Membrane Transport Mechanisms of Mizoribine in the Rat Intestine and Human Epithelial LS180 Cells

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The aim of the present study was to characterize membrane transport mechanisms of mizoribine in the intestinal epithelial cells. We evaluated the contribution of Na⁺-dependent and -independent membrane transporters to mizoribine absorption in the rat intestine using an *in situ* closed loop method. In addition, we evaluated the effects of structurally related compounds, extracellular Na⁺ concentrations, and an inhibitor of Na⁺-independent equilibrative nucleoside transporter, nitrobenzylmercaptapurine ribonucleoside (NBMPR), on the uptake of mizoribine in human intestinal epithelial LS180 cells. In the presence and also absence of Na⁺ in rat intestinal loops, more than 60% of the administered dose (50 µg at the concentration of 100 µg/ml=386 µM) of mizoribine was absorbed in 40 min. In the LS180 cells, ribavirin and inosine reduced the uptake of 400 µM mizoribine with the increasing concentrations (from 5 to 50 mM) of the inhibitors. The cellular uptake of mizoribine in the absence of extracellular Na⁺ decreased to 72.7% of the uptake in the presence of extracellular Na⁺, whereas 100 µM NBMPR decreased the uptake of mizoribine markedly to 34.7% of that without NBMPR. These findings suggest that Na⁺-independent nucleoside transporters are largely responsible for absorption of mizoribine in the intestine.

Key words mizoribine; intestinal absorption; nucleoside transporter; LS180 cell

Mizoribine is an orally available immunosuppressive agent, which has been on the market since 1984 in Japan for the prevention of rejection in renal transplantation.¹⁾ In contrast to other immunosuppressive agents (e.g., azathioprine), mizoribine has been shown in animal experiments to lack oncogenicity and exhibited a clinically low incidence of severe adverse drug reactions (such as myelosuppression and hepatotoxicity), making it useful in long-term immunosuppression therapy.²⁾ Orally administered mizoribine is absorbed rapidly despite its hydrophilic property; therefore, specific membrane transporters can be responsible for the intestinal absorption of the drug.³⁾

Concentrative nucleoside transporter (CNT) 1 and CNT2 are Na⁺-dependent, and the movement of nucleoside regardless of its concentration gradient is coupled to that of the sodium ion.^{4,5)} CNT1 and CNT2 are localized on the apical side of enterocytes, and these transporters differ in their substrate specificities: CNT1 transports pyrimidine-nucleoside (e.g., thymidine) preferentially, whereas CNT2 transports purine-nucleoside (e.g., inosine) preferentially.^{4–6)} Okada *et al.* evaluated the intestinal absorption of mizoribine using the rat intestinal closed loop.⁷⁾ Inosine inhibited the absorption of mizoribine in the rat intestinal loop; therefore, they thought that the nucleoside transporter was involved in the intestinal absorption of mizoribine in rats, and that CNT2 was a plausible candidate responsible for apical uptake of the drug in intestinal epithelial cells.⁷⁾ On the other hand, Mori *et al.* investigated the effect of thymidine on the intestinal absorption of mizoribine in rats.⁸⁾ Thymidine, as well as inosine and ribavirin, decreased the intestinal absorption of mizoribine; therefore, they thought that intestinal absorption of mizoribine was mediated by CNT1 as well as CNT2.⁸⁾

Equilibrative nucleoside transporter (ENT) 1 and ENT2 are Na⁺-independent, and mediate nucleoside transport in both directions depending on the nucleoside concentration gradient.^{5,9)} ENT1 and ENT2 transport both pyrimidine- and purine-nucleoside, and they are inhibited by 6-[(4-nitro-

benzyl)-thio]-9-β-D-ribofuranosyl-purine (NBMPR).^{5,9)} Recently, Govindarajan *et al.* investigated the expression of nucleoside transporters in the human intestine, liver, kidney, and placenta.⁶⁾ In the human intestine, immunohistochemical analysis showed the presence of ENT1 and ENT2 protein staining in the crypt regions. The intensity seemed higher than the respective immunoreactivity observed in the enterocytes in the same histological section on the same slide. In addition, a certain degree of ENT1 and ENT2 staining was always observed in all the villi cells, with almost equal intensity at the apical and basolateral side of the enterocytes.⁶⁾ On the other hand, it is still unclear whether ENTs are involved in the apical membrane transport of mizoribine in the intestinal epithelial cells.

