

Laboratory of Biopharmaceutics and Pharmacokinetics¹, Faculty of Pharmaceutical Sciences, Hiroshima International University, Hiroshima; Asahi Kasei Pharma Corporation², Tokyo, Japan

Increased intestinal absorption of mizoribine, an immunosuppressive agent, in cholestatic rats

N. MORI¹, T. YOKOOJI¹, Y. KAMIO², T. MURAKAMI¹

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Dr. Teruo Murakami, Laboratory of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hiro-koshingai, Kure, Hiroshima 737-0112, Japan
t-muraka@ps.hirokoku-u.ac.jp

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The intestinal absorption of mizoribine, an imidazole nucleoside, is mediated by concentrative nucleoside transporter (CNT)1 and CNT2 in rat. Previously, bile and bile salts such as sodium glycocholate were found to suppress the intestinal absorption of mizoribine. In the present study, the contribution of bile on the intestinal absorption of mizoribine was further evaluated in rats. Cholestatic states were induced by an intraperitoneal injection (2 ml/kg) of 50% carbon tetrachloride (CCl₄) dissolved in olive oil, or oral administration (100 mg/2 ml/kg) of α -naphthylisothiocyanate (ANIT) dissolved in olive oil. The animals were subjected to absorption studies 24 h after treatment. Cholestatic states were confirmed by measuring plasma concentrations of bile acids and bile flow rates. When oral bioavailability of mizoribine was estimated by the recovery amount in the urine, rats under cholestatic states exhibited significantly higher oral bioavailabilities than untreated control rats. In contrast, the intestinal absorption percentages of mizoribine from *in-situ* lavaged intestinal loops were the same magnitudes among untreated control, CCl₄- and ANIT-treated rats. These results indicated that the increased oral bioavailability of mizoribine in cholestatic rats was not ascribed to the modulation of nucleoside transporter's expression. In conclusion, various diseased states accompanied with cholestasis may increase the oral bioavailability of mizoribine, possibly due to its less amounts of bile in the intestinal lumen.

1. Introduction

Mizoribine (or Bredinin®), an imidazole nucleoside, has long been used as an orally available immunosuppressive agent in human renal transplantation in Japan. Recently, mizoribine was found to have a potent efficacy as anti-hepatitis C virus (HCV) reagent in combination with interferon- α , as well as ribavirin (Naka et al. 2005). In clinical practice, mizoribine is administered orally, and the bioavailability is reportedly 65–100% in fasted human (Honda et al. 2006; Stypinski et al. 2006). Mizoribine is not metabolized in human body and excreted into urine as an intact form. Thus, the oral bioavailability of mizoribine can be estimated by measuring the recovered amount of mizoribine in the urine after oral administration. Nucleoside analogues are hydrophilic, and their intestinal absorptions are mediated by Na⁺-dependent concentrative nucleoside transporters (CNTs) expressed in the brush-border membrane of absorptive epithelia and Na⁺-independent equilibrative nucleoside transporters (ENTs) expressed in the basolateral membrane of absorptive epithelia, in which CNT family is an active transport system and ENT family is a facilitated diffusion system. CNT family contains three members (CNT1, CNT2, CNT3), and CNT1 and CNT2 are expressed in a proximal-to-distal gradient along the intestine. The expression level of CNT3 is low in rats (Casado et al. 2002; Lu et al. 2004). Recently, we examined the contribution of CNT1 and CNT2 in the intestinal absorption of mizoribine in rats, and found that the intestinal absorption of mizoribine is mediated by both CNT1 and CNT2. Nucleoside-

derived drugs such as gemcitabine (a pyrimidine nucleoside analogue, a CNT1 substrate) and ribavirin (a purine nucleoside analogue, a CNT2 substrate) significantly suppressed the intestinal absorption of mizoribine (Mori et al. 2008a, b). In that study, we also observed that the washing of the intestinal lumen with physiological saline, or removal of bile from the intestinal lumen, prior to the absorption study significantly increased the *in-situ* intestinal absorption of mizoribine. In addition, the co-administration of sodium cholate or sodium glycocholate (both 10 mM) significantly suppressed the *in-situ* intestinal absorption of mizoribine (Mori et al. 2008a).

