Effect of Salt Intake on Bioavailability of Mizoribine in Healthy Japanese Males

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Summary: Bioavailability of mizoribine in subjects with the concentrative nucleoside transporter 1 (CNT1, SLC28A1) 565-A/A allele is significantly lower than that in subjects with the SLC28A1 565-G/G allele. The aims of the present study were to investigate the cellular uptake of mizoribine in CNT1- and CNT2-expressing Madin-Darby canine kidney type II (MDCKII) cells, and to evaluate the effect of salt intake on bioavailability of mizoribine in healthy Japanese volunteers with SLC28A1 565-A/A and -G/A alleles. Eight healthy males participated in the present study, and took 150 mg mizoribine concomitantly with/without 300 mg salt. Bioavailability of mizoribine was estimated by total cumulative urinary excretion of the drug. Mizoribine was taken up Na+-dependently into not only CNT1-expressing but also CNT2-expressing MDCKII cells, indicating that mizoribine is a substrate for both CNT1 and CNT2. Mean bioavailability of mizoribine taken with salt (83.8%) was significantly higher than that taken without salt (73.0%). These findings suggest that the salt intake is expected to improve the bioavailability of mizoribine in patients with insufficient intestinal absorption.

Keywords: mizoribine; bioavailability; salt intake; CNT1; CNT2; nucleoside transporters

Introduction

Mizoribine, an orally available immunosuppressive agent, has been approved in Japan since 1984 for the prevention of rejection of kidney transplants.1,2 Mizoribine has subsequently been approved for the treatment of lupus nephritis, rheumatoid arthritis, and nephrotic syndrome.3,4 Mizoribine is a highly hydrophilic and weakly acidic compound, and is not metabolized in the body. Mizoribine binds poorly to plasma proteins (2.3%), and is excreted predominantly in the urine (81.8% at 6 h after oral administration of 100 mg mizoribine in healthy subjects);5 therefore, renal function has been considered to be one of the main causes of the pharmacokinetic variability of mizoribine. On the other hand, Ihara et al. evaluated the pharmacokinetics of mizoribine in 14 kidney transplant recipients.5 The cumulative urinary excretion of mizoribine (bioavailability) in the patients was variable, and ranged from 12% to 81% of the dose (mean: 41%).6 The findings suggested that the extent of intestinal absorption is another major cause of the pharmacokinetic variability of mizoribine.

Naito et al. evaluated the effect of polymorphisms of concentrative nucleoside transporter 1 (CNT1/SLC28A1) on bioavailability of mizoribine in Japanese kidney transplant recipients, and reported that bioavailability of mizoribine in patients with SLC28A1 565-G/G and -A/A alleles was markedly lower than that in patients with the SLC28A1 565-G/G allele.7 In addition, we previously evaluated the effect of a genetic polymorphism of SLC28A1 on bioavailability (intestinal absorption) of mizoribine in healthy Japanese males.8 Bioavailability of mizoribine in subjects with the SLC28A1 565-A/A allele was significantly lower than that in subjects with the SLC28A1 565-G/G allele.9 These results indicated that CNT1 in intestinal epithelial cells is at least partly involved in the absorption of mizoribine.8,9

Not only CNT1 but also CNT2 is Na+-dependent, and the movement of nucleoside regardless of its concentration gradient is coupled to that of the sodium ion.9,10 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., cytidine), whereas CNT2 transports purine-
nucleoside (e.g., inosine) preferentially.8-11 An antiviral drug, ribavirin, is the well-known substrate for CNT2,12-14 and the chemical structure of ribavirin is very close to that of mizoribine. Therefore, mizoribine may be a substrate for not only CNT1 but also CNT2. If so, the bioavailability (intestinal absorption) of mizoribine could be improved by salt intake even in patients with the SLC28A1 565-A/A allele.

The aim of the present study was to evaluate the cellular uptake of mizoribine in CNT1- and CNT2-expressing Madin-Darby canine kidney type II (MDCKII) cells. We also evaluated the effect of salt intake on the bioavailability of mizoribine in healthy Japanese volunteers with SLC28A1 565-A/A and -G/A alleles.

Methods

Materials: Mizoribine and [14C]mizoribine (12.9 mCi/mmol) were kindly donated by Asahi Kasei Pharma (Tokyo, Japan). [3H]Cytidine (24.9 Ci/mmol) and [3H]inosine (34 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA). [3H]Mannitol (20 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest purity available.