In the present study, we evaluated the contribution of Na⁺-dependent and -independent nucleoside transport to rat intestinal absorption of mizoribine using *in situ* closed loop methods. In addition, we performed pharmacokinetic analysis of cellular uptake of mizoribine in human intestinal epithelial LS180 cells.^{10–13)} We further evaluated the effects of structurally related compounds, extracellular Na⁺ concentrations, and NBMPR on the cellular uptake of mizoribine in LS180 cells.

MATERIALS AND METHODS

Materials Mizoribine and [¹⁴C]mizoribine (12.9 mCi/mmol) were kindly donated by Asahi Kasei Pharma (Tokyo, Japan). [³H]Mannitol was purchased from Moravec Biochemicals Inc. (Brea, CA, U.S.A.). Ribavirin was obtained from MP Biochemicals, LLC. (Illkirch, France). Inosine and NBMPR were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available.

Animals Male Wistar rats (190–230 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). Rats were housed in a temperature- and humidity-controlled room with free ac-

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cess to water and standard rat chow. All experiments were performed in accordance with the Guidelines for Animal Experiments of University of Toyama.

LS180 Cells LS180 cells at passage 38 were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). LS180 cells were seeded at a density of 5×10^5 cells/cm² on a 3.8 cm² plastic dish using a Falcon® multiwell™ plate (BD Bioscience, Bedford, MA, U.S.A.). They were maintained with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biowest, Nuallé, France) in atmosphere of 5% CO₂–95% air at 37 °C for 7 d.^{12,13} All experiments were carried out between passages 55–66.

Intestinal Absorption of Mizoribine in Rats Rats were fasted for 20 h with free access to water before the subsequent experiment. The rat was anesthetized with 50 mg/kg sodium pentobarbital, and body temperature was maintained with appropriate heating lamps. The abdominal cavity was opened, and silicon tubes were inserted at the end of the pylorus and the middle of the small intestine. The lumen of the gut was washed with an aqueous buffer solution (30 ml) using a pump (Masterflex® digital console L/S pump® drive; Core-Parmer Instrument Co., Barrington, IL, U.S.A.). The aqueous buffer solution consisted of 101.0 mM NaCl, 20.1 mM Na₂HPO₄·12H₂O, and 47.0 mM KH₂PO₄ (pH 6.4). In order to evaluate intestinal absorption of mizoribine under Na⁺-free condition, NaCl and Na₂HPO₄·12H₂O in the aqueous buffer solution were replaced with choline chloride and K₂HPO₄, respectively. After the lumen of the gut was washed, a 15-cm-long intestinal loop under the opening of bile duct was prepared by ligation with a silk suture. The intestinal loop was put back into the body, and then the rats were kept still for 5 min. A volume (0.5 ml) of aqueous buffer solution containing mizoribine (100 µg/ml = 386 µM) and phenol red (1 mg/ml) was injected into the intestinal loop. After 40 min, the content of an intestinal loop was collected with the fresh buffer solution. The collected solution was filtered using an Ultrafree®-MC centrifugal filter unit (0.45 µm, Millipore Co., Bedford, MA, U.S.A.) by centrifugation at 12000 *g* for 3 min, and stored at –30 °C until assay.

The amount of mizoribine in the sample solution was measured with a high-performance liquid chromatography (HPLC) method. A 50-µl aliquot of the sample was injected into a HPLC system. The column was Develosil™ RPAqueous (4.6 mm i.d.×250 mm, 5 µm, Nomura Chemical Co., Ltd., Japan). Mizoribine was eluted using a gradient of the mobile phase A (50 mM ammonium acetate adjusted to pH 5.6 with acetic acid), and the mobile phase B containing 80% 50 mM ammonium acetate (pH 5.6) and 20% acetonitrile (v/v). The gradient elution of mobile phase B was 0% in 0–8 min, 5% for 8–12 min, 100% for 12–17 min, and 0% for 17–35 min. The flow rate was 0.9 ml/min, and the column temperature was 40 °C. The peaks were monitored at 280 nm.