In the present study, we further examined the effect of bile on the intestinal absorption of mizoribine in rats. Rats with significantly decreased bile flow rates, or rats under cholestatic states, were induced by an intraperitoneal injection of 50% carbon tetrachloride (CCl₄) dissolved in olive oil (2 ml/kg) and administration of α -naphthylisothiocyanate (ANIT) dissolved in olive oil (100 mg/2 ml/kg) orally.

2. Investigations and results

2.1. Effect of food on oral bioavailability of mizoribine in control rats

Untreated control rats were divided into two groups (4 rats each) prior to the absorption study, and the first group rats were fasted overnight, and the second group rats were fed ad libitum, with

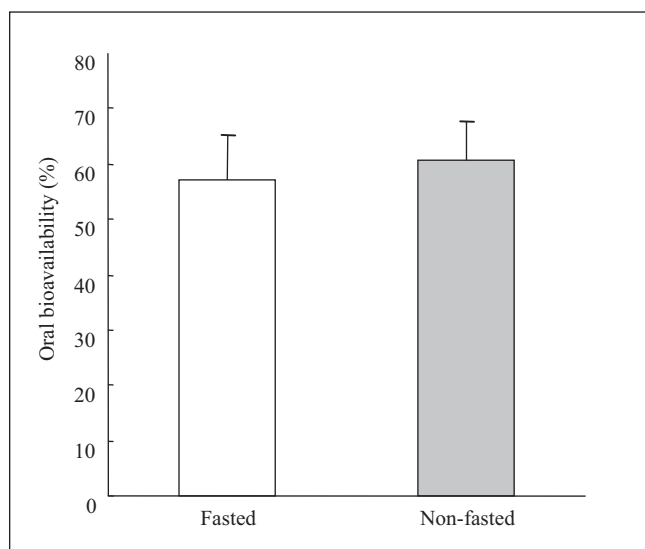


Fig. 1: Oral bioavailabilities, or urinary excretion percentages, of mizoribine administered at a dose of 5 mg/kg in rats under fasted and non-fasted conditions. Urine was collected for 24 h after oral administration of mizoribine. Each value represents the mean \pm S.E.M. of 4 trials

free access to water. These two group rats received mizoribine orally at a dose of 5 mg/kg. The urinary excretion percentages of mizoribine during 24 h after oral administration were approximately 60% of the dosed amounts in both group rats (Fig. 1), indicating that food intake exerted no significant effect on the oral bioavailability of mizoribine.

2.2. Effect of cholestatic states on oral bioavailability of mizoribine

Induction of cholestatic states in rats was evaluated by measuring several biochemical parameters 24 h after treatments with CCl₄ or ANIT (Table). CCl₄-treated rats showed significantly higher plasma activities of GOT and GPT compared with control rats, indicating the induction of acute hepatic failure (Sugihara et al. 1992; Huang et al. 2001; Yumoto et al. 2003; Yokooji et al. 2006). In contrast, in ANIT-treated rats, the increase in plasma activities of GOT and GPT was relatively small as compared with those in CCl₄-treated rats. Both CCl₄- and ANIT-treated rats exhibited significantly higher plasma concentrations of total bile acids. Also, the bile flow rates in CCl₄-treated rats decreased by approximately 50% that in control rats, and in ANIT-treated rats almost completely. These data suggested the induction of cholestatic states in both CCl₄- and ANIT-treated rats.

Using these cholestatic rats, the effect of cholestatic states on oral bioavailability of mizoribine *in vivo* was evaluated by measuring urinary excretion percentages during 24 h after

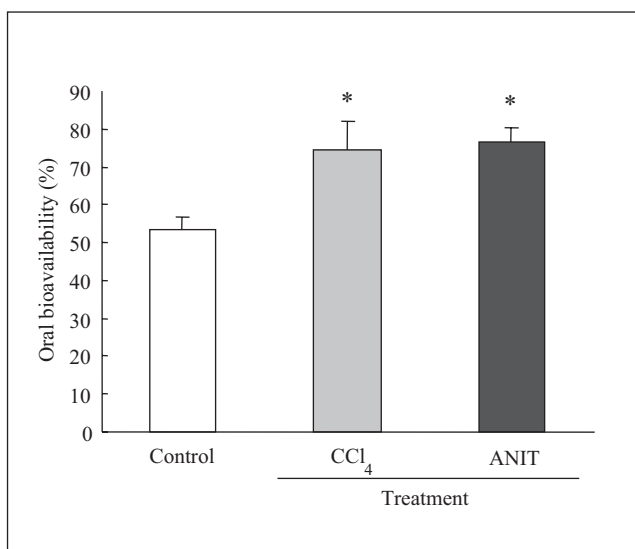


Fig. 2: Oral bioavailabilities, or urinary excretion percentages, of mizoribine administered at a dose of 5 mg/kg in control, CCl₄-treated and ANIT-treated rats. Urine was collected for 24 h after oral administration of mizoribine. Each value represents the mean \pm S.E.M. of 4 trials. * $P < 0.05$, significantly different from the value of control

