A Study of the Intestinal Absorption of an Ester-Type Prodrug, ME3229, in Rats: Active Efflux Transport as a Cause of Poor Bioavailability of the Active Drug

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ABSTRACT
The intestinal absorption of a prodrug is affected by a number of factors, such as its membrane permeability, stability in the gut lumen, and conversion to the parent drug in enterocytes. We evaluated the absorption of ME3229, an ester-type prodrug of a hydrophilic glycoprotein IIb/IIIa receptor antagonist, ME3277, synthesized at Meiji Seika Kaisha, Ltd. The structures of these compounds are shown in Fig. 1. ME3277 was not orally active, presumably because of its low lipophilicity, and its bioavailability (BA) was shown in Fig. 1. ME3277 was not orally active, presumably because of its low lipophilicity, and its bioavailability (BA) was 10% of the dose was absorbed in rats. To clarify this unexpected outcome, we evaluated the rate of its disappearance from the gut lumen (V1), its degradation in the gut lumen (V2), uptake into enterocytes (Vupake), and appearance in the mesenteric vein (V2) by using a single-pass perfusion technique in combination with an in vitro metabolism study. Our data suggested that ME3229 crossed the apical membrane and was taken up into enterocytes at a rate compatible with its lipophilicity, but that only a small fraction of the metabolites formed in enterocytes reached the mesenteric vein, primarily attributable to efflux into the intestinal lumen. Transport of the main metabolite across rat intestinal tissue mounted on an Ussing chamber suggested that an active efflux system pumped out any ionized metabolite(s) present.

The use of a prodrug is a common approach to improve oral absorption of hydrophilic compounds exhibiting poor membrane permeability. However, there are many other factors that affect the oral absorption of prodrugs, such as hydrolysis of the ester pro moiety in the gut lumen and conversion to the active drug in enterocytes. ME3229 is an ester-type prodrug of a glycoprotein IIb/IIIa receptor antagonist, ME3277, synthesized at Meiji Seika Kaisha, Ltd. The structures of these compounds are shown in Fig. 1. ME3277 was not orally active, presumably because of its low lipophilicity, and its bioavailability (BA) was approximately 1% in rats. After i.v. administration, ME3277 was eliminated primarily in urine in the unchanged form, with a plasma half-life of 0.5 and 2.2 h in rats and humans, respectively (our unpublished data). ME3229 was designed to provide enhanced oral absorption by increasing the lipophilicity. After esterification, the octanol/water distribution coefficient (log D) at pH 7.4 increased from less than −3 to 1.27. Although there are variations, lipophilic drugs with a log D value greater than 0 generally exhibit high membrane permeability, and this membrane permeability is known to be a good predictor of oral absorption (Artursson, 1990; Artursson and Karlsson, 1991; Rubas et al., 1996; Yee, 1997). Therefore, ME3229 was expected to be well absorbed in vivo. In fact, its oral absorption was improved compared with ME3277, but the average urinary and biliary excretion of radioactivity was only 8.69 and 1.28%, respectively, after oral administration of [14C]ME3229 to rats, suggesting that the oral absorption was still lower than that of compounds with comparable lipophilicity.

This study was performed to clarify the mechanism responsible for this low oral absorption and BA. Determinants of intestinal absorption were evaluated using various techniques. Membrane permeability was measured in a transport study using Caco-2 cell monolayers. Conversion of prodrug to the active metabolite and other metabolites was evaluated in vitro metabolism studies using plasma and the S9 fraction of liver and the small intestinal mucosa of rats. In addition, the absorption parameters were determined in a single-pass perfused rat small intestine experiment in which absorption into enterocytes, absorption into the mesenteric vein, and degradation in the gut lumen were evaluated separately.

Materials and Methods

Chemicals. ME3229, its metabolites, and EF5081 (internal standard for the HPLC assay) were synthesized at Meiji Seika Kaisha Ltd. (Yokohama, Japan). [14C]ME3229 with a specific activity of 1.91 MBq/mg was synthesized at Nycomed Amersham plc (Buckinghamshire, UK). [14C]mannitol (11.5 MBq/mg) was obtained from Nycomed Amersham, warfarin from Nakalai Tesque Inc. (Kyoto, Ja-

ABBREVIATIONS: log D, octanol/water distribution coefficient; BA, bioavailability; Peff, permeability rate constant; DMSO, dimethyl sulfoxide; DPBS, Dulbecco’s PBS.
pan), and phenol red from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NADP⁺, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were from Oriental Yeast Co. Ltd. (Tokyo, Japan). All reagents were of analytical grade.