Uptake of Mizoribine in LS180 Cells The cellular uptake of [¹⁴C]mizoribine was examined using LS180 cells grown on plastic dishes of multiwell plates. The composition of the incubation medium was as follows: 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES), and 400 µM unlabeled mizoribine (pH 7.4). The cells were first pre-incubated for

60 min at 37 °C with 2 ml of incubation medium. The incubation medium was replaced with 500 µl of fresh incubation medium 10 min before addition of [¹⁴C]mizoribine (0.2 µCi/well). After the cells were incubated with [¹⁴C]mizoribine for 5–60 min, they were immediately washed with ice-cold phosphate buffer, and collected. To evaluate efflux of mizoribine from the cells, the incubation medium was replaced with [¹⁴C]mizoribine-free incubation medium 30 min after addition of [¹⁴C]mizoribine, and then the cells were incubated for another 15–30 min. The amount of [¹⁴C]mizoribine in the cells were determined using a liquid scintillation counter. To estimate extracellular trapping of mizoribine, the amount of [³H]mannitol in the cells was also determined.¹⁴

The cellular uptake of mizoribine analyzed in a model-dependent manner using NONMEM software running on a mainframe UNIX machine at the Kyoto University Data Processing Center, as described previously.^{14,15} The following mass balance equations were prepared for the pharmacokinetic analysis:

$$\frac{dX_C}{dt} = \frac{CL_{M \rightarrow C}}{V_M} \cdot X_M - \frac{CL_{C \rightarrow M}}{V_C} \cdot X_C \quad (1)$$

$$\frac{dX_C}{dt} = \frac{CL_{M \rightarrow C}}{V_M} \cdot X_M - \frac{CL_{C \rightarrow M}}{V_C} \cdot X_C \quad (2)$$

where X_M and X_C are the amount of mizoribine in the incubation medium and the cells determined at time t , respectively. V_M indicates the volume of the incubation medium (500 µl). V_C indicates the cell volume (2.62 µl/cm²), measured with sulfanilamide as described previously.^{11,12} The influx and efflux clearance of mizoribine was designated as $CL_{M \rightarrow C}$ and $CL_{C \rightarrow M}$, respectively.

In order to evaluate cellular uptake of [¹⁴C]mizoribine under the Na⁺-free condition, NaCl in the incubation medium was replaced with choline chloride. In addition, to evaluate the effect of structurally related compounds and NBMPR on cellular uptake of [¹⁴C]mizoribine, the incubation medium was replaced with 500 µl of fresh incubation medium containing 5–50 mM structurally related compounds or 100 µM NBMPR 5–10 min before addition of [¹⁴C]mizoribine. The cells were incubated with [¹⁴C]mizoribine for 5–45 min, and the amounts of [¹⁴C]mizoribine in the samples were determined as described above.

Real-Time Polymerase Chain Reaction Assay of mRNA of Transporters in LS180 Cells Total RNA was isolated from LS180 cells using an RNeasy® Mini Kit, QIAshredder, and RNase-Free DNase Set (QIAGEN, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Reverse transcription of extracted total RNA was performed using an Omniscript® RT Kit (QIAGEN) and random hexamer (QIAGEN) according to the manufacturer's instructions. PCR was carried out on the MX3000P® QPCR System (Stratagene, La Jolla, CA, U.S.A.) using SYBR® Premix Ex Taq™ (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Primer sequences for CNT1, CNT2, ENT1, ENT2, multidrug resistance protein (MDR) 1, multidrug resistance-associated protein (MRP) 1, MRP2, MRP4, MRP5, breast cancer resistance protein (BCRP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported elsewhere.^{12,16,18–20} Cycling conditions were 1 cycle for 30 s at 95 °C, followed

by 45 cycles of 5-s denaturation at 95 °C, 20-s annealing at 60 °C, and 15-s extension at 72 °C. The mRNA level of nucleoside and efflux transporters was normalized according to GAPDH mRNA level, and the ratio was presented using a common logarithm.

Data Analysis Values are expressed as the mean $\pm$ S.E. Multiple comparisons were performed using Scheffé's test following one-way ANOVA provided that the variances of groups were similar. If this was not the case, a Scheffé-type test was applied following Kruskal–Wallis analysis. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Intestinal Absorption of Mizoribine in Rats We first evaluated the intestinal absorption of mizoribine in rats using an *in situ* closed loop method. The intestinal absorption of mizoribine was estimated by calculating percentage of the dose (50 μ g at the concentration of 100 μ g/ml = 386 μ M) of mizoribine disappeared from rat intestinal loops for 40 min. Mizoribine was absorbed much faster than phenol red (a representative hydrophilic compound), and the absorption rate of mizoribine in the presence of 141 mM Na⁺ was 64.2% of the dose (Fig. 1). In addition, absorption of mizoribine in the absence of Na⁺ (63.7% of the dose) was comparable to that in the presence of 141 mM Na⁺ (Fig. 1). These findings indicated that Na⁺-independent transporters are mainly responsible for the intestinal absorption of mizoribine in rats.