administration. In both CCl₄- and ANIT-treated rats, the oral bioavailabilities of mizoribine were significantly higher than that in control rats (Fig. 2).

2.3. In-situ intestinal absorption of mizoribine in CCl₄- and ANIT-treated rats

To examine the actual function of nucleoside transporters in the intestine, the intestinal absorption of mizoribine was measured by using lavaged intestinal loops. The disappearance percentages from the intestinal loops, or intestinal absorption percentages, of mizoribine were the same magnitudes among control, CCl₄- and ANIT-treated rats (Fig. 2). These results indicate that the increased oral bioavailability of mizoribine under cholestatic states *in vivo* was not ascribed to the modulation of the expression of nucleoside transporters.

3. Discussion

The contribution of bile on the intestinal absorption of mizoribine was further examined in rats, since bile and bile salts such as sodium cholate and sodium glycocholate have been found to suppress the *in-situ* intestinal absorption of mizoribine (Mori et al. 2008a). Firstly, the influence of food intake, or fast and non-fast, on intestinal absorption of mizoribine was examined. However, food intake exerted no significant effect on the oral bioavailability of mizoribine (Fig. 1). There is no gallbladder

Table: Biochemical parameters in control, CCl₄-treated, and ANIT-treated rats

Parameters	Control	Treatment	
		CCl ₄	ANIT
GOT (IU/L)	23.6 \pm 1.9	262.0 \pm 40.3 ^a	63.5 \pm 18.1 ^b
GPT (IU/L)	6.6 \pm 1.8	163.0 \pm 37.6 ^a	19.9 \pm 4.8
Total bile acids (μ mol/L)	6.8 \pm 2.0	35.7 \pm 9.6 ^b	427.0 \pm 13.3 ^a
Bile flow rate (ml/h)	1.32 \pm 0.07	0.63 \pm 0.28 ^b	n.d.
Liver weight (g/100 g B.W.)	3.26 \pm 0.19	4.18 \pm 0.10 ^a	3.83 \pm 0.10 ^a

Each parameter was determined 24 h after treatments. Values are expressed as the mean \pm S.E.M. (n=4)

^a $P < 0.01$

^b $P < 0.05$; significantly different from the value of control. n.d.; not detected

in rats, therefore, bile is always and constantly secreted into the intestinal lumen in rats, independent of food intake. Thus, the no effect of food intake on oral bioavailability of mizoribine in rats was considered due to the continuous flow of bile both under fasted and un-fasted conditions.