Cell Culture. A Caco-2 cell line was obtained from American Type Culture Collection (Manassas, VA). Caco-2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% l-glutamine (200 mM), and 1% NEAA (10 mM) in culture flasks in a humidified air, 5% CO₂ atmosphere. Cells were harvested with trypsin-EDTA and seeded on 24-mm Transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA) and cultured for 14 to 19 days before starting the transport experiments. Cell passages between 30 and 37 were used in the experiments.

Animals. Male Sprague-Dawley rats, 7 to 8 weeks old (for the in vivo study) and 9 to 11 weeks (for the single-pass perfusion study and Ussing chamber study) were supplied by Charles River UK Limited (Margate, UK) and Japan SLC Co. (Shizuoka, Japan), respectively. Animals were treated humanely, and the animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

In Vivo Study. After an overnight fast, 1 mg Eq/kg [¹⁴C]ME3229 was administered orally to rats. At designated times, blood samples were collected by venesection of the tail vein and transferred to heparinized tubes. Plasma was then obtained by centrifugation. Plasma samples for chromatographic analysis were extracted with acetonitrile and/or water. In a separate group of rats, 1 mg Eq/kg [¹⁴C]ME3229 was administered, and urine was collected. For the biliary excretion study, the bile duct of each rat was cannulated with a catheter and fixed with a ligature under anesthesia using a mixture of isoflurane and oxygen. The rats then received 1 mg Eq/kg [¹⁴C]ME3229, and bile samples were collected.

In Vitro Metabolism of ME3229 in Rat Small Intestine, Liver, and Plasma. Liver from a rat anesthetized with diethyl ether was perfused with ice-cold saline, then removed, and minced. The small intestine was removed and cut along the axis, and the mucosa was removed by scraping. Liver and intestinal mucosa were homogenized in a Potter-Elvehjem-type Teflon homogenizer in 0.25 M sucrose, 1 mM EDTA, 25 mM Tris-HCl buffer (pH 7.4). S9 fractions were prepared. [¹⁴C]ME3229 was incubated with the S9 fraction of intestinal mucosa and liver in the presence and absence of an NADPH-generating system at 37°C. The final concentrations of protein and substrate were 2 mg/ml and 30 μM, respectively. Metabolism in fresh plasma was also studied at a substrate concentration of 2.5 μM. At designated times, the reaction was terminated by adding acetone. The supernatant was separated by centrifugation and dried under a stream of nitrogen gas.

Transport across Caco-2 Cell Monolayers. For the study of drug transport across Caco-2 cell monolayers, apical medium (1.5 ml) was used, consisting of Dulbecco’s PBS (DPBS), 25 mM arabinose, 5 mM NaHCO₃, 11 mM MES, and 1 mM CaCl₂ (pH 6.5). Serosal medium (2.6 ml) consisted of DPBS, 25 mM glucose, 5 mM NaHCO₃, 11 mM HEPES, and 1 mM CaCl₂ (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.

Single-Pass Perfusion of Small Intestinal Segments. Before these experiments, rats were fasted overnight, but water was available ad libitum. Rats were anesthetized with Nembutal (Abbott, Chicago, IL). The femoral vein was cannulated with polyethylene tubing (PE-50) to transfuse blood collected previously from donor rats. The abdominal cavity was opened, and a 5-cm intestinal segment was prepared at the upper part of the ileum. After flushing out the intestinal contents with 5 ml of ice-cold saline followed by 1 ml of air, the segment was cannulated with silicon tubing (Tygon tube, 1.15 mm i.d. × 3.2 mm o.d.) connected to a perfusion pump. The outflow from the segment was led to the sampling tube via polyethylene tubing (1.4 mm i.d., 1.57 mm o.d.) 3 cm in length. To collect venous outflow, silicon tubing (Silascone no. 0, 1.0 mm i.d., 1.5 mm o.d.) was inserted into the mesenteric vein draining the segment via the superior mesenteric vein. To avoid blood loss, transfusion was

Fig. 1. Chemical structure of ME3229 and proposed metabolic pathway.
started immediately at 0.2 ml/min, and then the rate was adjusted to match the venous outflow. The segment was perfused with DPBS (pH 6.5) containing 50 μg/ml phenol red as a nonabsorbable marker and 400 μM ME3229 or 500 μM warfarin as test compounds. Dimethyl sulfoxide (DMSO) used to dissolve ME3229 did not exceed 0.5% of the perfusate volume. The perfusion flow rate was 0.2 ml/min for ME3229 and 0.3 ml/min for warfarin. The perfusate from the intestinal segment and the mesenteric venous outflow were collected at 4-min intervals. The blood samples were incubated at 37°C for 1 h before separation of plasma. After the perfusion period, the contents of the intestinal segment were drained with 1 ml of saline. The volume of the contents was calculated from the dilution of phenol red. In some experiments, perfusion with ME3229-containing perfusate was terminated at 20 min, and the contents of the segment were washed out with ice-cold buffer. Perfusion was resumed immediately with drug-free buffer, and sampling of the venous blood and perfusate was continued.

