Polarized Efflux of Mono- and Diacid Metabolites of ME3229, an Ester-Type Prodrug of a Glycoprotein IIb/IIIa Receptor Antagonist, in Rat Small Intestine

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Accepted for publication July 26, 2000 This paper is available online at http://www.jpet.org

ABSTRACT
ME3229 is an ester-type prodrug of a glycoprotein IIb/IIIa receptor antagonist ME3277. In our previous study, it was shown that only a small part of the drug taken up into the enterocytes reached the mesenteric vein, mainly due to transporter-mediated efflux of its hydrolyzed metabolites formed in the cells. To characterize the efflux transport system for the metabolites, the transport of the diacid metabolite ME3277 and the monoacid metabolites PM-10 and PM-11 were studied. ME3277 and PM-10 were preferentially transported in the serosal-to-mucosal direction across the rat small intestine in the presence of glucose. Permeability of ME3277 across monolayer of Caco-2 cells with P-glycoprotein (P-gp) and indomethacin-sensitive efflux pump expression did not show any directionality and verapamil, an inhibitor of P-gp, and indomethacin did not affect the permeability of ME3277 across rat intestinal tissue. Directional transport was not site specific and was observed in the Eisai hyperbilirubinemic rat whose canalicular multispecific organic anion transporter/multidrug resistance-associated protein (cMOAT/MRP2) is hereditarily defective as well as in normal rats. The efflux transport of ME3277 was inhibited by 1-naphthol, 1-choloro-2,4-dinitrobenzene, and sulfobromophthalein, and efflux of ME3277 and monoacid metabolites from intestinal tissue preloaded with ME3229 fell in the presence of 1-naphthol and sulfobromophthalein. These results demonstrate that mono- and diacid metabolites of ME3229 were pumped out into the gut lumen by an energy-dependent transport system located on the mucosal membrane of intestinal tissue and distinct from either P-gp, indomethacin-sensitive efflux pump or canalicular multispecific organic anion transporter/MRP2. An inhibition study suggested that this unknown transporter has a substrate specificity similar to that of MRP transporter families.

ME3229 is an ester-type prodrug of a GP IIb/IIIa antagonist, ME3277, synthesized at Meiji Seika Kaisha, Ltd., Yokohama, Japan. The structures of these compounds are shown in Fig. 1. In our previous study, we analyzed the characteristics of the intestinal absorption of ME3229, to clarify the mechanism behind its poor oral absorption. It was found that the prodrug was taken up into enterocytes at a rate compatible with its lipophilicity (log \( D \equiv 1.27 \)). However, only a small part of the hydrolyzed metabolites formed in the enterocytes reached the mesenteric vein, mainly due to efflux into the intestinal lumen, which was suggested to be one of the mechanisms for its low bioavailability. Moreover, the permeation of the major metabolite across rat small intestinal tissue was significantly greater in the serosal-to-mucosal direction, suggesting that some efflux transport system contributes to the efflux of the metabolites into the gut lumen (Okudaira et al., 2000).

The prodrug approach has been adopted to enhance the oral absorption of zwitterionic GP IIb/IIIa antagonists. However, this approach proved unsuccessful until a dual prodrug, with masking of both acidic and basic groups, was introduced (Kamm et al., 1998; Wittke et al., 1999). Thus, it is important to characterize the efflux transport of ME3277 and identify the site in the molecule recognized by the efflux transport system.

Recently, a variety of efflux transport systems have been shown to exist on the mucosal membrane of enterocytes and to limit intestinal absorption of some drugs. P-glycoprotein (P-gp) and canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2 (cMOAT/MRP2) are well characterized efflux transporters. P-gp is an ATP-dependent, efflux membrane transporter with broad sub-

