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Modulation in concentrative nucleoside transporters-mediated intestinal absorption of mizoribine, an immunosuppressive agent, in lipopolysaccharide-treated rats

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The characteristics of intestinal absorption of mizoribine and cephalexin, that are mediated by concentrative nucleoside transporters (CNTs) and PEPT1, respectively, was examined in lipopolysaccharide (LPS)-treated rats. LPS treatment is known to modify the expression of some transporters and induce cholestasis. At 24 h after the LPS treatment, averaged concentrations of IL-6 and total bile acids in plasma were 15-fold and 2-fold that in untreated control rats, respectively, and bile flow rate decreased by 40% of control, indicating the induction of inflammatory and cholestatic states. The oral bioavailability, estimated by urinary excretion percentage of unchanged form, of mizoribine in LPS-treated rats was 1.5-fold higher than that in control rats, whereas the bioavailability of cephalexin remained unchanged. When mizoribine and cephalexin were administered into *in-situ* jejunum loops, there were no differences in the absorption rates between control and LPS-treated rats. These results indicated that the functional expression of CNT1, CNT2, and PEPT1 were not modulated by LPS treatment. When mizoribine (a CNT1/CNT2 substrate) and gemcitabin (a CNT1 substrate) were administered as a solution dissolved in bile into the intestinal loop, their absorption rates decreased significantly. In contrast, the absorption rate of ribavirin (a CNT2 substrate) remained unchanged. In conclusion, LPS treatment exerted no significant effect on the expression of CNT1 and CNT2 in the intestine. Bile was found to suppress the CNT1-mediated intestinal absorption of mizoribine and gemcitabin. The increased oral bioavailability of mizoribine in LPS-treated rats could be ascribed to the less amount of bile or bile acids in the intestine under cholestatic state of rats.

1. Introduction

Mizoribine (or Bredinin®), an imidazole nucleoside, has long been used as an orally available immunosuppressive agent in human renal transplantation in Japan. Mizoribine also possesses a potent efficacy as an anti-hepatitis C virus (HCV) agent in combination with interferon-α, like ribavirin (Naka et al. 2005). The relatively high and steady oral bioavailability of mizoribine in healthy subjects has been recognized, in which the excretion percentages of unchanged mizoribine were 65–100% in fasted human (Honda et al. 2006; Stypinski et al. 2006). Mizoribine was absorbed efficiently to the same extents from various sites along the small intestine, and the altered gastric emptying rates exerted no significant effects on the oral bioavailability of mizoribine in rats (Mori et al. 2010a). The intestinal absorption of mizoribine is mediated by concentrative nucleoside transporter (CNT)1 and CNT2, and therefore co-administration of nucleoside-derived drugs such as gemcitabine, a substrate for CNT1, and ribavirin, a substrate for CNT2, can suppress the intestinal absorption of mizoribine (Mori et al. 2008). Bile and bile salts such as sodium glycocholate also decreased the intestinal absorption of mizoribine. When mizoribine was administered into a lavaged intestinal *in-situ* loop, the intestinal absorption rate of mizoribine was higher than that in un-lavaged intestinal loop. In addition, the oral bioavailabilities of mizoribine under cholestatic states induced by carbon tetrachloride and α-naphthylisothiocyanate were higher than those in untreated control rats (Mori et al. 2010a).

In the present study, the effect of lipopolysaccharide (LPS) treatment on intestinal absorption of mizoribine was examined in rats, by employing cephalexin for comparison. Cephalexin, a β-lactam antibiotic, is known as a substrate for proton-coupled oligopeptide transporter (PEPT1) and the significant contribution of PEPT1 in the intestinal absorption of cephalexin in rats is well recognized (Tsuji and Tamai 1996; Terada et al. 1997; Tamai et al. 1997; Chu et al. 2001). LPS is generally used to induce inflammatory and cholestatic states in rodents. LPS treatment is also known to modulate mRNA and/or protein levels of various metabolizing enzymes, solute carrier (SLC) transporters, and ABC efflux transporters, *via* the increased cytokine levels such as interleukin (IL)-6 (Sukhai et al. 2000; Hartmann et al. 2001, 2002 and 2005; Cherrington et al. 2004; Kalitsky-Szirtes et al. 2004; Fakhoury et al. 2006; Morgan et al. 2008; Petrovic et al. 2007 and 2008; Le Vee et al. 2008 and 2009). Regarding the LPS-induced cholestatic states, Arab et al. (2009) reported that injection of LPS induced a significant decrease of bile flow (−24%), biliary bile salts (−40%) and

glutathione excretion (−70%), as well as a significant decrease in Ntcp (−90%) and Mrp2 (−80%) protein levels. In the present study, the evaluation of intestinal absorption of mizoribine and cephalexine was made by measuring their urinary excretion rates or by measuring the remained amount in the *in-situ* intestinal loops, since these two compounds are not metabolized in the body (Murase et al. 1978).