To determine the degradation rate of ME3229 in the gut lumen, perfusate was collected at the exit of the segment. ME3229 was added to the perfusate at various concentrations and incubated at 37°C. At designated times, aliquots were taken and deproteinized with an equivalent volume of ice-cold acetonitrile. Samples were centrifuged, and the supernatant was dried under N2 gas and stored frozen until required for assay.

**Ussing Chamber Method.** Rats were anesthetized with diethyl ether and sacrificed by exanguination from the abdominal aorta. The upper part of ileum was then removed and rinsed with ice-cold saline. Segments (approximately 1 cm in length) not containing Peyer’s patches were cut open and mounted on the pins of the Ussing chamber cells, and half-cells were clamped together. To both compartments, 3 ml of DPBS (pH 7.4) containing 3 g/l glucose was added. After a preincubation period of 10 min, PM-10 dissolved in DMSO was added to the donor chamber at a concentration of 50 μM. The final concentration of DMSO was 0.5%. The temperature of the diffusion cells was kept at 37°C, and the fluids in both compartments were circulated by a stream of O2/CO2 (95:5). At designated times, 1-ml aliquots were taken from the receiving chamber and replaced with the same volume of drug-free medium. Specimens were mixed with an equivalent volume of acetonitrile, dried under N2 gas, and stored frozen until required for assay.

**Assay.** Radioactivity in the samples was determined in liquid scintillation analyzer. Scintillation fluids, Quickscint 1 (Zinger Analytic, Maidenhead, UK) or Atomlight (NEN Life Science Products, Boston, MA), were used for analyzing samples from the in vivo study or the in vitro transport and metabolism studies, respectively.

Concentrations of ME3229, ME3277, PM-5, PM-10, and warfarin were determined by HPLC. The HPLC system used involved two pumps, a UV detector, a system controller, an autosampler, a column oven, and an integrator. The columns and composition of the mobile phase used for each assay are listed in Table 1, conditions A through D. To analyze the incubation media of Caco-2 cells and intestinal perfusate, samples were injected directly into the HPLC system. Plasma samples for the analysis of ME3277 and PM-5 underwent solid-phase extraction using Bond ElutC18 and Bond ElutSAX columns (Varian, Harbor City, CA). Warfarin was extracted from plasma samples into diethyl ether under acidic conditions by liquid-liquid extraction. Samples from transport experiments using Ussing chambers were dissolved in methanol/water (1:1) and injected into the HPLC system.

For the chromatographic analysis of samples from the in vivo study (extracts of plasma, urine, and bile samples) and the in vitro metabolism study, these were dissolved in appropriate solvents and injected into the HPLC system equipped with two pumps, a system controller, an autosampler, a column oven, and a fraction collector. A linear gradient system that could successfully separate PM-5, ME3277, PM-10, PM-11, and ME3229 was used (Table 1, condition E). The flow rate was 1 ml/min. Radioactivity in the eluent from the column was monitored in a liquid scintillation counter.

Phenol red was determined by spectrophotometry. Perfuse (100 μL) was made alkaline with 1.5 ml of 1 N NaOH, and the absorbance at 555 nm was measured.

**Data Analysis.** The apparent permeability coefficient ($P_{app}$) across Caco-2 cell monolayers was calculated according to the following equation:

$$P_{app} (cm/s) = \frac{dA/dt}{S \cdot C_0}$$

where $dA/dt$ is the flux of drug across the monolayers (nmol/s), $S$ is the surface area of the cell monolayers (4.526 cm²), and $C_0$ is the initial drug concentration in the donor medium (μM). Absorption parameters in the in situ perfusion experiment were obtained as shown below. The disappearance rate of test compounds from the gut lumen (V1) was calculated according to eq. 2:

$$V1 = F \times C_{in} \times \left(1 - \frac{C_{out}}{C_{in}}\right)$$

where $F$ is the perfusate flow rate (0.2 ml/min for ME3229 and 0.3 ml/min for warfarin), and $C_{in}$ and $C_{out}$ are the concentrations of test compound at the entrance and exit of the intestinal segment, respectively. The ratio $C_{out}/C_{in}$ was corrected with the concentration of phenol red. The appearance rate of test compounds in the venous outflow (V2) was calculated as:

$$V2 = Q \times C_b$$

where $Q$ and $C_b$ are blood flow rate draining the segment and the total concentration of the test compound and metabolites in the venous blood, respectively. Because the in vitro metabolism study showed that esters are hydrolyzed to their corresponding diacids in plasma, the concentrations of ME3277 and PM-5 were determined and $C_b$ was calculated, assuming that their distribution to red blood cells was negligible and the hematocrit value was 0.45.

