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Research Paper

Modulated pharmacokinetics and increased small intestinal toxicity of methotrexate in bilirubin-treated rats

Tomoharu Yokooji, Nobuhiro Mori and Teruo Murakami

Laboratory of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hiro-koshingai, Kure, Hiroshima, Japan

Abstract

Objectives The effect of bilirubin treatment on the pharmacokinetics and small intestinal toxicity of methotrexate was evaluated in rats, since bilirubin and its glucuronide conjugates can suppress multidrug resistance-associated protein-mediated transport.

Methods Rats were treated intravenously with bilirubin and the various clearances and tissue distribution of methotrexate were estimated under a steady-state plasma concentration. Intestinal toxicity induced by methotrexate was also evaluated by measuring the leakage of alkaline phosphatase (ALP) activity. Probenecid, an inhibitor for multidrug resistance-associated protein and organic anion transporters, was used for comparison.

Key findings The treatment with bilirubin increased the steady-state plasma concentration and reduced biliary excretion clearance, urinary excretion clearance and intestinal exsorption clearance of methotrexate, as did treatment with probenecid. The intestinal absorption and jejunum distribution of methotrexate also significantly increased in bilirubin- and probenecid-treated rats. A greater leakage of ALP activity to the luminal fluid, with a lower ALP activity in the intestinal mucosal membrane after intestinal perfusion of methotrexate, was observed in bilirubin- and probenecid-treated rats.

Conclusions Hyperbilirubinemia, which is involved under various disease states, may increase the small intestinal accumulation and toxicities of methotrexate, since high plasma concentrations of conjugated bilirubin can suppress the function of multidrug resistance-associated proteins, which facilitate the efflux of methotrexate out of cells.

Keywords bilirubin; methotrexate; multidrug resistance-associated proteins; pharmacokinetics; toxicity

Introduction

Methotrexate, a folic acid antagonist, is widely used in clinical practice as a chemotherapeutic agent to treat rheumatoid arthritis and other malignancies.[1–3] The clinical application of methotrexate, however, is limited due to its gastrointestinal toxicity, with effects such as shortened villi and/or dysfunction of the villi, which may cause changes in the absorptive and biochemical functions of the small intestine.[4,5] As regards the mechanism(s) of methotrexate-induced gastrointestinal toxicity, the roles of nitrosative stress,[6,7] oxidative stress[8] and decreased content of polyamines such as spermine and spermidine in the intestinal mucosa[9] have been reported. Methotrexate is known as a substrate for multiple transporters, including multidrug resistance-associated protein (Mrp) 2, breast cancer resistance protein, reduced folate carrier 1, proton-coupled folate transporter, organic anion transporting polypeptide 1a3, and organic anion transporters (Oats).[10–16] The modulation of such transporters' function can alter the pharmacokinetics and pharmacodynamics of methotrexate.[14–17] The adverse side effects of methotrexate are also altered under modulated transporter function. For example, with methotrexate-induced toxicities it is reported that changes in the bone marrow, spleen and intestines after multiple dosing are more severe in Eisai hyperbilirubinemic rats (EHBRs), which hereditarily lack Mrp2, as compared with those in normal rats.[18]

Previously, we examined the effect of bilirubin treatment on the pharmacokinetics of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), an Mrp substrate, after intravenous administration of 1-chloro-2,4-dinitrobenzene (CDNB), a precursor of DNP-SG, in rats, and found that the jejunum efflux and biliary excretion of DNP-SG were almost completely suppressed in bilirubin-treated rats.[19] When bilirubin was administered intravenously at a dose of

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

85.5 μmol/kg, plasma concentrations of unconjugated/conjugated bilirubin were in a range from 70 to 140 μM.[19] Bilirubin is known as a substrate for Oatp1a1 and bilirubin glucuronides formed *in vivo* are substrates and inhibitors for Oatp1a1 and Mrp2.[20–22] Also, the accumulation of DNP-SG in the brain, liver, jejunum and skeletal muscle after intravenous administration of CDNB was significantly increased in bilirubin-treated rats, as well as in probenecid-treated rats.[23] Hyperbilirubinemia exhibiting high plasma concentrations of unconjugated/conjugated bilirubin is frequently observed in various disease states, including obstructive jaundice caused by cancer and liver grafts.[24–27]

In the present study, the effect of bilirubin and probenecid treatment on the pharmacokinetics of methotrexate and methotrexate-induced intestinal toxicity were evaluated in rats, since bilirubin and its glucuronide conjugates are suspected of suppressing the Mrps-mediated transport of methotrexate *in vivo*.