2. Investigations and results

2.1. Biochemical parameters in untreated control and LPS-treated rats

To ensure the induction of inflammatory and cholestatic states, the concentrations of IL-6 and total bile acids in plasma and bile flow rates were determined after the treatment with LPS in rats (Table). The plasma concentration of IL-6 increased by 15-fold of control in average 24 h after LPS treatment, though the observed values were scattered among foŭr different rats as follows: 3563, 2256, 13460, and 1732 pg/ml. The plasma concentrations of total bile acids 24 h and 48 h after LPS treatments were significantly higher than those in untreated control rats. In addition, the bile flow rates 24 h after LPS treatment were significantly decreased by approximately 40% of control. These biochemical parameters indicated the induction of inflammatory and cholestatic states in all LPS-treated rats.

2.2. Oral bioavailability of mizoribine and cephalexin in LPS-treated rats

Mizoribine and cephalexin were administered orally as a solution, and the oral bioavailability was evaluated by measuring their urinary excretion percentages of unchanged intact form (Fig. 1). The bioavailabilities of mizoribine, a substrate for CNT1 and CNT2, in LPS-treated rats were significantly higher than those in untreated control rats. In contrast, the bioavailabilities of cephalexin remained unchanged even after LPS treatment.

2.3. In-situ intestinal absorption of mizoribine and cephalexin in LPS-treated rats

Mizoribine and cephalexin were administered as a solution into jejunum loop after the luminal content was washed out to evaluate their intestinal absorption percentages during 1 h (Fig. 2). The intestinal absorption percentages, estimated by the remained amount in the intestinal lumen, of both mizoribine and cephalexin were comparable between untreated control and LPS-treated rats. The result indicated that the functional expression of transporters such as CNT1, CNT2, and PEPT1 in the intestine was not altered by LPS treatment.

2.4. Effect of bile on in-situ intestinal absorption of mizoribine, gemcitabin, and ribavirin

For comparison with mizoribine (a substrate for CNT1 and CNT2), gemcitabin (a substrate for CNT1) and ribavirin (a substrate for CNT2) were employed. These compounds were dissolved in freshly collected rat's bile to apply as a solution into jejunum loop (Fig. 3). The intestinal absorption percentages of mizoribine and gemcitabin were significantly decreased by 23% and 40% of control, respectively. In contrast, the intestinal absorption percentage of ribavirin dissolved in bile was remained unchanged, suggesting the suppressing effect of bile on CNT1-mediated transport.

3. Discussion

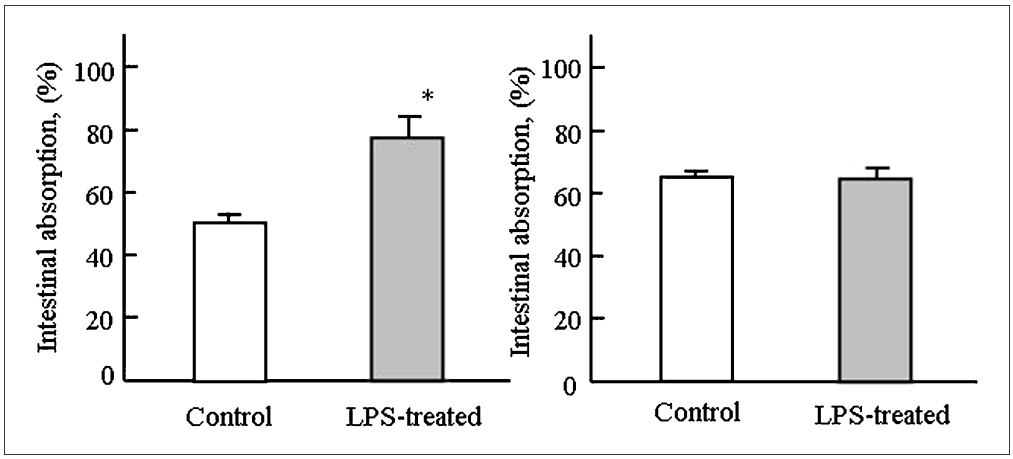
In the present study, we examined the effect of LPS treatment on transporter-mediated intestinal absorption of mizoribine, a substrate for CNT1 and CNT2, by using cephalexin, a substrate for PEPT1, for comparison in rats.