Next, the contribution of bile to the intestinal absorption of mizoribine was examined by using rats in the cholestasis state. Intrahepatic cholestasis involves impaired excretion of bile via the hepatobiliary system, and the majority of adult patients with chronic cholestasis have primary biliary cirrhosis or primary sclerosing cholangitis (Feuer and Di Fonzo 1992; Poupon et al. 2000; Jansen and Sturm 2003; Gershwin and Mackay 2008). CCl_4 has been widely used to induce liver damage, especially as a model of primary hepatic cirrhosis (Tuñón et al. 2009). The administration of CCl_4 in rats induces both severe damage processes and hepatic regeneration simultaneously, depending on the CCl_4 dose, administration route of CCl_4 , and exposure time (Murakami et al. 2002; Weber et al. 2003; Taniguchi et al. 2004). The administration of ANIT orally to rats produces transient intrahepatic cholestasis, necrosis of the bile duct endothelium, and areas of focal injury to the hepatocytes in periportal areas of the liver (Desmet et al. 1968; Chisholm and Dolphin 1996; Orsler et al. 1999). The induction of liver cirrhosis is evaluated by the activity of aminopyrine N-demethylation, serum bile acids, extravascular albumin space, hepatocellular volume, and so on (Reichen et al. 1988). In the present study, plasma concentrations of total bile acids and bile flow rates were determined 24 h after treatments. Both treated rats showed higher plasma bile acids concentrations and lower or negligible bile flow rates, indicating the induction of cholestatic states in these treated rats (Table). In such cholestatic rats, the oral bioavailabilities of mizoribine were approximately 1.4-fold higher than those in untreated control rats. However, no significant difference was observed in the increased oral bioavailability of mizoribine between CCl_4 - and ANIT-treated rats, though the bile flow rates were fairly different between them (Fig. 2, Table). Further studies are necessary to clarify the relationship between the severity of cholestatic states and intestinal absorption rates of mizoribine. In contrast, when the absorption study of mizoribine was carried out in lavaged intestinal loops *in-situ*, no difference was observed in the oral bioavailabilities of mizoribine between normal and cholestatic rats (Fig. 3). In the present study, the closed intestinal loops were made at the proximal intestine, since the absorption percentages of mizoribine and ribavirin have been found to be the same between the proximal and distal intestines in our previous study (Mori et al. 2008b). Collectively, results suggested that the increased oral bioavailability of mizoribine in CCl_4 - and ANIT-treated rats was due to the less amount of bile in the intestinal lumen, but not due to the modulation of CNT1 and/or CNT2 expression in the intestine. Cholestasis and/or cholestatic states are known to affect the expression of intestinal transporters such as apical sodium-dependent bile acid transporter (ASBT), P-glycoprotein, and multidrug resistance-associated protein (MRP)2 in rats (Sauer et al. 2000; Huang et al. 2001; Murakami et al. 2002; Yumoto et al. 2003; Kamisako and Ogawa 2005; Yokooji et al. 2006; Martínez-Augustín and de Medina 2008). However, the modulating effect of bile on the expression of nucleoside transporters would be ruled out in the present study, since the suppressing effect of bile on the intestinal absorption of mizoribine was observed only in the case of coexistence of mizoribine and bile. Mizoribine is a water soluble hydrophilic compound with a very low lipophilicity (log P is -2.87 , when calculated by Crippen's fragmentation method) (Ghose and Crippen 1987). The direct interaction of mizoribine such as micelle formation with bile salts may also be ruled out, because of the hydrophilic property. Various bile salts at appropri-

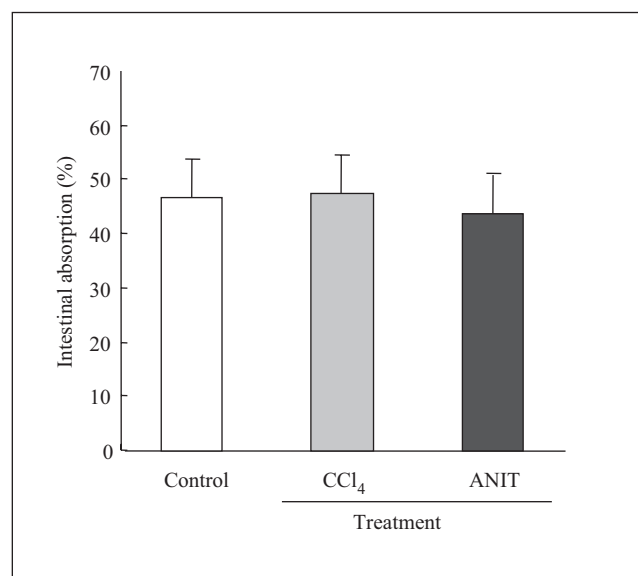


Fig. 3: Intestinal absorption percentages of mizoribine from *in-situ* lavaged intestinal loops prepared in control, CCl_4 -treated and ANIT-treated rats. Mizoribine (1 mg/kg) was administered into a 10 cm-long jejunal loop, and the remained amount of mizoribine in the loop 60 min after administration was determined. Each value represents the mean \pm S.E.M. of 4 trials

ate concentrations are known to increase the permeability of biomembranes via paracellular routes (Murakami et al. 1984, 2000). However, the addition of sodium glycocholate (10 mM) significantly decreased the CNT1- and CNT2-mediated intestinal absorption of mizoribine (Mori et al. 2008a, b). Thus, the contribution of the paracellular route in the decreased oral bioavailability of mizoribine by bile will also be ruled out. Further studies are necessary regarding the suppressive mechanism of bile and bile salts on the intestinal absorption of mizoribine. In conclusion, various disease states accompanied by cholestatic states, such as primary biliary cirrhosis, primary sclerosing cholangitis, drug-induced cholestasis and genetic cholestasis (Hofmann 1999; Poupon et al. 2000; Jansen and Sturm 2003; Davit-Spraul et al. 2009), may increase the oral bioavailability of mizoribine, possibly due to low amounts of bile in the intestinal lumen.