The rate of degradation during passage through the segment was calculated assuming that the degradation activity in the perfusate could be described in terms of the distance from the entrance ($x$) as

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**TABLE 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ME3277, PM-5</td>
<td>L-column ODS³</td>
<td>UV 280 nm</td>
</tr>
<tr>
<td>B</td>
<td>ME3229, PM-10, PM-11</td>
<td>Inertsil ODS-2²</td>
<td>UV 280 nm</td>
</tr>
<tr>
<td>C</td>
<td>ME3229</td>
<td>Inertsil ODS-2²</td>
<td>UV 280 nm</td>
</tr>
<tr>
<td>D</td>
<td>Warfarin</td>
<td>Inertsil ODS-2²</td>
<td>UV 308 nm</td>
</tr>
<tr>
<td>E</td>
<td>ME3277, PM-5, PM-10, PM-11, ME3229</td>
<td>Inertsil ODS-2² (isocratic) UV 308 nm</td>
<td>Radioactivity</td>
</tr>
</tbody>
</table>

³ 4.6 mm Φ × 150 mm (Chemicals Inspection and Testing Institute, Tokyo, Japan).
² 4.6 mm Φ × 250 mm (GL Sciences Inc., Tokyo, Japan).
² 4.6 mm Φ × 150 mm (GL Sciences Inc.).
\[ K_{\text{deg}}(x) = K_{\text{deg, out}} \cdot \frac{x}{L} \]  

(4)

where \( L \) and \( K_{\text{deg, out}} \) are the length of the intestinal segment and the degradation rate constant of ME3229 in the perfusate at the exit of the segment, respectively. It was assumed that the concentration of ME3229 in the intestinal segment could be described as a function of \( x \):

\[ C(x) = C_{\text{in}} \cdot \text{Exp}(-\alpha \cdot x) \]  

(5)

The degradation rate in the segment between \( x - (\Delta x/2) \) and \( x + (\Delta x/2) \) is described as:

\[ \Delta V_{\text{deg}} = C(x) \cdot K_{\text{deg}}(x) \cdot \Delta \text{vol} \]  

(6)

where \( \Delta \text{vol} \) is the volume of perfusate in the segment between \( x - (\Delta x/2) \) and \( x + (\Delta x/2) \).

The degradation rate in the segment is given as:

\[ V_{\text{deg}} = \frac{K_{\text{deg, out}} \cdot V}{L} \cdot \left\{ -\frac{L + 1}{\alpha + 1} \cdot \text{Exp}(-\alpha \cdot L) + \frac{1}{\alpha} \right\} \]  

(7)

where \( V \) is the volume of perfusate in the segment, calculated from the concentration of phenol red.

The uptake clearance was calculated as follows:

\[ \text{CL}_{\text{uptake}} = \frac{V1 - V_{\text{deg}}}{L} \int_{0}^{L} C(x) \text{dx}/L \]  

(8)

For comparison of the absorption parameter with the reported value, the permeability rate constant (\( P_{\text{app}} \)) was calculated as:

\[ P_{\text{app}} = Q \cdot \frac{1 - C_{\text{out}}/C_{\text{in}}}{2 \pi RL} \]  

(9)

where \( R \), the radius of the segment, was assumed to be 0.178 cm (Yamashita et al., 1997). Data are expressed as mean ± S.E.

**Results**

**In Vivo Study.** After oral administration of \([^{14}\text{C}]\text{ME3229}\), the maximum concentration of radioactivity (\( C_{\text{max}} \)) was 0.235 ± 0.033 μg Eq/ml, and this was observed at 0.4 ± 0.1 h after administration (\( n = 5 \)); thereafter, it declined with a half-life of 1.0 ± 0.2 h. The cumulative excretion in urine and bile was 8.69 ± 0.62 and 1.28 ± 0.11% of the dose, respectively (\( n = 5 \)). ME3229 and monoester metabolites were not detected in plasma and urine, and ME3277 and PM-5 were the major metabolites. The percentage of PM-5 increased with time. ME3277 and PM-5 accounted for 89.2 and 10.8% of the total radioactivity in plasma at 0.5 h postdose and 68.5 and 31.5% at 1.5 h postdose.

