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Role of intestinal efflux transporters in the intestinal absorption of methotrexate in rats

Tomoharu Yokooji, Teruo Murakami, Ryoko Yumoto, Junya Nagai and Mikihisa Takano

Abstract

The role of intestinal efflux transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs) in intestinal absorption of methotrexate was examined in rats. In everted intestine, the mucosal efflux of methotrexate after application to serosal side was higher in jejunum than ileum, and the efflux in jejunum was suppressed by pantoprazole, a BCRP inhibitor, and probenecid, an MRP inhibitor, but not by verapamil, a P-gp inhibitor. The mucosal methotrexate efflux in ileum was suppressed by pantoprazole, but not by other inhibitors. On the other hand, the serosal efflux of methotrexate after application to mucosal side was greater in ileum than jejunum, and was suppressed by probenecid. In in-vivo rat studies, the intestinal absorption of methotrexate was significantly higher when methotrexate was administered to ileum than jejunum. Pantoprazole increased methotrexate absorption from jejunum and ileum. Probenecid increased the absorption of methotrexate from jejunum but decreased the absorption from ileum, as evaluated by peak plasma methotrexate levels. In conclusion, BCRP and MRPs are involved in the regional difference in absorption of methotrexate along the intestine, depending on their expression sites.

Introduction

Methotrexate, a folic acid antagonist, is a potent inhibitor of dihydrofolate reductase (DHFR) and is used as a chemotherapeutic agent to treat neoplastic disease and autoimmune disease, such as rheumatoid arthritis (Evans et al 1986; Giannini et al 1992). The use of methotrexate, however, is limited due to the induction of drug resistance and side effects such as the suppression of bone marrow, gastrointestinal dysfunction and hepatotoxicity.

In the treatment of rheumatoid arthritis, methotrexate is administered in a low oral dose intermittent regimen (Gispen et al 1987; Kremer et al 1988; Weinblatt et al 1988). Though the mean bioavailability of methotrexate is relatively high (approximately 73%), the plasma concentrations of methotrexate exhibit wide variability (Oguey et al 1992; Lebbe et al 1994). The scattered oral bioavailability of methotrexate would not be due to food intake or renal failure, such as low glomerular filtration rate (Hamilton & Kremer 1995; Murry et al 1995). Because methotrexate causes severe cytotoxicity, it is important to investigate the mechanism of the variability in oral bioavailability of methotrexate.

Reduced folate carrier (RFC) is expressed both on the apical and basolateral membranes, and proton coupled folate transporter/haem carrier protein (PCFT/HCP1) is expressed on the apical membranes of enterocytes (Selhub & Rosenberg 1981; Said & Redha 1987; Said et al 1987; Qiu et al 2006). These carriers transport folate and its analogues, including methotrexate, by using the H^+ gradient as a driving force (Selhub & Rosenberg 1981; Said & Redha 1987; Said et al 1987; Mason et al 1990; Chiao et al 1997; Li et al 2003; Nakai et al 2007). The intestinal absorption of methotrexate could be mainly mediated by RFC or PCFT/HCP1, or both (Selhub & Rosenberg 1981; Qiu et al 2006). In addition to these influx transporters, methotrexate is reportedly a substrate of ATP-dependent efflux transporters (ABC transporters), including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs) (Norris et al 1996; Masuda et al 1997; Gifford et al 1998; Bebawy et al 1999; Hirohashi et al 1999; Breedveld et al 2004). These efflux transporters are responsible for conferring multidrug resistance by

Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Tomoharu Yokooji, Ryoko Yumoto, Junya Nagai, Mikihisa Takano

Laboratory of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Hiroshima International University, Kure, Hiroshima, Japan