The relationship between methotrexate-induced intestinal toxicity and the function of efflux transporter(s) in the intestine is not yet well understood. In our pharmacokinetic studies of methotrexate, the plasma and tissue concentrations, biliary excretion, urinary excretion and intestinal exsorption (secretion to luminal fluid from the blood circulation) of methotrexate at steady state were determined. The intestinal toxicity was evaluated by measuring the leakage of intestinal alkaline phosphatase (ALP) activity as an intracellular marker enzyme.[5] For comparison, rats treated with probenecid, an inhibitor for Mrps and Oats, were also used.

Materials and Methods

Materials

Methotrexate was obtained from Wako Pure Chemicals (Osaka, Japan). Probenecid and bilirubin were obtained from Sigma Chemical Co. Ltd (St Louis, USA). All other chemicals used were of the highest purity available.

Animals

Male Sprague–Dawley rats aged 7–9 weeks old were fasted overnight with free access to water before the experiments. The protocol of the experiments was reviewed and approved in advance and 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 the Science Council of Japan.

Clearances and tissue accumulation of methotrexate under steady state in rats

The *in-vivo* clearance and tissue accumulation of methotrexate under a steady-state plasma concentration of methotrexate were estimated as pharmacokinetic parameters in the untreated control, probenecid-treated and bilirubin-treated rats, respectively. Rats were fasted overnight, anesthetized with pentobarbital (30 mg/kg, i.p. injection) and affixed supine on a surface kept at 37°C to maintain their body temperature at around at 36°C. Cannulation (polyethylene (PE) tubing) was made at a femoral vein (PE-50) for the administration of drug, a femoral artery (PE-50) for the sampling of blood, a bile duct (PE-10) for the sampling of bile and a bladder (PE-50) for the sampling of urine, respectively. The intestinal lumen was flushed with approximately 20 ml of saline prewarmed at 37°C, and a 20-cm long jejunum loop was made from 5 cm below the bile duct opening to estimate the intestinal exsorption of methotrexate. The jejunum loop was perfused with pH 6.5 phosphate buffered saline (20.4 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 129 mM NaCl, 1.5 mM KCl, 14 mM glucose, 1 mM $CaCl_2$) in a single perfusion at a rate of 0.2 ml/min to collect intestinal effluents serially. Methotrexate was dissolved at a concentration of 3.61 or 2.34 μmol/ml in saline containing 5% mannitol to increase the urine flow. This solution was injected intravenously at a dose of 3.61 μmol/ml/kg, followed by constant infusion at a rate of 4.68 μmol/2 ml/h via a cannula inserted into a femoral vein. After a steady-state plasma concentration of methotrexate was achieved (approximately 1 h after the initiation of methotrexate infusion), saline was injected intravenously via the tail vein at a volume of 0.5 ml/kg as the control study. In the inhibition studies, on the other hand, either probenecid or bilirubin was injected intravenously at a dose of 175.2 μmol/0.5 ml/kg or 85.5 μmol/0.5 ml/kg, respectively, instead of saline. After a 10-min stabilization, the intestinal effluent, bile and urine were collected every 10 min, and blood was collected at an intermediate time point between each biological fluid collection. In our previous study, treatments with probenecid and bilirubin 10 min before CDNB administration were found to suppress the intestinal DNP-SG efflux almost completely.[19,23] At the end of this study (100 min after initial methotrexate dosing), rats were exsanguinated by decapitation, a sufficient amount of ice-cold saline was injected into the portal vein to remove blood and the following 11 tissues were isolated: brain, heart, lung, liver, kidney, jejunum, ileum, colon, spleen, testis and skeletal muscle.