Gemcitabin and ribavirin were also employed partly for comparison with mizoribine. LPS treatment is known to modify various transporters' expression and induce cholestasis (Sukhai et al. 2000; Hartmann et al. 2001, 2002 and 2005; Cherrington et al. 2004; Kalitsky-Szirtes et al. 2004; Fakhoury et al. 2006; Morgan et al. 2008; Petrovic et al. 2007 and 2008; Le Vee et al. 2008 and 2009). For example, Cherrington et al. (2004) reported that hepatic mRNA levels of Mrp2, Mrp6, Mdr1a, Oatp1, Oatp2, Oatp4, Ntcp, bile salt export pump, Oct1, and Oat3 were dramatically decreased, beginning approximately 6 h after LPS administration. In accordance with the modulated expression of various transporters, the pharmacokinetics of substrate compounds are modulated. Kalitsky-Szirtes et al. (2004) reported that LPS injection decreased mRNA levels of mrp2 and mdr1a by approximately 50% in rat intestine, and decreased the basal to apical efflux of digoxin, amiodarone, and 5-carboxyfluoresceine in the jejunum. Hartmann et al. (2005) reported that mRNA for Mdr1a, Mdr1b, Mdr2, and Bsep in the liver significantly decreased in mice, and LPS-treated mice exhibited a significant decrease (50%) in biliary clearance and 3-fold increased renal clearance of doxorubicin. In general, modulation of transporter expression and induction of cholestasis resulted in significant modification of the pharmacokinetics of substrates (Kalitsky-Szirtes et al. 2004; Hartmann et al. 2005; Fakhoury et al. 2006; Petrovic et al. 2007). In the present study, the treatment with LPS increased plasma IL-6 concentrations greatly and increased plasma concentrations of total bile acids significantly in all 4 rats, indicating LPS-treated rats induced inflammatory and cholestesis (Table). LPS treated rats exhibited significantly higher intestinal absorption percentage of mizoribine than untreated control rats, whereas the absorption percentages of cephalexin were comparable between untreated control and LPS-treated rats (Fig. 1). To clarify the mechanism for the higher oral bioavailability of mizoribine in LPS-treated rats, studies were made from the following two viewpoints: (1) effect of LPS treatment on the expression of relating transporters (CNT1, CNT2, and PEPT1), and (2) effect of cholestatic state, or bile, on nucleoside transporter-mediated intestinal absorption of mizoribine. Under normal conditions, CNT1 and CNT2 are expressed efficiently along the small intestine, and regional difference was not observed in the intestinal absorption of mizoribine in rats (Mori et al. 2008, 2010a, 2010b). Soler et al. (2001a and 2001b) reported that the treatment with LPS up-regulated CNT1 and CNT2 transport activity and expression in murine bone marrow macrophages. Wojtal et al. (2009) determined mRNA expression levels of solute carrier transporters in the terminal ileum and colon of imflammatory bowel disease (IBD) patients (Crohn's disease and ulcerative colitis), and found that, in the ileum of IBD patients, mRNA levels of Na^+-independent equilibrative nucleoside transporter (ENT)1 and ENT2 were significantly elevated, and, in inflamed colon of IBD patients, mRNA levels of ENT1, ENT2, and CNT2 were significantly higher. Regarding PEPT1, it was reported that the expression level of PEPT1 on brush border membrane determined by Western blot analyzed in 24-h fasted rats was longitudinally almost constant in rat small intestine, different from the case of humans (Hironaka et al. 2009). In human, PEPT1 is reportedly expressed mainly in the proximal intestine such as duodenum and jejunum (Terada et al. 2005; Kim et al. 2007). With respect to the effect of LPS on PEPT1 expression, Shu et al. (2002) reported that LPS decreased mRNA for PepT1 by

