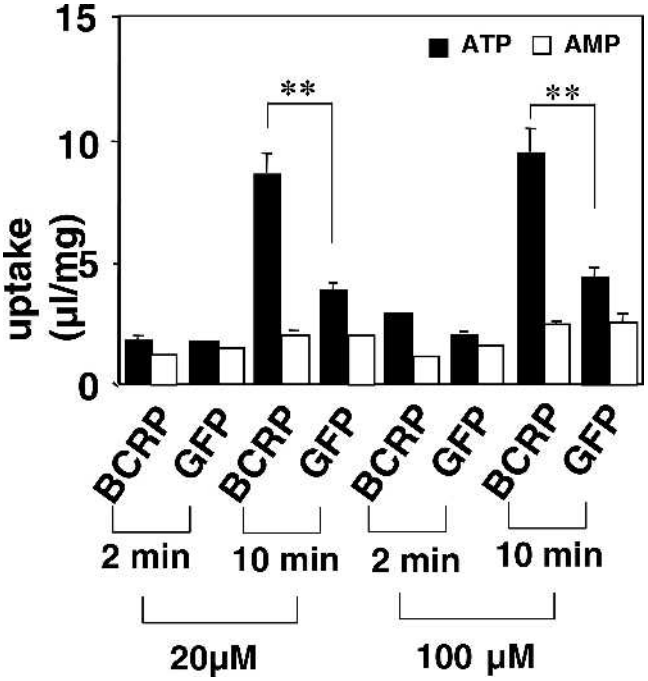

Fig. 2. The transport of ME3277 by human BCRP. The uptake of ME3277 by membrane vesicles (8 μg protein) prepared from BCRP or GFP-expressing LLC-PK1 cells was examined for 2 min or 10 min at 37°C in a medium containing 20 μM and 100 μM ME3277 in the presence or absence of ATP. **Significantly higher in BCRP-expressing membrane vesicles ($p < 0.01$). Data expressed as mean ± SE; $n = 3$.

