Alteration of Methotrexate Biliary and Renal Elimination during Extrahepatic and Intrahepatic Cholestasis in Rats

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Methotrexate (MTX), an important anticancer and immunosuppressive agent, has been suggested for the treatment of primary biliary cirrhosis. However, the drug’s pharmacodynamics and toxicity is dependent on its concentrations in plasma which in turn are directly related to MTX’s elimination in the liver and kidney. Therefore, the aim of this study was to evaluate changes in MTX biliary and renal excretion during either intrahepatic or obstructive cholestasis in rats. The steady state pharmacokinetic parameters of MTX were evaluated in rats one (BDO1) or seven (BDO7) days after bile duct obstruction (BDO) or 18 h after administration of lipopolysaccharide (LPS). In comparison to the respective control groups, biliary and total clearances of MTX were decreased to 12% and 49% in the BDO1 group, to 5% and 56% in the BDO7 animals, and to 42% and 43% in the LPS group, respectively. Renal clearance of MTX was unchanged in BDO groups, but decreased to 23% of controls in the LPS animals. The serum biochemistry and expression of main hepatic MTX transporters (Mrp2, Mrp3, Mrp4, Bcrp, Oatpl1a1, Oatpl1a4 and Oatplb2) confirmed the pathological cholestatic changes in the liver and partly elucidated the cause of changes in MTX pharmacokinetic parameters. In conclusion, this study is the first describing marked alteration of MTX hepatic and renal elimination induced by cholestasis in rats. Moreover, the reported changes in MTX pharmacokinetics and respective transporter expression suggest important mechanistic differences between the two widely used cholestatic models.

Key words methotrexate; cholestasis; pharmacokinetics; conjugated bilirubin; drug transporter

Cholestasis is a reduction in bile flow, which may result either from the failure of the secretory transport in hepatocytes or ductular cells (intrahepatic cholestasis), or from blockage of the biliary tract outside the liver (extrahepatic cholestasis). The consequence is hepatic and systemic accumulation of potentially toxic biliary compounds such as bilirubin, which is associated with progressive liver damage and jaundice. In an effort to limit hepatic impairment produced by intracellular accumulation of these anionic compounds, spontaneous anticholestatic defense mechanisms develop in hepatocytes. They comprise down-regulation of sinusoidal uptake transporters (Ntcp—sodium taurocholate cotransporting polypeptide, Oatps—organic anion transporting polypeptides) and canalicular efflux transporters (Mrp2—multidrug resistance-associated protein 2, Bcrp—breast cancer resistance protein) together with induction of basolateral export pumps (e.g. Mrp3 and Mrp4—multidrug resistance-associated protein 3 and 4). During extrahepatic cholestasis, the compensatory mechanism also includes changes in the kidneys, where efflux transporters (e.g. Mrp2) for the organic anions are up-regulated, and thus facilitate the excretion of toxic substrates into urine. However, these adaptive changes are too weak to prevent ongoing liver injury.

Lipopolysaccharide (LPS)-induced cholestasis is associated with changes in the expression of hepatic transporters in the similar manner as during extrahepatic cholestasis. These changes are thought to be mediated by proinflammatory cytokines, such as tumor necrosis factor α, interleukin 1β and interleukin 6 produced by Kupffer cells, and formation of nitric oxide in Kupffer cells and hepatocytes, as a result of LPS-induced nuclear factor (NF)-κB activation. Hepatocyte responds to these cytokines by the alteration of gene expression, mainly at the transcriptional level.

Moreover, serious liver impairment produced either by acute toxic insult (intrahepatic endotoxin-induced cholestasis) or long-term severe cholestatic damage, may also be associated with alteration of renal elimination, an effect ascribed to the reduced glomerular filtration and impairment of proximal tubular cells. Hence, pharmacokinetics of compounds that are substrates for the abovementioned transporters could be altered during cholestasis by several mechanisms. This is especially important for drugs which are considered for the treatment of cholestatic diseases. In such cases, changes in the pharmacokinetics of the drug could be partly predicted from the results of the detailed studies of transporter expression in the liver and kidneys and from in vitro studies in isolated cells. Nevertheless, the existence of other mechanisms involved in the pathophysiology of cholestasis such as paracellular leakage through an impaired blood–biliary barrier (tight-junction connection of hepatocytes) or decreased perfusion of organs makes such predictions less accurate and raises the need for data from in vivo kinetic studies.