Table: Biochemical parameters in untreated control and LPS-treated rats

		LPS treatment	
	Control	24 h	48 h
Concn. of IL-6 in plasma (pg/ml)	357 ± 100	5252 ± 2762	365 ± 21
Concn. of total bile acids in plasma (mmol/L)	5.0 ± 0.4	10.9 ± 1.6[a]	9.0 ± 1.8[b]
Bile flow rate (ml/h)	1.32 ± 0.07	0.77 ± 0.17[b]	–

Values are expressed as the mean±S.E.M. (n = 4). —: not determined.
[a] $P < 0.01$,
[b] $P < 0.05$; Significantly different from the value of control.

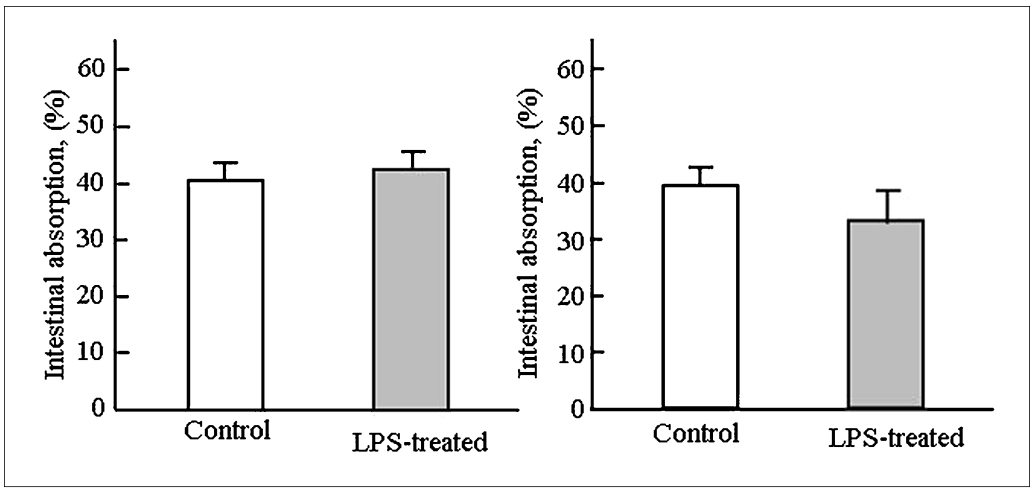


(A) (B)

Fig. 1: Oral bioavailabilities of mizoribine (A) and cephalexin (B) in untreated control and LPS-treated rats. Rats fasted 24 h received either mizoribine or cephalexin at a dose of 5 mg/kg by stomach intubation, and the rats were housed in metabolic cages to collect urine for 24 h. Each value represents the mean±S.E.M. of 4 trials. * $P < 0.05$, significantly different from the value in untreated control rats

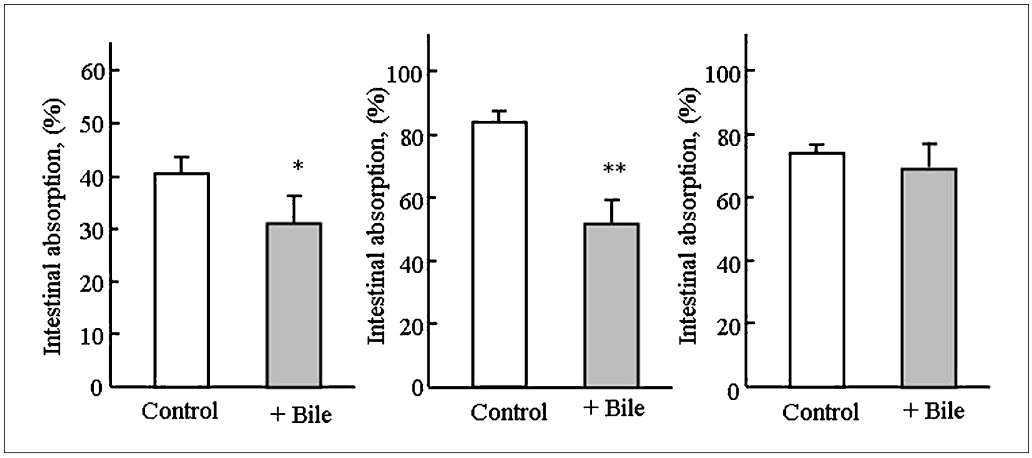
32–62% of control rats, and the protein level of PepT1 in the jejunum was consistent with the mRNA level. In this study, the effect of LPS treatment on the expression of CNT1, CNT2 and PEPT1 was evaluated functionally by measuring the intestinal absorption rates of mizoribine and cephalexin in *in-situ* intestinal loop study. As shown in Fig. 2, LPS treatment exerted no significant effect on the intestinal absorption rates of both mizoribine and cephalexin. The discrepancy in the effect of LPS on PEPT1 expression between previous reported results (Soler et al. 2001a and 2001b; Wojtal et al. 2009; Shu et al. 2002) and present studies would be mostly derived from the exposure time of the host to cytokines, at least partly. For example, rats were treated with LPS daily for 3 days in the study reported by Shu et al. (2002). Recently, Hironaka et al. (2009) reported that the contribution of PEPT1 in oral absorption of cephalexin is around a half of total absorption. Accordingly, in case of CEX, it is also reported that the function of PEPT1 can be compensated by passive diffusion (Hironaka et al. 2009). Regarding the effect of LPS, and/or cytokines, on the expression of CNTs and ENTs in the intestine *in vivo*, further study is necessary.

