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Study on absorption sites of quinidine and methotrexate in rat intestine

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Influx or efflux transporter(s), or both, are frequently involved in the intestinal absorption of various therapeutic drugs. In the present study, the effects of altered gastric emptying rates (GER) on intestinal absorption of quinidine (a substrate for P-glycoprotein, P-gp) and methotrexate (a substrate for multiple-transporters including proton-coupled folate transporter, PCFT) were examined to find their main absorption sites along the intestine employing rats. In untreated control rats, quinidine administered orally was rapidly absorbed from the proximal intestine, where P-gp is less expressed. Increased GER, which transferred an unabsorbable model compound to the middle intestine within 15 min after oral administration, exerted no significant effects on the extent of oral bioavailability of quinidine, whereas it increased the initial absorption rate greatly. Decreased GER, in which more than 50% of a model compound administered was retained in the stomach even 1 h after administration, decreased the onset time of intestinal absorption, but not the extent of oral bioavailability of quinidine. In untreated control rats, methotrexate was absorbed efficiently from the proximal intestine under acidic conditions, where PCFT is abundantly expressed. Increased GER significantly decreased, and decreased GER slightly increased the oral bioavailability of methotrexate. In conclusion, altered GER was found to affect the transporter-mediated intestinal absorption of drugs in different manners, depending on the solubility, membrane permeability, luminal concentration of the drug, luminal pH, substrate specificity, and the expression sites of transporter.

1. Introduction

It is known that riboflavin has a narrow absorption window in the upper part of the small intestine, and therefore, administration after meal or gastro-retentive formulation such as floating pellet, which can reduce the gastric emptying rate (GER) of riboflavin, can increase the oral absorption of riboflavin (Levy and Jusco 1966; Hewitt and Levy 1971; Hamdani et al. 2006; Kagan et al. 2006; Ahmed and Ayres 2007). In general, the reduced delivery rate of a substrate drug to the absorption site, where the influx transporter is regionally expressed, will decrease the unabsorbed fraction by avoiding the saturation in the transporter-mediated transport. The colon also exhibits a carrier-mediated transport system for riboflavin in rats, however, the transport capacity in the colon is lower than that in the upper intestine, as evaluated by Michaelis-Menten constant (K_m) and maximal transport rate (J_{max}) in riboflavin transport (Yuasa et al. 2000; Tomei et al. 2001). Such site specific intestinal absorption has also been reported with various ions and nutrients, including thiamine hydrochloride (Hewitt and Levy 1971; Sklan and Trostler 1977) and bile acids (Lack 1979; McClintock and Shiau, 1983; Nakashima et al. 1989; Kramer et al. 1993). Anthone et al. (1992 and 1993) reported that ileal water and electrolyte absorption exceeds jejunal absorption in both the basal and meal-stimulated states. Calcium is reported to be absorbed by a transcellular active process in the duodenum and upper jejunum and a paracellular, passive process throughout the length of the intestine, though the contribution of these transport mechanisms varied depending on the intake amount of calcium, in the mammalian small intestine (Bronner 2003).

In addition to electrolytes and nutrients, the site specific intestinal absorption is also reported for chemically synthesized therapeutic drugs. Kaneniwa et al. (1986) assessed the optimal absorption site of cimetidine in rats and suggested that cimetidine is completely absorbed in the duodenum and ileum during its passage through these intestinal sites, but in the jejunum an unabsorbed fraction of cimetidine passes to the ileum, where it is absorbed completely. Suttle and Brouwer (1995) examined the ranitidine absorption in isolated segments of rat small intestine, and found that ranitidine was absorbed from the entire small intestine, however, the terminal ileum was the optimal site of gastrointestinal absorption. Lindahl et al. (1998) investigated the mechanisms of fluvastatin transport across the intestinal mucosa in various regions of the intestine in rats, and found that fluvastatin had the lowest permeability in the jejunum and the highest permeability in the colon, although they also reported that neither the dose nor the intestinal region influenced the oral bioavailability of fluvastatin significantly in human (Lindahl et al. 2004).

In general, the site-specific intestinal absorption, or site-specific intestinal un-absorption, of a drug will be closely related with the site-specific expression levels of the transporter. In such case, the modulation of delivery rate of a substrate drug to the absorption site would greatly affect the absorption kinetics of the drug, as observed in riboflavin absorption (Levy and Jusco 1966). The function of the stomach such as acid and endocrine secretion,

