Effects of Folic Acid and Pyrimethamine, a Dihydrofolate Reductase Inhibitor, on Intestinal Absorption of Folates in Rats

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ABSTRACT—Oral co-administration of folic acid (pteroylglutamic acid, PteGlu) potentiates the decrease of plasma 5-methyltetrahydrofolic acid (5-CH₃-H₄PteGlu) concentration induced by pyrimethamine (PYR) in rats. To clarify the mechanisms of this potentiated decrease, we examined the effects of PteGlu and PYR on intestinal absorption of folates in rat jejunal loops, because plasma 5-CH₃-H₄PteGlu concentration is maintained by enterohepatic circulation of folates. The intestinal absorption of 5-[¹⁴C]CH₃-H₄PteGlu was inhibited by PteGlu, but not by PYR. The absorption of [³H]PteGlu was inhibited by reduced folates that exist in bile. These findings indicate that PteGlu competes with the bile reduced folates for the intestinal transport system. The bile secretion of reduced folates was also examined to observe the conversion of absorbed PteGlu to reduced folates in the liver in the presence of PYR. The bile secretion of reduced folates increased drastically after the administration of PteGlu alone, but not after the administration of PteGlu with PYR. These facts suggest that the absorbed PteGlu was not converted to reduced folates in the liver due to PYR. In conclusion, the potentiated decrease of plasma 5-CH₃-H₄PteGlu concentration must have resulted from a combination of the following two factors: the inhibition of reabsorption of bile reduced folates by PteGlu and the inhibition of PteGlu conversion to reduced folates in the liver by PYR.

Keywords: Folate, Pyrimethamine, Intestinal absorption, Bile secretion

Folate deficiency (1–3) and inhibition of folate metabolism (4) can induce embryotoxicity in animals and humans, including fetal resorption, growth retardation and congenital malformations. Pyrimethamine (PYR), an antiprotozoan drug, is a dihydrofolate reductase (DHFR) inhibitor, and it has a teratogenic property in animals (5, 6). Our previous studies demonstrated that oral co-administration of folic acid (pteroylglutamic acid, PteGlu) markedly potentiated the teratogenicity of PYR in rats (7) and mice (8). 5-Methyl-tetrahydrofolic acid (5-CH₃-H₄PteGlu) is a principal active folate in the plasma of these animals (9), and the plasma concentration represents the folate status in the body (10). PYR decreases plasma 5-CH₃-H₄PteGlu concentration (11, 12), which means that there is a folate deficiency. However, we found that oral co-administration of PteGlu potentiated the decrease of plasma 5-CH₃-H₄PteGlu concentration induced by PYR (11, 12). We considered, therefore, that the potentiation of the teratogenicity of PYR by co-administration of PteGlu resulted from the potentiated decrease of plasma 5-CH₃-H₄PteGlu concentration, in other words, the potentiation of folate deficiency.

Enterohepatic circulation of folates is a major source of plasma folates, and this plays an important role in folate homeostasis (13–15). The interruption of enterohepatic circulation of folates, therefore, can influence the plasma folate concentration. For example, a rapid decrease of plasma folate was induced by bile drainage in rats (13, 15). In rat bile, there are several reduced folate derivatives including tetrahydrofolic acid (H₄PteGlu), 5-CH₃-H₄PteGlu, 5,10-methylenetetrahydrofolic acid (5,10-CH₂-H₄PteGlu) and 10-formyltetrahydrofolic acid (10-CHO-H₄PteGlu). The plasma concentration of 5-CH₃-H₄PteGlu is maintained by the enterohepatic circulation of these bile folates (15). It has been reported that some folate derivatives including PteGlu and methotrexate share a carrier-mediated intestinal transport system (16,}

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If the bile reduced folates and PteGlu hold an intestinal transport system in common, PteGlu administered orally can inhibit the intestinal reabsorption of bile reduced folates and cause the interruption of enterohepatic circulation of folate.

In the present study, we conducted the following two experiments to clarify the mechanism for the potentiated decrease of plasma 5-CH3-H4PteGlu concentration by oral co-administration of PteGlu with PYR to rats. The first experiment examined the competition in intestinal transport between PteGlu and reduced folates that exist in bile by using the in situ rat jejunum loops method. In the second experiment we examined the bile secretion of reduced folates after administration of PYR and PteGlu to rats to monitor the conversion of absorbed PteGlu to reduced folates.