ABBREVIATIONS: GP IIb/IIIa antagonist, glycoprotein IIb/IIIa receptor antagonist; P-gp, P-glycoprotein; cMOAT/MRP2, canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2; EHBR, Eisai hyperbilirubinemic rat; DNP-SG, 2,4-dinitrophenyl-S-glutathione; SD rat, Sprague-Dawley rat; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; BSP, sulfobromophthalein; CDNB, 1-choloro-2,4-dinitrobenzene; 2-DG, 2-deoxyglucose; A, apical; S, serosal; M, mucosal; pe, permeability; 1-NG, 1-naphthol glucuronide.
strate specificity for a number of structurally diverse drugs (Hunter and Hirst, 1997; Arimori and Nakano, 1998; Wacher et al., 1998). Unlike P-gp, which preferentially transports lipophilic cations (Tanaka et al., 1996), substrates for cMOAT/MRP2 are anionic compounds, including glucuronide and glutathione conjugates (Oude Elferink et al., 1995; Keppler and König, 1997; Kusuhara et al., 1998; Suzuki and Sugiyama, 1998; König et al., 1999). Expression of P-gp and cMOAT/MRP2 in intestinal tissue has been documented (Thiebaut et al., 1987; Hsing et al., 1992; Hirohashi et al., 1998, 2000a; Gotoh et al., 2000). In addition, the impact on the bioavailability has been established in experiments using mdr1a/1b knockout mice and mutant rats, EHBR, whose cMOAT/MRP2 is hereditarily deficient. In mdr1a/1b knockout mice, the absorption of paclitaxel, a substrate of P-gp, was greater than that in wild-type mice (Schinkel et al., 1997; Sparreboom et al., 1997). In EHBR, the export of DNP-SG, a typical substrate of cMOAT/MRP2, across rat small intestinal tissue was significantly reduced compared with that in control rats (Gotoh et al., 2000). These findings suggest that those efflux transport systems limit the oral absorption of some drugs.

There are other transporters present in intestinal tissue, such as the indomethacin-sensitive efflux system for 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein and other fluorochromes, found in several intestinal epithelial cell lines (Allen et al., 1990; Collington et al., 1992) and MRP3, which mediates the cellular export of nonconjugated and conjugated organic anions (Hirohashi et al., 1998, 1999; König et al., 1999). However, MRP3 has recently been shown to be expressed on the basolateral membrane of small intestinal epithelial cells in humans (Hirohashi et al., 2000b).

As far as GP IIb/IIIa antagonists are concerned, the contribution of P-gp to the efflux transport of a cyclic peptide, DMP728, and carboxyester prodrug L-775,318 has been suggested, based on the enhanced transport across rat small intestinal tissue or Caco-2 cell monolayers, and enhanced intestinal absorption in an in situ perfusion study in the presence of verapamil (Aungst and Saitoh, 1996; Prueksaritanont et al., 1998).

This study was performed to characterize the efflux transport system for the metabolites of ME3229, which was shown to be the reason for the reduced absorption of ME3229 in the intestinal perfusion experiment. The transport of the diacid metabolite ME3277 and the monoacid metabolite PM-10 was characterized using the everted sac and Ussing chamber methods.

**Materials and Methods**

**Animals.** Male SD rats and EHBR aged 9 to 11 weeks were supplied by Japan SLC Co. (Shizuoka, Japan). Rats were purchased 1 week before the experiments and fed on standard laboratory animal chow (MF; Oriental Yeast Co. Ltd., Tokyo, Japan). They were treated in accordance with the guidelines provided by the Animal Care Committee of Meiji Seika Kaisha, Ltd.

**Chemicals.** ME3229, ME3277, PM-10, PM-11 (Fig. 1), and EF5139 (Fig. 4) were synthesized at Meiji Seika Kaisha, Ltd. Verapamil, probenecid, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Phenol red, BSP sodium hydrate, CDNB, and 1-naphthol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Warfarin and 2-deoxyglucose (2-DG) were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). All the other chemicals and reagents were commercial products and of analytical grade.

**Cell Culture.** The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD). Caco-2 cells were grown in the Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), and 1% nonessential amino acids (10 mM) in culture flasks in a humidified air-5% C02 atmosphere. Cells were harvested with trypsin-EDTA and seeded on a 24-mm Transwell, polycarbonate membrane, and cultured for 20 days before starting the transport experiments. The cell passage number was 30.