In-situ intestinal absorption of methotrexate

The effects of bilirubin and probenecid treatments on intestinal absorption of methotrexate were evaluated by the *in-situ* jejunum loop method. Briefly, anesthetized rats were cannulated with PE tubing (PE-50) at a femoral vein for the administration of probenecid or bilirubin. The luminal content of the jejunum was flushed with approximately 20 ml of saline prewarmed at 37°C. A 20-cm closed jejunum loop was made in the jejunum by ligating both ends of the loop. Probenecid (175.2 μmol/kg) or bilirubin (85.5 μmol/kg) was administered intravenously via a cannula inserted at a femoral vein, and 10 min later methotrexate (25 nmol/kg) was administered into the closed jejunum loop. At 60 min after administration of methotrexate, the amounts of methotrexate remaining in the jejunum loop, including the luminal fluid, were determined. The mucosal membrane of the jejunum loop was collected by scraping with a cover glass.

Evaluation of methotrexate-induced intestinal toxicity

Rats were fasted overnight, anesthetized with pentobarbital (30 mg/kg, i.p. injection) and affixed supine on a surface kept

at 37°C to maintain their body temperature around at 36°C. The luminal content of proximal intestine (a 20-cm long segment from 5 cm below the bile duct opening) was flushed with pre-warmed saline at 37°C and the lumen was perfused in a recirculating manner at a flow rate of 0.2 ml/min with pH 6.5 phosphate buffered saline, with or without methotrexate (25 μM). In the inhibition studies, instead of saline, either probenecid or bilirubin was injected intravenously, 10 min before the initiation of perfusion, at a dose of 175.2 μmol/0.5 ml/kg or 85.5 μmol/0.5 ml/kg, respectively. The intestinal perfusate was collected up to 60 min, and the intestinal mucosal membrane at the jejunum was collected by scraping with a cover glass at the end of this study (60 min after initiation of the perfusion study). The collected intestinal mucosa was homogenized in an appropriate volume of buffer solution consisting of 150 mM mannitol, 6 mM Tris-HCl (pH 7.1), 2.5 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid and 0.05 mM phenylmethylsulfonyl fluoride, using a Process Homogenizer PH91 (SMT Company, Tokyo, Japan). These analytical procedures were performed on ice, and the homogenate preparations were used immediately after preparation or stored in liquid nitrogen until use. Protein concentrations in homogenates were measured by the Bradford method, using bovine serum albumin as the standard.[28] The specific activity of ALP in both luminal fluids (intestinal perfusate) and membrane homogenates was measured in a manner reported previously.[29,30]

Sample analysis

The biological fluid samples containing methotrexate were deproteinized with 20% perchloric acid, in which the final concentration of perchloric acid in samples was more than 4%. Isolated tissues were homogenized in a 2- to 9-fold volume of 4% perchloric acid. All these deproteinized samples were kept on ice at least for 30 min, and centrifuged at 3000*g* for 10 min. The concentration of methotrexate was determined by HPLC using a column of Mightysil RP-18 (Kanto Kagaku, Tokyo, Japan), in a manner reported previously.[15] Briefly, the mobile phase used was a mixture of acetonitrile, methanol and 1% acetic acid in a ratio of 5 : 10 : 85 (v/v), and the flow rate of the mobile phase was 1 ml/min. Detection was made at a wavelength of 304 nm. The detection limit of methotrexate under the present analytical conditions was approximately 10 nM.

Total plasma clearance (CL_{total}, ml/min) of methotrexate was estimated by dividing the constant-infusion rate of methotrexate (4.68 μmol/h) by the steady-state plasma concentration (C_{pss}) of methotrexate. Clearances of biliary excretion (CL_{bile}, ml/min), urinary excretion (CL_{urine}, μl/min) and intestinal exsorption (CL_{exp}, μl/min) were estimated by dividing each excretion rate with C_{pss} of methotrexate, respectively. To evaluate the activity of efflux transporter-mediated efflux in biliary excretion, urinary excretion and intestinal efflux (jejunum), the intrinsic clearances ($*CL_{bile}$, $*CL_{urine}$ and $*CL_{exp}$) of methotrexate were also estimated by dividing the excretion rates of methotrexate into the bile, urine and intestinal perfusate by the concentration of methotrexate in the liver, kidney and jejunum, respectively. These intrinsic clearance values were normalized with the body weight (kg) of each rat.