Tomoharu Yokooji, Teruo Murakami

Correspondence: M. Takano, Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. E-mail: takanom@hiroshima-u.ac.jp

promoting the active efflux of chemotherapeutic agents out of cells, and limiting influx and facilitating efflux to prevent the intracellular accumulation of their substrates in normal tissues. Such efflux transporters are also expressed in the small intestine abundantly (Takano et al 2006). Both P-gp and BCRP are expressed on the apical brush-border membrane of enterocytes preferentially in the distal intestine (Terao et al 1996; Suzuki & Sugiyama 2000; Tanaka et al 2005). MRP1–6 are expressed in the small and large intestine of man and rodents (Prime-Chapman et al 2004; Zimmermann et al 2005; Johnson et al 2006; Maher et al 2006). In particular, MRP2 and MRP3 have greater roles than other MRPs, because of their higher expression levels. MRP2, localized in the brush-border membrane, is preferentially expressed at the proximal intestine, and MRP3, localized in the basolateral membrane, is at the distal intestine (Mottino et al 2001; Rost et al 2002; Chan et al 2004; Zimmermann et al 2005; Yokooji et al 2007). Because of the site-specific and membrane-specific expression of these efflux transporters in the intestine (Gotoh et al 2000; Tian et al 2002; Shoji et al 2004; Yokooji et al 2005), they could affect the intestinal bioavailability or pharmacokinetics of orally administered substrate drugs.

In this study, the role of efflux transporters, such as P-gp, BCRP and MRPs, in the intestinal absorption of methotrexate was examined in rats by considering the localization of expression sites of efflux transporters along the intestine.

Materials and Methods

Materials

Methotrexate and verapamil were obtained from Wako Pure Chemicals (Osaka, Japan). Probenecid and pantoprazole were purchased from Sigma Chemical Co. Ltd (St Louis, MO) and LKT Laboratories, Inc. (St Paul, MN), respectively. BXP-21, a monoclonal antibody for BCRP, was from Monosan (Uden, Netherlands) and a secondary antibody, peroxidase-labeled affinity antibody to mouse IgG (H+L), was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). All other chemicals used were of the highest purity available.

Animals

Male Sprague–Dawley (SD) rats, 7–9 weeks old, were used. Experiments with rats were performed in accordance with the Guide for Animal Experimentation from Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University.

Expression analysis of BCRP in rat intestine

The expression of BCRP in rat intestine was evaluated by Western blot analysis using brush-border membrane (BBM). BBM samples of upper and lower intestine were prepared by a magnesium/ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) precipitation method as reported previously (Yokooji et al 2006). The concentration of BCRP proteins in BBM samples was evaluated by Western blot analysis after sodium dodecylsulfate-polyacrylamide gel electrophoresis as described previously by using BXP-21 (1:50 dilution) (Yokooji et al 2006).

In-vitro efflux of methotrexate in rat everted intestine

Ten-cm long everted jejunum and ileum were prepared from SD rats. Methotrexate was dissolved at a concentration of 10 μM in pH 7.4 Dulbecco's phosphate-buffered saline (D-PBS) (composition in mM: 1.5 KH_2PO_4, 8 Na_2HPO_4, 137 NaCl, 3 KCl, 5 glucose, 1 $CaCl_2$, 0.5 $MgCl_2$) containing 4% dimethyl sulfoxide (DMSO), where DMSO was used to increase the solubility of methotrexate. The pH of 7.4 was selected to minimize the activity of influx transporters for methotrexate in the intestine (Chiao et al 1997; Kneuer & Honscha 2004; Qiu et al 2006; Nakai et al 2007). The drug solution (1 mL) was applied to the serosal side of the closed everted sac. The sac was then immersed in 8 mL of pH 7.4 D-PBS containing 4% DMSO pre-warmed at 37°C and pre-oxygenated with 5% CO_2–95% O_2 gas. The bubbling of the incubation medium with CO_2–O_2 gas was continued throughout the efflux study. The mucosal efflux of methotrexate across the everted intestine was measured by sampling the mucosal medium periodically for 120 min. In an inhibition study, verapamil (300 μM), pantoprazole (300 μM) or probenecid (1 mM) was added to the mucosal medium to make an appropriate final concentration as a typical inhibitor for P-gp, BCRP and MRPs, respectively.