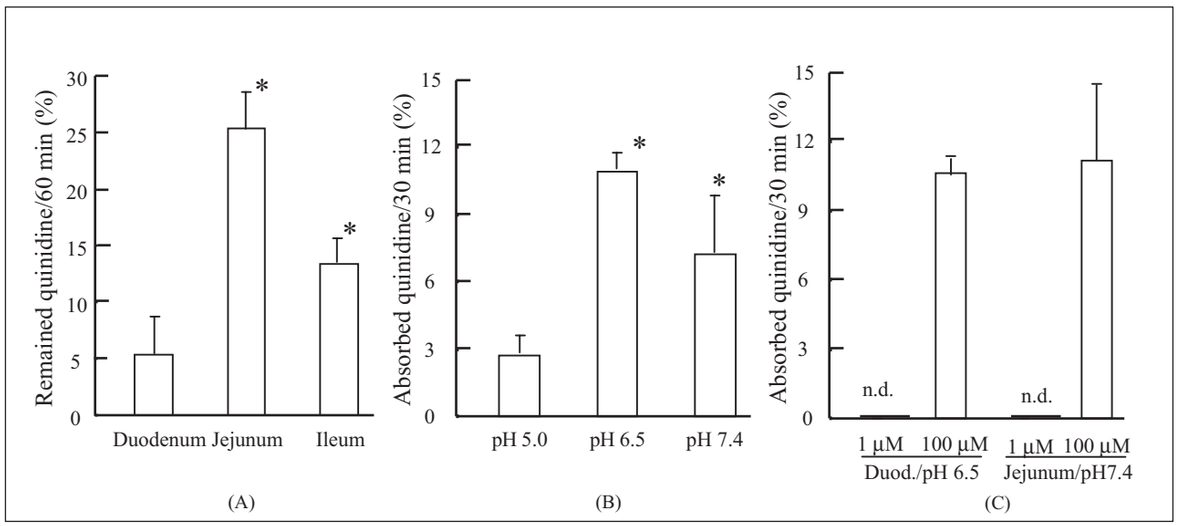

Fig. 1: Effect of administration sites along the intestine (A), effect of luminal pH at the duodenum (B), and effect of luminal concentration of quinidine at the duodenum (Duod.) and jejunum on the intestinal absorption of quinidine. (A) quinidine dissolved in distilled water was administered at a dose of 3 mg/ml/kg to each 10 cm long loop. (B) Quinidine solution (100 μM) with different pHs was perfused at the 15 cm long duodenum region in a recirculating manner. (C) Quinidine solution (1 μM, 100 μM; pH 6.5 for duodenum and pH 7.4 for jejunum) was perfused at the 15 cm long duodenum or jejunum region in a recirculating manner. The remained amounts of quinidine were determined in the loop 60 min after administration (A) or in the intestinal perfusate 30 min after initiation of the perfusion (B, C). Each value represent the mean ± S.D. (n = 3). *: $P < 0.05$

initiation of digestion, holding and crushing of ingested food, gastric motility and gastric emptying rate (GER) is modulated under various diseased states (Pohle and Domschke 2003; Hardoff et al. 2001; Castell et al. 2004). For example, in patients with Parkinson's disease, the GER is delayed compared with control healthy volunteers (Hardoff et al. 2001; Nyholm and Lennernäs 2008), and some patients with Parkinson's disease develop response fluctuations due to a significant delay in GER after several years of chronic treatment with levodopa (Djaldetti et al. 1996; Hardoff et al. 2001).

In this study, we examined the effect of altered GERs on the intestinal absorption of quinidine and methotrexate to assess their main absorption site in the intestine. Quinidine is known to be a substrate for P-glycoprotein (P-gp), and methotrexate is a substrate for multiple transporters including proton-coupled folate transporter (PCFT). Altered GER was induced by injecting metoclopramide or scopolamine butylbromide subcutaneously and evaluated by measuring gastro-intestinal distribution of FITC-dextran of molecular weight 10,000 (FD-10) after oral administration in the same manner as reported previously (Mori et al. 2008).

2. Investigations, results and discussion

2.1. Factors affecting the intestinal absorption of quinidine

The intestinal epithelial membrane expresses various ATP-binding cassette (ABC) transporters such as P-gp, multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP), in addition to various solute carriers (SLC). Among these ABC efflux transporters, P-gp is considered the most important efflux transporter in pharmacotherapy, since P-gp transports a variety of clinically important structurally and pharmacologically unrelated, neutral and positively charged, membrane-permeable hydrophobic compounds, and can suppress their intestinal absorption, more or less, by inhibiting the influx and facilitating the efflux of such compounds out of cells (Takano et al. 2006; Murakami and Takano 2008). In human and rodent intestine, P-gp is expressed on the apical surface of superficial columnar epithelial cells, and in general, higher expression of P-gp is observed at the ileum and colon, as compared with those in the jejunum, duodenum and stomach (Ho et al. 2003). Mouly and Paine (2003) determined P-gp expression by western blotting along the entire length of the human small intestine, and found that relative P-gp levels increased progressively from the proximal to the distal region (approximately 2-fold) in each donor intestine. Higher expression and/or function of intestinal P-gp in the ileal region in comparison with other regions in human and rodent intestine are also reported by many investigators (von Richter et al. 2004; Makhey et al. 1998; Yumoto et al. 1999; Stephens et al. 2001).

Quinidine is a substrate for P-gp (Műller et al. 1994; Kusuhara et al. 1997; Emi et al. 1998). Adachi et al. (2003) reported that the higher permeability-surface are (PS) product of quinidine in mdr1a/1b (−/−) mice was much higher than that in P-gp-expressing normal mice. Mizuno et al. (2003) reported a higher Kp (brain-to-plasma partition coefficient) value ratio of quinidine in mdr1a/1b (−/−) than that in mdr1a/1b (+/+) mice, indicating a higher contribution of P-gp in the brain distribution of quinidine. In addition, quinidine is also known as a potent inhibitor for P-gp, as well as verapamil and cyclosporine A (Suzuyama et al. 2007). At a higher concentration, like more than 30 μM, quinidine can inhibit the P-gp-mediated basolateral-to-apical efflux transport of rhodamine 123 across Caco-2 cell monolayers *in vitro*, and serosal-to-mucosal efflux transport of rhodamine 123 in rat intestine *in vivo* (Yumoto et al. 1999).