Statistical analysis

Differences among group mean values were assessed by Kruskal–Wallis or ANOVA tests followed by post-hoc test (Dunn's test) or Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

Results

Effects of probenecid and bilirubin treatments on methotrexate clearance under steady states

The effects of probenecid and bilirubin treatment on methotrexate clearance were evaluated under a steady-state plasma concentration of methotrexate in rats (Table 1). Methotrexate was mostly excreted into the bile and urine in an unchanged form, and the contributions of CL_{bile} and CL_{urine} to CL_{total} were 54 and 40%, respectively, in the untreated control rats.

Table 1 Effects of probenecid and bilirubin treatments on *in-vivo* methotrexate clearance under a steady-state plasma concentration of methotrexate in rats

	Control	+Probenecid	+Bilirubin
C_{pss} (μM)	24.4 ± 0.8	46.2 ± 1.7*	40.3 ± 2.7*
CL_{total} (ml/min/kg)	12.2 ± 0.4	6.1 ± 0.1*	7.7 ± 0.7*
CL_{bile} (ml/min/kg)	6.6 ± 0.1	1.7 ± 0.1*	2.1 ± 0.2*
CL_{urine} (ml/min/kg)	4.9 ± 0.3	3.6 ± 0.2*	4.1 ± 0.3*
CL_{exp} (μl/min/kg)	20.5 ± 1.1	1.5 ± 0.1*	8.8 ± 1.4*
Concn. in liver (nmol/g tissue)	22.7 ± 1.9	15.7 ± 2.2	21.7 ± 1.3
$*CL_{bile}$ (ml/min/kg)	10.1 ± 1.1	5.0 ± 0.4*	3.0 ± 0.2*
Concn. in kidney (nmol/g tissue)	40.4 ± 4.2	30.9 ± 3.8	58.6 ± 3.6**
$*CL_{urine}$ (ml/min/kg)	3.0 ± 0.2	5.6 ± 0.7*	1.9 ± 0.1
Concn. in jejunum (nmol/g tissue)	1.7 ± 0.0	3.0 ± 0.2**	3.5 ± 0.4**
$*CL_{exp}$ (μl/min/kg)	300.4 ± 33.3	25.9 ± 4.7*	94.7 ± 15.5*

Methotrexate was administered at a dose of 3.6 μmol/kg by intravenous bolus injection, followed by a constant rate infusion (4.7 μmol/h) to settle C_{pss} of methotrexate in rats. CL and*CL of methotrexate were estimated by dividing each excretion rate with C_{pss} and tissue methotrexate concentration, respectively. Probenecid and bilirubin were administered intravenously at a dose of 175.2 and 85.5 μmol/kg 10 min before collecting each biological sample, respectively. Each value represents the mean ± SE of results from four rats. $**P < 0.01$, $*P < 0.05$: significantly different from the value for control.