Cell culture: MDCKII cells expressing CNT1 and CNT2, which were previously characterized, were used.11 The cells were seeded at a density of 6.0 × 10⁴ cells/well on a 24-well plate (USA Scientific, Ocala, FL, USA), and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gemini Bio-products, West Sacramento, CA, USA) and 100 µg/mL G418 (Gemini Bio-products) an atmosphere of 5% CO2–95% air at 37°C until they reached 90–100% confluence.

Cellular uptake of cytidine, inosine, and mizoribine in CNT1- and CNT2-expressing MDCKII cells: The cellular uptake of [3H]cytidine, [3H]inosine, and [14C]mizoribine was examined using CNT1- and CNT2-expressing MDCKII cells grown on plastic dishes of 24-well plates. The composition of the incubation buffer was as follows: 130 mM N-methyl-D-glucamine (NMDG), 3 mM K2HPO4, 5 mM d-glucose, 2 mM CaCl2, 1.2 mM MgCl2, 6H2O, and 20 mM Tris-HCl (pH 7.4). Cytidine, inosine, and mizoribine are substrates for equilibrative nucleoside transporters (ENTs); therefore, 10 µM NBMPR, an ENT-specific inhibitor, was added to the incubation buffer to evaluate the CNT-specific uptake of these compounds.15-18 In order to evaluate the cellular uptake of cytidine, inosine, and mizoribine in the presence of extracellular Na+, NMDG in the incubation buffer was replaced with NaCl. The cells were washed 3 times with incubation buffer, and then pre-incubated for 20 min at 37°C with 500 µL incubation buffer, which was replaced with 500 µL fresh incubation buffer containing [3H]cytidine (1 µCi/well = 0.080 µM), [3H]inosine (1 µCi/well = 0.059 µM), or [14C]mizoribine (0.323 µCi/well = 50 µM). After the cells had been incubated with incubation medium containing the radio-labeled compound for 30–120 min, they were immediately washed 3 times with ice-cold incubation buffer. The cells were solubilized by 1.0 mL of 2% sodium dodecyl sulfate (SDS). To estimate non-specific binding of mizoribine to the cells, 50 µM mannitol containing 50 nM [3H]mannitol (1 µCi/well) was added to the incubation buffer.15,16 We also evaluated the effect of non-labeled mizoribine on the cellular uptake of [3H]cytidine and [3H]inosine. That is, the incubation buffer was replaced with 500 µL fresh incubation buffer containing 5 µM-50 mM non-labeled mizoribine.

The amounts of radio-labeled compounds in the samples were determined using a liquid scintillation counter. Protein concentration of the cells was measured with a BCA Protein Assay Reagents Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions.

Subjects and study protocols: Eight healthy Japanese males with the SLC28A1 565-A/A or -G/A allele9 participated in the present study. The ranges of age and weight were 22–38 years old (mean: 25.6 years old) and 53–72 kg (mean: 62.4 kg), respectively. All subjects gave written informed consent to participate in the clinical trial, which was approved by the ethics committee of University of Toyama. They drank 500 mL water on awakening, and were given 150 mg (three 50-mg tablets, the maximal daily dose permitted in Japan) mizoribine (Bredinin® tablet; Asahi Kasei Pharma, Tokyo, Japan) with a glass of water under fasting conditions at least 1 h after awakening. Urine samples were collected at 1, 2, 3, 4, 6, 8, 10, and 12 h after the dose, and all subjects drank a glass of water (about 200 mL) at the time of each urine collection to ensure adequate urine flow. In addition, all subjects had a light breakfast at least 2 h after the dose because the time to reach the maximum concentration (tmax) of mizoribine ranges 2.24–2.67 h in healthy volunteers.19 In order to evaluate the effect of salt intake on the pharmacokinetics of mizoribine, they were also given 150 mg mizoribine (579 µmol) concomitantly with 300 mg salt (5,133 µmol NaCl); The Salt Industry Center of Japan, Tokyo, Japan) in a hydroxypropylmethyl cellulose capsule (Matsuya Co., Ltd., Osaka, Japan). The amount of mizoribine in each urine sample was measured using high-performance liquid chromatography (HPLC) as described previously.15