Bidirectional efflux (mucosal and serosal effluxes) of methotrexate from the intestinal epithelial cells was examined in the same manner as reported previously (Yokooji et al 2007). Briefly, methotrexate was dissolved at a concentration of 10 μM in pH 7.4 D-PBS containing 4% DMSO and was kept at 4°C as a drug solution. For transport studies of methotrexate, 10-cm long everted jejunum and ileum were prepared, in which both ends of the everted intestine were catheterized with polyethylene tubing to collect the inner serosal (basolateral) fluid of the sac. A sample (1 mL) of the drug solution was applied to the serosal side and the sac was immersed in 20 mL of the same drug solution kept at 4°C for 40 min to preload methotrexate. Then, both serosal and mucosal (apical) sides of the everted sac were washed carefully with ice-cold PBS without methotrexate. The serosal side of the sac was again filled with 1 mL of PBS, and then the sac was immersed in 20 mL of PBS pre-warmed at 37°C and pre-oxygenated with 5% CO_2–95% O_2. The bubbling of the incubation medium with CO_2–O_2 gas was continued throughout the transport study. Samples of both mucosal and serosal media were taken periodically for 120 min. In inhibition study, probenecid was added to both sides of membranes in the intestine at a concentration of 1 mM in PBS with 4% DMSO.

In-vivo intestinal absorption study of methotrexate in rats

Rats were fasted overnight, anaesthetized with pentobarbital (30 mg kg^{-1}, i.p., injection) and affixed supine on a surface kept at 37°C to maintain the body temperature at approximately 36°C. Jejunum (a 20-cm long segment from 5 cm

below the bile duct opening) and ileum (a 20-cm long segment above the ileocaecum) were used to elucidate the regional difference of intestinal methotrexate absorption in rats. Each intestinal segment was perfused with 20 mL of pH 7.4 D-PBS containing 4% DMSO and 10 μM methotrexate in a re-circulating perfusion manner at a rate of 1 mL min^{-1}. In an inhibition study, 300 μM pantoprazole or 1 mM probenecid was added to the intestinal perfusate. The intestinal perfusate was sampled periodically to determine the concentration of methotrexate.

In a separate experiment, cannulation (polyethylene tubing, PE-50) was made at the femoral vein for the administration of probenecid, and the femoral artery for the sampling of blood, respectively. A 20-cm long loop of jejunum or ileum was made, and methotrexate was administered into the closed intestinal loop at a dose of 1.25 μmol kg^{-1}. In an inhibition study, probenecid was administered intravenously at a dose of 175.2 μmol kg^{-1} 10 min before methotrexate administration. A dose of probenecid, 175.2 μmol kg^{-1}, was found to suppress the MRP2-mediated mucosal efflux of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), an MRP substrate, almost completely in rats in our previous study (Yokooji et al 2005). After administration of methotrexate, blood (0.2-mL samples) was collected with time for 1 h to measure plasma methotrexate concentrations.

Protein binding of methotrexate in 10% intestinal tissue homogenates and plasma

The luminal contents of whole small intestine were thoroughly washed out with a sufficient amount of ice-cold saline, and the intestine was divided into two parts of the same length. The mucosal surface of the upper and lower half of intestine was scraped off with a cover glass. The intestinal mucosa collected was homogenized in pH 7.4 D-PBS by means of a glass–Teflon Potter homogenizer (1000 rev min^{-1}, 10 strokes). The homogenate was centrifuged at 2040 rev min^{-1} for 10 min, and the supernatant was used for protein binding of methotrexate. Blood was collected by heart puncture to obtain plasma from rats. The binding of methotrexate to 10% intestinal tissue homogenate and plasma was determined by ultrafiltration method at 37°C by using ULTRAFREE-MC (cutoff level of molecular weight at 10 000; Millipore Corporation, Bedford, MA). The concentration of methotrexate in these biological samples was 10 μM.

Analysis

Blood samples were centrifuged to obtain plasma samples, then 50 μL of pH 7.4 D-PBS and 50 μL of 20% perchloric acid were added to each 100-μL plasma sample. Intestinal perfusate, transport medium and other biological samples were diluted with an equal volume of 10% perchloric acid. All these biological samples were kept on ice for at least 30 min, and centrifuged at 3 000 rev min^{-1} for 10 min. The concentration of methotrexate in the supernatants of various biological samples was determined by HPLC, using a column of TSKgel ODS-80TM (Tosoh, Tokyo, Japan). Briefly, mobile phases used was acetonitrile–methanol–1% acetic acid (5:10:85 v/v), and the flow rate of mobile phase was 1 mL min^{-1}. Detection was made at a wavelength of 304 nm. Differences among group mean values were assessed by the Kruskal–Wallis test followed by a post-hoc test (Dunn's test) or Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