**Permeation across Caco-2 Monolayers.** To study drug transport across Caco-2 cell monolayers, transport medium [apical (A), 1.5 ml; serosal (S), 2.6 ml] was used consisting of 1 mM CaCl2, 5.3 mM KCl, 0.4 mM KH2PO4, 0.5 mM MgCl2, 0.4 mM MgSO4, 3.3 mM
Na$_2$HPO$_4$, 137 mM NaCl, 25 mM HEPES, and 25 mM d-glucose (pH 7.4). After preincubation for 10 min, drug solution was applied to the donor compartment. At designated times, aliquots were taken from the receiver compartment. All samples were kept frozen until required for assay. The permeability coefficient across the Caco-2 cell monolayers ($P_{\text{app}}$) was calculated according to the following equation:

$$P_{\text{app}}(\text{cm/s}) = \frac{(dA/dt)(S_{\text{cell}} \cdot C_0)}{A}$$

where $dA/dt$ is the flux of drug across the monolayers (nmol/s), $S_{\text{cell}}$ is the surface area of the cell monolayers (4,526 cm$^2$), and $C_0$ is the initial drug concentration in the donor medium (μM).

**Permeation across Rat Small Intestinal Tissue.** The Ussing chamber and everted gut sac methods were used to evaluate permeation across rat small intestinal tissue. For Ussing chamber studies, side-by-side diffusion chambers (NaviCyte, Inc., Sparks, NV) were used. The volume of each half chamber was 3 ml and the exposed tissue surface area was 0.636 cm$^2$. Rats were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta. Jejunum, midgut (the upper part of ileum), and ileum (the lower part of ileum) were taken and the luminal contents were flushed out with ice-cold saline. Strips of intestinal tissue of approximately 1.5-cm length were excised and then cut open along the mesenteric border. Tissues were mounted onto the pins of the diffusion chambers. Care was taken to avoid Peyer’s patches. To the tissue in the Ussing chamber study and to the mucosal side in the everted sac study. To study energy dependence, glucose was replaced with equimolar 2-DG.

**Assay.** Concentrations of ME3277, PM-10, and PM-11 were determined by HPLC. The HPLC system consisted of an LCSS-900 controller, AS-950 autosampler, 2 PU-980 pumps, and UV-970 detector (Jasco Corporation, Tokyo, Japan) and a Shodex oven AO-30C (Showa Denko, KK, Tokyo, Japan) connected to a C-R7A plus integrator (Shimadzu, Inc., Kyoto, Japan). Compounds were separated on an Inertsil ODS-2 column (5 μm, 4.6 mm Φ × 250 mm; GL Sciences Inc., Tokyo, Japan) at 40°C, using a gradient procedure with 0.5% acetic acid and acetonitrile. The detection wavelength was 280 nm.

**Statistics.** Statistical comparisons were made using $t$ tests.

### Results

**Permeation of PM-10 across Rat Small Intestinal Tissue.** The directionality and energy dependence of PM-10 permeation were investigated in the Ussing chamber study. PM-10 was added to the mucosal or serosal side of the midgut isolated from SD rats. In both cases, PM-10 and ME3277 were detected in the receiving chamber. Figure 2 shows the flux of PM-10 and ME3277. The flux of both PM-10 and ME3277 was greater when PM-10 was added to the serosal side compared with that observed when PM-10 was added to the mucosal chamber. In the absence of glucose, the compounds were added to the mucosal surface at a concentration of 50 μM. Aliquots (200 μl) of serosal medium were collected at designated times and replaced with fresh medium.

In the inhibition studies, inhibitors were added to the both sides of the tissue in the Ussing chamber study and to the mucosal side in the everted sac study. To study energy dependence, glucose was replaced with equimolar 2-DG.

