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Nonlinear absorption of methylprednisolone by absorptive and secretory transporters

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ABSTRACT

The intestinal absorption rate constant of methylprednisolone (MP) evaluated by the loop method increased significantly with increasingly higher concentrations of the drug up to 500 μM in a nonlinear fashion but did not increase further at higher concentrations. Mucosal-to-serosal directed permeation of MP across rat jejunal sheets also increased in a nonlinear fashion in a low concentration range (100–150 μM), followed by a decrease as the concentration increased further, whereas serosal-to-mucosal directed permeation decreased in a concentration-dependent manner. Vectorial transport of MP across Caco-2 cell monolayers was observed, with greater transport in the basolateral-to-apical direction at 37 °C. These observations suggest that MP is taken up in the intestinal epithelial cells by a carrier-mediated transport mechanism. The absorptive and secretory clearance of MP increased and decreased with P-glycoprotein (P-gp) inhibitors, respectively. These results strongly suggest that MP is secreted into the intestinal lumen predominantly by P-gp. We conclude that intestinal transport of MP involves P-gp or some other transporters in both the absorptive and secretory directions, and complex nonlinear intestinal absorption characteristics can be ascribed to the existence of multiple transport mechanisms.

1. Introduction

The bioavailability of drugs after oral administration depends nonlinearly on concentration in some cases, which shows a dose-dependent increase and/or a decrease. Understanding the mechanism causing such nonlinear behavior would be helpful in the development of orally active drugs, as well as in their clinical applications. Nonlinear phenomena in intestinal absorption can be caused by several factors. A decrease of bioavailability with increase of dose can be accounted for by limited solubility of the drug in the intestinal lumen or by capacity-limited permeation across the intestinal epithelial membranes, such as specialized carrier-mediated transport mechanism. Recent studies on drug transport in the intestine have demonstrated the presence of many carrier-mediated transport systems for various drugs as well as natural compounds. On the other hand, a dose-dependent increase of bioavailability can be mainly ascribed to saturable first-pass metabolism in the liver and/or gastrointestinal tract. The finding of drug-metabolizing enzymes such as the cytochrome P-450 isozyme CYP3A, in the small intestine has contributed to a mechanistic understanding of alterations of drug bioavailability arising from causes other than hepatic metabolism (Ducharme et al., 1995; Watkins et al., 1987). Another possible cause of dose-dependent changes in bioavailability is the contribution of an intestinal luminal secretory system. P-glycoprotein (P-gp), which was originally found in multidrug-resistant tumor cells as an anticancer drug efflux pump, is also present in the luminal membrane of intestinal epithelial cells (Thiebaut et al., 1987) and acts as a secretory transporter into the intestinal lumen from the cells (Hsing et al., 1992), in addition to its role as a component of the blood–brain barrier, transferring drugs out of the brain (Tsuji et al., 1992). P-gp thus functions as an absorption barrier to various drugs, resulting in a lower absorption rate than would be expected from the lipophilicity of the drug molecules. If such secretory transporters, including P-gp, become saturated at high doses of drugs, an apparent increase of bioavailability would be expected at high drug concentrations.

Adrenocortical steroid hormones are widely used in the treatment of various inflammatory and immunologic diseases due to their broad pharmacological activities (Fauci et al., 1976). They have been considered to be well-absorbed from the gastrointestinal tract by passive diffusion because of their relatively high lipophilicity (Georgitis et al., 1982). Therefore, detailed information has not been available on their mechanism of absorption in spite of their expanded clinical use since the 1950s. Recently, several reports have described that various endogenous steroid hormones interact with P-gp as substrates or modulators (Rao et al., 1994; Wolf and Horwitz, 1992). Although P-gp was first found to be one of the most important factors involved in the multidrug resistance

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(MDR) of tumor cells Juliano and Ling, 1976, it is regarded to serve as a kind of transporter that secretes endogenous steroid hormones into blood (Borst et al., 1993). Intestinal P-gp was also found to be expressed on the luminal membrane (Hsing et al., 1992) and capable of restricting the absorption of various compounds (Tsuji and Tamai, 1996). These findings imply that intestinal P-gp may significantly interfere with the absorption of some steroid hormones. Therefore, the intestinal absorption of various steroid hormones should be reevaluated in detail.

