Regular Article

**Improvement of Intestinal Absorption of P-glycoprotein Substrate by D-tartaric Acid**

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**Summary:** The purpose of the present experiment was to examine the effects of d-tartaric acid (TA) on intestinal drug absorption under both *in situ* and *in vitro* experimental conditions. In the *in vitro* diffusion chamber experiments, TA (10 mM) added to the mucosal side of rat colon significantly decreased rhodamine123 (Rho 123) transport from the serosal to mucosal side. Since TA has been shown to change the integrity of the epithelial tight junctions in rat colon at low pH conditions, resulting in improved paracellular drug transport, the effect of TA on membrane resistance was examined at pH 7.4 in the present study. It was found that membrane resistance, an indicator of paracellular integrity, did not change at pH 7.4. In the *in situ* loop method, TA (20 mM) increased the absorption of Rho123 in both ileum and colon but not in jejunum. TA (20 mM) also increased the absorption of daunorubicin in the ileum, but TA (20 mM) did not change the expression level of P-glycoprotein (P-gp). TA (20 mM) significantly inhibited excretion of i.v.-administered Rho123 and daunorubicin into the ileal lumen. In conclusion, for the first time we demonstrated that TA increases the intestinal absorption of P-gp substrates Rho123 and daunorubicin, possibly by modulating the P-gp function without changing the expression level of P-gp in the rat intestine.

**Key words:** d-tartaric acid; P-glycoprotein; jejunum; ileum; colon; absorption improvement

**Introduction**

Tartaric acid is a dicarboxylic acid considered to have no pharmacological effect except for shortening intestinal transit time at larger doses. For this reason, this organic acid has been widely used as a drug excipient in the pharmaceutical industry.

In our previous study, we demonstrated that d-tartaric acid (TA) has an effect of changing the integrity of the epithelial tight junctions in rat colon at low pH. This signifies that use of TA at low pH possibly enhances intestinal absorption of drugs such as hydrophilic macromolecules. In the rat, oral administration of TA together with a low molecular weight polyphenol, catechin, significantly increased the serum concentration of catechin glucuronide, indicating that TA increased the intestinal absorption of catechin. It was also suggested that tea catechins are substrates of P-glycoprotein (P-gp). These results suggest the possibility that TA may increase the intestinal absorption of catechin either by increasing the transepithelial transport resulting from the changed integrity of tight junctions or by inhibiting the eflux of catechin from the cell to lumen resulting from the modulation of P-gp function.

If TA has an effect to increase the intestinal absorption of P-gp substrates other than catechin, this compound may have a great impact on the improvement of drug therapy. To evaluate this possibility, we examined the effects of TA on the intestinal absorption in both the *in vitro* and *in situ* experimental conditions. Our results demonstrated for the first time that TA increases intestinal absorption of P-gp substrates possibly by directly modulating the P-gp function.

**Materials and Methods**

**Materials:** d-tartaric acid, rhodamine 123 (Rho123), daunorubicin, verapamil and FITC-dextran 40000 (FD40) were purchased from Sigma Aldrich Co. Ltd. All other reagents were of analytical grade or higher.
Animals and experimental design: Male Wistar rats (eight weeks old) were purchased from Japan SLC Ltd. (Shizuoka, Japan). All of the animal experiments were performed according to the guidelines of Tokyo University of Pharmacy and Life Science. The animals were fasted for 18–20 hours before starting the experiment. Water was freely given while fasting.