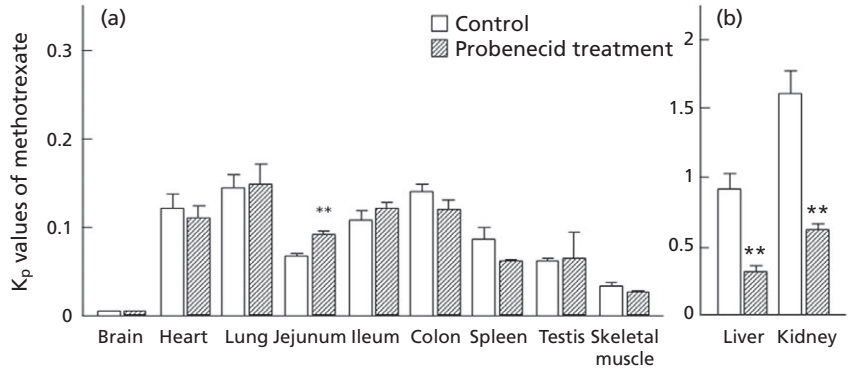

Figure 1 Effect of probenecid treatment on tissue accumulation of methotrexate under a steady-state plasma concentration of methotrexate in rats. Methotrexate was administered at a dose of 3.6 μmol/kg by intravenous bolus injection, followed by a constant rate infusion (4.7 μmol/h) in rats. Probenecid was administered intravenously at a dose of 175.2 μmol/kg 10 min before collecting each biological sample. Each value represents the mean ± SE of results from four rats. $^{**}P < 0.01$: significantly different from the value for control.

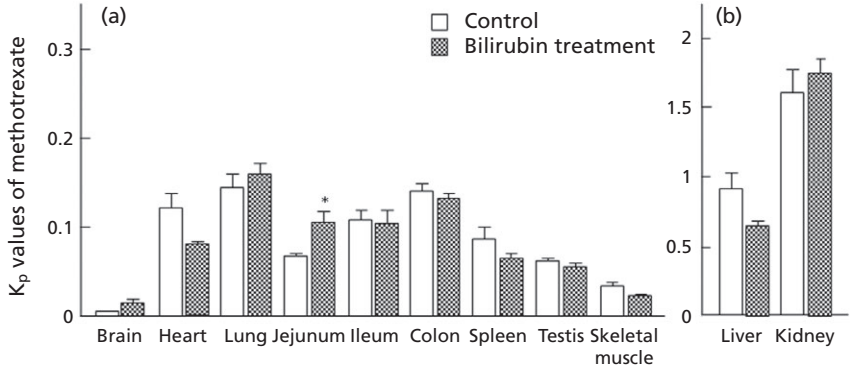

Figure 2 Effect of bilirubin treatment on tissue accumulation of methotrexate under a steady-state plasma concentration of methotrexate in rats. Methotrexate was administered at a dose of 3.6 μmol/kg by intravenous bolus injection, followed by a constant rate infusion (4.7 μmol/h) in rats. Bilirubin was administered intravenously at a dose of 85.5 μmol/kg 10 min before collecting each biological sample. Each value represents the mean ± SE of results from four rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from the value for control.

Intestinal exsorption of methotrexate was also observed, although the contribution of CL_{exp} to CL_{total} was quite small (0.084% of total clearance per 20-cm long jejunum). Treatment with probenecid increased C_{pss} of methotrexate approximately 1.9-fold over the control rats, and decreased CL_{bile} by 74%, CL_{urine} by 27% and CL_{exp} by 93% compared to control, respectively. In bilirubin-treated rats, the plasma concentrations of conjugated bilirubin were more than 70 μM as reported previously,[19] and clearance of methotrexate was decreased to almost the same extent with probenecid treatment. The concentration of methotrexate in the jejunum membrane was significantly higher in both probenecid- and bilirubin-treated rats, whereas concentrations of methotrexate in the kidney were different between bilirubin and probenecid-treated rats. To normalize the different tissue concentrations of methotrexate between probenecid- and bilirubin-treated rats, the intrinsic clearances of methotrexate in the liver, kidney and jejunum ($^{*}CL_{bile,}$ $^{*}CL_{urine}$ and $^{*}CL_{exp}$) were also estimated by dividing the excretion rates of methotrexate by tissue methotrexate concentrations (Table 1). The decreased $^{*}CL_{bile,}$ and $^{*}CL_{exp}$ values of methotrexate indicate that the function of efflux transporters for methotrexate in the liver and intestine is greatly suppressed in bilirubin-treated rats, as it is in probenecid-treated rats.