In the present study, we examined the absorptional behavior including the absorptive and secretory transport system of methylprednisolone (MP) in the rat jejunal membrane *in situ*, in vitro and in Caco-2 cell monolayers, we reveal the participation of its multiple transport mechanisms. Studies on MP metabolism have been limited to the liver (Lu et al., 2008; Villikka et al., 2001), and none have involved the small intestine. Thus, the intestinal metabolism of MP can be considered to have been ignored. The transport mechanism can be exclusively examined using the above-mentioned experimental systems.

2. Materials and methods

2.1. Materials

Methylprednisolone, verapamil and fluorescein isothiocyanate dextran 40,000 (FD40) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were commercial products of reagent grade.

2.2. Measurement of intestinal absorption by the in situ loop method

Male Wistar/ST rats (8-weeks old) were purchased from Japan SLC Ltd. (Shizuoka, Japan). All of the experiments with the animal were performed according to the guidelines of Tokyo University of Pharmacy and Life Sciences. The animals were fasted for 18 h before starting the experiments. Water was freely given while fasting. Rats were anesthetized with somnopentyl (pentobarbital sodium, 50 mg/kg). All the experiments were performed under Somnopentyl-induced anesthesia, and body temperature was maintained at 37 °C with a heat lamp. Intestinal absorption of methylprednisolone (MP) was evaluated by the loop method (Tomita et al., 2009). The jejunum of male Wistar/ST rats weighing 200–250 g (Japan SLC, Hamamatsu, Japan) was exposed by a midline abdominal incision, and two L-shaped glass cannulas (i.d. 2 mm, o.d. 4 mm) were inserted through small slits at the proximal and distal ends (7 cm). The proximal end and distal end of the cannulas were inserted into a point 5 cm and 12 cm from the Treitz ligament, respectively. Each cannula was secured by ligation with a silk suture, and the intestine was returned to the abdominal cavity to maintain its integrity. A 4 cm portion of Tygon tubing (i.d. 3 mm, o.d. 5 mm) was attached to the exposed end of each cannula, and a 10 ml hypodermic syringe fitted with a connecting tube and containing perfusion solution warmed at 37 °C was attached to the proximal cannula. To clear the gut, saline was passed slowly through it to the distal cannula and discarded until the effluent was clear. The remaining perfusion solution was carefully expelled from the intestine by means of air pumped through the syringe, and 5 ml of MP solution was immediately introduced into the intestine. The distal cannula was connected to a 10 ml syringe fitted with a three-way stopcock. At 15, 30, 45 and 60 min after administration of a drug solution, a 0.5 ml aliquot of luminal solution was removed through the attached syringe. The test solution was composed of 126 mM NaCl, 5.0 mM KCl, 1.4 mM $CaCl_2$, 3.5 mM $NaHCO_3$, 4.85 mM $NaH_2PO_4{\cdot}2H_2O$, 0.95 mM Na_2HPO_4 and 2 g/L D(+)-glucose at pH 6.5, and the solution was gassed with 95% O_2/5% CO_2 before and during the transport experiment. MP concentrations in the solution used in this study were 100–1000 μM. MP was soluble in the above buffer solution containing 1% dimethylsulfoxide to make 1000 μM or more. The absorption rate constant was evaluated from the slope of decline of the concentration in the luminal fluid with time. Here, the change in volume of water in the intestinal lumen was corrected with a measurement of the change in concentration of an unabsorbable marker, fluorescein isothiocyanate dextran 40,000 (FD-40), administered simultaneously with MP. The concentration of FD-40 used in this study was 0.1%.