Measurement of transepithelial transport of Rho123 and membrane resistance (Rm) in the in vitro diffusion chamber methods: The methods used in this study followed those previously described by Tomita et al. The transepithelial transport of Rho123 in rat colon was examined using diffusion chamber method. The serosal and mucosal reservoirs were filled with 5 mL Krebs Henseleit bicarbonate buffer (KHBB) solution, which was continuously circulated and oxygenated by mixed gas (95% O₂/5% CO₂) to maintain tissue viability at pH 7.4 and 37°C throughout the experiments. After 20 min, the KHBB solution was exchanged with 5 mL KHBB solution containing Rho123 in the serosal side (donor side) and with 5 mL KHBB solution without Rho123 in the mucosal side (acceptor side). In the same experiments, Rho123 was added to the mucosal side (donor side) but not to the serosal side (acceptor side). To examine the effect of TA, the mucosal reservoir was filled with KHBB solution containing TA (5 or 10 mM). The pH of KHBB solution containing TA was adjusted to 7.4 using sodium hydroxide. The samples were taken from the acceptor side at intervals of 10 min. Permeation clearance (CLp) was obtained as follows.

\[ \text{CLp} = \frac{dQ}{dt} / (A \times C_0) \]

The \( dQ/dt \) is the transport rate (\( \mu \)g/min) and corresponds to the slope of the regression line between the transport amounts and time. \( C_0 \) is the initial concentration in the donor chamber (\( \mu \)g/mL), and \( A \) is the area of the membrane (0.64 cm²).

Simultaneously, the Rm of the rat colon was calculated from the membrane potential difference measured under the load of a small external current (0.1 mA and 0.01 mA) according to Ohm’s law.

Absorption experiments using the in situ loop method: Intestinal absorption was evaluated by the method described in our previous report. A 5-mL KHBB solution containing Rho 123 (26 \( \mu \)M), daunorubicin (26 \( \mu \)M), or FD40 (0.1%) with or without TA or verapamil was administered into the loop. One milliliter was then sampled from the loop at 0 and 30 min after administration. The residue was collected at 60 min.

The concentrations of Rho123 or daunorubicin as a model compound of P-gp substrate, and FD40 as a volume indicator were measured by a fluorescent spectrophotometer (HITACHI FP-6500, Tokyo, Japan). The absorption clearance was calculated by the method described in our previous report.

Western blotting: An in situ ileum loop (18 cm length) was prepared in each rat and KHBB solution with or without TA (20 mM) (pH 7.4) administered into the loop (5.0 mL). At 60 min after administration, the crude membrane fractions were prepared from the ileum using magnesium chloride precipitation. Briefly, the ileal luminal contents were thoroughly washed out with a sufficient amount of ice-cold saline and the ileum was then divided into several parts of the same length. The ileum’s mucosal surface was scraped off with a slide glass. The mucosa collected was homogenized in a buffer containing 0.05 mg/mL phenylmethylsulfonylfluoride, 300 mM mannitol, 12 mM Tris, and 5 mM EGTA (pH 7.1) with a tissue homogenizer. The 10 mM magnesium chloride aqueous solution was added to the homogenate. The homogenate was centrifuged at 3,000 g for 10 min. The supernatant was then centrifuged at 42,000g for 30 min. The pellet was re-suspended in 300 mM mannitol, 20 mM HEPES, 10 mM Tris, and 4mM magnesium chloride (pH 7.4). The protein expression levels of P-gp in crude membrane fraction were evaluated by Western blotting. Western blotting using C219 monoclonal antibody (Alexis) for P-gp was performed as reported previously.

Excretion experiments: Rats were anesthetized with pentobarbital (50 mg/kg i.p. injection) and placed supine under the heater to maintain their body temperature. Cannulation (silicone tubing, Silascon® Kaneka Medix Co.) was made at a jugular vein for administration and sampling. Also, the ileal lumen (7 cm length) was flushed with saline pre-warmed at 37°C, and the proximal end of the lumen was catheterized with an in-flow glass cannula, which was connected to the perfusion system. The distal end of the ileum was also catheterized with an out-flow glass cannula to collect intestinal effluents serially. The single perfusion of KHBB solution into the ileal lumen was started at a rate of 1 mL/min. After a 10 min perfusion, saline containing Rho123 (260 \( \mu \)M) or daunorubicin (520 \( \mu \)M) was administered by i.v. injection as a bolus with a volume of 2.8 mL/kg, followed by a saline injection at the same volume via the cannula inserted at the jugular vein. Blood samples (300 \( \mu \)L) were drawn before and 1.5, 3, 5, 10, 20, 30 and 60 min after administration. The intestinal effluent samples were then collected every 10 min. Excretions of Rho123 and daunorubicin from blood to ileal lumen were expressed as total excreted amounts for 60 min. Whole blood samples were centrifuged for 5 min at 12,000 rpm. After centrifuge, the supernatant of samples (100 \( \mu \)L) were dissolved into purified water in order to make the volume sufficient for assay.