Drug flux was obtained from the slope of the amount appearing in the receiving medium versus time plot. In the Ussing chamber study, permeability coefficients from the S-to-M chamber [Pe(S → M)] and from the M-to-S chamber [Pe(M → S)] were calculated by dividing the drug flux in the direction of S-to-M and M-to-S by the initial donor concentration, respectively, and normalized by the surface area. In the everted sac study, Pe(M-to-S) was calculated by dividing the drug flux from the M-to-S surface by the tissue weight.

**Inhibition studies.** The inhibition studies were performed using PM-10, ME3277, and PM-11 at concentrations of 10 μM, 100 μM, and 1 mM, respectively. In the inhibition studies, inhibitors were added to the both sides of the tissue in the Ussing chamber study and to the mucosal side in the everted sac study. To study energy dependence, glucose was replaced with equimolar 2-DG.

Drug flux was obtained from the slope of the amount appearing in the receiving medium versus time plot. In the Ussing chamber study, permeability coefficients from the S-to-M chamber [Pe(S → M)] and from the M-to-S chamber [Pe(M → S)] were calculated by dividing the drug flux in the direction of S-to-M and M-to-S by the initial donor concentration, respectively, and normalized by the surface area. In the everted sac study, Pe(M-to-S) was calculated by dividing the drug flux from the M-to-S surface by the tissue weight.

**Statistics.** Statistical comparisons were made using $t$ tests.

**Fig. 2.** Directionality and energy dependence of PM-10 permeability across rat small intestinal tissue in the M-to-S and S-to-M directions (Ussing chamber study). Initial concentration of PM-10 in the donor compartment was 50 μM. Data represent flux appearing as PM-10 (a) and ME3277 (b) per unit of exposed surface area. Mean ± S.E., $n = 3$. ■, medium contained 3 g/l D-glucose; □, d-glucose was replaced by equimolar 2-deoxyglucose. *$P < .05$. **$P < .01$. **
directionality of the flux became minimal. Flux(S → M) for PM-10 fell significantly, but Flux(M → S) did not change significantly. In contrast, Flux(M → S) of ME3277 significantly increased 5-fold, but the fall in Flux(S → M) was not significant.

Transport Characteristics of ME3277. In the next part of our study, the transport characteristics of a metabolically stable metabolite ME3277 were studied. Figure 3 represents the effect of glucose depletion on the transport of ME3277 in the S-to-M and M-to-S direction observed in the Ussing chamber study. In the presence of D-glucose, Pe(S → M), Pe(M → S), and the ratio [Pe(S → M)/Pe(M → S)] were 3.55 ± 0.29 μm/min (mean ± S.E., n = 5), 0.55 ± 0.17 10 μm/min (n = 5) and 6.5, respectively. When D-glucose was replaced with 2-DG, Pe(S → M), Pe(M → S), and the ratio [Pe(S → M)/Pe(M → S)] became 1.93 ± 0.30 μm/min (mean ± S.E., n = 5), 1.21 ± 0.22 μm/min (n = 4), and 1.6, respectively.

The transport of ME3277 from the M-to-S surface of the everted gut sac in the presence and absence of glucose was determined. The Pe(M → S) in the presence and absence of D-glucose was 0.41 ± 0.09 μl/min/g of tissue (mean ± S.E., n = 3) and 1.82 ± 0.13 μl/min/g of tissue (n = 3), respectively.

The effect of the structurally related compound EF5139 on the flux of ME3277 in the M-to-S direction was investigated in the everted sac study. As shown in Fig. 4, amount permeating during the first 20 min of the incubation period significantly increased in the presence of EF5139. The effect of EF5139 disappeared after an incubation period longer than 40 min.

The effect of some compounds known to be inhibitors of a number of active transport systems on the transport of ME3277 was studied. Table 1 shows the effect of verapamil, indomethacin, and CDNB, a precursor of DNP-SG, on the transport of ME3277 in the S-to-M direction observed in the Ussing chamber study. In the presence of 0.5mM verapamil, Pe(S → M) fell to 57% of the control. Indomethacin did not affect the permeability of ME3277. In the presence of CDNB, at concentrations of 0.1, 0.2, and 0.5 mM, Pe(S → M) fell to 56, 51, and 39% of the control, respectively.