Estimation of pharmacokinetic parameters of mizoribine in individual subjects: The pharmacokinetic parameters of mizoribine were estimated as described previously.9 Briefly, to estimate the pharmacokinetic parameters of mizoribine in the subjects, we assumed a 1-compartment model with first-order absorption. The urinary excretion rate of mizoribine (Xu) can be described by the following equation:

\[ X_u = k_e \cdot A = k_e \cdot \frac{D \cdot F \cdot k_a}{k_a - k_e} \cdot (e^{-k_e \cdot t} - e^{-k_a \cdot t}) \]  

(1)

where \( k_e \) and \( k_a \) are the elimination and absorption rate constants, respectively, \( A \) is the amount of mizoribine in the body at time \( t \), \( D \) is the dose (150 mg), and \( F \) is bioavailability of mizoribine. The urinary excretion rate (Xu) inter- and extrapolated from the data in the terminal elimination phase (at 6–12 h after the dose) can be described by the following equation:

\[ X'_u = k_e \cdot \frac{D \cdot F \cdot k_a}{k_a - k_e} \cdot e^{-k_e \cdot t} \]  

(2)

Therefore, the \( k_e \) value was estimated by the following equation:

\[ \ln X'_u = \ln \left( k_e \cdot \frac{D \cdot F \cdot k_a}{k_a - k_e} \right) - k_e \cdot t \]  

(3)

The amount of mizoribine in the urine after the final collection of urine (\( A_{12-\infty} \)), the cumulative urinary excretion (\( A_{0-12} + A_{12-\infty} \)), and the \( F \) value of mizoribine were assessed by the following equations:

\[ A_{12-\infty} = \frac{X_u_{12}}{k_e} \]  

(4)
where \(X_{0,12}\) is the estimated urinary excretion rate at 12 h after the dose, and \(A_{0,12}\) is the total amount of mizoribine in the urine until 12 h after the dose. Furthermore, we estimated the \(k_a\) value using the urinary excretion rate in the early absorption phase (at 0–3 h after the dose). That is, from Eqs. (1) and (2), the difference between \(X_a\) and \(X_a'\) is described as follows:

\[
X_a' - X_a = k_e \frac{D \cdot F \cdot k_a}{k_a - k_e} e^{-k_a t}
\]

Therefore, the \(k_a\) value was estimated by the following equation:

\[
\ln(X_a' - X_a) = \ln \left( \frac{D \cdot F \cdot k_a}{k_a - k_e} \right) - k_a \cdot t
\]

In addition, absorption lag time (ALAG) was estimated by the intersection of Eqs. (3) and (7).

**Statistical analysis:** Values are expressed as the mean ± S.E. In all figures, when error bars are not shown, they are smaller than the symbol. Differences in the pharmacokinetic parameters of mizoribine between with and without salt intake were evaluated using the Wilcoxon signed-rank test. On the other hand, differences in the cellular uptake of cytidine, inosine, and mizoribine between in the presence and absence of extracellular Na⁺ were evaluated using Student’s t-test if the variance of the groups was similar. If this was not the case, the Mann-Whitney U-test was used. \(p < 0.05\) was considered to be statistically significant.

**Results**

**Inhibitory effect of mizoribine on cellular uptake of cytidine and inosine in CNT1- and CNT2-expressing MDCKII cells:** We first evaluated the cellular uptake of \(^{[3]H}\)cytidine and \(^{[3]H}\)inosine in CNT1- and CNT2-expressing MDCKII cells, respectively. Figure 1A shows the time course of the cellular uptake of 0.080 µM (1 µCi/well) \(^{[3]H}\)cytidine in CNT1-expressing MDCKII cells. The uptake of cytidine in the presence of extracellular Na⁺ was time-dependent, and was significantly higher than that in the absence of extracellular Na⁺ (Fig. 1A). To estimate the IC\(_{50}\) value of mizoribine for the uptake of cytidine, we also evaluated the effect of mizoribine on the 30-min uptake of \(^{[3]H}\)cytidine in CNT1-expressing MDCKII cells (Fig. 1B). Mizoribine inhibited the Na⁺-dependent uptake of cytidine in a concentration-dependent manner, and 50 mM mizoribine completely inhibited the uptake of cytidine (2.9% of the control). The IC\(_{50}\) value was estimated to be 2.5 ± 0.3 mM (Fig. 1B).