Results

Western blot analysis for BCRP expression in rat intestine

The expression level of BCRP in rat intestine was evaluated by Western blot analysis using BBM and a monoclonal antibody for BCRP, BXP-21 (Figure 1). A band of approximately 140 kDa, corresponding to the molecular size of BCRP dimer (Asashima et al 2006), was observed in both jejunum and ileum. The band density of BCRP was comparable between jejunum and ileum.

Contribution of efflux transporters to the mucosal methotrexate efflux in-vitro

The effect of various efflux transporter inhibitors on the mucosal efflux of methotrexate after application to serosal side was examined using everted intestine in-vitro (Figure 2). The efflux of methotrexate for 120 min was followed in a zero-order-rate fashion with no lag time in either jejunum or ileum (data not shown). In the absence of transporter inhibitors, the efflux rate of methotrexate in jejunum was approximately 1.3-fold higher than that in ileum. Pantoprazole, a BCRP inhibitor, significantly suppressed the efflux of methotrexate in both jejunum and ileum. Probenecid, an MRP inhibitor, suppressed the efflux of methotrexate in jejunum, but not in ileum. Verapamil, a P-gp inhibitor, did not show any significant effect on methotrexate efflux in either jejunum or ileum.

Bidirectional efflux of methotrexate from rat everted intestine in-vitro

To evaluate the mucosal and serosal efflux of methotrexate from the intracellular compartment simultaneously, methotrexate was pre-loaded in rat everted intestine at 4°C. The uptake amount of methotrexate into the intestinal sac during a

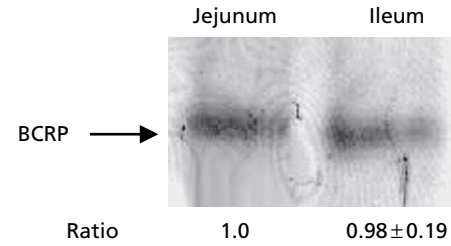

Figure 1 Western blot analysis of BCRP protein in rat jejunum and ileum. For the detection of BCRP in the intestine, brush-border membrane was used. Each value of relative staining intensity (Ratio) represents the mean ± s.e. of three determinations.

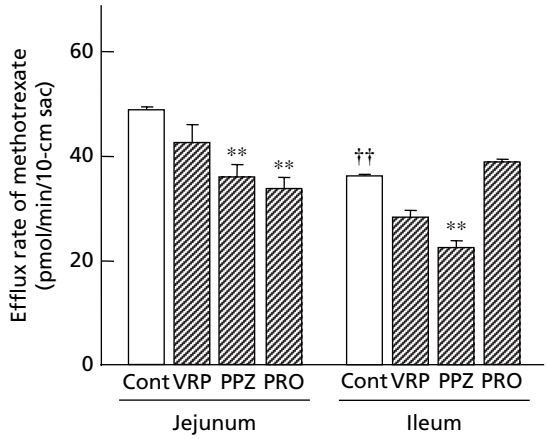

Figure 2 Effect of various transporter inhibitors on mucosal efflux of methotrexate in everted jejunum and ileum of rats. Methotrexate (10 nmol) was applied to the serosal side of a 10-cm long everted sac. Cont, control; VRP, verapamil (300 μM); PPZ, pantoprazole (300 μM); PRO, probenecid (1 mM). Each value represents the mean ± s.e. of three determinations. $^{**}P < 0.0$, vs control; $^{\dagger\dagger}P < 0.01$, vs jejunum mucosal efflux in the absence of probenecid (control).

40-min incubation was approximately 25% of the loaded amount, and the amount was almost the same between the jejunum and ileum. The addition of probenecid into the medium exerted no significant effect on the uptake amount of methotrexate. In jejunum, the total amount of methotrexate effluxed from the everted sac without probenecid was approximately 15% of uptake amount during a 120-min incubation at 37°C. The efflux of methotrexate was observed preferentially in the mucosal side and the effluxed amount was several-fold higher than that in serosal side (Figure 3A). Probenecid significantly suppressed the mucosal efflux of methotrexate by approximately 30% of control in jejunum.