MATERIALS AND METHODS

Chemicals

The magnesium salts of 5,10-CH2-H2PteGlu and H2PteGlu were purchased from Dr. B. Schircks' Laboratories (Jona, Switzerland) and the disodium salt of 5-CH3-H2PteGlu from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). 10-CHO-H2PteGlu was prepared from the calcium salt of 5-formyltetrahydrofolic acid (5-CHO-H2PteGlu) (Lederle Japan Co., Ltd., Tokyo) using the method described by Scott (18). PteGlu and PYR were purchased from Wako Pure Chemical Industries (Osaka). All reduced folate derivatives were dissolved in 0.2% sodium ascorbate solution except for 5,10-CH2-H2PteGlu, which was dissolved in 0.2% sodium ascorbate solution containing 3 × 10⁻³% paraformaldehyde. These solutions were stored at −80°C. All of the following radioactive materials were purchased from Amersham Life Science (Buckinghamshire, UK): [3',5',7',9'-3H]PteGlu potassium salt (radioactive purity, 96.9%; radioactivity, 962 MBq/nmol) dissolved in 1% sodium ascorbate solution; 5-[14C]CH3-H2PteGlu barium salt (radioactivity purity, 90.4%; radioactivity, 2.04 MBq/nmol); [3H]-inulin (radioactive purity, 98.1%; radioactivity, 40.7 MBq/nmol); and [carboxyl-14C]inulin (radioactive purity, 98.6%; radioactivity, 220 kBq/nmol).

Animals

Female Wistar rats (SLC Co., Ltd., Shizuoka) were housed in wire-mesh cages with a light cycle from 08:00 to 20:00 hr and with controlled temperature (23±2°C) and humidity (55±15%). Rats were quarantined for more than 2 weeks before the experiment, and the weight of the used animals was 150–195 g. During the quarantine period, the rats were fed a pellet diet (F-2; Funabashi Farm, Chiba) and water ad libitum. Rats were fasted for a night before the experiment.

Intestinal absorption

Under anesthesia with an intraperitoneal injection of pentobarbital sodium (40 m/kg), the small intestine was exposed via an abdominal incision. Starting from about 2 cm distal to the ligament of Treitz, two or three segments of jejunum, each 3-cm-long, were ligated with silk ties, taking care to preserve the vasculature of each segment. Then, 0.3 ml of test solution was injected into each loop. The test solution of 5-CH3-H2PteGlu contained 5-[14C]-CH3-H2PteGlu of 1.0 µM and [3H]inulin with or without PteGlu (0.5–50 µM), PYR (100 µM) or unlabeled 5-CH3-H2PteGlu (5 µM) in Krebs-Ringer phosphate buffer (NaCl 120 mM, KCl 4.8 mM, MgSO4 1.20 mM, glucose 10.8 mM, sodium phosphate 16.3 mM) containing 0.2% sodium ascorbate at a final pH of 6.3. The amount of 5-[14C]CH3-H2PteGlu injected into a loop (0.3 nmol) was almost equal to that of 5-CH3-H2PteGlu secreted into the bile for 10 min in rats (15). The test solution of PteGlu contained [3H]PteGlu of 0.1 µM and [14C]inulin with or without one bile-reduced folate, which was H2PteGlu, 5-CH3-H2PteGlu, 5,10-CH2-H2PteGlu, 10-CHO-H2PteGlu or unlabeled PteGlu (20 µM, respectively), in Krebs-Ringer phosphate buffer containing 0.2% sodium ascorbate at a final pH of 6.3.