Recently methotrexate (MTX), a folic acid antagonist, has been used for the treatment of primary biliary cirrhosis, the cholestatic condition characterized by pathophysiological changes similar to those described in experimental models of cholestasis. Moreover, widespread use of the agent in cytostatic or immunosuppressive therapy also increases the probability of its administration in other types of cholestatic diseases. Importantly, elimination of MTX from the organism is significantly influenced by the activity of several transport proteins in the liver, expression of which is profoundly altered by cholestasis (e.g. organic anion transporting...
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polypeptide 1a1 (Oatp1a1), Scl0a1; Organic anion transporting polypeptide 1a4 (Oatp1a4), Scl0a4; Organic anion transporting polypeptide 1b2 (Oatp1b2); Oatp1b2, Scl0b2; members of the multidrug resistance-associated protein subfamily Mrp2, Mrp3, Mrp4, (Abcc2, Abcc3, Abcc4); and Breast cancer resistance protein—Bcrp, Abcg2). As the outcome of MTX therapy is highly determined by its pharmacokinetics, an increase in its disposition is associated with the symptoms of drug toxicity. This is commonly seen with impaired MTX elimination as the consequence of the drug–drug interaction produced by inhibition of involved transport proteins at the basolateral and apical membranes of hepatocytes and proximal tubular cells. 

The aim of the present study was to describe changes in the pharmacokinetics of MTX during cholestasis in rats. A detailed clearance study of MTX was performed using the in vivo rat model of both intrahepatic (lipopolysaccharide-induced) and extrahepatic (bile duct obstruction-induced; acute and chronic) cholestasis with special focus paid to biliary excretion. In parallel, the kinetics of endogenous conjugated bilirubin was evaluated in the same animals to further extend the information about disposition of this organic anion in these two models of cholestasis in rats. Verification of cholestatic liver impairment was performed by serum biochemistry and analysis of mRNA and protein expression of the principal anionic drug uptake and efflux transporters which are at the same time main transporters for MTX in the liver. 

MATERIALS AND METHODS

Materials Methotrexate was purchased from Ebewe Pharma (Ebewe Pharma GmbH. Nfg. KG, Austria). Mouse monoclonal antibodies M_{II}15 and BXP-21, directed to the Mrp2 (170—180 kDa) and Bcrp (70 kDa) were obtained from Signet Laboratories, Inc. (Dedham, MA, U.S.A.). Rabbit polyclonal antibodies anti-Oatp1a1 and anti-Oatp1a1 and anti-Mrp4, directed to the Oatp1a4 (75 kDa), Oatp1a1 (80 kDa) and Mrp4 (150 kDa) were obtained from Millipore (Billerica, MA, U.S.A.) and Mrp4 from Abcam plc (Cambridge, U.K.). Mouse monoclonal antibody M_{II}1-21 directed to Mrp3 (180—190 kDa) was obtained from Alexis Co. (Lausen, Switzerland). Goat polyclonal antibody anti-Oatp1b2 directed to Oatp1b2 (85 kDa) was purchased from Santa Cruz Biotechnology, Inc. (CA, U.S.A.). As the loading control for Western blot, rabbit polyclonal anti-β-actin antibody (42—45 kDa) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Horseradish peroxidase-linked sheep anti-mouse and donkey anti-rabbit immunoglobulin G were purchased from GE Healthcare (Prague, Czech Republic) and horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G was obtained from Pierce Biotechnology (Rockford, U.S.A.). All other reagents and supplies were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Electrophoresis GmbH (Heidelberg, Germany), respectively, and were of the highest purity available.

Animal Model Male Wistar rats (n=6 in each group) weighing 280 to 330 g (Konarowice, Czech Republic) were used throughout the study. In these animals, extrahepatic cholestasis was induced by bile duct obstruction (BDO) under general anesthesia (pentobarbital sodium 50 mg/kg body weight intraperitoneally (i.p.)). The cannula was inserted into the bile duct, closed and fixed subcutaneously. Obstruction lasted for either one (BDO1) or seven days (BDO7). Intrahepatic cholestasis was induced by the administration of endotoxin (lipopolysaccharide isolated from Salmonella Typhimurium; 4 mg/kg i.p.) 18 h before the experiment. Control rats were either sham-operated (the bile duct was only manipulated and left unobstructed) or injected i.p. with physiological saline (LPS-control). Rats were housed under controlled environmental conditions (12-h light-dark cycle; temperature, 22±2°C) with a freely available commercial food diet and water. The study protocol was approved by the animal welfare committee of the Charles University in Prague, Faculty of Medicine in Hradec Kralove.

In Vivo Clearance Study Under the anesthesia induced by pentobarbital (50 mg/kg, i.p.), the bile duct was either cannulated (sham-operated and LPS/LPS-control animals) or its obstruction was removed (BDO animals) by cutting the free tip of the biliary cannula, and bile was consequently collected at 10 min intervals. In addition, all rats were cannulated with polyethylene tubes in the right jugular vein for drug administration, the left carotid artery for blood sampling, and the urinary bladder for urine collection. Body temperature was maintained at 37°C with a heated platform.