Table 2 shows the effect of some compounds on the permeation of ME3277 in the everted sac study. In the presence of BSP (2.5 mM), 1-naphthol (1 mM), and CDNB (0.5 mM), Pe(M → S) of ME3277 increased significantly by 2.81, 3.77, and 3.53-fold, respectively. The change in the Pe(M → S) of ME3277 produced by the other compounds was less than double.

In the Ussing chamber study, the permeation of ME3277 across the jejunum, midgut, and ileum isolated from SD rats and EHR were compared (Fig. 5). In both SD rats and EHR, Pe(S → M) was significantly greater than Pe(M → S) at any of the sites in the small intestine. No site dependence was observed in SD rats or EHR. Except for the ileum, where Pe(S → M) in EHR was significantly greater, no difference was observed between EHR and SD rats.

Transport of ME3277 across Caco-2 Cell Monolayers. The permeation coefficients of ME3277 across Caco-2 cell monolayers in the A-to-S and S-to-A direction were 7.51 × 10⁻⁸ ± 0.76 × 10⁻⁸ and 8.81 × 10⁻⁸ 10 ± 1.08 × 10⁻⁸ cm/s (mean ± S.E., n = 3), respectively. The permeability coefficients in the A-to-S and S-to-A direction were not statistically different.

Inhibitory Effect of 1-Naphthol and BSP on the Efflux of Other Metabolites of ME3229. To investigate whether ME3277 and the other metabolites of ME3229 are transported into the gut lumen by the same efflux transport system, an everted gut sac of rat small intestine was pre-loaded with ME3229 and then the intestinal sac was transferred to medium containing 0.5mM 1-naphthol or 2.5 mM
creased, and so the increase in the Flux(M → S) for PM-10 was suppressed, the fraction of PM-10 hydrolyzed to ME3277 increased, and so the increase in the Flux(M → S) for PM-10 and the reduction in the Flux(S → M) for ME3277 became less pronounced. The energy dependence of the transport of metabolically stable ME3277 across rat intestinal tissue was confirmed in the experiments using Ussing chamber and everted sac methods. Figure 3 shows that Pe(M → S) increased and Pe(S → M) decreased in the absence of glucose. An increase in Pe(M → S) in the absence of glucose was also detected in the everted sac study. These findings suggest that a transport system located on the brush-border membrane contributes to the efflux of ME3277.

We have evaluated the contribution of some known efflux transporters located on the brush-border membrane to drug efflux to the gut lumen. Among them are P-gp, indomethacin-sensitive efflux system, and cMOAT/MRP2. Table 1 shows the effect of verapamil, indomethacin, and CDNB, known inhibitors of each transport system, on Pe(S → M) of ME3277 in Ussing chamber study. Substrates of P-gp are generally lipophilic neutral or cationic drugs. Verapamil reduced Pe(S → M) at 0.5 mM but not at 0.2 mM. Even at 0.5 mM, Pe(M → S) determined by the everted sac method did not increase. The efflux transport of rhodamine 123, a substrate of P-gp, was reported to be inhibited completely in the presence of 0.5 mM verapamil in an experiment using everted gut sacs (Hsing et al., 1992). Indomethacin did not affect Pe(M → S) determined by the everted sac method.

### Discussion

This study was performed to characterize the efflux transport system for the metabolites of ME3229, an ester-type prodrug. In our previous study, we found that the metabolites formed in the intestinal tissue were pumped out into gut lumen, which was suggested to be one of the mechanisms for its low bioavailability (Okudaira et al., 2000).