At 10 min after the injection of test solution, the loops were detached from the mesentery and from each other and rinsed with saline. Then the loops were cut open and their luminal contents were washed with 7 ml saline. A suitable volume (1 ml/100 mg tissue) of Soluene-350 solubilizing agent (Canberra Packard, Berkshire, UK) was added to the empty intestinal loops, and then the loops were incubated for 24 hr at 55°C. Each aliquot (1 ml) of the saline wash and tissue solution was mixed with 10 ml of the liquid scintillant (Aquasol-II; Du Pont NEN Research Product, Boston, MA, USA and Hyonicfluor; Canberra Packard) and the radioactivity was counted in a liquid scintillation counters programmed for simultaneous counting of [14C] and [3H]. The disappearance of 5-[14C]CH3-H2PteGlu or [3H]PteGlu from the lumen was calculated from the counts in the saline wash corrected for inulin recovery (75–100%). Net accumulation of 5-[14C]CH3-H2PteGlu or [3H]PteGlu in the intestine was determined from the radioactivity in the tissue solution corrected for the inulin space. Systemic absorption of 5-[14C]CH3-H2PteGlu or [3H]PteGlu was estimated from the difference of radioactivity between luminal disappearance and accumulation in the intestine.

Bile secretion

Under anesthesia with an intraperitoneal injection of urethane (0.8 g/kg), a polyethylene catheter (0.28 mm
I.D., SP-10; Natsume Co., Ltd., Tokyo) was cannulated into the rat bile duct via an abdominal incision. Then PYR, PteGlu and PYR plus PteGlu suspended in 0.5% sodium carboxymethylcellulose (CMC) solution was injected into the duodenum. The control was injected with only 0.5% CMC solution into the duodenum. The dose of PYR was 1.6 mg/kg and that of PteGlu was 50 mg/kg. This combination produces the potentiated decrease of plasma 5-CH3-H4PteGlu concentration in rats (11, 12).

Bile samples from the catheter were collected into test tubes containing 0.4% sodium ascorbate solution (bile : ascorbate solution= 1 : 1 v/v) for 30 min before the injection and for 4 hr after the injection, at intervals of 30 min. The test tubes were placed on ice. After measuring the volume, the bile samples were stored at -80°C until the analysis.

**HPLC analysis of concentrations of reduced folates in bile**
The bile concentrations of H4PteGlu, 5-CH3-H4PteGlu, 5,10-CH2-H4PteGlu and 10-CHO-H4PteGlu were determined by HPLC with an electrochemical detector (ECD) as previously described (19). The analytical column was a phenyl-bonded phase (Nova-pak phenyl, 100 × 8 mm; Waters Assoc., Milford, MA, USA). The mobile phase was a mixture of 20 mM acetate buffer (pH 5.0) containing 0.1 mM EDTA and acetonitrile (95 : 5 v/v), and the applied potential of ECD was +350 mV. Bile samples were diluted with 0.2% sodium ascorbate solution containing 1.8 × 10⁻⁵% paraformaldehyde (1 : 10, v/v), and the mixtures were centrifugated at 5000 × g for 2 min. The supernatant was filtered with a 0.45-μm microfilter (Chromatodisk; Biofield, Osaka), and an aliquot of the filtered supernatant was injected into the column.

**Statistical analyses**
All data are presented as means ± S.D. Intestinal absorption data were statistically analyzed by Student's t-test or Dunnett's multiple comparison procedure. A P value of < 0.05 was considered to indicate a significant difference.

**RESULTS**

**Intestinal absorption**

Figure 1 shows the effects of PteGlu on the systemic absorption of 5-[¹⁴C]CH₃-H₄PteGlu. PteGlu dose-dependently decreased the systemic absorption of 5-[¹⁴C]CH₃-H₄PteGlu and reduced it by a half at 5 times the amount of 5-CH₃-H₄PteGlu. The inhibition of 5-[¹⁴C]CH₃-H₄PteGlu transport by PteGlu was similar to that by unlabeled 5-CH₃-H₄PteGlu at the same dose. Figure 2 shows the effects of PYR, PteGlu and PYR plus PteGlu on the systemic absorption of 5-[¹⁴C]CH₃-H₄PteGlu. PYR affected neither the systemic absorption of 5-[¹⁴C]CH₃-H₄PteGlu nor the inhibition of systemic absorption of 5-[¹⁴C]CH₃-H₄PteGlu by PteGlu, even at 100 times the amount of 5-CH₃-H₄PteGlu. Table 1 shows the inhibitions of systemic absorption of [³H]PteGlu by the addition of H₄PteGlu, 5-CH₃-H₄PteGlu, 5,10-CH₂-H₄PteGlu, 10-CHO-H₄PteGlu or unlabeled PteGlu. No significant differences were observed in the percent inhibition among the added folates. These results show that PteGlu and
reduced folates secreted in the bile were absorbed through the same intestinal transport system, and the affinity of PteGlu for the transport system is similar to those of the reduced folates.