Figure 2A shows the time course of the cellular uptake of 0.059 µM (1 µCi/well) \(^{[3]H}\)inosine in CNT2-expressing MDCKII cells. The uptake of inosine in the presence of extracellular Na⁺ also exhibited time-dependence, and was significantly higher than that in the absence of extracellular Na⁺ (Fig. 2A). In order to estimate the IC\(_{50}\) value of mizoribine for the uptake of inosine, we further evaluated the 30-min uptake of \(^{[3]H}\)inosine in CNT2-expressing MDCKII cells (Fig. 2B). Mizoribine inhibited the Na⁺-dependent uptake of inosine in a concentration-dependent manner; however, the uptake of inosine was not completely inhibited even though the concentration of mizoribine increased up to 50 mM (24.5% of the control). The IC\(_{50}\) value was estimated to be 8.8 ± 1.5 mM (Fig. 2B).

**Cellular uptake of mizoribine in CNT1- and CNT2-expressing MDCKII cells:** We next evaluated the uptake of 50 µM (0.323 µCi/well) \(^{[14]C}\)mizoribine in CNT1- and CNT2-expressing MDCKII cells (Fig. 3). The 120-min uptake of mizoribine in CNT1-expressing MDCK cells in the presence of extracellular Na⁺ was 3.4-fold higher than that in the absence of extracellular Na⁺ (Fig. 3A). On the other hand, the 120-min uptake of mizoribine in CNT2-expressing MDCKII cells in the presence of extracellular Na⁺ was 1.8-fold higher than that in the absence of extracellular Na⁺ (Fig. 3B). These findings suggest that mizoribine is a low affinity substrate for both CNT1 and CNT2.

**Effect of salt intake on the pharmacokinetic parameters of mizoribine in healthy Japanese males:** We further evaluated the effect of salt intake on bioavailability (intestinal absorption) of mizoribine in 8 healthy Japanese males. Three subjects had the SLC28A1 565-A/A allele, and 5 subjects had the SLC28A1 565-G/A allele (Table 1). Figure 4 shows the urinary excretion rate of mizoribine in 2 typical volunteers, and Table 1 shows the pharmacokinetic parameters of mizoribine calculated by the urinary excretion rate of the drug in all 8 volunteers. The ALAG (mean: 0.389 h) and \(k_e\) (mean: 0.271 h⁻¹) of mizoribine taken with salt were not different from the ALAG (mean: 0.422 h) and \(k_e\) (0.295 h⁻¹) of the drug taken without salt. On the other hand, the \(k_d\) (mean: 1.081 h⁻¹) and \(F\) (mean: 83.8%) of mizoribine taken with salt were significantly higher than the \(k_d\) (mean: 0.850 h⁻¹) and \(F\) (mean: 73.0%) of the drug taken without salt (Table 1). These findings indicated that salt intake affects intestinal absorption but not renal elimination of mizoribine.
Discussion

Mizoribine was taken up Na\(^+\)-dependently into not only CNT1-expressing but also CNT2-expressing MDCKII cells (Fig. 3). In addition, bioavailability of mizoribine in subjects with the SLC28A1 565-A/A or 565-G/A allele was increased by salt intake (Table 1 and Fig. 4), indicating that CNT2 as well as CNT1 are at least partly involved in the intestinal absorption of mizoribine in humans. The concentration of 150 mg mizoribine in the intestine is about 2.3 mM, if the volume of the intestinal fluid is assumed to be 250 mL. In the present study, CNT-mediated intestinal absorption of mizoribine was unlikely to be saturated (Table 1 and Fig. 4), because salt intake improved intestinal absorption of mizoribine. That is, the affinity of mizoribine for CNT1 and CNT2 seemed to be low. Indeed, the IC\(_{50}\) values of mizoribine for cytidine (2.5 mM) and inosine (8.8 mM) uptake were relatively higher than the assumed concentration of mizoribine in the intestine (Figs. 1B and 2B).

Bioavailability of mizoribine was significantly increased by salt intake (Table 1, Fig. 4). However, the effect of salt intake on bioavailability of the drug seemed to be less (Table 1), because bioavailability of the drug in healthy volunteers was relatively high.

Fig. 2. The uptake of 0.059 \(\mu\)M (1 \(\mu\)Ci/well) [\(^{14}\)C]inosine in CNT2-expressing MDCKII cells

A: The cells were incubated with [\(^{14}\)C]inosine for 30–120 min in the absence or presence of extracellular Na\(^+\). B: [\(^{14}\)C]inosine was incubated with CNT2-expressing MDCKII cells for 30 min in the absence and presence of unlabeled mizoribine. Closed triangles indicate Na\(^+\)-dependent uptake. Each symbol represents the mean ± S.E. of 6 experiments. The IC\(_{50}\) values were estimated by KaleidaGraph 4.1 (Synergy Software, Reading, PA, USA). \(\ast p < 0.05\); significantly different from the uptake in the absence of extracellular Na\(^+\).