**In Vitro Metabolism of ME3229.** Figure 2 depicts the composition of metabolites after incubation of \([^{14}\text{C}]\text{ME3229}\) with the S9 fraction of the small intestinal mucosa and liver. After incubation of ME3229 with small intestinal S9 fraction, barely any unchanged drug was detected and the monoester metabolites PM-10 and PM-11 were predominant, even at the first sampling time (5 min). During continuous incubation, the monoesters decreased, and the reduced forms of PM-10 and PM-11 (PM-12 and PM-13, respectively) and further hydrolyzed metabolites (ME3277 and PM-5) increased in a time-dependent manner. After 60 min of incubation, the predominant metabolite was PM-12. In the absence of NADPH, ME3229 was converted to PM-10, PM-11, and ME3277, but not to the reduced form. In contrast, ME3229 was converted exclusively to ME3277 by the liver S9 fraction (Fig. 2). In rat plasma, ME3229 and other ester metabolites were quite unstable. They were hydrolyzed to the corresponding acids with half-lives of less than 1 min. Hydrolysis of ME3229 in plasma and small intestinal fluid was reduced effectively by dinitrophenylphosphate (data not shown). The proposed metabolic pathway is shown in Fig. 1.

**Passage of ME3229 and ME3277 across Caco-2 Cell Monolayers.** \( P_{\text{app}} \) for ME3277 was approximately one-third that of mannitol. After esterification of ME3277, \( P_{\text{app}} \) increased 100-fold. After addition of ME3229 to the apical medium, PM-10 was the predominant compound in the serosal medium. ME3229, ME3277, PM-11, and a trace of PM-5 also appeared in the serosal medium (Table 2).

**Single-Pass Perfusion of Rat Ileum.** Figure 3 shows the disappearance rate of ME3229 from the perfusate and the appearance rate of ME3277 and PM-5 in the venous outflow during single-pass perfusion of rat ileum with the perfusate containing 400 μM ME3229. Steady state was achieved after perfusion for 20 min, in which the rate of venous appearance was 0.34 nmol/min and the ME3277 concentration in the perfusate at the exit of the segment was 67.7% of the input concentration. Compounds detected in the venous blood were ME3277 and PM-5, and PM-5 accounted for approximately 7% of that appearing in the venous blood. The average disappearance rate of ME3229 during passage to the serosal side was 0.12 ± 0.08 μmol/min (20 min).

**Permeation of ME3229, ME3277, and Mannitol across a Caco-2 cell monolayer in the apical-to-serosal direction**

<table>
<thead>
<tr>
<th>Compound (mol. wt.)</th>
<th>( P_{\text{app}} ) (× 10⁻⁶ cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME3229 (560.7)</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>ME3229b</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>ME3277 (446.5)</td>
<td>0.079 ± 0.040</td>
</tr>
<tr>
<td>Mannitol (182.2)</td>
<td>0.085 ± 0.003</td>
</tr>
</tbody>
</table>

a The radiolabeled compound was added to the mucosal medium, and the flux of radioactivity appearing in the serosal medium was used as \( \text{dA/dt} \) in eq. 1.

b Unlabeled ME3229 was added to the mucosal medium, and the flux of each metabolite appearing in the serosal medium was used as \( \text{dA/dt} \) in eq. 1.
through the segment (V1) was calculated to be 25.8 nmol/min, which was 76-fold greater than that of the appearance in the venous blood (V2) (Table 3).

The same experiment was performed with warfarin, a compound considered to be absorbed in unchanged form. Steady state was achieved after perfusion for 10 min. In contrast to ME3229, V1 and V2 for warfarin at steady state were very similar (Table 3). \( P_{\text{eff}} \) (eq. 9) was calculated to be \( 7.7 \times 10^{-3} \) cm/min.