Statistical analysis: All the results were expressed by
mean value ± standard error (Mean ± SE). Statistical significance between two groups was analyzed using Dunnett’s test, and P value less than 0.05 was considered to be significantly different.

**Results**

**Effects of TA on Rho123 transport and membrane resistance (Rm) using in vitro diffusion chamber method:** The effects of TA (10 mM) on permeation clearance (CLp) of Rho123 in the serosal to mucosal direction (S to M) across rat colonic epithelia at pH 7.4 were measured. The results indicated that, when TA was added to the mucosal side, TA (10 mM) significantly decreased the Rho123 excretion (Fig. 1a). However, TA (5 mM) did not show such effects. No marked change was observed in Rm in the presence of TA at pH 7.4 (Fig. 1b).

The effects of TA (10 mM) on CLp of Rho123 in the mucosal to serosal direction (M to S) through rat colonic epithelia at pH 7.4 were also measured. The results indicated that TA (10 mM) significantly increased the Rho123 absorption. However, TA (5 mM) did not show such effects (Fig. 1b). No marked change was observed in Rm of each condition. Data represent mean ± S.E. (n = 3 – 23). N.S.: not significant, *: p < 0.05 compared with control.

**Absorption site dependency of the effects of TA and verapamil on absorption clearance of P-gp substrates using in situ loop method:** We examined the effects of TA (20 mM) on the absorption of rhodamine123 and daunorubicin from the loop of rat jejunum, ileum and colon. TA (20 mM), added to the lumen of the loop, significantly increased the absorption of Rho123 both in ileum and colon but not in jejunum (Fig. 2a). On the other hand, TA (10 mM) did not change the absorption clearance of Rho123 (data not shown). In ileum but not in jejunum and colon, TA (20 mM) significantly increased the absorption of daunorubicin (Fig. 2b).

Effects of verapamil on the absorption of Rho123 in rat jejunum, ileum and colon were examined using the in situ loop method. Verapamil (1 mM), added to the lumen of the loop, significantly increased the absorption of Rho123 and daunorubicin both in jejunum and ileum but not in colon (Fig. 2).

**Effect of TA in mucosal side on the luminal excretion of i.v.-administered P-gp substrates:** Total excreted amounts of Rho123 and daunorubicin intravenously...
administered via the jugular vein to the ileal lumen were measured. When TA (20 mM) or verapamil (1 mM) was applied to the ileal lumen, excretion of Rho123 was significantly inhibited (Fig. 3a). Also, excretion of daunorubicin tended to be decreased by TA (20 mM) or verapamil (1 mM) (Fig. 3b).

Effect of TA on expression of P-gp protein: In the ileum, TA (20 mM) did not change the expression of P-gp protein as measured with Western blotting (Fig. 4).

Discussion

In the first series of experiments, we examined the effects of TA on Rho123 transport across rat colonic epithelium using the in vitro diffusion chamber method. It was found that TA (10 mM), added to the mucosal side at pH 7.4, significantly decreased the Rho123 transport from the serosal to mucosal side (S to M) (Fig. 1a), but increased the transport from the mucosa to serosal side (M to S) (Fig. 1b).