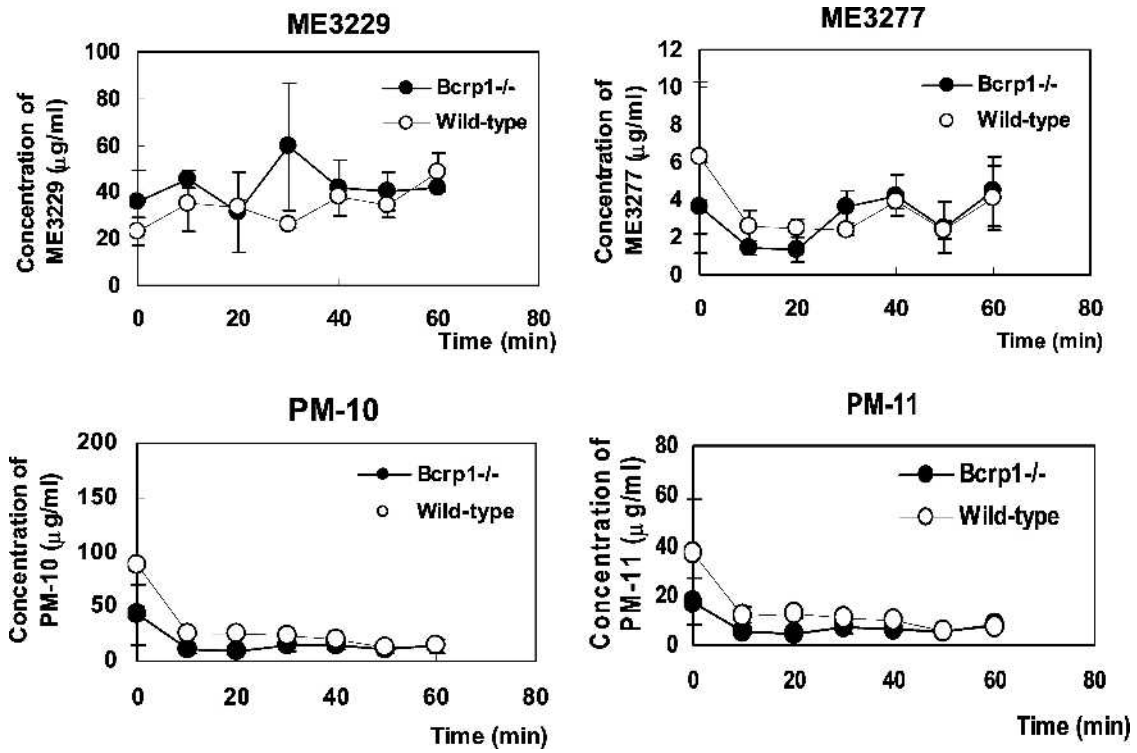

Fig. 3. Concentration of ME compounds in the perfusate. A segment of mouse jejunum was perfused with 112 μg/ml ME3229 for 60 min. The length of the segment and the perfusion flow rate were 7 cm and 0.1 ml/min, respectively. Each point represents the mean ± SE of three mice.

the samples was comparable with that obtained from single-pass perfusion experiments in rats (1). ME3229 was the major compound and its concentration was 36.2 ± 3.8 and 43.0 ± 16.1 μg/ml in wild-type mice and Bcrp1$^{-/-}$ mice, respectively. This is approximately 50% of the input concentration. The concentration of its active metabolite, ME3277, was 3.06 ± 0.69 and 3.21 ± 1.17 μg/ml in wild-type mice and Bcrp1$^{-/-}$ mice, respectively. The concentrations of PM-10 and PM-11 were 19.2 ± 2.1 and 9.11 ± 1.16 μg/ml in wild-type mice and 12.2 ± 3.7 and 6.47 ± 1.18 μg/ml in Bcrp1$^{-/-}$ mice, respectively. ME3277 and PM-5 were determined in plasma. Figure 4 shows the plasma concentration of these compounds and, after perfusion for 60 min, the plasma levels of ME3277 in Bcrp1$^{-/-}$ mice increased about 2-fold while those of PM-5 increased about 3-fold compared with the wild-type mice ($p < 0.05$). Therefore, Bcrp1 plays an important role in the disposition of ME3277 in the body after oral administration. There are two possibilities to explain this result. One is the decreased intestinal efflux, and the other is the reduced total body clearance of ME3277 and PM-5. In addition, it has been reported that BCRP contributes to the renal elimination of some compounds (14). *In vivo* pharmacokinetic analysis of ME3277 after i.v. administration was performed and most of ME3277 was excreted in urine as unchanged drug (80%)

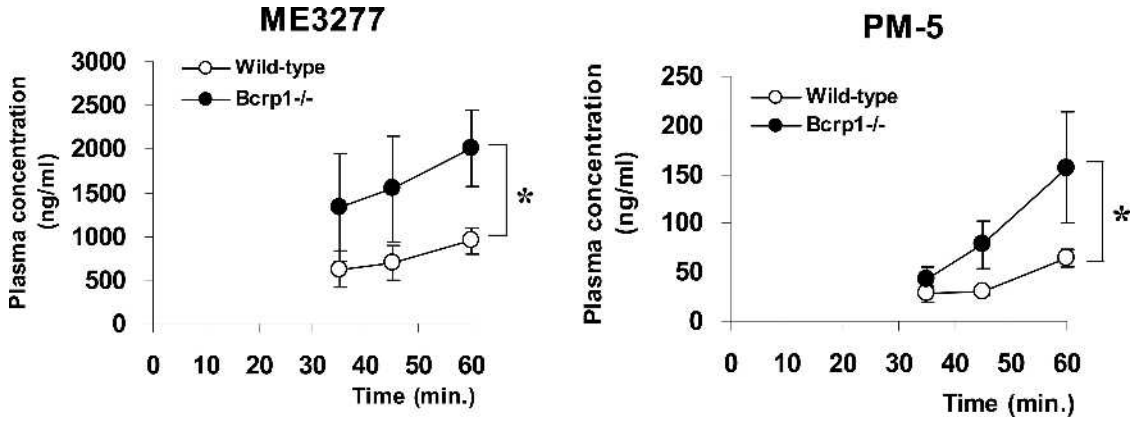

Fig. 4. Concentration of ME3277 and PM-5 in plasma. A segment of mouse jejunum was perfused with 112 μg/ml ME3229 for 60 min. At 35, 45, 60 min, blood samples were collected from the jugular vein and juglar vein and plasma samples were prepared. Concentration of ME3277 and PM-5 in plasma were determined by LC-MS/MS method. Each point represents the mean ± SE of three mice. *$p < 0.05$.