To explain the discrepancy between V1 and V2 of ME3229, the contribution made by degradation in the lumen of the intestinal segment before absorption was estimated. The degradation rate constant of ME3229 in the perfusate at various time periods of perfusion ranged from 0.0288 min\(^{-1}\) to 0.0352 min\(^{-1}\) during the study period, and it was dependent on the concentration of ME3229. The degradation rate constants at the initial concentrations of 100, 200, 300, and 400 \( \mu \)M were 0.288 \( \pm \) 0.212, 0.115 \( \pm \) 0.082, 0.085 \( \pm \) 0.032 min, and 0.070 \( \pm \) 0.009 min\(^{-1}\), respectively (\( n = 3-4 \)). ME3229 was quite stable in fresh perfusate. Taking into consideration that the concentration of ME3229 was 400 and 270 \( \mu \)M at the entrance and exit of the intestinal segment, respectively, the average degradation rate constant at the initial concentration, ranging from 250 to 300 \( \mu \)M (0.0854 min\(^{-1}\)), was used as \( K_{\text{deg,out}} \) in eq. 7. The degradation rate was calculated to be 2.91 nmol/min, which accounted for only approximately 10\% of the disappearance rate (V1). The volume of perfusate in the segment (V) was 0.22 \( \pm \) 0.03 ml (\( n = 9 \)).

In the second set of experiments, the intestinal segment was perfused with 400 \( \mu \)M ME3229 for 20 min, followed by perfusion with drug-free perfusate for another 20 min. Figure 4 shows the concentration of ME3229 and the appearance rate of total metabolites in the perfusate. The absorption rate into venous blood is shown in Fig. 5. ME3229 disappeared from the perfusate soon after withdrawal of the drug. However, the monoesters PM-10 and PM-11 continued to appear in the perfusate after withdrawal of ME3229. The concentration of ME3277 in the perfusate persisted even longer. The appearance rate in the mesenteric vein also declined slowly. Twenty minutes after switching the medium, absorption of the metabolites into the venous outflow continued. The amount of the metabolites appearing in the perfusate and in the venous blood during this last 20-min period is shown in Table 4.

**Ussing Chamber Experiments.** When PM-10 was added to the donor chamber, both PM-10 and the hydrolyzed metabolite ME3277 appeared in the receiving chamber. Figure 6 shows the flux of ME3277 and PM-10 into the receiving chamber when PM-10 was added to the mucosal (mucosal to serosal) and serosal (serosal to mucosal) sides. The flux of both ME3277 and PM-10 in the serosal-to-mucosal direction was significantly greater than that in the mucosal-to-serosal direction.

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**Figure 3.** Absorption of ME3229 in the single-pass-perfused rat ileal segment. Rat ileal segment was perfused with 400 \( \mu \)M ME3229. The length of the segment and the perfusion flow rate were 5 cm and 0.2 ml/min, respectively. a, degradation rate of ME3229 from the segment; b, appearance rate of ME3277 and PM-5 in the mesenteric vein draining the segment. Based on the metabolism study in rat plasma, the sum of ME3277 and PM-5 was considered to be \( C_b \) in eq. 2. Each point represents mean \( \pm \) S. E. of five rats.

**Table 3.** Absorption parameters for ME3229 and warfarin

<table>
<thead>
<tr>
<th>Input Rate</th>
<th>( C_{\text{out}}/C_{\text{in}} )</th>
<th>Disappearance Rate (V1)</th>
<th>Absorption Rate (V2)</th>
<th>Degradation Rate (Vdeg)</th>
<th>Uptake Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/min</td>
<td></td>
<td>nmol/min</td>
<td>nmol/min</td>
<td>nmol/min</td>
<td>( \mu )l/min</td>
</tr>
<tr>
<td>ME3229(^a)</td>
<td>80</td>
<td>0.677 ( \pm ) 0.018</td>
<td>25.8 ( \pm ) 1.5</td>
<td>0.34 ( \pm ) 0.15</td>
<td>2.91</td>
</tr>
<tr>
<td>Warfarin(^b)</td>
<td>150</td>
<td>0.856 ( \pm ) 0.006</td>
<td>21.6 ( \pm ) 0.9</td>
<td>21.8 ( \pm ) 2.6</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Calculated by eq. 2.
\(^b\) Calculated by eq. 3.
\(^c\) Calculated by eq. 7.
\(^d\) Calculated by eq. 8.
\(^e\) Mean \( \pm \) S. E. of five rats.
\(^f\) Mean \( \pm \) S. E. of four rats.
ME3229 is an ester-type prodrug of ME3277, an Arg-Gly-Asp (RGD) peptidemimetic glycoprotein IIb/IIIa receptor antagonist. After i.v. administration, ME3277 was excreted in urine primarily as unchanged drug (our unpublished data). Based on a distribution volume of approximately 0.3 l/kg in experimental animals, its distribution was considered to be limited to the extracellular space. Although ME3277 itself was not orally absorbed because of its hydrophilicity and resultant poor membrane permeability, increasing the membrane permeability by masking the carboxylic acid groups of ME3277 seemed to improve the oral absorption and, consequently, the oral BA.