2.3. Transport experiments with diffusion chambers

Rat jejunum tissue sheets were prepared as described previously (Eto et al., 2006). Tissue preparation, consisting of the mucosa and most of the muscularis mucosa, was made by removing the submucosa and tunica muscularis with fine forceps. Tissue sheets were mounted vertically in a diffusion chamber that provided an exposed area of 0.64 cm^2. The volume of the bathing solution on the mucosal and serosal sides was 5 ml, and the solutions temperature was maintained at 37 °C in a water-jacketed reservoir. The test solution was composed of 126 mM NaCl, 5.0 mM KCl, 1.4 mM $CaCl_2$, 3.5 mM $NaHCO_3$, 4.85 mM $NaH_2PO_4{\cdot}2H_2O$, 0.95 mM Na_2HPO_4 and 2 g/L D(+)-glucose at pH 6.5, and the solution was gassed with 95% O_2/5% CO_2 before and during the transport experiment. The concentration of MP in test solution on each side was determined as described above. Verapamil was used as a specific P-gp inhibitor (Bansal et al., 2009; Tomita et al., 2008; Panchagnula et al., 2005; Varma and Panchagnula, 2005). To examine the inhibitory effect of verapamil, the mucosal reservoir was filled with test solutions of containing various concentrations of verapamil. The samples were taken from the acceptor side at intervals of 30 min. Permeation clearance was obtained as follows:

$$\text{Permeation clearance} = \frac{dQ/dt}{A \times C_0}$$

dQ/dt is the rate of transport (μg/min) determined from the slope of the linear regression line between the transport amount and time. C_0 is the initial concentration in the donor chamber (μg/ml), and A is the area of the membrane (0.64 cm^2).

Simultaneously, the membrane resistance (Rm) of the rat jejunum tissue sheets was calculated from the membrane potential difference measured under the load of a small external current (0.1 mA and 0.01 mA) according to Ohm's law using a Short Circuit Current Amplifer (CEZ-9100, Nihon Koden, Japan). The change in Rm during the transport experiment (2 h) was maintained within 20% of the initial value (about 50 Ω cm^2).

2.4. Cultivation of Caco-2 cell monolayers

Caco-2 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% non-essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin, as described previously (Tomita et al., 2002). The cells were passaged upon reaching 80% confluency using trypsin–EDTA and plated at densities of 1:5 in 170 cm^2 T flasks. Caco-2 cells (passage numbers 40–66) were seeded at a density of 60,000 cells/cm^2 on snapwell polycarbonate membranes of Snapwell (1 cm^2, 0.4 μm pore size, Costar, Cambridge, MA, USA). The medium was changed the day after seeding and every other day thereafter. For the transport experiments, Caco-2 cells were grown on Snapwell microporous polycarbonate membranes (Costar, Bedford, MA) and cultured for about 3 weeks before use for transport experiments.

2.5. Transport experiments with Caco-2 cell monolayers

The transport experiments with Caco-2 cells grown on Snapwell membranes were performed as described (Nagira et al., 2006). The confluent cells were washed with Modified Krebs Ringer Phosphate Bicarbonate Buffer (MKRPB) (0.645 mM $CaCl_2{\cdot}2H_2O$, 4.83 mM KCl, 1.214 mM KH_2PO_4, 1.205 mM $MgSO_4{\cdot}7H_2O$, 113.3 mM NaCl, 10.18 mM Na_2HPO_4, 5 mM D-glucose and 16.96 mM $NaHCO_3$, pH 7.4, osmolarity was 315 mOsm/kg), and 1.5 ml and 2.6 ml of MKRPB were added on the apical and basolateral sides, respectively, of a cell insert. To measure apical-to-basolateral or basolateral-to-apical flux, a test compound was included in the apical and basolateral side, respectively. At the designated time, 0.1 ml of basolateral or 0.2 ml of apical side solution was withdrawn and replaced with an equal volume of MKRPB. The permeation of MP at increasingly higher concentrations of MP across Caco-2 cell monolayers was measured in both the apical-to-basolateral and basolateral-to-apical directions, and permeation clearance was obtained as the slope of the time course of appearance of intact MP in either the basal or apical bathing solution.

Simultaneously, Rm of the cell was obtained in the same manner as in the rat jejunal sheet. The change in the TEER during the transport experiment (2 h) was maintained within 10% of the initial value (about 500 $\Omega\,cm^2$).