As reported previously, the intestinal absorption of mizoribine was higher when the luminal concentration of bile or bile salts was low. Also, the administration of mizoribine to lavaged intestine and to CCl_4- and ANIT-treated rats exhibiting



(A) (B)

Fig. 2: Intestinal absorption of mizoribine (A) and cephalexin (B) in untreated control and LPS-treated rats. Mizoribine and cephalexin were dissolved in saline and administered to a 10 cm-long jejunum loop at a dose of 1 mg/2 ml/loop. The intestinal absorption (%) was estimated by measuring the remained amount of mizoribine in the loop 60 min after administration. Each value represents the mean±S.E.M. of 4 trials



(A) (B) (C)

Fig. 3: Effect of bile on intestinal absorption of mizoribine (A), gemcitabin (B) and ribavirin (C) in untreated control in untreated control rats. Mizoribine, gemcitabin and ribavirin were dissolved at a concentration of 0.5 mg/ml in bile freshly corrected from rats. Each drug solution was administered into a 10 cm-long jejunum loop at a volume of 2 ml/kg (corresponding to a dose of 1 mg/kg). The intestinal absorption (%) was estimated by measuring the remained amount of mizoribine in the loop 60 min after administration. Each value represents the mean±S.E.M. of 4 trials. ** $P < 0.01$ and * $P < 0.05$, significantly different from the value in untreated control rats

cholestatic states *in vivo* showed a higher intestinal absorption as compared with those in untreated control rats (Mori et al. 2008, 2010a, 2010b). The effect of bile on the function of CNT1 and CNT2 was evaluated by measuring transporter-mediated intestinal absorption of mizoribine (a CNT1/CNT2 substrate), gemcitabin (a CNT1 substrate) and rivabirin (a CNT2 substrate) (Fig. 3). The intestinal absorption percentages, evaluated by remained amount in the intestinal loop, of mizoribine and gemcitabin were significantly lower, when drugs were administered as a solution dissolved in bile, whereas ribavirin absorption remained unchanged. These results suggested that bile suppresses the CNT1-mediated, but not CNT2-mediated, intestinal absorption of substrate compounds. Regarding the interaction between bile or bile salts and CNT1, further study is necessary. The study on the direct physicochemical interaction between bile salts and nucleoside analogues is also important. In clinical practice, inflammation and cholestasis will be frequently observed. Under inflammatory states in humans, the pharmacokinetics of transporter substrates are frequently modulated. For example it is reviewed that the plasma levels of P-glycoprotein substrates such as cyclosporine, amitriptyline, verapamil increased; the clearance of ciprofloxacin, an MRP2 substrate, decreased; and the volume of distribution of arbekacin, a P-gp substrate, increased (Petrovic et al. 2007). Fakhoury et al. (2006) compared CYP3A and P-gp mRNA expression in 19 noninflammed duodenal biopsies from children with Crohn disease with 19 normal biopsies, and found that CYP3A, CYP3A5, and P-gp levels were significantly higher in the Crohn disease group than in the control group.