In the first part of this study, we investigated the energy dependence of the transport of PM-10 by comparing the permeability in the presence and absence of glucose. As shown in Fig. 2, directionality observed in the presence of glucose became minimal for both PM-10 and ME3277 when glucose was replaced with 2-DG, and the efflux transport system was shown to be energy dependent. However, the change in the flux for PM-10 and ME3277 differed. This difference was explained by the change in the hydrolysis rate. In the absence of glucose, where efflux transport of PM-10 was suppressed, the fraction of PM-10 hydrolyzed to ME3277 increased, and so the increase in the Flux(M → S) for PM-10 and the reduction in the Flux(S → M) for ME3277 became less pronounced. The energy dependence of the transport of metabolically stable ME3277 across rat intestinal tissue was confirmed in the experiments using Ussing chamber and everted sac methods. Figure 3 shows that Pe(M → S) increased and Pe(S → M) decreased in the absence of glucose. An increase in Pe(M → S) in the absence of glucose was also detected in the everted sac study. These findings suggest that a transport system located on the brush-border membrane contributes to the efflux of ME3277.

We have evaluated the contribution of some known efflux transporters located on the brush-border membrane to drug efflux to the gut lumen. Among them are P-gp, indomethacin-sensitive efflux system, and cMOAT/MRP2. Table 1 shows the effect of verapamil, indomethacin, and CDNB, known inhibitors of each transport system, on Pe(S → M) of ME3277 in Ussing chamber study. Substrates of P-gp are generally lipophilic neutral or cationic drugs. Verapamil reduced Pe(S → M) at 0.5 mM but not at 0.2 mM. Even at 0.5 mM, Pe(M → S) determined by the everted sac method did not increase. The efflux transport of rhodamine 123, a substrate of P-gp, was reported to be inhibited completely in the presence of 0.5 mM verapamil in an experiment using everted gut sacs (Hsing et al., 1992). Indomethacin did not affect Pe(S → M) at 0.5 mM. Furthermore, although P-gp and indomethacin-sensitive efflux system are reported to be expressed on the brush-border membrane of Caco-2 cells (Hosoya et al., 1996; Makhey et al., 1998), the transport of ME3277 across Caco-2 cell monolayers did not show any directionality. Thus, the contribution of P-gp and indomethacin-sensitive efflux system was considered to be minimal. cMOAT/MRP2 is a drug efflux transporter whose substrates are glucuronide and glutathione conjugates and non-conjugated organic anions. Northern blot analysis indicated that cMOAT/MRP2 is expressed in the small intestine but to a lesser extent compared with liver and in Caco-2 cells and

### Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>n</th>
<th>Control Pe(M → S) μm/min</th>
<th>Treated Pe(M → S) μm/min</th>
<th>% Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil (0.2 mM)</td>
<td>4</td>
<td>3.53 ± 0.22</td>
<td>3.83 ± 1.00</td>
<td>108</td>
<td>0.8197</td>
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<tr>
<td>Verapamil (0.5 mM)</td>
<td>3</td>
<td>3.71 ± 0.20</td>
<td>2.11 ± 0.33</td>
<td>57</td>
<td>0.0783</td>
</tr>
<tr>
<td>Indomethacin (0.5 mM)</td>
<td>3</td>
<td>2.79 ± 0.57</td>
<td>2.45 ± 0.31</td>
<td>88</td>
<td>0.6288</td>
</tr>
<tr>
<td>CDNB (0.1 mM)</td>
<td>3</td>
<td>3.43 ± 0.28</td>
<td>1.92 ± 0.17</td>
<td>56</td>
<td>0.0723</td>
</tr>
<tr>
<td>CDNB (0.2 mM)</td>
<td>3</td>
<td>3.43 ± 0.28</td>
<td>1.74 ± 0.17</td>
<td>51</td>
<td>0.0627</td>
</tr>
<tr>
<td>CDNB (0.5 mM)</td>
<td>4</td>
<td>3.53 ± 0.22</td>
<td>1.36 ± 0.29</td>
<td>39*</td>
<td>0.0166</td>
</tr>
</tbody>
</table>

*P < .05 compared with control.