2.6. Statistical analysis

All results are expressed as the mean ± standard error (mean ± S.E.). Statistical significance between the two groups was analyzed using Dunnett's test. Differences were considered to be significant at a level of $P < 0.05$.

3. Results

3.1. Concentration-dependence of intestinal absorption of methylprednisolone in rats

The relationship between the first-order absorption rate constant of methylprednisolone (MP) and its concentration was evaluated by the loop method. When 100–1000 μM MP was perfused into the intrajejunal loop, the intestinal absorption rate constant was changed nonlinearly as shown in Fig. 1. There was an increase in the constant from 100 to 500 μM, but not at 1000 μM. The absorption rate constant was significantly higher at 500 μM than at 100 μM ($P < 0.05$). This result suggests that at least two nonlinear events are involved in the intestinal absorption of MP.

The transport of MP in rat intestinal tissue was further examined using the diffusion chamber method. Permeation at various concentrations of MP across a rat jejunal sheet was measured in both the mucosal-to-serosal and serosal-to-mucosal directions, and the absorptive and secretory permeation coefficients were obtained as the slope of the time course of appearance of intact MP in the serosal and mucosal bathing solution, respectively. As is clearly shown in Fig. 2, the serosal-to-mucosal permeation coefficient significantly decreased in a concentration-dependent manner, whereas the mucosal-to-serosal permeation coefficient increased up to 150 μM, and at the higher concentrations, tended to decrease. Furthermore, in the presence of verapamil, serosal-to-mucosal transport of 100 μM MP tended to decrease, whereas the mucosal-to-serosal transport increased significantly (Fig. 2). Vectorial transport of MP across the rat jejunal membrane was not observed above 150 μM of MP (Fig. 2) and verapamil effect is disappeared (data not shown).

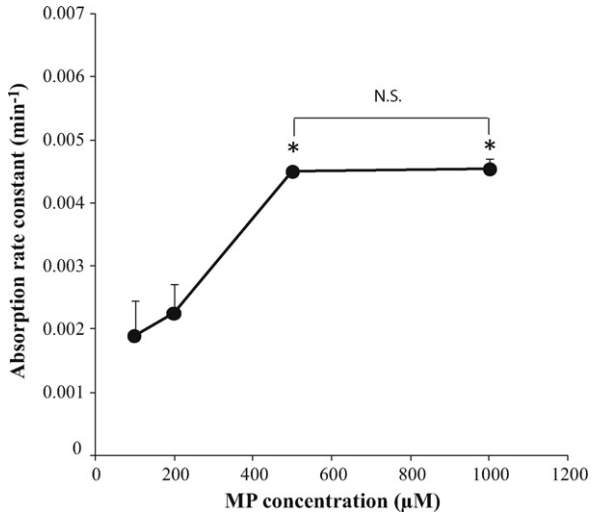

Fig. 1. Concentration-dependence of jejunal absorption of methylprednisolone in rats. The intestinal absorption rate constant (k_a) of methylprednisolone (100–1000 μM) was evaluated from the time course of the decrease in the luminal methylprednisolone concentration measured by the *in situ* loop method. Each datum represents the mean ± S.E. (n = 3–5 for each condition). * Significantly different from the value of absorption rate constant at 100 μM methylprednisolone ($P < 0.05$). N.S. not significantly different from the absorption rate constant at 500 μM methylprednisolone.

3.2. Transcellular transport of methylprednisolone across Caco-2 cell monolayers

The transport of MP in Caco-2 cell monolayers was also examined using the diffusion chamber method. Permeation with increasingly higher concentrations of MP across Caco-2 cell monolayers was measured in the apical-to-basolateral and basolateral-to-apical directions. Fig. 3 indicates that the basolateral-to-apical permeation clearance significantly decreased in a concentration-dependent manner, whereas the apical-to-basolateral permeation clearance increased up to 500 μM, and at higher concentrations, tended to decrease. The basolateral-to-apical permeation of 100 μM MP was significantly inhibited by the metabolic inhibitor NaN_3 (data not shown). In contrast, in the