In ileum, approximately 9% of pre-loaded methotrexate amount was recovered in the mucosal and serosal media during a 120-min incubation. In ileum, the mucosal efflux of methotrexate was lower, whereas serosal efflux was slightly higher than in jejunum. The presence of 1 mM probenecid exerted no significant effect on mucosal methotrexate efflux in ileum, but significantly suppressed the serosal efflux of methotrexate (Figure 3B).

Role of efflux transporters in intestinal methotrexate absorption

The effect of pantoprazole and probenecid on methotrexate transport was evaluated in-vivo by measuring the disappearance profile of methotrexate from the re-circulating intestinal perfusate. Methotrexate disappeared from the perfusate with a zero-order rate constant and the adsorption of methotrexate to the tubing for intestinal perfusate and intestinal tissue of rats was not observed. The disappearance rate, or intestinal absorption rate, of methotrexate in jejunum was lower by approximately 40% than in ileum (Figure 4). The addition of pantoprazole to the perfusate increased the disappearance rate of methotrexate by approximately 1.6 fold in jejunum, and 1.4 fold in ileum. Probenecid increased the disappearance rate of methotrexate in jejunum by approximately 1.4 fold, but did not show any effect in ileum.

Role of MRPs in intestinal absorption of methotrexate

The role of MRPs (MRP2 in BBM and MRP3 in basolateral membrane) in intestinal absorption of methotrexate was further examined by measuring the plasma concentrations of methotrexate in an in-vivo intestinal loop method. The plasma concentration of methotrexate after intra-ileum administration was significantly higher than that after intra-jejunum administration (Figure 5). Some pharmacokinetic

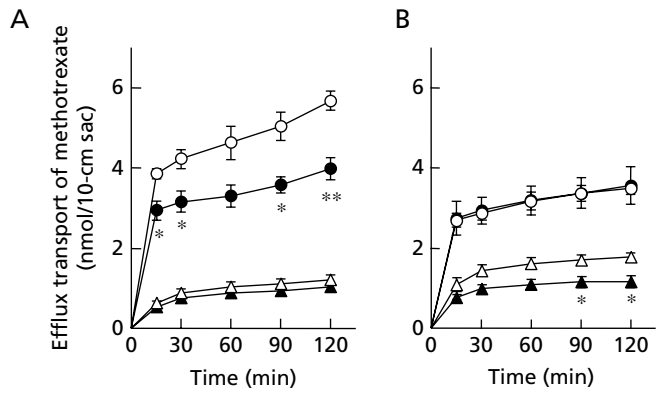

Figure 3 Bidirectional efflux of methotrexate after pre-loading in everted rat jejunum (A) and ileum (B) in the absence or presence of probenecid. Everted intestine was incubated in methotrexate solution (10 μM) at 4°C for 40 min to pre-load methotrexate and, after washing, the efflux transport study of methotrexate was carried out at 37°C. Opened circles represent the efflux to mucosal side, and opened triangles represent the efflux to serosal side in the absence of probenecid (control). Closed circles and triangles represent the efflux to mucosal and serosal side, respectively, in the presence of probenecid (1 mM). Each value represents the mean ± s.e. of three determinations. $^{**}P < 0.01$, $^{*}P < 0.05$, vs control.