### Table 2

<table>
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<tr>
<th>Inhibitor</th>
<th>n</th>
<th>Control Pe(M → S) μl/min/g tissue</th>
<th>Treated Pe(M → S) μl/min/g tissue</th>
<th>% Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol red (10 mM)</td>
<td>5</td>
<td>0.30 ± 0.07</td>
<td>0.53 ± 0.08</td>
<td>177*</td>
<td>0.0496</td>
</tr>
<tr>
<td>Probenecid (10 mM)</td>
<td>5</td>
<td>0.29 ± 0.04</td>
<td>0.46 ± 0.08</td>
<td>155</td>
<td>0.0927</td>
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<tr>
<td>Warfarin (5 mM)</td>
<td>4</td>
<td>0.27 ± 0.05</td>
<td>0.44 ± 0.13</td>
<td>163</td>
<td>0.3949</td>
</tr>
<tr>
<td>DIDS (1 mM)</td>
<td>5</td>
<td>0.38 ± 0.11</td>
<td>0.40 ± 0.08</td>
<td>105</td>
<td>0.8660</td>
</tr>
<tr>
<td>BSP (2.5 mM)</td>
<td>6</td>
<td>0.26 ± 0.05</td>
<td>0.75 ± 0.16</td>
<td>281*</td>
<td>0.0305</td>
</tr>
<tr>
<td>Verapamil (0.5 mM)</td>
<td>4</td>
<td>0.44 ± 0.11</td>
<td>0.42 ± 0.06</td>
<td>95</td>
<td>0.9078</td>
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<tr>
<td>1-Naphthol (0.5 mM)</td>
<td>6</td>
<td>0.48 ± 0.09</td>
<td>0.74 ± 0.15</td>
<td>154*</td>
<td>0.0309</td>
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<tr>
<td>1-Naphthol (1 mM)</td>
<td>5</td>
<td>0.30 ± 0.07</td>
<td>1.13 ± 0.23</td>
<td>377*</td>
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<td>CDNB (0.5 mM)</td>
<td>3</td>
<td>0.49 ± 0.12</td>
<td>1.73 ± 0.21</td>
<td>353*</td>
<td>0.0332</td>
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*P < .05 compared with control.
contributes to the secretion of organic acids, such as DNP-SG (Ito et al., 1997; Gotoh et al., 2000; Hirohashi et al., 2000a). Site-specific expression of cMOAT/MRP2 in rat intestine was observed, i.e., jejunum > duodenum > ileum > colon. The transport of DNP-SG across the jejunum, in the S-to-M direction in SD rats, is greater than that in EHBR whose cMOAT/MRP2 is hereditary defective (Gotoh et al., 2000). As far as the transport of ME3277 is concerned, neither site specificity nor reduced Pe(S → M) was observed in EHBR compared with SD rats (Fig. 5). In the ileum, Pe(S → M) was even greater in EHBR. These findings suggest that cMOAT/MRP2 is not responsible for the efflux transport of ME3277 either. A contribution from MRP3/Mrp3 may not be probable because MRP3 has been found to be expressed on the basolateral membrane of human epithelial cells and its localization in rats was considered to be similar (Hirohashi et al., 1999).

Inhibition of the efflux transport of ME3277 by some other compounds was studied. Figures 3 and 4 suggest that inhibition of efflux transport could be detected in both in the Ussing chamber and everted sac studies. Because the membrane permeability of ME3277 is low, the everted sac method in which the permeated compound accumulated in 0.7 ml of serosal medium was favorable from an analytical point of view. In the presence of a structurally similar compound, EF5139, the M-to-S flux during the first 20-min period of incubation significantly increased (Fig. 4). EF5139 is a diester compound that is hydrolyzed to mono- and diacid in the intestinal tissue. Metabolic pathway and hydrolysis rate were similar to those for ME3229 (data not shown), and our preliminary experiment suggested its metabolites formed in the intestinal tissue were pumped out into mucosal medium. The data shown in Fig. 4 suggest that efflux of ME3277 is inhibited by the metabolite(s) of EF5139. However, the effect disappeared after a longer incubation period. We consider the concentration of the metabolites of EF5139 in the tissue was not maintained because of the reduced concentration of EF5139 in the medium due to hydrolysis and the efflux of the metabolites. In fact, peaks corresponding to the metabolites appeared on the chromatogram of apical medium.