**Bile secretion of reduced folates**

5-CH₃-H₄PteGlu, 5,10-CH₂-H₄PteGlu, H₄PteGlu and 10-CHO-H₄PteGlu were found as the reduced folates in rat bile after intraduodenal injection of PYR and/or PteGlu. As shown in Fig. 3, the secretion of total reduced folates increased drastically after the injection of PteGlu alone, but no drastic increase was found after the injection of PteGlu with PYR. Each reduced folate also showed a similar secretion profile. There were no significant differences in secreted bile volumes among the treatments.

**DISCUSSION**

After oral administration, PteGlu absorbed from the intestine is rapidly converted to reduced folates by DHFR in the liver, and the plasma 5-CH₃-H₄PteGlu concentration increases. On the other hand, PYR inhibits the DHFR in the liver and reduces the plasma 5-CH₃-H₄PteGlu concentration. These facts suggest that PteGlu may alleviate the decrease of plasma 5-CH₃-H₄PteGlu induced by PYR. In the previous study, however, oral coadministration of PteGlu markedly potentiated the decrease of plasma 5-CH₃-H₄PteGlu concentration induced by PYR in rats (11, 12). This fact suggests that orally administered PteGlu, under the condition of inhibited DHFR activity in tissue, can disturb the folate homeostasis in the body.

Enterohepatic circulation of folates is important for folate homeostasis in rats, because the interruption of enterohepatic circulation, such as bile drainage, rapidly decreased plasma 5-CH₃-H₄PteGlu concentration in rats (13, 15). Shin et al. have demonstrated that the intestinal reabsorption of reduced folates in bile, which contain nonmethylated tetrahydrofolates as well as 5-CH₃-H₄PteGlu, plays an important role in the regulation of plasma 5-CH₃-H₄PteGlu concentration (15). In the present study, we demonstrated that PteGlu and the reduced folates found in rat bile (H₄PteGlu, 5-CH₃-H₄PteGlu, 5,10-CH₂-H₄PteGlu and 10-CHO-H₄PteGlu) were absorbed through the same intestinal transport system. Further-

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**Table 1. Inhibition of systemic absorption of [³H]PteGlu from rat jejunum loops by bile reduced folates**

<table>
<thead>
<tr>
<th>Folate (20 µM)</th>
<th>Percent inhibition* (%)</th>
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<tbody>
<tr>
<td>H₄PteGlu</td>
<td>79.8 ± 12.2</td>
</tr>
<tr>
<td>5-CH₃-H₄PteGlu</td>
<td>80.0 ± 10.6</td>
</tr>
<tr>
<td>5,10-CH₂-H₄PteGlu</td>
<td>65.1 ± 34.7</td>
</tr>
<tr>
<td>10-CHO-H₄PteGlu</td>
<td>59.5 ± 23.6</td>
</tr>
<tr>
<td>Unlabeled PteGlu</td>
<td>73.9 ± 7.2</td>
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</tbody>
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*Percent inhibition = (1 - (systemic absorption of [³H]PteGlu in the presence of each folate / systemic absorption of [³H]PteGlu)) × 100.
more, the affinity of PteGlu for the intestinal transport system was similar to those of these reduced folates. These data show that PteGlu competes with the bile reduced folates for the intestinal transport system, which means orally administered PteGlu inhibits the intestinal reabsorption of bile reduced folates, leading to the interruption of the enterohepatic circulation of folates (Fig. 4).

In our previous study, the oral dose of PteGlu was 50 mg/kg, which potentiated the decrease of plasma 5-CH₃-H₄PteGlu concentration induced by PYR in rats (11, 12). This dose was about 300 times as much as the total reduced folates secreted in bile for a day (15). In the present study, we demonstrated that the absorption of 5-CH₃-H₄PteGlu was reduced by a half by the addition of PteGlu at 5 times the amount of 5-CH₃-H₄PteGlu. Thus, we considered that the dose of PteGlu administered in the previous study (50 mg/kg) was high enough to inhibit completely the intestinal reabsorption of bile reduced folates.