Effects of probenecid and bilirubin treatments on tissue accumulation of methotrexate under steady state

The effects of probenecid and bilirubin treatments on the tissue accumulation of methotrexate were examined in the following 11 tissues: brain, heart, lung, liver, kidney, jejunum, ileum, colon, spleen, testis and skeletal muscle. This was done by estimating the tissue-to-plasma partition coefficients (K_p values) of methotrexate at the steady state (Figures 1 and 2). In probenecid-treated rats, the K_p value of methotrexate for the jejunum increased approximately 1.4-fold over the control, whereas the K_p values for the liver and kidneys decreased by approximately 65% of control. In bilirubin-treated rats, the K_p value of methotrexate for the jejunum also increased significantly, although the K_p values for the liver and kidneys remained unchanged.

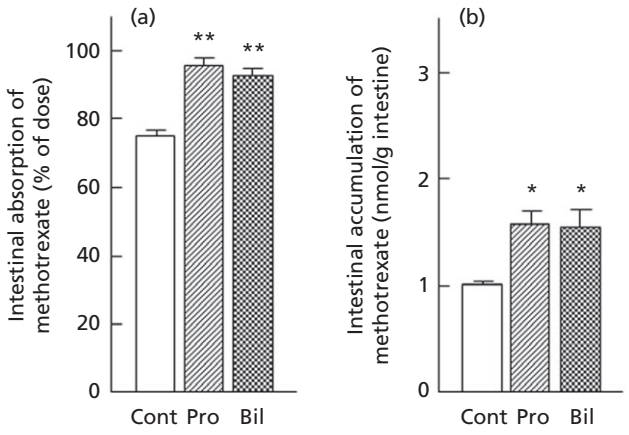

Figure 3 Effects of probenecid and bilirubin treatments on intestinal absorption (a) and accumulation (b) of methotrexate administered to jejunum loop at 60 min in rats. Methotrexate was administered into 20-cm long jejunum loop at a dose of 25 nmol/kg. Probenecid (Pro) was administered intravenously at a dose of 175.2 μmol/kg 10 min before collecting each biological sample. Bilirubin (Bil) was administered intravenously at a dose of 85.5 μmol/kg 10 min before collecting each biological sample. Each value represents the mean ± SE of results from four rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from the value for control. Cont, control.

Effects of probenecid and bilirubin treatments on intestinal absorption of methotrexate

The effects of probenecid and bilirubin treatments on intestinal absorption of methotrexate were examined by measuring the disappearance amount of methotrexate from the intestinal loop after administration into the jejunum loop (Figure 3). In untreated control rats, the disappeared amount, or absorbed amount, of methotrexate was approximately 75% of the dose 60 min after administration (Figure 3a). In bilirubin-treated rats, the intestinal absorption of methotrexate significantly increased to the same levels as those in probenecid-treated rats. In addition, treatment with both probenecid and bilirubin increased the accumulation of methotrexate in the jejunum mucosal tissue approximately 1.5-fold compared to the control (Figure 3b).

Effects of probenecid and bilirubin treatments on methotrexate-induced intestinal toxicity

The leakage of ALP activity into the jejunum perfusate and remaining ALP activity in the jejunum mucosa were determined to evaluate the effect of probenecid and bilirubin treatments on methotrexate-induced intestinal toxicity (Figure 4). In this study, bilirubin treatment alone, without administering methotrexate, slightly but significantly increased the leakage of ALP activity into the jejunum perfusate. A greater leakage of ALP activity into the jejunum perfusate after administration of methotrexate was observed in probenecid- and bilirubin-treated rats than in untreated control rats (Figure 4a). In accordance with this observation, the ALP activity remaining in the jejunum mucosa was significantly lower in probenecid- and bilirubin-treated rats (Figure 4b). These results indicate that treatment with bilirubin significantly increases methotrexate-induced intestinal toxicity, as does treatment with probenecid.