Okada *et al.* evaluated the intestinal absorption of mizoribine which was dissolved in Na⁺-free distilled water (30 μ g at the concentration of 100 μ g/ml).⁷⁾ They reported that the 30-min absorption of mizoribine in rat intestinal loop (about 6–9-cm loop from the end of the pylorus) was 52.9% of the dose, and that inosine significantly decreased the absorption rate of the drug.⁷⁾ On the other hand, Mori *et al.* investigated the absorption of mizoribine dissolved in saline (1 mg/kg at the concentration of 500 μ g/ml).⁸⁾ They reported that the 60-min absorption of mizoribine in rat intestinal loop (10-cm loop from 5 cm below the bile duct opening) was about 45% of the dose, and that inosine, thymidine, and ribavirin significantly decreased the absorption rate of the drug.⁸⁾ The results in the present study seemed to be in line with those previous findings of Okada *et al.* and Mori *et al.*^{7,8)}

Pharmacokinetic Analysis of Mizoribine Uptake in LS180 Cells To characterize the membrane transport of mizoribine in the intestine, we next evaluated the uptake of mizoribine in human intestinal epithelial LS180 cells grown on plastic dishes. The cellular uptake of mizoribine in the presence of extracellular 125 mM Na⁺ was time-dependent; that is, the uptake of [¹⁴C]mizoribine increased rapidly, and reached approximately 0.56% of dose at 30 min (Fig. 2). In the case that the extracellular medium containing [¹⁴C]mizoribine was replaced with [¹⁴C]mizoribine-free incubation medium 30 min after the addition of radio-labeled drug, intracellular [¹⁴C]mizoribine moved rapidly into extracellular medium (Fig. 2). We performed the pharmacokinetic analysis of the data on cellular mizoribine accumulation using a 2-compartment (cell and medium) model. The influx and efflux clearance of mizoribine were estimated to be 118 and 424 nl/min/cm², respectively. The finding indicated that influx of mizoribine was not greater than efflux of the drug in

Fig. 1. Intestinal Absorption of Mizoribine for 40 min in Rats

Mizoribine solution (100 μ g/ml) was injected into rat intestinal closed loop. Open and hatched columns represent 40-min absorption of phenol red and mizoribine, respectively. Each column represents the mean $\pm$ S.E. for 7 rats.

Fig. 2. Time Course of 400 μ M Mizoribine (Containing 31.0 μ M [¹⁴C]Mizoribine) Uptake in LS180 Cells in the Presence of Na⁺

Closed circles represent intracellular mizoribine after the incubation medium was replaced with [¹⁴C]mizoribine-free incubation medium at 30 min after the addition of the radio-labeled drug. Solid line represents a simulation curve obtained by pharmacokinetic analysis of mizoribine accumulation. Influx and efflux clearance was estimated to be 118 and 424 nl/min/cm², respectively.

LS180 cells, even in the presence of Na⁺.

Effect of Structurally Related Compounds on Uptake of Mizoribine in LS180 Cells We evaluated the inhibitory effect of structurally related compounds on the cellular uptake of mizoribine in the presence of extracellular 125 mM Na⁺. Figure 3 shows the uptake of 400 μ M mizoribine for 45 min in the presence of 5–50 mM structurally related compounds. Unlabeled mizoribine at concentrations of 5 and 50 mM did not significantly inhibit the apparent uptake of [¹⁴C]mizoribine (Fig. 3). On the other hand, ribavirin inhibited the uptake of [¹⁴C]mizoribine with increasing concentrations (from 5 to 50 mM), although the inhibitory effects of 5 mM ribavirin were not statistically significant (Fig. 3). The inhibitory effects of 5 and 50 mM inosine on the uptake of [¹⁴C]mizoribine were stronger than that of 50 mM ribavirin. These findings indicated that specific nucleoside transporters are involved in the uptake of mizoribine in LS180 cells.