Several authors have also reported that the intestinal transports of some folate derivatives were inhibited by other folate derivatives, and there is a shared carrier-mediated transport system of folate (17, 20-22). Another report states that the intestinal folate transport system exists on the brush border membrane (BBM) of epithelial cells of the intestine, and the affinity of PteGlu for BBM is stronger than those of other folate derivatives (23).

The inhibition of 5-CH₃-H₄PteGlu transport by PYR

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**Fig. 3.** Bile secretion of total reduced folates after intraduodenal injection of PYR at 1.6 mg/kg (○), PteGlu at 50 mg/kg (●), and PYR (1.6 mg/kg) plus PteGlu (50 mg/kg) (□) to rats. The total reduced folates are the sum of H₄PteGlu, 5-CH₃-H₄PteGlu, 5,10-CH₂-H₄PteGlu and 10-CHO-H₄PteGlu. The data are presented as the percent of the folate secretion rate for 30 min before the injection (basal secretion rate). Each point represents a mean±S.D. (n=4–5). △: Control.

**Fig. 4.** Effects of PteGlu and PYR on enterohepatic circulation of folate. (1): The interruption of intestinal reabsorption of endogenous bile folates by PteGlu. (2): The inhibition of conversion of absorbed PteGlu to reduced folates in the liver by PYR. R-H₄PteGlu: 5-CH₃-H₄PteGlu, 5,10-CH₂-H₄PteGlu and 10-CHO-H₄PteGlu; EHC: enterohepatic circulation.
was not observed in this study, although Zimmerman et al. reported that PYR inhibits folate transport (24). In their study, however, the intestinal folate transport was inhibited by a dose as much as 20 times our dose. This suggests the inhibition of folate transport by PYR is negligible compared with that by PteGlu. Accordingly, we didn’t consider that PYR was important for the inhibition of intestinal reabsorption of bile reduced folates.

To confirm whether the absorbed PteGlu is converted into reduced folates in the body in the presence of PYR, we also examined bile secretion of reduced folates after administration of PteGlu with PYR, because the DHFR activity of the liver is the highest among tissues (25), and the bile folates reflect the folate metabolism in the liver (26). The bile secretion of total reduced folates markedly increased after administration of PteGlu alone, which shows the conversion of absorbed PteGlu to reduced folates in the liver. On the other hand, the drastic increase of bile secretion of total reduced folates was not found after administration of PteGlu with PYR. These results suggest that absorbed PteGlu was not converted to reduced folates in the liver in the presence of PYR because of the inhibition of DHFR activity by PYR (Fig. 4).

Strum (27) has reported the biliary secretion of 5-CH₃-H₄PteGlu using the isolated perfused rat liver. They demonstrated that the biliary secretion of 5-CH₃-H₄PteGlu occurred by an energy-dependent, carrier-mediated process, and it was inhibited by PteGlu and 10-CHO-H₄PteGlu. These characteristics are similar to those of the common folate transport system in the intestine (16, 17). In view of the finding that PYR did not inhibit the intestinal folate transport, we consider that PYR would also have no effect on the biliary secretion of reduced folates including 5-CH₃-H₄PteGlu. This hypothesis is also supported by our result that the bile secretion of total reduced folates did not decrease by administration of PYR alone.

The enterohepatic circulation of folate consists of the secretion of reduced folates into the bile and those taken up by intestinal reabsorption. Since co-administration of PteGlu with PYR did not decrease the bile secretion of total reduced folates, we consider that the co-administration of PteGlu interrupts the enterohepatic circulation of folate by inhibiting intestinal reabsorption, but not by inhibition of the bile secretion of reduced folates.

In conclusion, as the mechanism by which the decrease of plasma 5-CH₃-H₄PteGlu concentration is potentiated by oral co-administration of PteGlu to rats that received PYR, a combination of the following two factors is considered: one is the interruption of enterohepatic circulation of endogenous bile folates by PteGlu, and the other is the inhibition of conversion of absorbed PteGlu to reduced folates in the liver due to the DHFR inhibition by PYR.

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