To elucidate the effect of cholestasis on clearance parameters of MTX, all rats (both control and cholestatic animals) were infused with methotrexate solution (0.65 mM in 2.5% mannitol) into the right jugular vein as a bolus of 10 mg/kg, followed by constant infusion at a rate of 15 ml/h·kg⁻¹ (Perfusor Compact; Braun, Prague, Czech Republic) to attain a steady-state plasma concentration (Cₜₛ). After Cₜₛ of MTX was achieved (60 min after administration of MTX infusion), bile and urine samples were collected at 10 and 20 min intervals, respectively, over 1 h. Blood samples were taken at the midpoint of the bile and urine collection periods. Plasma samples were obtained by centrifugation of the blood samples at 3000 g for 5 min. The volume of bile and urine samples was measured gravimetrically, with specific gravity assumed to be 1.0. All plasma, bile and urine samples were stored at −80°C until analysis. At the end of the experiments, animals were exsanguinated and the liver and kidneys were immediately removed, frozen by liquid nitrogen and stored at −80°C until analysis.

Analytical Procedures The concentrations of MTX in plasma, urine and bile were determined by high performance liquid chromatography (HPLC) after deproteination of samples according to a previously described method. Briefly, the instrument was an Agilent 1100 series (Agilent, Palo Alto, CA, U.S.A.) chromatograph provided with a fluorescence detector (excitation, 350 nm; emission, 430 nm). Separation was achieved at 30°C using a Gemini C₁₈, 110 Å, 4.6×150 mm column and a Gemini C₁₈, 4×3 mm pre-column (Phenomenex, Torrance, CA, U.S.A.). The mobile phase flowing at the rate of 0.6 ml/min was ammonium acetate and acetonitrile (87%:13%, v/v). The concentrations of bilirubin and creatinine in plasma, bile and urine were measured on Cobas Integra® 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

Pharmacokinetic Analysis Pharmacokinetic parameters—total body clearance (CL_{tot}), biliary clearance (CL_{bile})
and renal clearance \( (CL_R) \) of MTX and conjugated bilirubin—were calculated by the following equations:

\[
\begin{align*}
CL_{\text{tot}} &= I/C_{ss} \\
CL_{\text{bile}} &= R_{\text{bile}}/C_{ss} \\
CL_R &= R_R/C_{ss}
\end{align*}
\]

where \( I \) is the infusion rate (\( \text{nmol/\text{min} \cdot \text{kg}^{-1} \)), \( C_{ss} \) is the plasma concentration at steady state (\( \text{C_{ss}} \) of methotrexate—60—120 min after the initiation of MTX infusion—\( \mu \text{M} \)), \( R_{\text{bile}} \) is the biliary excretion rate at steady state (\( \text{nmol/\text{min} \cdot \text{kg}^{-1} \)) and \( R_R \) is the renal excretion rate at steady state. The glomerular filtration ratio (GFR) was evaluated as the clearance of endogenous creatinine (\( CL_{\text{CRI}} \)). The renal clearance ratio of methotrexate was calculated as \( (CL_{R}/f_u) \cdot CL_{\text{CRI}}^{-1} \), where \( f_u \) represents the unbound fraction of the drug. Each parameter was calculated using the mean value of three points during 60 min of steady state.

**Real Time RT-PCR Analysis**  
Total RNA was isolated from liver and kidney tissue samples using TRIzol reagent (Invitrogen, U.S.A.) and converted into cDNA via an iScript reverse transcription kit (Bio-Rad Laboratoires, Hercules, U.S.A.). 10 ng cDNA were loaded into one reaction, all processed in triplicate. The amplification was completed using the TaqMan® Fast Universal PCR Master Mix, pre-designed TaqMan® Gene Expression Assay kit for the following genes: Mtp2 (Abcc2, Rn00563231_m1), Bcrp (Abcg2, Rn00710585_m1), Mrp3 (Abcc3, Rn00589786_m1), Mrp4 (Abcc4, Rn01465702_m1), Oatp1a1 (Slco1a1, Rn00755148_m1), Oatp1a4 (Slco1a4, Rn00756233_m1), Oatp1b2 (Slco1b2, Rn06686231_m1), and GAPDH rat endogenous control kit (4352338E) all provided by Applied Biosystems (Foster City, U.S.A.). The time—temperature profile used in the “fast” mode was: 95 °C for 20 s; 40 cycles: 95 °C for 3 s, 60 °C for 30 s. The relative expression ratio was then calculated according to Pfaffl, 2001:

\[
R = 2^{\Delta \Delta C_{\text{target}}}
\]

where \( E_{\text{target}} \) and \( E_{\text{housekeeping}} \) are the effectivity values determined from the calibration curve slopes for each gene, and \( \Delta C_{\text{target}} \) and \( \Delta C_{\text{housekeeping}} \) are the differences in threshold values (\( C_t \)) between control and cholestatic samples for each of the targets, \( i.e. \) Mtp2, Bcrp, Mrp3, Mrp4, Oatp1a1, Oatp1a4, Oatp1b2 and the housekeeping (GAPDH) gene.