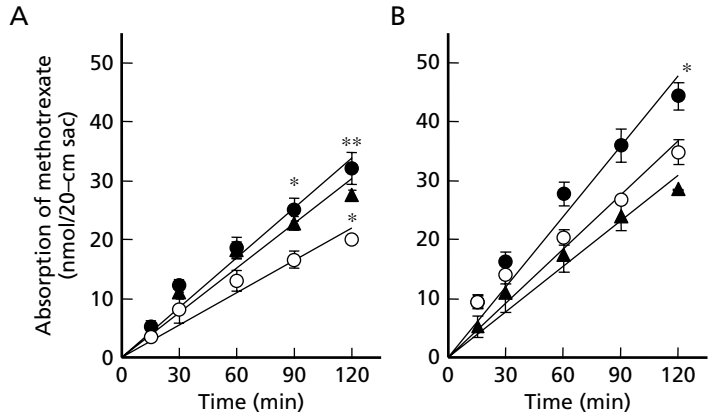

Figure 4 Effect of various transporter inhibitors on the intestinal absorption of methotrexate from 20-cm long jejunum (A) and ileum (B) loops. The absorbed amount of methotrexate was assumed to be the same magnitude as the disappeared amount from the perfusate. Open circles represent the control. Closed circles and triangles represent the methotrexate absorption in the presence of pantoprazole (300 μM) and probenecid (1 mM), respectively. Each value represents the mean ± s.e. of three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, vs control.

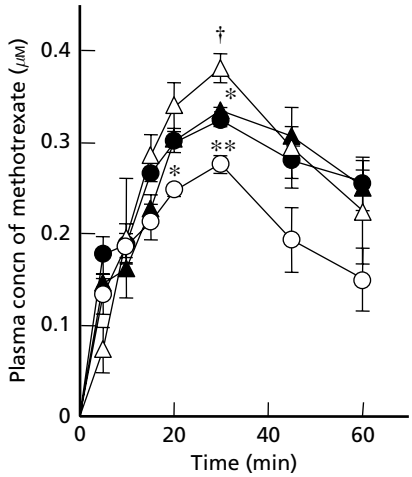

Figure 5 Plasma concentration–time profile of methotrexate administered to jejunum and ileum loops in rats. The dose of methotrexate to each loop was 1.25 μmol kg^{-1}. Open and closed symbols represent the control and probenecid treatment (175.2 μmol kg^{-1}, i.v.), respectively. Circles and triangles are results after intra-jejunum and intra-ileum loop administrations (a 10-cm-long loop), respectively. Each value represents the mean ± s.e. of three determinations. $^{**}P < 0.01$, $^{*}P < 0.05$, vs control; $^{\dagger}P < 0.05$ vs jejunum.

parameters, such as the peak plasma concentration (C_{max}) and the area under the arterial plasma concentration–time curve from 0 to 60 min ($AUC_{0–60}$), of methotrexate are summarized in Table 1. The C_{max} of methotrexate given from the ileum was approximately 1.4-fold higher than that given from the jejunum. In rats treated with probenecid, the C_{max} of methotrexate given from jejunum increased significantly, and that given from ileum decreased significantly, resulting in the comparable plasma concentrations of methotrexate between the jejunum and ileum.

Binding of methotrexate to 10% intestinal tissue homogenates and plasma

Methotrexate is known to bind intracellular proteins, such as DHFR, which results in accumulation in cells (Nozaki et al 2004). Accordingly, the effects of pantoprazole and probenecid on the protein binding of methotrexate were examined using 10% intestinal tissue homogenate and plasma (Table 2). The binding of methotrexate to the intestinal tissue homogenates was similar in jejunum and ileum. Pantoprazole and probenecid exerted no significant effect on the protein binding of methotrexate in the intestinal tissue homogenates and plasma.

Discussion

Recently, we evaluated the mucosal and serosal efflux of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), a typical MRP substrate, in rat jejunum and ileum, and found a marked regional difference between jejunum and ileum. The jejunum exhibited a higher mucosal MRP2 and a lower basolateral MRP3 expression than ileum, and DNP-SG efflux to mucosal surface was significantly greater in jejunum, while serosal efflux was greater in ileum. These results suggested the possible variability in intestinal bioavailability of MRP-related compounds, depending on their absorption sites or regional expression profiles of MRPs along the intestine (Yokooji et al 2007). In this study, we further studied the possible site-specific intestinal absorption of an MRP-related drug, methotrexate.