Because the metabolites of ME3229 secreted into the gut lumen have a carboxyl group, the effect of organic acids was investigated. As shown in Table 2, probenecid and warfarin did not change Pe(M → S) significantly. DIDS, an anion exchange inhibitor, had no effect either. In the presence of phenol red, Pe(M → S) was significantly increased, but by 77% at most. The presence of an active efflux transport system for phenol red, which is inhibited by probenecid, has been reported (Saitoh et al., 1996). Our result suggested that ME3277 does not share the efflux system with phenol red and probenecid. On the other hand, BSP (2.5 mM), 1-naphthol (1 mM), and CDNB (0.5 mM) increased Pe(M → S) significantly by 2.8-fold or more (Table 2). The effect of CDNB was confirmed in the Ussing chamber study, where Pe(S → M) fell markedly (Table 1). These compounds are metabolized to glutathione or glucuronide conjugates. BSP, BSP-glutathione, DNP-SG, and 1-NG are all substrates for cMOAT/MRP2 (Kusuhara et al., 1998; Suzuki and Sugiyama, 1998). The excretion of 1-NG into bile and intestinal lumen has been compared in Wistar rats and cMOAT/MRP2-deficient TR rats. In contrast to the reduced biliary excretion in TR rats, excretion into the intestinal lumen was almost the same in both strains, suggesting that 1-NG is secreted by an organ-specific system distinct from cMOAT/MRP2 or is secreted by more than one system with a minimal contribution from cMOAT/MRP2 (de Vries et al., 1989). Based on the data shown in Fig. 5, the possibility that cMOAT/MRP2 is the main transport system for efflux of ME3277 may be excluded. Our results from the inhibition study suggest the existence of some other transporter(s) with a very similar substrate specificity to cMOAT/MRP2. These may include some other unknown transporters belonging to MRP families.

Finally, ME3229 was preloaded into everted gut sacs in the absence of inhibitors. As shown in Fig. 6, the efflux of monocarboxylic acids fell significantly in the presence of BSP and 1-naphthol. The effect on ME3277 was variable and not significant, possibly because the inhibition of efflux transport affected the formation rate of ME3277 as discussed in conjunction with Fig. 2. These results suggest that 1-naphthol and BSP inhibit the efflux of both ME3277 and monocarboxylic acids and that metabolites with one or two carboxyl groups in the molecule share the same transport system(s).

GP IIb/IIIa antagonists are Arg-Gly-Asp tripeptide mimet-
ics. As already mentioned, the bioavailability of simple carboxydrates of GP IIb/IIIa antagonists is generally low. Masking both anionic and cationic groups in the molecule led to improved oral absorption (Kamm et al., 1998; Wittke et al., 1999). In this study, we have demonstrated that the anionic group in the molecule of ME3277 is important for recognition by the efflux transport system. Although it is not clear whether the cationic group is essential for this system, designing a prodrug whose carboxydrate is not hydrolyzed in the intestinal tissue may be a useful approach to improve the oral absorption of this zwitterionic drug. In this context, improved oral absorption of some dual prodrugs of GP IIb/III a antagonists might be explained, at least in part, by the hypothesis that cleavage of the carboxydrate is delayed compared with the single prodrug and formation of metabolites preferentially exported by the transporter for anionic drugs is reduced. To validate this hypothesis, further research is needed to clarify whether the efflux transport system we have demonstrated in this study contributes to the efflux of other GP IIb/IIIa antagonists.

In conclusion, the efflux transport system for the metabolites of ME3229, which was responsible for its poor oral absorption rate, was characterized. Transport of PM-10, the major metabolite, and ME3277, the active drug, was shown to be energy dependent and the inhibition study suggested that metabolites of ME3229, both monocarboxylic acid and diacid, were pumped out into the gut lumen by a transport system with a similar substrate specificity to cMOAT/MRP2. The efflux transport system was shown to be distinct from either P-gp or cMOAT/MRP2.

References


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