Discussion

In the present study, the effect of an intravenous bolus injection of bilirubin on the pharmacokinetics and intestinal toxicity of methotrexate was evaluated in rats, since bilirubin treatment is suspected of suppressing Mrp-mediated transport of methotrexate.[10–16] Probenecid treatment was also examined. Probenecid is an inhibitor of multiple organic anion transporters such as Mrps, Oatps and Oats.[31–33] Bilirubin is known as a substrate for Oatps and bilirubin glucuronides are reportedly substrates and inhibitors for Oatp1a1 and Mrp2.[20–22] Hyperbilirubinemia accompanied by obstructive jaundice is frequently observed under various disease states such as hepatoma, liver graft and cholelithiasis – the plasma concentrations of unconjugated/conjugated bilirubin often reach more than 100 μM.[24–27] Recently, we observed that bilirubin treatment significantly suppresses the jejunum efflux, biliary excretion and urinary excretion of DNP-SG, a typical Mrp substrate, and significantly increases the tissue concentrations of DNP-SG in the brain, liver, jejunum and skeletal muscle.[19,23] In a preliminary study, at 60 min after intravenous administration of bilirubin (86.5 μmol/kg), concentrations of conjugated bilirubin were 70 nmol/ml, 160 nmol/g and 20 nmol/g in the plasma, liver and jejunum, respectively. The Michaelis constant (K_m value) of bilirubin monoglucuronide in Mrp2-mediated transport is reported to be 0.8 μM, and that of bilirubin bisglucuronide is 0.5 μM.[34] The plasma protein binding of conjugated bilirubin reportedly is 4.0%.[35] This consideration suggests that high plasma concentrations of conjugated bilirubin, which are observed under various disease states accompanied by hyperbilirubinemia, may suppress Mrp2 function systemically.

In the present study, the effect of bilirubin treatment on clearance and tissue distribution of methotrexate was first examined under a steady-state plasma concentration of methotrexate. As shown in Table 1, bilirubin treatment significantly suppresses $^{*}CL_{bile}$ and $^{*}CL_{exp}$ of methotrexate, as does probenecid treatment, indicating that the efflux transport of methotrexate from the intracellular compartment to the bile duct and intestinal lumen, respectively, are suppressed in bilirubin- and probenecid-treated rats. In accordance with this observation, the intestinal accumulation of methotrexate in bilirubin-treated rats increased approximately 1.5-fold over that in untreated control rats. The effect was similar in probenecid-treated rats. The decreased intrinsic clearances and increased intestinal accumulation of methotrexate in bilirubin- and probenecid-treated rats may be mainly due to the suppression of Mrp2-mediated efflux transport of methotrexate by bilirubin glucuronide(s) formed by UGT1A1 in the tissue.[20,36–38] In this study, a clear regional difference was observed between the jejunum and ileum in the effects of bilirubin and probenecid treatments, that is, the accumulation of methotrexate increased in the jejunum, but not in the ileum. In our previous studies, the expression and function of Mrp2 in the brush-border membrane of enterocytes was high in the jejunum but low in the ileum. In contrast, the expression and function of Mrp3 in the basolateral membrane of the ileum was high, but low in the jejunum.[39] Thus, the jejunum, with higher levels of Mrp2, was subject to a greater suppressive effect on treatment with bilirubin, although further study is