In colon, TA (10 mM) at low pH (pH 3–4) has been reported to change the integrity of tight junctions and to significantly decrease Rm, an indicator of the integrity of tight junctions. To examine the similar TA effect at neutral pH, we measured Rm in the presence of TA (10 mM) at pH 7.4. The results indicated that TA (10 mM) did not change Rm at pH 7.4 (Fig. 1a, b), suggesting that TA changes the integrity of tight junctions at low pH but not at neutral pH. It was suggested that the effect of TA (10 mM) to change the transport of Rho123 is not attributable to the changes in the integrity of tight junction, at least at pH 7.4.

Considering the fact that Rho123 is a P-gp substrate, a possible explanation for the TA-induced decrease in Rho123 excretion is that TA modulates P-gp function. Although TA also increased Rho123 absorption in the diffusion chamber experiments, this effect is attributable to effects neither on tight junction nor P-gp function. Further experiments are necessary to clarify the mechanism of this effect.

To further examine the effects of TA, we used the in situ loop method in the rat intestine. TA (20 mM) significantly increased the absorption of Rho123 both in the ileum and colon (Fig. 2a), supporting the in vitro results.

In this experiment, 20 mM TA was necessary to increase the absorption, whereas in the in vitro experiment 10 mM TA inhibited Rho123 transport from the serosal to mucosal side. The difference in the effective concentrations of TA may possibly be attributable to the different experimental conditions.

To examine whether or not TA increases the absorption of P-gp substrate other than Rho123 (Fig. 2a), we obtained the effect of TA on the transport of daunorubicin (Fig. 2b), because it has been reported that excretion of daunorubicin depends more strongly on P-gp than Rho123.

In colon, TA increased the absorption of Rho123 but not of daunorubicin (Fig. 2a, b). The result that verapamil did not increase the absorption of daunorubicin in the colon (Fig. 2b) indicates that the role of P-gp is small in the colon. Also, it is suggested that the effect of TA on Rho123 absorption is not only attributable to the inhibition of P-gp, but also to the activation of the absorption transporters for Rho123. It can also be considered that an excretion mechanism other than P-gp is inhibited by TA. It has been reported that Rho123 is
transported by a transporter, a family of an organic cation transporter that transports Rh123 from the serosal side into the cell.\textsuperscript{12} Further experiments are necessary to evaluate these possibilities.

We have found that \(\alpha\)-tartaric acid effectively inhibited the Rh123 transport from the serosal to mucosal side in rat colon, although \(\beta\)-tartaric acid was ineffective (unpublished observation). Such a structure-dependent difference in the action of TA supports the suggestion that TA is transported by a specific membrane transporter. This transporter may be the Na\textsuperscript{+}-dependent dicarboxylate transporter which has been found in both the small intestine and kidney.\textsuperscript{13–15} TA did not increase the P-gp substrate absorption in jejunum. Such a regional difference may possibly be due to the different distribution of dicarboxylate transporter in the intestine.

The possibility that the inhibitory effect of TA is due to inhibition of P-gp protein expression was ruled out by the Western blotting method (Fig. 4), indicating that TA directly modulates the P-gp function.

To further confirm this possibility, we administered Rh123 and daunorubicin intravenously and determined the amount of Rh123 and daunorubicin excreted into the ileal lumen (Fig. 3). TA (20 mM) significantly inhibited the excretion of Rh123. Because of the relatively large standard error, the difference was not significant, but TA also inhibited daunorubicin excretion. These results, together with the \textit{in vitro} diffusion chamber experiments, support the suggestion that the effect of TA is to inhibit excretion of P-gp. Also, the results obtained by \textit{ex vivo} experiments in the ileum are consistent with those shown by absorption experiments in the \textit{in situ} ileal loop method.

In conclusion, for the first time we demonstrated that TA increases the intestinal absorption of P-gp substrates, Rh123 and daunorubicin, possibly by modulating the P-gp function without changing the expression of P-gp protein in the rat ileum and colon. Our results suggest the usefulness of TA as a pharmaceutical excipient to increase the absorption of P-gp substrates in the intestine.

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References