It will be generally considered that the efficient oral bioavailability of quinidine could not be expected due to the P-gp-mediated efflux in the intestine. In the Biopharmaceutical Classification System (BCS) defined by the FDA, however, quinidine, as well as some other P-gp-related compounds such as dilitiazem and verapamil, is categorized as a class 1 compound with high solubility and high permeability (Amidon et al. 1995; Wu and Benet 2004). In the present study, at first, we examined the effect of administration sites of quinidine in segmental small intestine, the effect of luminal pH, and the effect of luminal concentration of quinidine on the intestinal absorption of quinidine, since P-gp expression is site specific and the concentration of a substrate at the absorption site is considered

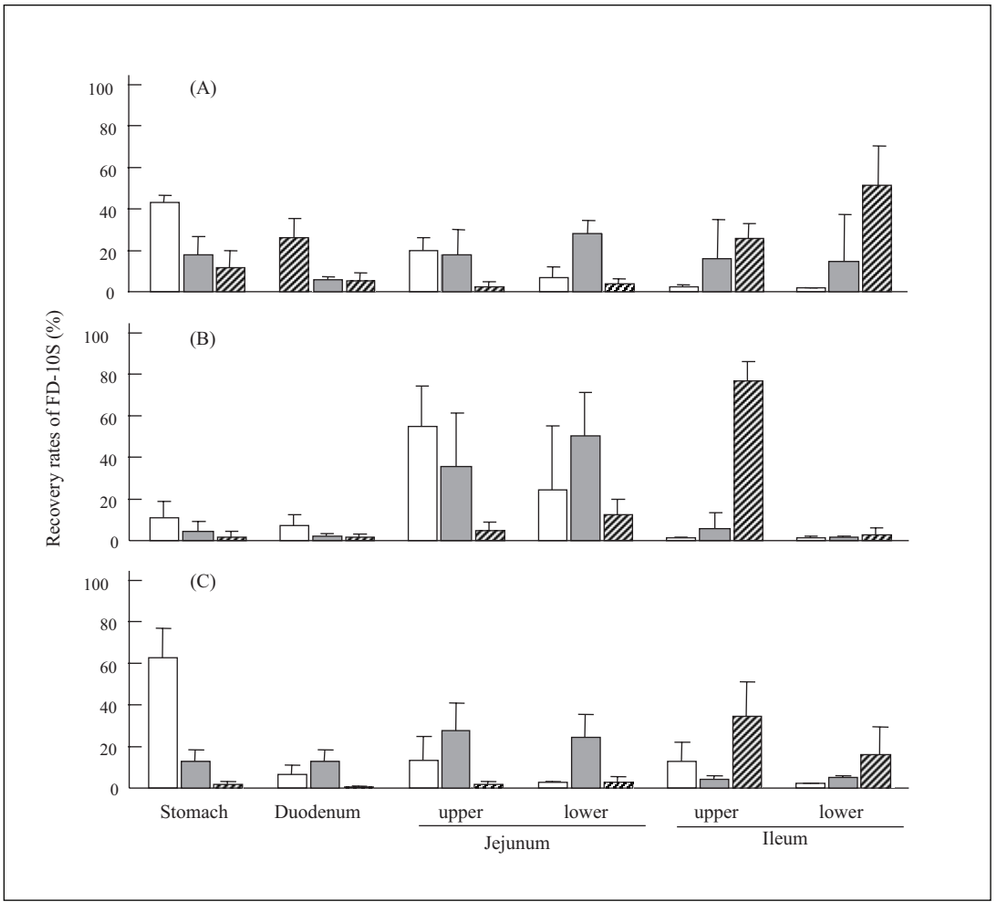

Fig. 2: Time profiles for the remaining amounts of FD-10S in the intestine after oral administration in rats with normal GER (A), with increased GER (B) and with decreased GER (C). GERs were altered by injecting methoclopamide (B) or scopolamine butylbromide (C) subcutaneously. FD-10S was administered orally at a dose of 5 mg/ml/kg 15 min after the treatment. Animals were then sacrificed 15 min (opened column), 30 min (dotted column) and 60 min (slashed column) for (A) and (B), or 1 h (opened column), 2 h (dotted column) and 4 h (slashed column) for (C) after oral administration, to evaluate the regional distribution of FD-10S in the entire length of the small intestine. Each value represents mean ± S.D. of 3-4 trials