Effect of Na⁺ and NBMPR on Uptake of Mizoribine in LS180 Cells We further investigated the initial (5-min) uptake of mizoribine to characterize the influx transporter of the drug in LS180 cells. Figure 4 shows the uptake of 400 μ M mizoribine for 5 min in the presence and absence of extracellular Na⁺. The removal of extracellular Na⁺ significantly de-

Fig. 3. Effect of Structurally Related Compounds on Uptake of 400 μ M Mizoribine (Containing 31.0 μ M [14 C]Mizoribine) in LS180 Cells in the Presence of Na^+

The cells were incubated with [14 C]mizoribine for 45 min in the presence or absence of structurally related compounds. Each column represents the mean $\pm$ S.E. for 5–11 measurements. * $p < 0.05$; significantly different from control.

Fig. 4. Effect of Na^+ and NBMPR on Uptake of 400 μ M Mizoribine (Containing 31.0 μ M [14 C]Mizoribine) in LS180 Cells

The cells were incubated with [14 C]mizoribine for 5 min in the presence or absence of Na^+ and NBMPR. Each column represents the mean $\pm$ S.E. for 8 measurements. * $p < 0.05$; significantly different from Na^+ (+), NBMPR (-).

creased the uptake of mizoribine; however, the decrease in the uptake of mizoribine was only 27.3%. Figure 4 also shows the effect of 100 μ M NBMPR, a specific inhibitor of ENTs, on the 5-min uptake of mizoribine in LS180 cells. The uptake of mizoribine in the presence of NBMPR was decreased to 34.7% of that without NBMPR (Fig. 4). These findings suggested that Na^+ -independent nucleoside transporters (probably ENTs) are largely responsible for the uptake of mizoribine in LS180 cells.

Expression of mRNA of Transporters in LS180 Cells

We finally evaluated the expression of the mRNA of membrane transporters in LS180 cells. Figure 5 shows the mRNA expression level of nucleoside transporters (CNT1, CNT2, ENT1, and ENT2) in LS180 cells. The expression level of CNT1 mRNA was much lower than that of CNT2 mRNA, whereas the expression level of ENT1 and ENT2 mRNA was comparable to that of CNT2 mRNA (Fig. 5). Figure 5 also

Fig. 5. Expression of the mRNA of Various Transporters in LS180 Cells
Each column represents the mean $\pm$ S.E. for 4 measurements.

shows the mRNA expression level of efflux transporters (MDR1, MRP1, MRP2, MRP4, MRP5, and BCRP) in LS180 cells. The expression level of MDR1, MRP1, MRP4, and MRP5 mRNA was much higher than that of MRP2 and BCRP mRNA (Fig. 5).

The preferred substrates for MDR1 are lipophilic compounds, such as cyclosporine and tacrolimus, whereas that for MRP1 are organic anions, *e.g.*, drugs conjugated to glutathione (GSH), glucuronate, or sulfate.^{21,22} On the other hand, MRP4 and MRP5 transport nucleobase, nucleoside, and nucleotide analogs including cyclic nucleotides (cAMP and cGMP).^{22,23} Reid *et al.* investigated the transport activity of MRP4 and MRP5 for antiviral and anticancer drugs, using MRP4- or MRP5-transfected human embryonic kidney (HEK) 293 cells.²³ They reported that MRP4 transports 9-(2-phosphonomethoxyethyl)adenine (PMEA), 9-(2-phosphonomethoxyethyl)guanine (PMEG), thioguanine, and cladribine, but that MRP5 transports PMEA and thioguanine, but not PMEG and cladribine.²³ It is still unclear whether MRP4 and/or MRP5 is involved in the membrane transport of mizoribine in LS180 cells. Further studies will be needed to clarify which influx/efflux transporters are responsible for brush-border membrane transport of mizoribine in the human intestine.

CONCLUSION

In the present study, we evaluated the contribution of Na^+ -dependent and -independent nucleoside transporters to mizoribine absorption in the rat intestine and human intestinal epithelial LS180 cells. The present findings indicated that Na^+ -independent nucleoside transporters (probably ENTs) are largely responsible for the intestinal absorption of mizoribine in the rat intestine and LS180 cells. To our knowledge, this is the first report of involvement of Na^+ -independent transporters in the apical membrane transport of mizoribine in the intestine.

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