Methotrexate is a substrate of MRPs such as MRP2 and MRP3, though this compound is also known as a substrate of P-gp, BCRP and influx transporters RFC-1 and PCFT/HCP1 (Norris et al 1996; Masuda et al 1997; Hirohashi et al 1999; Breedveld et al 2004; Qiu et al 2006; Nakai et al 2007). The intestinal absorption of methotrexate would be mainly mediated by H^{+}-coupled RFC-1 or PCFT/HCP1 (Selhub & Rosenberg 1981; Chiao et al 1997; Qiu et al 2006; Nakai et al 2007). Accordingly, to minimize the activity of these influx transporters,

Table 1 Pharmacokinetic parameters of methotrexate administered into the jejunum and ileum with or without probenecid in rats

	Jejunum		Ileum	
	Control	+ Probenecid	Control	+ Probenecid
C_{max} (μM)	0.28 ± 0.01	0.32 ± 0.01**	0.38 ± 0.02†	0.34 ± 0.01**
T_{max} (min)	30	30	30	30
AUC_{0-60} (μM·min)	12.0 ± 0.6	15.6 ± 0.3	16.2 ± 2.0	15.6 ± 0.2
CL_{total}/F (mL min^{-1} kg^{-1})	104.8 ± 5.6	80.4 ± 1.7	80.2 ± 11.5	80.1 ± 0.8

Each value represents the mean ± s.e. of three determinations. CL_{total}/F was estimated by dividing the dose of methotrexate by AUC_{0-60}. **$P < 0.01$, †$P < 0.05$, vs value for control and jejunum, respectively.

Table 2 Effect of pantoprazole and probenecid on the protein binding of methotrexate to 10% intestinal homogenates and plasma of rats

	10% Jejunum	10% Ileum	Plasma
Control	49.9 ± 1.5	44.4 ± 0.8	53.5 ± 1.8
Pantoprazole			
150 μM	47.0 ± 0.5	ND	ND
300 μM	48.0 ± 0.4	ND	49.5 ± 2.3
Probenecid			
500 μM	46.6 ± 0.9	ND	ND
1 mM	47.4 ± 1.0	ND	46.1 ± 0.9

The concentration of methotrexate was 10 μM in biological samples. Protein binding (%) was determined by ultrafiltration method. ND, not determined. Each value represents the mean ± s.e. of three determinations.

we performed all studies with methotrexate in-vitro and in-vivo at pH 7.4. The localized expression of efflux transporters along rat intestine is well documented. P-gp is expressed on the brush-border membrane of enterocytes, especially in the distal intestine (Tian et al 2002). MRP2 is on the brush-border membrane, mostly in the proximal intestine, and MRP3 is on the basolateral membrane at a higher level in the distal region in rodents (Mottino et al 2001; Rost et al 2002; Yokooji et al 2007). The protein level of BCRP in the brush-border membrane fractions was comparable between jejunum and ileum in this study (Figure 1), though a higher expression of BCRP mRNA in distal intestine is reported in male and female rats (Tanaka et al 2005).

To evaluate the role of efflux transporters in the mucosal or serosal efflux of methotrexate in jejunum and ileum, the effect of transporter inhibitors on methotrexate transport were examined (Figure 2, 3). In an inhibition study, verapamil (300 μM), pantoprazole (300 μM) and probenecid (1 mM) were used as typical inhibitors for P-gp, BCRP and MRPs, respectively. In addition, verapamil (300 μM) and pantoprazole (300 μM), respectively, can suppress BCRP and P-gp (Pauli-Magnus et al 2001; Ozvegy-Laczka et al 2004). In this study, verapamil did not show any significant effect on methotrexate transport either in jejunum or ileum, suggesting the low inhibitory effect on BCRP function, if any. The inhibitory effect of pantoprazole on P-gp function could be ruled out, since the participation of P-gp in methotrexate transport was not observed in the inhibition study with verapamil, as described below.

The mucosal efflux of methotrexate was higher in jejunum than ileum, and the efflux was inhibited significantly by pantoprazole and probenecid, but not by verapamil. In ileum, only pantoprazole suppressed the mucosal efflux of methotrexate. Thus, it was found that both BCRP and MRP2 are involved in the mucosal efflux of methotrexate in jejunum by approximately 30% each, whereas BCRP alone contributes to the mucosal efflux by approximately 40% in ileum. The contribution of P-gp-mediated efflux of methotrexate was not observed even in ileum, where functional P-gp is abundantly expressed (Yumoto et al 1999). The serosal efflux of methotrexate was suppressed by probenecid in ileum, but not in jejunum (Figure 3), suggesting the participation of MRP3-mediated basolateral efflux, in good agreement with MRP3 expression site (Yokooji et al 2007). Accordingly, the site-specific bidirectional efflux pattern of methotrexate was well accounted for by the site-specific expression of efflux transporters of BCRP, MRP2 and MRP3, as schematically illustrated in Figure 6.