to be a rate determining factor in transporter-mediated transport system. The effect of luminal pH was also examined to evaluate the absorption characteristics of quinidine, since quinidine is a basic compound with a pKa value of 8.57 and therefore the uncharged fraction, or apparent lipophilicity, of quinidine varies depending on the luminal pH of small intestine. Normal human subjects have pH values of 1.0–2.5 in the stomach, pH 6.6 ± 0.5 in the proximal small intestine, and pH 7.4–7.5 in the mid and distal intestine, as evaluated with a pH sensitive radiotelemetry capsule in 66 normal subjects (Evans et al. 1988). Thus, the effect of luminal quinidine concentrations was evaluated at pH 6.5 in duodenum and pH 7.4 in jejunum. Fig. 1A shows the site specific intestinal absorption of quinidine, in which the amounts of quinidine remained in the loop 60 min after administration into the segmented intestinal loop are compared among three different regions. The proximal intestine showed a smaller remaining amount of quinidine compared with those in the middle and distal intestine, indicating a greater absorption rate of quinidine at the proximal intestine, possibly due to the lower expression of P-gp in this region. The lower absorption rates of quinidine at the middle and distal intestine, irrespective of the higher luminal pH than that in duodenum physiologically, may be explained by the higher expression of P-gp. The absorption percentages of quinidine at pH 6.5 and pH 7.4 were significantly higher than that at pH 5.0, possibly due to the higher fraction of uncharged quinidine at a higher luminal pH (Fig. 1B). When quinidine was perfused at a concentration of 1 μM in a recirculating manner, the intestinal absorption of quinidine was not detected even at the duodenum, where quinidine was absorbed efficiently (Fig. 1C). The undetectable absorption of quinidine at 1 μM at the duodenum could be due to the expression of P-gp at a low level. When 100 μM quinidine was used, quinidine was efficiently absorbed even in the jejunum, and the extent of the quinidine absorption was the same extent between duodenum and jejunum, probably due to the inhibitory action of quinidine against P-gp function. At a fixed pH, the absorption rate of quinidine would decrease with the decrease in luminal concentration of quinidine, due to the decrease in the inhibitory action against P-gp-mediated efflux. Verma and Panchagnula (2005) reported that the functional activity of P-gp is almost the same at lowest concentration of quinidine at pH 4.5 and pH 7.4, though it reduced significantly with relatively small increments in quinidine concentration at pH 7.4. Thus, all three factors examined in the present study, that is, the absorption sites, luminal concentration, and luminal pH were found to be important factors in determining the intestinal absorption of quinidine.

2.2. Effect of GERs on the oral bioavailability of quinidine

Previously, we evaluated the absorption site of quinidine after oral administration in untreated control rats, and found that quinidine can be absorbed rapidly in the proximal intestine after discharged from the stomach, escaping the barrier function of

P-gp, because P-gp is mostly expressed in the distal intestine (Mori et al. 2008; Murakami and Takano 2008). In the present study, the effect of altered GERs on the intestinal absorption of quinidine was further examined. The altered GER was evaluated by measuring the time-dependent regional intestinal distribution of FD-10S after oral administration, in the same manner as reported previously (Mori et al. 2008). Fig. 2 shows the time profiles of FD-10S amounts distributed along the gastrointestinal tract in untreated control rats (A), rats with increased GER (B) and rats with decreased GER (C). The data in untreated control rats were cited from our previous study (Mori et al., 2008). The total recovery rate of FD-10S from the whole intestinal lumen was almost 100% at all three time points examined, except the case of 4 h in rats with decreased GER, in which some fraction of FD-10S administered was transferred to the caecum and colon (data not shown). In untreated control rats, FD-10S was remained mostly at the proximal region, including the stomach at 15 min after administration. At 30 min, FD-10S was distributed evenly throughout the gastrointestinal tract, and at 60 min, most of FD-10S was accumulated in the distal small intestine (Fig. 2A). In rats with increased GER, approximately 90% of FD-10S administered was discharged from the stomach within 15 min after administration, and most of it (approximately 80% of the dosed amount) were retained in the middle and proximal small intestine (Fig. 2B). In rats with decreased GER, more than 50% of FD-10S administered was retained in the stomach even 1 h after administration, and thereafter, FD-10S was discharged from the stomach and distributed throughout the entire length of the intestine.