4. Experimental

4.1. Materials

Mizoribine (dry powder, purity: 99.8%) was a gift from Asahi Kasei Pharma Corporation (Tokyo, Japan). Carbon tetrachloride (CCl_4), α -naphthylisothiocyanate (ANIT), and olive oil were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other chemicals used were of the highest purity available.

4.2. Methods

4.2.1. Animal treatment

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. Cholestatic states were induced by intraperitoneal injection of a mixture of CCl_4 and olive oil (50% v/v) at a dose of 2 ml/kg, or oral administration of ANIT (100 mg) dissolved in 2 ml olive oil at a dose of 2 ml/kg. Control rats received the same volume of olive oil alone, respectively. These rats were fasted overnight and subjected to absorption study 24 h after the treatments. Cholestatic states were evaluated by measuring plasma concentrations of bile acids and bile flow rates, in addition to plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities.

4.2.2. *In-vivo absorption study*

Mizoribine was dissolved in distilled water at a concentration of 5 mg/ml, and rats fasted overnight or without fasted received mizoribine orally at a dose of 5 mg/kg. These rats were kept in a metabolic cage at 23 °C to collect urine for 24 h after administration of mizoribine. To the urine, an equal amount of acetonitrile was mixed for deproteinization, and the suspension was centrifuged at 1,000 g for 5 min to collect supernatant.

4.2.3. *In-situ intestinal loop study*

Rats fasted overnight were anaesthetized with pentobarbital (30 mg/kg, i.p. injection). The *in-situ* intestinal loop study was carried out in the same manner as reported previously (Mori et al. 2008). Briefly, bile duct was ligated and the intestinal lumen was washed with a sufficient amount of saline prewarmed at 37 °C after cannulating polyethylene tubings at the upper duodenum and lower ileum of the small intestine. Then, a 10 cm-long intestinal loop was made by ligating both ends of the intestinal loop at proximal small intestine of anesthetized rats (a segment from 5 cm below the bile duct opening). Mizoribine was dissolved in saline at a concentration of 0.5 mg/ml and was administered to the loop via the polyethylene tubing (PE 10) inserted into the loop at a volume of 2 ml/kg (corresponding to a dose of 1 mg/kg). One hour after the administration of mizoribine, rats were killed by injecting a sufficient amount of saturated KCl solution to the heart. The intestinal loop containing mizoribine was isolated, and the isolated loop was weighed and homogenized with the tissue homogenizer (21,000 rpm, 2 min) after adding 9-fold volume of distilled water. To the 10% intestinal homogenate (0.5 mL), 0.5 ml of acetonitrile was added and the suspension was centrifuged at 1,000 g for 5 min to obtain the supernatant.

4.2.4. *Analysis*

Blood samples were centrifuged at 4,000 × g to obtain plasma samples. Plasma activities of GOT and GPT, and concentrations of total bile acids in plasma were measured with commercially available analytical kits: Transaminase CLI-Test Wako (Wako Pure Chemicals, Osaka, Japan) for GOT and GPT, and Total bile acids-Test Wako (Wako Pure Chemicals, Osaka, Japan) for bile acids, respectively.

Concentrations of mizoribine in the supernatants of biological samples (intestinal tissue homogenates, urine) were determined by HPLC according to a reported method (Hosotsubo et al. 1988). Briefly, the column used was a Shimpack CLC-NH₂ (6.0 mm I.D. × 150 mm, Shimadzu Corporation, Kyoto, Japan) and mobile phase was a mixture of 1/15 M phosphate buffer (pH 2.5) and acetonitrile, in a ratio of 27.5: 72.5 (v/v). The flow rate of mobile phase was 1.3 ml/min, and detection was made at wavelength of 280 nm.

Data were expressed as the mean ± S.E.M. Differences among group mean values were assessed by the Kruskal-Wallis or ANOVA test followed by a post-hoc test (Tukey test). A difference of $P < 0.05$ was considered statistically significant.

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