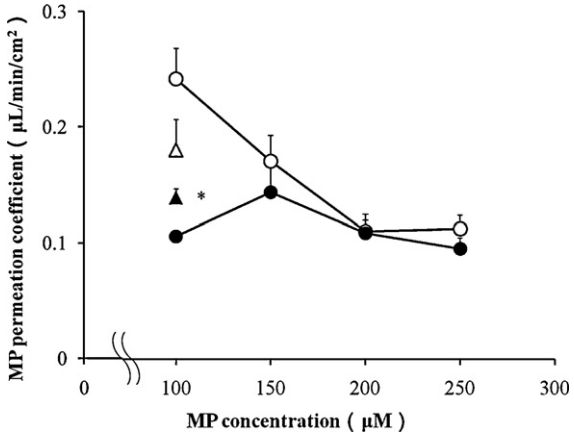

Fig. 2. Concentration-dependence of methylprednisolone's clearance across rat jejunal tissue. The permeation of methylprednisolone was evaluated using a diffusion chamber mounted with the rat jejunum. Open and closed circles represent the permeation clearance in the serosal-to-mucosal and mucosal-to-serosal direction, respectively. Open and closed triangles represent the serosal-to-mucosal and mucosal-to-serosal transfer of 100 μM methylprednisolone in the presence of 500 μM verapamil, respectively. Each datum represents the mean ± S.E. for seven experiments. * Significantly different from the influx value in the absence of verapamil ($P < 0.05$).

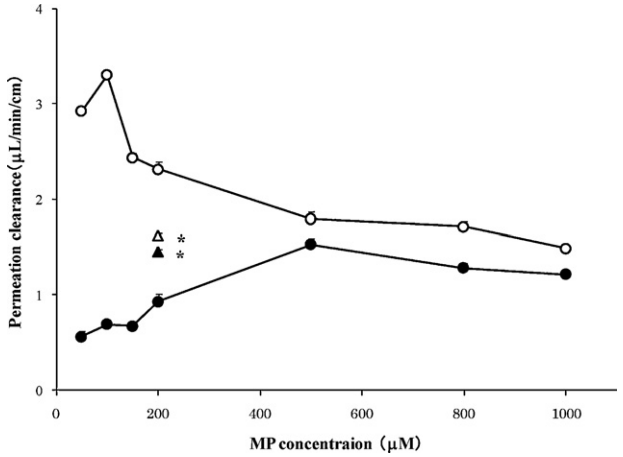

Fig. 3. Concentration-dependence of methylprednisolone's permeation clearance across Caco-2 cell monolayers. The permeation of methylprednisolone was evaluated using a diffusion chamber mounted with Snapwell. Open and closed circles represent the clearance in the basolateral-to-apical and apical-to-basolateral direction, respectively. Open and closed triangles represent the basolateral-to-apical and apical-to-basolateral transfer of 200 μM methylprednisolone in the presence of 500 μM verapamil, respectively. Each datum represents the mean ± S.E. for seven experiments. * Significantly different from data of influx or efflux value without verapamil ($P < 0.05$).

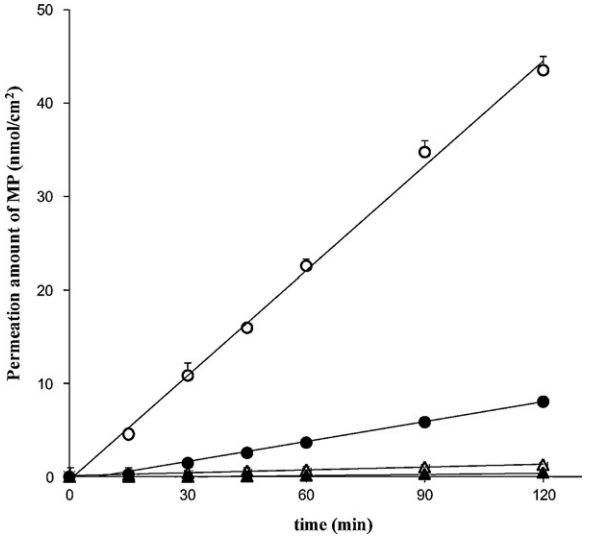

Fig. 4. Effect of temperature on the polarized permeation of methylprednisolone across Caco-2 cell monolayers. The concentration of methylprednisolone used in this study was 50 μM. Circle and triangle represent the data obtained at 37 °C and 4 °C, respectively. Open and closed symbols represent the data for the basolateral-to-apical and apical-to-basolateral directions, respectively. Each datum represents the mean ± S.E. for seven experiments.