The permeability of ME3229 across a Caco-2 cell monolayer was approximately 30 times greater than that of mannitol (Table 2), which allows us to expect almost complete absorption based on the reported correlation between $P_{app}$ and oral absorption (%) (Artursson and Karlsson, 1991; Rubas et al., 1996; Yee, 1997). However, when $[^{14}C]$ME3229 was administered orally to rats, only 10% of the dose was absorbed. In addition, a considerable amount of the reduced metabolite PM-5 was detected in the plasma and urine of rats.

The formation of an unexpected metabolite was explained based on the in vitro metabolism study as follows. The in vitro metabolism study using the small intestinal S9 fraction showed that the intermediate metabolites PM-10 and PM-11 were metabolized to the reduced metabolites PM-12 and PM-13 (Fig. 2). When ME3229 was absorbed into enterocytes, PM-10 and PM-11 were formed, and possibly retained in the cell (as discussed below) because of the relatively low membrane permeability, and exposed to the reduction enzyme(s). As a result, they were reduced to PM-12 and PM-13 and then hydrolyzed to PM-5 in enterocytes, venous blood, and/or liver and then appeared in the systemic circulation. On the other hand, when ME3277 was administered i.v., its distribution was restricted in the plasma compartment because of its low membrane permeability, and it was not exposed to the reduction enzyme(s) in the tissue cells. Thus, reduction to PM-5 was a minor elimination pathway.

**Characteristics of ME3229 Absorption Evaluated Using the Single-Pass Perfusion Method.** To clarify the

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**Fig. 4.** Concentration of ME3229 and its metabolites in the perfusate during perfusion of a rat ileal segment with ME3229 and after withdrawal of ME3229 from the perfusate. Rat ileal segment was perfused with 400 μM ME3229 for 20 min, and then the perfusate was switched to drug-free buffer. Each point represents mean ± S.E. of three rats.

**Fig. 5.** Appearance rate of ME3277 and PM-5 in the venous outflow draining the intestinal segment during perfusion of rat ileal segment with ME3229 and after withdrawal of ME3229 from the perfusate. Rat ileal segment was perfused with 400 μM ME3229 for 20 min, and then the perfusate was switched to drug-free buffer. Each point represents mean ± S.E. of three rats.

**TABLE 4**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Amount Appearing (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME3277</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>PM-5</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>PM-10</td>
<td>17.6 ± 0.5</td>
</tr>
<tr>
<td>PM-11</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>ME3277</td>
<td>4.3 ± 1.0</td>
</tr>
</tbody>
</table>

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**Discussion**

ME3229 is an ester-type prodrug of ME3277, an Arg-Gly-Asp (RGD) peptidemimetic glycoprotein IIb/IIIa receptor antagonist. After i.v. administration, ME3277 was excreted in urine primarily as unchanged drug (our unpublished data). Based on a distribution volume of approximately 0.3 l/kg in experimental animals, its distribution was considered to be limited to the extracellular space. Although ME3277 itself was not orally absorbed because of its hydrophilicity and resultant poor membrane permeability, increasing the membrane permeability by masking the carboxylic acid groups of ME3277 seemed to improve the oral absorption and, consequently, the oral BA.

The permeability of ME3229 across a Caco-2 cell monolayer was approximately 30 times greater than that of mannitol (Table 2), which allows us to expect almost complete absorption based on the reported correlation between $P_{app}$ and oral absorption (%) (Artursson and Karlsson, 1991; Rubas et al., 1996; Yee, 1997). However, when $[^{14}C]$ME3229 was administered orally to rats, only 10% of the dose was absorbed. In addition, a considerable amount of the reduced metabolite PM-5 was detected in the plasma and urine of rats.

The formation of an unexpected metabolite was explained based on the in vitro metabolism study as follows. The in vitro metabolism study using the small intestinal S9 fraction showed that the intermediate metabolites PM-10 and PM-11 were metabolized to the reduced metabolites PM-12 and PM-13 (Fig. 2). When ME3229 was absorbed into enterocytes, PM-10 and PM-11 were formed, and possibly retained in the cell (as discussed below) because of the relatively low membrane permeability, and exposed to the reduction enzyme(s). As a result, they were reduced to PM-12 and PM-13 and then hydrolyzed to PM-5 in enterocytes, venous blood, and/or liver and then appeared in the systemic circulation. On the other hand, when ME3277 was administered i.v., its distribution was restricted in the plasma compartment because of its low membrane permeability, and it was not exposed to the reduction enzyme(s) in the tissue cells. Thus, reduction to PM-5 was a minor elimination pathway.