The intestinal distribution of quinidine given orally, expressed as % of administered amount, in these GER modulated rats are summarized in Table 1, together with the data in untreated control rats. In untreated control rats, quinidine remained mostly at the proximal intestine, regardless of the elapsed time, and only a small amount of quinidine reached the distal intestine. Approximately 75% of quinidine administered was discharged from the stomach into the intestinal lumen and 70% of them were absorbed at the proximal intestine within 30 min. A small amount of the unabsorbed quinidine at the proximal intestine was transferred to the middle and distal intestine, where P-gp is abundantly expressed. The lower intestinal absorption rate of quinidine at the distal region could be due to P-gp-mediated efflux (Mori et al. 2008; Murakami and Takano 2008). In rats with increased GER, 95% of quinidine administered was discharged from the stomach and 80% of quinidine administered was absorbed within 15 min, indicating that the increased GER increased the initial absorption rate of quinidine greatly. In these rats, some parts of quinidine may have been transferred to the middle and/or distal intestine immediately after oral administration, as was with FD-10S. However, the amount of quinidine remained in the middle intestine was quite small, suggesting that most of quinidine was absorbed even from the middle intestine rapidly. Quinidine acts as a P-gp substrate at a lower concentration, but acts as a potent P-gp inhibitor at a higher concentration such as $> 30\ \mu M$, though there are marked species differences both in P-gp transport activity and the inhibitory effects on P-gp-mediated drug transport (Műller et al 1994; Yumoto et al. 1999; Katoh et al. 2006; Takeuchi et al. 2006; Suzuyama et al. 2007), suggesting that the intestinal absorption of quinidine at a higher luminal concentration could not be limited by P-gp. Accordingly, when the luminal concentration of quinidine decreased to a lower level, like $5\ \mu M$ (corresponding to the Km value in rat mdr1-mediated quinidine transport, Műller et al 1994), due to the intestinal absorption, thereafter, the intestinal absorption of quinidine can be suppressed by P-gp. In rats with decreased GER, approximately 60% of quinidine administered remained in the stomach even for 1 h after administration. However, only a small amount of quinidine was recovered from the whole small intestine, suggesting the discharged quinidine from the stomach was effectively absorbed in rats with decreased GER (Fig. 2C). These results indicated that the decreased GER decreased the onset of intestinal absorption, however, it did not decrease, rather increased, the extent of oral bioavailability of quinidine, because quinidine discharged from the stomach was almost completely absorbed in the proximal intestine. In the present study, it was found that the altered GERs did not exert a significant effect on the extent of quinidine absorption, though the initial absorption rates were affected depending on the altered GER. In BCS, quinidine is categorized as a class 1 compound with high solubility and high permeability (Wu and Benet 2005). In good accordance, quinidine was absorbed efficiently from any sites of the small intestine regardless of the expression of P-gp. Other BCS class 1 P-gp substrates such as dilitiazem and verapamil would also be absorbed efficiently from the entire small intestine, because they are also substrates at a lower concentration but inhibitors at a higher concentration against P-gp. In contrast, the intestinal absorption of other orally administered P-gp substrates categorized in BCS class 2-4 with low solubility or low permeability would be affected by the altered GER. The oral bioavailability of some class 2 P-gp substrates such as digoxin, cyclosporine A and tacrolimus are known to be affected by the amounts of P-gp expressed in the small intestine (Lown et al.

Table 1: Intestinal distribution of quinidine, expressed as % of dose, in the entire length of small intestine after oral administration at a dose of 3 mg/kg in rats with normal GER, increased GER, and decreased GER

Time after administration	Stomach (%)	Duodenum (%)	Jejunum (%)		Ileum (%)		Total recovery (%)
			upper	lower	upper	lower	
			Rats with normal GER				
15 min	50.9 ± 24.3	12.2 ± 4.2	2.9 ± 0.7	0.5 ± 0.4	0.1 ± 0.1	0.0 ± 0.1	66.6
30 min	24.5 ± 15.5	7.2 ± 2.3	3.1 ± 1.0	3.1 ± 1.9	2.5 ± 1.1	0.1 ± 0.0	40.5
60 min	16.2 ± 10.9	3.1 ± 2.0	3.0 ± 1.6	3.3 ± 2.4	2.5 ± 0.4	0.8 ± 0.5	28.8
			Rats with increased GER				
15 min	5.4 ± 3.3	3.2 ± 1.3	4.7 ± 2.7	4.9 ± 6.8	0.4 ± 0.3	0.3 ± 0.2	18.8
30 min	4.8 ± 4.7	2.2 ± 0.6	5.1 ± 2.0	4.0 ± 4.2	1.1 ± 1.0	0.5 ± 0.3	17.6
60 min	0.8 ± 0.9	0.8 ± 0.9	1.2 ± 0.9	1.0 ± 0.3	0.9 ± 0.3	0.4 ± 0.0	5.1
			Rats with decreased GER				
60 min	49.3 ± 10.6	3.2 ± 0.7	0.7 ± 0.9	0.2 ± 0.1	0.4 ± 0.3	0.1 ± 0.1	53.9

GER was increased by injecting methoclopamide or decreased by injecting scopolamine butylbromide, subcutaneously. Quinidine was administered orally 15 min after the treatment. Rats with normal GER and increased GER were then sacrificed at 15 min, 30 min or 60 min. Rats with decreased GER was sacrificed 60 min after the administration to evaluate the regional distribution of FD-10S in the entire length of the small intestine. Each value represents mean ± S.D. of 3–4 trials.

1997; Greiner et al. 1999; Masuda et al. 2000; Murakami and Takano 2008). If they were transferred to the middle or distal intestine immediately after the oral administration without being dissolved, their membrane permeability could be greatly suppressed by the P-gp efflux. Recently, Dahan and Amidon (2009) investigated the role of P-gp efflux in the *in vivo* intestinal absorption process of cimetidine and famotidine, both being BCS class 3 P-gp substrates, and they found their segmental dependent permeability through the gut wall with decreased P(eff) in the distal ileum in comparison to the proximal regions of the intestine. To gain steady and reliable bioavailability of orally administered P-gp substrates in BCS classes 2–4, the increase in the solubility and permeability would be essential to escape the P-gp-mediated efflux (Murakami and Takano 2009).