(Okudaira *et al.* unpublished data). The renal clearance was 290–340 ml h^{-1} kg^{-1}, which is comparable with the f_u*GFR (210–320 ml h^{-1} kg^{-1}) taking the unbound fraction (f_u, 0.4) of ME3277 in plasma into consideration. Thus, the renal elimination of ME3277 appears to be accounted for primarily by glomerular filtration. Therefore, it is unlikely that knockout of Bcrp1 affects the elimination of ME3277 from the body. Also, the increased concentration of ME3277 and PM-5 in the plasma of Bcrp1$^{-/-}$ mice indicates that the absorption of ME3229 is increased more than 2-fold in the absence of Bcrp1.

PM-10 and PM-11, precursor monoesters of ME3277, are quite unstable in plasma, and are hydrolyzed to ME3277 with half-lives of less than 1 min in rats (1). Taking this into consideration, it is possible that the increased plasma concentrations of ME3277 in Bcrp1$^{-/-}$ mice may be partly due to a reduced efflux of PM-10 and PM-11. This is also suggested by the slightly reduced recovery of PM-10 and PM-11 in the outflow of the perfusate in Bcrp1$^{-/-}$ mice (Fig. 3). Our previous research has shown that a compound which inhibits the efflux transport of ME3277 also inhibits the efflux of PM-10 and PM-11 in the gut lumen of rats (2). This result suggests that ME3277 and its precursor monoesters are accepted by the same transporter. Further studies are necessary to confirm whether these metabolites are indeed substrates of Bcrp1.

Recently, a synergistic role of metabolic enzymes and efflux transporters in reducing the oral bioavailability was described (15). The most important examples are the role of "CYP3A/3A4 and P-gp" and "conjugative metabolism and efflux transporters such as MRP2" (16–18). Carboxylesterases (CES) catalyze the conversion of a carboxylic ester to an alcohol and a carboxylic acid. In addition to carboxylic acid hydrolysis, the enzyme also hydrolyzes amides, thioesters, phosphoric acid esters, and acid anhydrides. To date, four families of CES have been identified and CES1 and CES2 play a major role in converting the prodrug to active metabolites. In mice, CES1 is widely distributed throughout the body, with high levels in the liver, kidney, brain, macrophage, lung, and plasma while CES2 is expressed in the small intestine and liver (19). Satoh and Hosokawa used prodrugs of ACE inhibitors, such as temocapril, as well as propranolol and flurbiprofen, to identify the substrate specificity of CES1 and CES2 (19). They found that the most of the temocapril and flurbiprofen molecules were hydrolyzed in the liver, whereas propranolol was hydrolyzed in both the small intestine and liver. These results indicate that the substrate specificities of CES1 and CES2 differ from each other (19). Taking this into consideration, it is possible that one of the factors to account for the increase in the oral bioavailability of ACE inhibitors after administration of respective ester-type prodrugs may be the minimal intestinal hydrolysis. In contrast, the low bioavailability of ME 3277 even after administration of ME3229 may be accounted for by the efficient hydrolysis of ME3229 in enterocytes and subsequent excretion of its metabolites via BCRP.

Therefore, it is possible that, for certain compounds such as ME3229, BCRP acts synergistically with CES to reduce the oral bioavailability. This is the first report to provide evidence for such a role. *In situ* and *in vivo* studies using BCRP knockout mice have suggested an interplay between BCRP and enzymes for conjugation, such as UDP-glucuronosyl transferases and sulfate transferase, has been suggested. In BCRP knockout mice of which the intestinal lumen was perfused with medium containing 4-methylumbelliferone (4MU) and E3040, the efflux rate of glucuronide of 4MU and E3040 into the outflow was significantly decreased, and that of sulfate of 4MU was diminished (unpublished observation). In addition to the intestine, BCRP plays a major role in the renal clearance of E3040 sulfate (14). Such interplay between efflux transporters and metabolic enzymes constitutes efficient detoxification system in the body and protects the body from the invasion of xenobiotics.

In conclusion, ME3277 is a substrate of BCRP, and the perfusion study in Bcrp1$^{-/-}$ mice demonstrated that efflux of ME3277, and probably other metabolites, by Bcrp1 limits its BA after oral administration of ME3229. Although developing a prodrug is one of efficient modification to improve the oral absorption of hydrophilic compounds, it is necessary to take into consideration where metabolites are formed and how they behave until they reach the target site.

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