In a bidirectional efflux study using everted intestine (Figure 3), the total amount of methotrexate effluxed from the everted sac was quite low; approximately 9–15% of uptake amount during a 120-min incubation at 37°C. Methotrexate is known to bind intracellular proteins, such as DHFR, expressed in the intestine (Sirotnak et al 1984). Also, the binding ratio of methotrexate was relatively high, approximately 50%, even in 10% intestinal homogenates (Table 2). Thus, the low efflux of methotrexate from intestinal sac may be due to the high intracellular binding of methotrexate, though further study is necessary to clarify the mechanism.

In good accordance with the above-described in-vitro studies, the intestinal absorption of methotrexate given from ileum was approximately 1.3- to 1.6-fold higher than that from jejunum (Figure 4, 5, Table 1). Pantoprazole and probenecid increased the intestinal absorption of methotrexate by 1.6 fold and 1.4 fold, respectively, in jejunum. Pantoprazole also increased the ileal absorption of methotrexate by approximately 1.4 fold of control. The higher intestinal absorption of methotrexate from ileum than jejunum would be, at least partly, due to the lower MRP2 expression on the brush-border membrane in the ileum. Also, the increase in the intestinal methotrexate absorption by pantoprazole in jejunum and ileum and by probenecid in jejunum would be due to the suppression of the mucosal efflux of methotrexate. In contrast, in ileum, probenecid decreased the absorption of

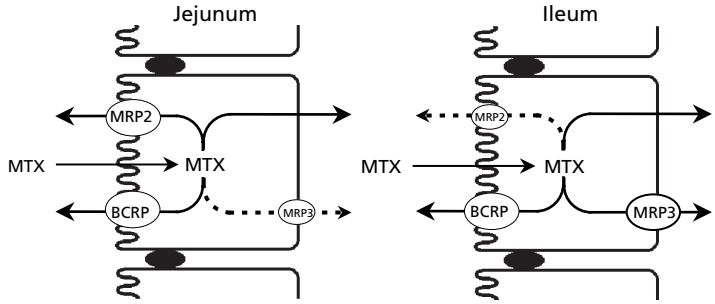

Figure 6 Schematic presentation for efflux transporter-mediated transport of methotrexate in rat intestine.

methotrexate. This would be due to the suppression of MRP3 in the basolateral membrane. This result suggests that MRP3 normally functions as an absorption transporter for MRP substrates in the intestine, as schematically represented in Figure 6. In this experiment, the change in C_{max} values between the absence and presence of probenecid was significant, but not in AUC values, possibly due to the variety of AUC values (Table 1). The intravenously administered probenecid can suppress not only the MRPs expressed in the intestine but also those in the liver, as demonstrated previously using DNP-SG (Yokooji et al 2006). Thus, probenecid may also affect the hepatic first-pass effect by suppressing biliary excretion of methotrexate, suggesting the alteration in systemic disposition profile of methotrexate in probenecid-treated rats. To evaluate the role of MRP3 in the intestinal absorption of methotrexate quantitatively, further study is necessary.

Conclusion

The role of ABC transporters, such as P-gp, BCRP and MRPs, in the intestinal absorption of methotrexate and the expression of these efflux transporters were examined in rats. Also, the regional difference in intestinal BCRP and MRP function was evaluated pharmacokinetically from the viewpoint of methotrexate transport. In good agreement with the regional difference in BCRP, MRP2 and MRP3 expression, a marked site-specific bidirectional efflux pattern of methotrexate was observed in rat intestine. BCRP-mediated mucosal efflux was of a similar magnitude between jejunum and ileum. MRP2-mediated mucosal efflux was greater in jejunum, and MRP3-mediated basolateral efflux was greater in ileum. A significantly higher oral bioavailability of methotrexate was observed when methotrexate was administered into the ileum. These results suggest that the intestinal bioavailability of methotrexate could be variable in man, depending on the expression or function of BCRP and MRPs.

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