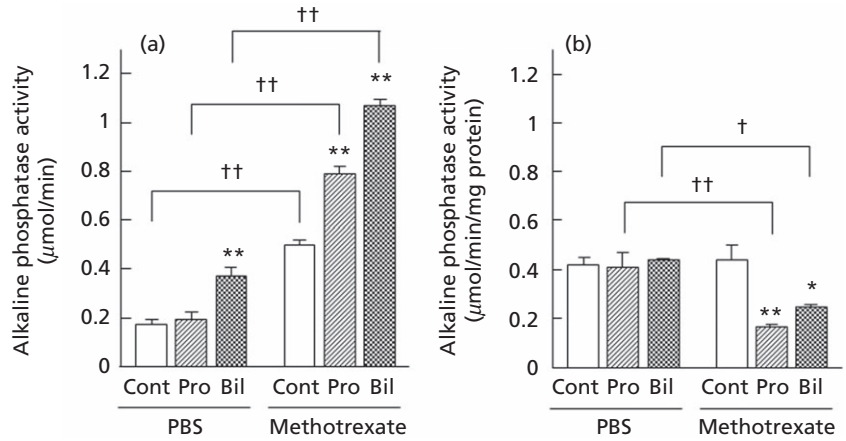

Figure 4 Effects of probenecid and bilirubin treatments on methotrexate-induced leakage of alkaline phosphatase activity into the intestinal perfusate (a) and remained activity in the mucosal membranes (b) in rats. Methotrexate was perfused in a re-circulating manner at a flow rate of 0.2 ml/min with pH 6.5 phosphate buffered saline with or without methotrexate (25 μM). Probenecid (Pro) or bilirubin (Bil) was administered intravenously at a dose of 175.2 μmol/kg or 85.5 μmol/kg 10 min before collecting each biological sample, respectively. Each value represents the mean ± SE of results from four rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from the value for control. $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$: significantly different from the value for control rats perfused with phosphate buffered saline (PBS). Cont. Control.

necessary. In addition, there was a marked discrepancy in the effects of probenecid and bilirubin treatments on the K_p values of methotrexate in the kidney and liver. The K_p value in bilirubin-treated rats remained unchanged, but probenecid-treated rats showed significantly lower K_p values for methotrexate in both kidney and liver (Figures 1 and 2, Table 1). In the kidney of rats, Oat1 and Oat3, which transport methotrexate in a bidirectional manner, are expressed in the basolateral membrane of renal proximal tubule cells, and Oatp1a1, Oatp1a3 (OAT-K1 and OAT-K2) and Oat2, in addition to Mrp2 and Mrp4, are expressed in the brush-border membranes.[11,40–42] In other words, probenecid would suppress both the influx and efflux of methotrexate in the kidney. On the one hand, bilirubin treatment is considered to mainly suppress the efflux of methotrexate, although the plasma concentrations of unconjugated/conjugated bilirubin and their inhibitory concentration (K_i value) on each transporter should also be taken into consideration in their inhibitory effects. Similarly, various influx and efflux transporters, including Mrps, Oatps and Oats, are expressed in rat hepatocytes. Thus, to differentiate the effects of unconjugated/conjugated bilirubin and probenecid on each influx and efflux transporter in the liver and kidney, further detailed experiments are necessary.

Oral administration of methotrexate is known to induce intestinal toxicity, leading to shortened villi and/or dysfunction, and resulting in changes in the absorptive and biochemical functions of the small intestine.[4,5] Regarding this methotrexate-induced intestinal toxicity, the contribution of Mrps in lowering intestinal toxicity has been suggested. Naba *et al*. reported that the methotrexate-induced toxicities, such as changes in bone marrow, spleen and intestines, which are caused by multiple dosing, are more severe in EHBRs lacking Mrp2 hereditarily than in normal rats.[18] Also, Kato *et al*. reported that higher accumulations of methotrexate in the intestinal proliferative cells, where Mrp1 is normally highly expressed, cause more severe gastrointestinal toxicity in *mrp1*$^{(-/-)}$ mice than in normal *mrp1*$^{(+/+)}$ mice.[43] In the present study, the intestinal toxicity induced by methotrexate was significantly higher in bilirubin- and probenecid-treated rats than in control rats, suggesting that Mrps expressed in the intestinal membrane can principally alleviate methotrexate-induced cytotoxicity (Figure 4).

Conclusion

In the present study, we evaluated the effect of bilirubin treatment on the pharmacokinetics of methotrexate and on methotrexate-induced intestinal toxicity in rats. Treatment with bilirubin, or high plasma concentrations of unconjugated/conjugated bilirubin, significantly decreased various clearances of methotrexate and increased tissue accumulation into the jejunum. In addition, bilirubin treatment significantly increased methotrexate-induced intestinal toxicity, possibly due to the suppression of Mrp function by bilirubin glucuronide(s). Hyperbilirubinemia accompanied by obstructive jaundice is caused by various disease states. Thus, special attention should be given to pharmacotherapy with MRP substrate drugs in hyperbilirubinemic patients, since MRP function can be suppressed systemically under hyperbilirubinemia.

Declarations

Conflict of interest

This Author(s) declare(s) that they have no conflicts of interest to disclose.

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