2.3. Effect of altered GERs on oral bioavailability of methotrexate

Methotrexate, a folic acid antagonist, is administered orally in the treatment of rheumatoid arthritis, and the plasma concentrations of methotrexate are known to exhibit wide variation in clinical practice, though the mean oral bioavailability of methotrexate is relatively high (approximately 75%) (Swierkot and Szechiński 2006; Gispen et al. 1987; Kremer and Lee 1988; Oguey et al. 1992; Lebbe et al. 1994; Hoekstra et al. 2004 and 2006). In a BCS, methotrexate is categolized as a class 3 or class 4 compound (Wu and Benet 2005). The variability of oral bioavailability of methotrexate was not ascribed to the food intake or renal failure such as the low glomerular filtration rate (Hamilton and Kremer 1995; Murry et al. 1995). In contrast, it was reported that the divided oral administration of methotrexate, like 30 mg by 8 h weekly, significantly increased the oral bioavailability in adult patients, in comparison with the single dose (Hoekstra et al. 2006). We supposed that the low and scattered oral bioavailability of methotrexate at a higher oral dose and the higher oral bioavailability at the divided oral administration may come from the involvement of multiple transporters including SLC and ABC transporters in the intestinal absorption of methotrexate. Based on such a consideration, we previously examined the contribution of MRP2, MRP3 and BCRP in intestinal absorption of methotrexate at pH 7.4 in rats (Yokooji et al. 2007). At pH 7.4, the contribution of PCFT, an influx SLC for methotrexate, can be ruled out, since PCFT is known to act only under acidic conditions with an optimal pH of 5.5 (Nakai et al. 2007; Inoue et al. 2008). In that study, it was found that MRP2, localized abundantly in brush-border membranes of the proximal small intestine, suppressed methotrexate absorption significantly, and MRP3, localized in the basolateral membranes of the distal small intestine, facilitated the intestinal absorption of methotrexate (Yokooji et al. 2007). In addition, BCRP that expressed in brush-border membrane along the whole small intestine suppressed the intestinal absorption of methotrexate in both proximal and distal small intestine. Like this, multiple ABC transporters such as MRP2, MRP3, and BCRP were found to be involved in the intestinal absorption of methotrexate, depending on their expression sites of transporters. Based on these observations, we next examined the contribution of PCFT in the intestinal absorption of methotrexate in rats at two different pHs, 5.5 and 7.4 (Yokooji et al. 2009). PCFT, expressed abundantly in the upper small intestine, has been identified as the molecular entity of the carrier-mediated transport system of folate and its analogues including methotrexate (Yuasa et al. 2009). In our study using everted intestine, the mucosal methotrexate influx rate in the proximal intestine at pH 5.5 was significantly greater than that at pH 7.4. Coadministration of folate or its analogues, such as folinate and 5-methyltetrahydrofolate, substrates

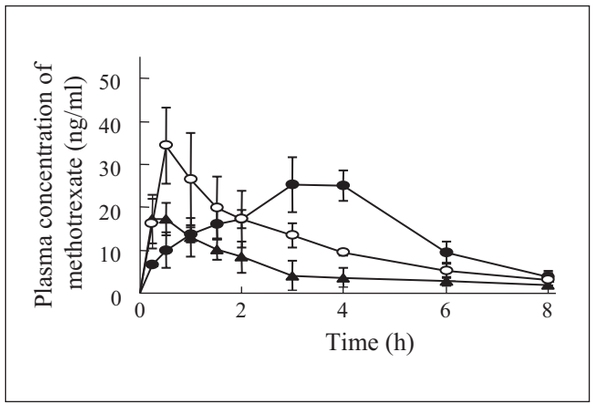

Fig. 3: Time profiles for plasma concentrations of methotrexate after oral administration at a dose of 1 mg/kg in rats with normal GER (opened circle), increased GER (solid triangle) and decreased GER (solid circle). GERs were altered by injecting methoclopamide (solid triangle) or scopolamine butylbromide (solid circle) subcutaneously. Metotrexate was administered orally 15 min after the treatment. Each value represents mean ± S.D. of 3–6 trials

for both PCFT and reduced folate carrier 1 (RFC1), significantly suppressed the methotrexate influx at pH 5.5. In contrast, in distal small intestine with a physiological pH of approximately 7.4 (Evans et al. 1988), methotrexate influx rate was low and was not pH dependent. Also, folate and its analogues exerted no significant effect on methotrexate absorption. These results suggested that, under physiological conditions, methotrexate would be absorbed mostly from the proximal intestine by PCFT, though the absorption may be suppressed partly by apical MRP2 (Yokooji et al. 2009).

In the present study, we examined the effect of altered GERs on the intestinal absorption of methotrexate after oral administration, to confirm the main absorption site of methotrexate in rats *in vivo* (Fig. 3). The dose of methotrexate employed was 1 mg/kg, which would be approximately two fold the clinical high oral dose regimen of methotrexate for humans (Hoekstra et al. 2006). In untreated control rats, methotrexate was absorbed rapidly, and disappeared from plasma in a first order rate constant. The increase in GER significantly decreased the values of peak plasma level (C_{max}) and area under the concentration-time curve from 0 to infinite (AUC) of methorexate to approximately half of those in control rats (Table 2). In contrast, the decreased GER delayed the time to reach C_{max} (T_{max}) significantly and increased AUC of methotreaxate by approximately 1.4-fold of that in control rats, though a significant difference