**Characteristics of ME3229 Absorption Evaluated Using the Single-Pass Perfusion Method.** To clarify the
absorption characteristics of ME3229, we examined the factors other than membrane permeability that might affect the oral absorption and BA of prodrugs, i.e., the stability of the drug in the gut lumen and the contribution of active efflux systems located on the apical membrane of enterocytes.

The hydrolysis rate of ME3229 in the perfusate recovered at the exit of the loop at various time periods of perfusion suggested that hydrolytic enzyme(s), presumably a carboxylesterase, is secreted continuously into the gut lumen. Thus, it was reasonable to consider that ME3229 was hydrolyzed to less permeable derivatives, i.e., monoesters and ME3277 in the gut lumen, and this was an important factor in reducing the oral absorption of this prodrug.

To characterize the intestinal absorption of ME3229 without the effect of hydrolysis in the gut lumen, we performed a single-pass perfusion experiment using rat ileum. There are two ways to determine the absorption rate, one based on the disappearance of the drug from the lumen as described in eq. 2, and the other based on the appearance of the drug in the venous outflow as described in eq. 3. We used warfarin as a reference compound, because it is absorbed primarily as unchanged form. The log D value of warfarin is reported to be 0.772 (Yamashita et al., 1997). As summarized in Table 3, in the case of warfarin, the absorption rates determined by the two methods were very similar, and $P_{\text{eff}}$ was comparable with the reported value. On the other hand, the V1 of ME3229 was much greater than the V2. This discrepancy could not be explained by degradation of the drug in the perfusate before absorption, which accounted for only 2.91 nmol/min of a disappearance rate of 25.8 nmol/min. Assuming that the brush-border esterase activity was negligible, the difference between $V_1$ and $V_{\text{dog}}$ (22.9 nmol/min) was considered to be the rate of uptake into enterocytes. The uptake clearance ($CL_{\text{uptake}}$) of ME3229 was comparable with or slightly greater than that of warfarin, suggesting that ME3229 was taken up into enterocytes at a reasonable rate in consideration of its lipophilicity, but only 1.5% reached the venous blood.

**Efflux of Metabolites into the Gut Lumen.** Recently, intestinal transport systems able to secrete drugs into the gut lumen have been extensively studied. In addition to P-glycoprotein, some other efflux systems expressed on the brush-border membrane of enterocytes may function as an absorption barrier (Collingston et al., 1991, 1992; Oude et al., 1993; Aungst and Saitoh, 1996; Saitoh et al., 1996; Hunter and Hirst, 1997; Arimori and Nakano, 1998; Gotoh et al., 2000; Hirohashi et al., 2000). As for prodrugs, L-775,318, a carboxyl ester prodrug, has been reported to be a substrate of P-glycoprotein, but its active metabolite is not. Because of active secretion, L-775,318 failed to be well absorbed orally (Prueksaritanont et al., 1998). The transport of a bis(pivaloyloxy)methyl ester of an antiviral agent and its metabolites has been studied using Caco-2 cells. The prodrug was shown to be a substrate for a P-glycoprotein-like carrier system, whereas its metabolites were transported by an efflux system that was inhibited by indomethacin (Annaert et al., 1997, 1998a,b). In our case, ME3229 was efficiently taken up into enterocytes, and thus the efflux system did not seem to function as an absorption barrier for the prodrug itself.

On the other hand, the perfusion study shown in Figs. 4 and 5 suggests that the hydrophilic metabolites formed in enterocytes are retained in the cell because of their relatively poor membrane permeability, and a fraction of them is transported to the gut lumen. Furthermore, as shown in the perfusion experiment in which ME3229 was perfused for 20 min and then the perfusate was switched to one that was drug-free, a directional appearance of the metabolites was observed. The recovery of any metabolite in the perfusate exceeded the total amount of metabolites recovered in the mesenteric vein (Table 4). More directly, permeation of PM-10 across rat ileum mounted on a diffusion chamber was greater in the serosal-to-mucosal direction (Fig. 6). This suggests the existence of a transport system that pumps out the metabolite(s) formed in enterocytes into the gut lumen, and this is considered to be the mechanism for the low absorption rate into the mesenteric vein (V2), in spite of the high membrane permeability of the prodrug. The identification of this efflux transport system will be the subject of additional studies.

**References**


Aungst BJ and Saitoh H (1996) Intestinal absorption barriers and transport mech-

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