presence of verapamil, basolateral-to-apical transport at 200 μM MP decreased significantly, whereas the apical-to-serosal transport increased significantly.

To determine whether the transport of MP across Caco-2 cell monolayers was dependent on temperature dependent, transport fluxes were measured at 4 °C and 37 °C conditions (Fig. 4). The permeation of MP was linear over 120 min with an initial lag time of a few minutes. The flux from the basolateral-to-apical side was about six times larger than the reverse flux (Fig. 4). The flux in at 4 °C, which is a measure of passive permeability, was significantly lower than that at 37 °C. Therefore, the transport of MP can be ascribed to active transcellular permeation.

To investigate whether MP interacts with P-gp present in Caco-2 cell monolayers and rat jejunal epithelial cells, the effect of verapamil on the transport of MP was examined. Since verapamil as a typical substrate for P-gp was used at a concentration of two times higher than that of MP, the transport of MP was significantly increased in the apical-to-basolateral direction, whereas it was significantly decreased in the basolateral-to-apical direction (Table 1).

4. Discussion

The concept that carrier-mediated intestinal absorption and luminal secretion mechanisms as well as intestinal tissue metabolic activity regulate the bioavailability of various drugs has been established previously (Benet et al., 1996; Tamai et al., 1997). Such a saturable physiological mechanism may sometimes produce nonlinear pharmacokinetic phenomena. The results obtained in the present study represent the first evidence to our knowledge that a complex pattern of nonlinear bioavailability of MP is indeed generated by the interaction of intestinal absorptive and secretory transport systems, when MP exists at a concentration beyond linear absorbability in the intestinal luminal fluid.

Intestinal absorption of MP assessed by the *in situ* loop method in rats exhibited distinctive nonlinearity, which showed a significant, apparently linear, increase in the absorption rate constant up to a drug concentration of 500 μM when compared with that at 100 μM, but did not increase further at higher concentration of 1000 μM (Fig. 1). Because the disappearance of intact MP from the intestinal luminal fluid was measured in this experiment, the above observations can be accounted for by saturable secretory and absorptive transport mechanisms, by a saturation of metabolism or by a limitation of solubility. Absorptive-directed (mucosal-to-serosal) flux of MP across jejunal tissue preparations mounted on a diffusion chamber increased at up to 200 μM of the drug (Fig. 2). This result is consistent with that obtained with the *in situ* loop method, and both can apparently be explained by the participation of a saturable secretory mechanism. This hypothesis is further supported by the measurement of serosal-to-mucosal flux using the diffusion chamber, which showed a marked decrease in the per-

Table 1
Effects of verapamil on MP transport in the apical-to-basolateral direction (influx) and basolateral-to-apical direction (efflux) across Caco-2 cell monolayers.

	Concentration of MP (μM)			
	50	100	150	200
Influx (μl/min/cm²)				
−verapamil	0.556 ± 0.06	0.694 ± 0.04	0.678 ± 0.05	0.932 ± 0.08
+verapamil	1.27 ± 0.07*	1.29 ± 0.05*	1.28 ± 0.12*	1.45 ± 0.03*
Efflux (μl/min/cm²)				
−verapamil	2.83 ± 0.19	3.31 ± 0.05	2.44 ± 0.04	2.32 ± 0.13
+verapamil	1.98 ± 0.01*	1.92 ± 0.07*	1.81 ± 0.04*	1.62 ± 0.03*

The concentrations of verapamil used in the present study were two times higher than the concentration of substrate in all the cases. Data represent the mean ± S.E. ($n = 3$–4 for each condition).