Fig. 3. The uptake of 50 \(\mu\)M (0.323 \(\mu\)Ci/well) [\(^{14}\)C]mizoribine in CNT1- (A) and CNT2- (B) expressing MDCKII cells

The cells were incubated with [\(^{14}\)C]mizoribine for 120 min. Open and hatched columns indicate the absence and presence of extracellular Na\(^+\), respectively. Each symbol represents the mean ± S.E. of 12 experiments. \(\ast p < 0.05\); significantly different from the uptake in the absence of extracellular Na\(^+\).

Table 1. Pharmacokinetic parameters of mizoribine in 8 volunteers who were administered mizoribine with or without salt

<table>
<thead>
<tr>
<th>ID</th>
<th>SLC28A1</th>
<th>A/LG (h)</th>
<th>(k_{a}) (h(^{-1}))</th>
<th>(k_{e}) (h(^{-1}))</th>
<th>F (%)</th>
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<tr>
<td></td>
<td>G565A</td>
<td>NaCl (-)</td>
<td>NaCl (+)</td>
<td>NaCl (-)</td>
<td>NaCl (+)</td>
</tr>
<tr>
<td>1</td>
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<td>0.725</td>
<td>1.367</td>
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<tr>
<td>2</td>
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<td>0.741</td>
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<tr>
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<td>4</td>
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<td>5</td>
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<td>0.377</td>
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<tr>
<td>6</td>
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<td>0.350</td>
<td>0.778</td>
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<td>7</td>
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<td>8</td>
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</table>

N.S, not significant.
even when the drug was taken without salt (mean: 73.0%, Table 1). One possible explanation for relatively higher bioavailability of mizoribine taken without salt may lie in the fact that Na⁺ is present in the intestinal lumen and/or mucosa even under fasting conditions, and that CNTs transport mizoribine into enterocytes. Indeed, the Na⁺ concentration in the mucosa of the intestine has been reported to be about 120 mM.20 Another explanation may be the involvement of other transporters as well as CNTs, and one plausible transporter may be the ENTs. ENT1 and ENT2 are Na⁺-independent, and mediate nucleoside transport in both directions depending on the nucleoside concentration gradient.10,23) ENT1 and ENT2 transport both pyrimidine- and purine-nucleoside, and are inhibited by NBMPR.10,21) We have previously reported that both CNTs and ENTs are involved in the uptake of mizoribine in LS180 cells.15) On the other hand, Govindarajan et al. have reported that ENT1 and ENT2 are only partially expressed on the apical membrane of the human enterocytes with the majority of the expression of these transporters being in other cells of the intestine.10 Therefore, the relative contribution of CNTs and ENTs (and/or other transporters) to the intestinal absorption of mizoribine in humans needs to be elucidated.

In the present study, the molar quantity of salt which the subjects took (300 mg = 5,133 μmol) was much higher than that of mizoribine (150 mg = 579 μmol). The average salt intake in most countries around the world is 9–12 g/day, with many Asian countries having a mean intake over 12 g/day.22) The World Health Organization (WHO) has set a worldwide target of a maximum salt intake of 5 g/day for adults.22) Salt intake of 300 mg (administered in the present study) is equal to about 2–6% of daily salt intake. The findings in the present study suggest that Na⁺ in daily foods and/or drinks could be one of the factors for nucleoside drug-food interactions. Theoretically, therefore, salt intake could be a method to improve the intestinal absorption of mizoribine in renal transplant recipients and patients with renal disease. However, daily salt intake is restricted in these patients, because there are some reports that salt restriction is effective in attenuating the progression of renal damage.23,24) Further studies are needed to ascertain whether a smaller amount of salt intake improves the intestinal absorption of mizoribine for adult and pediatric renal transplant recipients and patients with renal diseases.

In conclusion, the findings in the present study indicate that mizoribine is a low-affinity substrate for not only CNT1 but also CNT2. In addition, salt intake is expected to improve the bioavailability of mizoribine in patients with insufficient intestinal absorption. However, it is still unclear whether salt intake is useful for recipients of renal transplantation and patients with renal diseases to improve the pharmacokinetics and/or pharmacodynamics of mizoribine. Further studies will be needed to clarify the impact of salt intake on the treatment of these patients with mizoribine.

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