In conclusion, LPS treatment exerted no significant effect on the expression of CNT1 and CNT2, as well as PEPT1, in the intestine in rats. Bile was found to suppress the CNT1-mediated intestinal absorption of mizoribine and gemcitabin, different from the cases of CNT2-mediated intestinal absorption of ribavirin and PEPT1-mediated intestinal absorption of CEX. The increased intestinal absorption of mizoribine in LPS-treated rats could be ascribed to the cholestatic state of rats. Further study is necessary to clarify the suppressing mechanism of bile or bile acids on CNT1-mediated transport.

4. Experimental

4.1. Materials

Mizoribine was a gift from Asahi Kasei Pharma Corporation (Tokyo, Japan). Ribavirin (Rebetol®) was obtained from Schering-Plough K.K. (Osaka, Japan). Gemcitabine (Gemzar® Injection) was obtained from Eli Lilly Japan K.K. (Kobe, Japan). Cephalexin and LPS from *Escherichia coli* serotype O55:B5 were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Rat IL-6 ELISA kit, a commercially available analytical kit for IL-6 in rats, and Total bile acids –Test Wako, a clinically available analytical kit for total bile acids, were purchased from Thermo Scientific (IL, USA) and Wako Pure Chemical Industries, Ltd (Osaka, Japan), respectively. 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). The rats were fed a standard laboratory diet for rats (CE-2, Clea Japan, INC., Tokyo, Japan) and water for more than 1 week prior to experiments. Endotoxemia was induced by an intraperitoneal injection of LPS (5 mg/kg) dissolved in 0.5 ml of sterile saline in the same manner as reported by Kalitsky-Szirtes et al. (2004). LPS treated rats were then fasted for 24 h or 48 h with free access to drinking water. Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" from the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima International University, which is in accordance with the "Guidelines for proper conduct of animal experiments" from Science Council of Japan.

4.2.2. Evaluation of LPS-induced inflammation and cholestasis

Untreated control and LPS-treated rats were fasted for 24 h or 48 h and anaesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection. In case of 24 h-fasting, a polyethylene tubing (PE 10) was then inserted into the bile duct of both untreated control and LPS-treated rats and bile was collect for 1 h. Blood was taken from the jugular vein, and centrifuged at 3,000 rpm to obtain plasma samples. The concentrations of IL-6 and total bile salts in plasma were determined by using commercially available analytical kits, Rat IL-6 ELISA Kit and Total bile acids –Test Wako, respectively. The bile flow rates were estimated by weighing the bile collected and by assuming that the specific gravity of bile is unity.

4.2.3. In-vivo absorption study

Mizoribine and cephalexin were dissolved in distilled water at a concentration of 2.5 mg/ml. The 24-h fasted untreated control and LPS-treated rats received either mizoribine or cephalexin solution at a volume of 2 ml/kg by stomach intubation, and were housed in metabolic cages to collect urine for 24 h. 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 obtain the supernatant.

4.2.4. In-situ intestinal loop study

The 24-h fasted untreated control and LPS-treated rats were anaesthetized with pentobarbital (30 mg/kg) by an intraperitoneal injection. The *in-situ* intestinal loop study was carried out to evaluate the functional expression of transporters 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. A 10 cm-long intestinal loop was made by ligating both ends of the intestinal loop at the proximal small intestine (a segment from 5 cm below the bile duct opening). Mizoribine and cephalexin were dissolved in saline at a concentration of 0.5 mg/ml. In separate experiments, mizoribine, gemcitabin and ribavirin were dissolved in bile that was freshly corrected from untreated control rats at a concentration of 0.5 mg/ml. Each drug solution was administered into 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 hoŭr after the administration of mizoribine, gemcitabin or ribavirin, rats were killed by injecting a sufficient amount of saturated KCl solution to the heart. The intestinal loop containing a nucleoside-analogue drug 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.

4.2.5. Analysis

The concentrations of mizoribine in the supernatants of various biological samples including plasma, urine, and intestinal homogenates were determined by HPLC according to the reported method (Hosotsubo et al. 1988). The concentrations of cephalexin in various biological samples were determined by HPLC according to the method reported by Hironaka et al. (1988). The concentrations of gemcitabin and ribavirin in the supernatants of the luminal fluid and intestinal homogenate were measured by HPLC in the same manner as reported by Lanz et al. (2007) and Homma et al. (1999), respectively.

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) or Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

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