* $P < 0.05$ compared with no verapamil.

meation coefficient with an increase in the concentration of MP (Fig. 2). The break point observed in Figs. 1 and 2 was between 500 and 150 μM in both the experiments, although with the diffusion chamber method there is an increase of flux at a lower concentration (100–150 μM) than observed with the *in situ* loop method (100–500 μM). This small difference may be ascribed to the difference in thickness of the unstirred water layer in these experimental systems, because the loop method is expected to give a thicker unstirred water layer than that in the isolated jejunal sheet chamber method.

The decrease in the absorption rate constant at higher concentrations observed with the loop method can be explained by the participation of a saturable absorptive transport mechanism. The decrease in mucosal-to-serosal flux in the diffusion chamber at concentrations higher than 250 μM also supports the involvement of a saturable absorption mechanism. To confirm the results above, we used an *in vitro* method with cultured cells. Caco-2 cells, derived from human adenocarcinoma, have been shown to form an intestinal epithelial-like monolayer (Hilgers et al., 1990) and to have many of the small-intestinal membrane transport activities for amino acids (Hu and Borchardt, 1992), peptides (Dantzig and Bergin, 1990), hexoses (Riley et al., 1991), and monocarboxylic acids (Tsuji et al., 1994) and efflux transporter activity such as P-gp (Hilgers et al., 1990; Hsing et al., 1992). As it should be relatively easy to identify specialized transporters in their monolayers, Caco-2 cells were used in the present study.

Apical-to-basolateral flux of MP across the monolayers increased with concentration up to 500 μM, whereas the basolateral-to-apical clearance significantly decreased in a concentration-dependent manner beyond 100 μM. These results are also consistent with those obtained using the *in situ* loop method, and the findings both *in situ* and in vitro can apparently be explained by the participation of a saturable secretory mechanism. The vectorial transport of MP across Caco-2 cell monolayers was observed with a higher permeation constant in the basolateral-to-apical direction than in the reverse direction (Fig. 4). Accordingly, mechanisms of concentration dependency and vectorial transport may operate in Caco-2 cell monolayers similar to those observed in rat intestinal tissues. Here, apical-to-basolateral flux was significantly higher than the flux at 4 °C (Fig. 4), supporting the active transport of MP across the monolayers. We have shown the polarized transport of hydrophilic compounds such as fluorescein isothiocyanate dextran 4000, fluorescein disulfonic acid and lucifer yellow across rat mucosal tissues by a carrier-mediated transport mechanism (Tomita et al., 2000).

P-gp, an ATP-dependent drug efflux pump with broad substrate specificity, has been demonstrated to have a significant role as an intestinal secretory transporter (Hsing et al., 1992; Hilgers et al., 1990). Accordingly, the nonlinear increase in the absorptive transport of MP observed in the present study can be interpreted in terms of a contribution of P-gp. Several results in the present study support this idea: (1) The serosal-to-mucosal flux of MP in rat jejunal sheets mounted on a diffusion chamber was suppressed, but the mucosal-to-serosal flux was increased by verapamil with a high affinity for P-gp (Fig. 2). (2) Apical-to-basolateral transport of MP across Caco-2 cell monolayers was significantly increased in the presence of verapamil whereas basolateral-to-apical transport was reduced (Fig. 3, Table 1). (3) Markedly less permeation was observed at 4 °C when compared with 37 °C (Fig. 4), suggesting that P-gp makes a major contribution to the nonlinear increase in the bioavailability of MP delivered orally administration.

5. Conclusion

In conclusion, MP showed nonlinear intestinal absorption with an increase at the lower concentration ranges and subsequent decrease at the higher concentration range in absorption. Such nonlinearity can be explained partly by the operation of absorptive and secretory transporters in the intestine. It is considered that ABC transporter such as P-gp makes a substantial contribution to the secretory transport, though the absorptive transporter has not been identified yet. Clarification of the mechanistic and kinetic features of nonlinear intestinal absorption is important for prediction of drug–drug interactions in clinical use.

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