Table 2: Pharmacokinetic parameters of methotrexate after oral administration at a dose of 1 mg/kg in rats with normal GER (control), increased GER, or decreased GER

	Control rats	Increased GER	Decreased GER
C_{max} (ng/ml)	38.0 ± 2.52	18.2 ± 5.15**	24.8 ± 3.17
T_{max} (h)	0.67 ± 0.29	0.33 ± 0.14	3.67 ± 0.58**
AUC (ng h/ml)	107 ± 24.6	45.5 ± 11.4*	146 ± 13.7
Elimination rate constant (h^{-1})	0.31 ± 0.12	0.36 ± 0.24	0.46 ± 0.05
$T_{1/2}$ (h)	2.49 ± 0.91	2.71 ± 1.95	3.92 ± 1.56

GER was increased by injecting methoclopamide or decreased by injecting scopolamine butylbromide, subcutaneously. Methotrexate was administered orally 15 min after the treatment. Each value represents mean ± S.D. of 3–6 trials.
* $P < 0.05$
** $P < 0.01$, compared with control rats

was not detected between normal and decreased GER rats. These results may suggest that the intestinal absorption of methotrexate is mainly dependent on PCFT function, and PCFT expression is localized at the proximal intestine. The increased GER decreased the oral bioavailability of methotrexate possibly due to the saturation of PCFT and/or due to the passing by the main absorption site where PCFT is expressed.

2.4. Conclusion

In the present study, we examined the effect of altered GERs on the transporter-mediated intestinal absorption of quinidine and methotrexate to study their main absorption sites in the intestine, and obtained the following findings:

1) Quinidine is a highly soluble and permeable compound and categorized in class 1 in BCS. In good accordance, quinidine was mostly rapidly absorbed at the proximal intestine in rats with normal GER.
2) Absorption site, the luminal concentration of quinidine and luminal pH significantly affected the oral bioavailability of quinidine. For example, at a low luminal concentration of quinidine, the intestinal absorption of quinidine was not detected even in the proximal intestine, possibly due to the suppression by P-gp efflux.
3) The increased GER did not decrease the intestinal absorption of quinidine, rather increased the initial absorption rate, because quinidine at a high concentration, like > 30μM, can inhibit P-gp function, and will be absorbed at any intestinal sites until the luminal concentration of quinidine decreases to a very low concentration such as 5 μM (corresponding to Km value in mdr1-mediated quinidine transport).
4) In a BCS, methotrexate is categorized in class 3 or 4 with low membrane permeability. Multiple transporters such as MRP2, MRP3, BCRP and PCFT are involved in the intestinal absorption of methotrexate. The participation of multiple efflux transporters would cause variation in oral bioavailability of methotrexate among patients.
5) The intestinal absorption of methotrexate was mainly mediated by PCFT expressed in the proximal intestine. The increased GER significantly decreased, and decreased GER slightly increased the oral bioavailability of methotrexate.

Based on these findings, the main absorption sites of both quinidine and methotrexate under normal GER conditions were considered to be the proximal intestine, as well as for riboflavin. In general, it would be considered that the increased GER can decrease the oral bioavailability of BCS class 2-4 P-gp substrates, since the increased GER rapidly transfer the drug to the middle intestine, where P-gp is abundantly expressed. However, quinidine, a class 1 P-gp substrate, was efficiently absorbed at any sites of the intestine, possibly due to its high permeability and potent P-gp inhibitory effect. The intestinal absorption of methotrexate was primarily mediated by PCFT, which is highly expressed in the proximal intestine. The increased GER decreased the extent of oral bioavailability of methotrexate significantly. Like for riboflavin, gastroretentive formulation, which can reduce GER, may gain the steady and higher oral bioavailability of methotrexate in clinical practice.
In conclusion, altered GER was found to affect the transporter-mediated intestinal absorption of drugs in different manners, depending on the solubility, membrane permeability, luminal concentration of the drug, luminal pH, substrate specificity, and the expression sites of transporters.

3. Experimental

3.1. Chemicals and reagents

Quinidine was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Methotrexate was obtained from Wako Pure Chemicals (Osaka, Japan). Fluoresceine isothiocyanate-dextran with molecular weight of approximately 10,000 (FD-10S) was obtained from Sigma-Aldrich Japan K. K. (Tokyo, Japan). Metoclopramide (Primperan® Injection) and scopolamine butylbromide (Buscopan® Injection) were from Astellas Parma Inc. (Tokyo, Japan) and Nippon Boehringer Ingelheim Co., Ltd. (Tokyo, Japan), respectively. All other chemicals used were of the highest purity available.

3.2. Animals

Male Sprague-Dawley (SD) rats weighing about 250 to 350 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were fed a standard laboratory diet (CE-2, Clea Japan, INC., Tokyo, Japan) and water for more than 1 week prior to the experiments. Rats were fasted overnight with free access of water prior to the oral administration. Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" from the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima International University, which is in accordance with the "Guidelines for proper conduct of animal experiments" from Science Council of Japan.

3.2.1. Evaluation of the effects of the administration site, luminal pH and luminal concentration of quinidine

Quinidine dissolved in distilled water at a concentration of 3 mg/ml was administered into a 10-cm long intestinal loop, which was prepared at a region of 5 cm below the bile duct opening, middle center of the small intestine, or at a region of ileum (just above of ileocaecum). At 60 min later after administration, rats were lightly anesthetized with ethyl ether, and sacrificed by a heart puncture of an excess amount of sodium pentobarbital. The loop was isolated, weighed, added with 9-fold volumes of distilled water, and homogenized with a tissue homogenizer (21,000 rpm, 2 min). The homogenates were stored at -30 °C until analyzed. In the evaluation of the effect of luminal pH, quinidine was dissolved in pH 5.0, pH 6.5 or pH 7.4 isotonic phosphate buffered saline (PBS) at a concentration of 100 μM. The duodenum region (15 cm long) was perfused with quinidine solution (25 ml) in a recirculating perfusion manner at a rate of 3 ml/min. At 30 min after the initiation of the perfusion, the intestinal perfusate was recovered to determine quinidine concentration in perfusate. The effect of luminal concentration of quinidine was also evaluated by a recirculating perfusion manner. Quinidine solution at a concentration of 1 or 100 μM was prepared with pH 6.5 PBS for the duodenum region or pH 7.4 PBS for jejunum region, and the 15 cm long duodenum region or jejunum region of the small intestine was perfused with quinidine solution (25 ml) in a recirculating perfusion manner. At 30 min later, the perfusate was recovered to determine quinidine concentration in perfusate.

3.2.2. Evaluation of GER by intestinal distribution of FD-10S

FD-10S was dissolved in distilled water at a concentration of 5 mg/ml. The solution was administered orally by stomach intubation at a dosing volume of 1 ml/kg. Rats were anesthetized with ethyl ether, and sacrificed by a heart puncture of an excess amount of sodium pentobarbital at 1 min prior to the designated time (15 min, 30 min, or 60 min after the administration). The abdomen was opened and the whole small intestine was exposed. Ligation was made at the cardiac opening, the pyloric part, and the duodenum (15 cm down from the pyloric part), and the remaining small intestine was further divided into four parts of equal length (approximately 20 cm each). The resulting gastrointestinal loops were isolated, and each loop was weighed, added to 9-fold volumes of distilled water, and homogenized with a tissue homogenizer (21,000 rpm, 2 min). The homogenates were stored at −30 °C until analyzed.

3.2.3. Alteration of GER

Rats received metoclopramide (15 mg/kg) to increase GER or scopolamine butylbromide (10 mg/kg) to decrease GER, subcutaneously. FD-10S (5 ml/ml) or quinidine (3 mg/ml) dissolved in distilled water were administered orally 15 min after the treatment. The GER and intestinal distribution of FD-10S and quinidine were determined in the same manner as for FD-10S described above in the following time schedule: 15, 30 and 60 min after oral administration in untreated control rats and rats treated with metoclopamide, and 1, 2, and 4 h after oral administration in rats treated with scopolamine butylbromide.

3.2.4. Oral administration of methotrexate

Methotrexate, dissolved in saline at a concentration of 1 mg/ml, was administered orally at a dose of 1 ml/kg in untreated control, metoclopramide-treated, and scopolamine butylbromide-treated rats, 15 min after the treatment. Rats were lightly anesthetized with ethyl ether, and blood (0.2 ml each) was sampled from the jugular vein periodically at 20, 40, 60, 90, 120, 180, 240, 360 or 480 min. From each rat, blood samples were taken 4 or 5 times, and at least 3 blood samples were obtained for the each time point.

3.3. Analysis

To determine FD-10S concentration, the intestinal tissue homogenate was centrifuged at 10,000 rpm for 5 min, and the supernatant (50 μL) was properly diluted with distilled water. The fluorescence intensity was measured at wavelengths of 496 nm for excitation and 516 nm for emission. The concentration of quinidine in the tissue homogenate was determined by HPLC using the method described previously (Mori et al. 2008). A 1 ml of 0.1 M NaOH was added to 0.2 ml of tissue homogenate, and the suspension was extracted with 5 ml of ethylacetate. The ethylacetate layer (4 ml) was evaporated under reduced pressure and the residue was dissolved with 0.1 ml of HPLC mobile phase (acetonitrile/methanol/50 mM, pH 3.0 phosphate buffer = 4/2/4, v/v). For HPLC, a Lichrospher 100 RP-18(e) column (Cica-Merk, Tokyo, Japan) and a fluorescence detector operating at wavelengths of 310 nm (excitation) and 380 nm (emission) were used. Blood samples containing methotrexate were diluted appropriately with 20% perchloric acid. Intestinal mucosa samples were homogenized in an equal volume of 20% perchloric acid. The suspension was kept on ice for at least 30 min, and centrifuged at 3,000 rpm for 10 min. The concentrations of methotrexate in the supernatants were determined by HPLC using a column of Mightysil RP-18 (kanto Kagaku, Tokyo, Japan). The mobile phase used comprised a mixture of of acetonitrile, methanol and 0.1 M acetate buffer (pH 7.0) in a ratio of 3/6/91 (v/v). The flow rate of mobile phase was 1 ml/min, and detection of methotrexate was made at the wavelength of 304 nm.
Differences among group mean values were assessed by Kruskal-Wallis test or ANOVA test followed by post-hoc test (Tukey and Dunn's tests) or Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

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