**Membrane Preparation**  
Kidneys and livers were minced in ice-cold Tris–sucrose buffer (10 mmol/l Tris–HCl, 250 mmol/l sucrose, pH 7.6), containing 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 2 µg/ml aprotinin, 50 µg/ml benzamidine, and 40 µg/ml phenylmethylsulfonyl fluoride (PMSF) and then homogenized with a motor-driven teflon homogenizer (Braun Melsungen, Germany) operating at 1500 rpm. A membrane-enriched microsomal pellet was obtained from the postnuclear supernatant after a 100000 × g ultracentrifugation at 4 °C for 23 min. The pellet was resuspended in Tris–HCl buffer (50 mmol/l Tris–HCl, pH 7.4, protease inhibitors). The protein concentration was determined with the BCA assay (Pierce, Rockford, IL, U.S.A.) and samples were stored at −80 °C.

**Immunoblot Analysis**  
Crude membrane-containing homogenates (100 µg of liver or 50 µg of kidney proteins) from livers and kidneys were incubated with sample buffer at room temperature for 30 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 6.25% polyacrylamide gels. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.), the membrane was blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was then incubated with primary antibodies at the following concentrations: M2II6, M3II-21 and BXP-21 at 1:500; anti-Oatp1a1 and anti-Oatp1a4 at 1:5000, horseradish peroxidase-linked sheep anti-mouse antibody at 1:1000 or 1:500, donkey anti-rabbit IgG antibody at 1:5000 and rabbit anti-goat at 1:1000. After washing with TBST buffer, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare, Prague, Czech Republic) and subjected to autoluminography for 1—5 min. The immunoreactive bands on the exposed films were scanned with a densitometer ScanMaker i900 (UMAX, Prague, Czech Republic) and semiquantified using the QuantityOne imaging software (Bio-Rad). Equal loading of proteins onto the gel was confirmed by immunodetection of beta-actin.

**Statistical Analysis**  
Experiments were carried out in 6 animals per group. All experimental data are expressed as mean±S.E.M. Statistical significance was examined in groups of control and cholestatic animals with unpaired t-test using Graphpad Prism 5.0 software (Graphpad Software, Inc., San Diego, California, U.S.A.). A difference of \( p<0.05 \) was considered statistically significant.

**RESULTS**

**Effect of Cholestasis on in Vivo Steady-State Pharmacokinetics of Methotrexate**  
The effect of extrahepatic (1 or 7 d after BDO) and intrahepatic (18 h after administration of LPS) cholestasis on the steady-state pharmacokinetics of MTX is presented in Table 1. Time course of MTX plasma concentrations, biliary excretion rate and urinary excretion rate are shown in Fig. 1. Biliary and urinary flow were not significantly different between experimental and control groups. Cholestasis produced rise in MTX plasma \( C_{ss} \) (Fig. 1A) to 220%, 201% and 247% in the BDO1, BDO7, and LPS group, respectively. Analysis of elimination organ kinetics showed marked decrease in the biliary excretion rate of MTX to 19% and 8% of control values 1 and 7 d after BDO, respectively, but no change was observed in LPS animals (Fig. 1B). Nevertheless, calculation of MTX biliary clearance demonstrated its reduction in all cholestatic groups to 12%, 5% and 42% in BDO1, BDO7, and LPS animals, respectively. MTX urinary excretion (Fig. 1C) was significantly increased in both BDO groups (to 250% and 249% in rats 1 and 7 d after BDO, respectively), which was, however, not associated with significant increase in MTX renal clearance. Urinary excretion and renal clearance of MTX were both decreased in LPS animals and were 55% and 23% of control values, respectively. In agreement, the glomerular filtration rate remained unchanged in BDO1 and BDO7 rats, but was significantly decreased in the LPS group to 32% of control values, which corresponded to extend of reduction of \( CL_R \) in...
LPS animals. At the same time, total plasma protein, and albumin concentrations, and MTX unbound fraction in plasma were not affected by either type of cholestasis.

Effect of Cholestasis on Conjugated Bilirubin Kinetics

The influence of intrahepatic and extrahepatic cholestasis on pharmacokinetics (supposing steady-state conditions) of endogenous conjugated bilirubin is presented in Fig. 2. Plasma concentrations of conjugated bilirubin rose to 9873% and 14456% after 1 or 7 d of BDO, respectively, and increased to 173% in LPS rats, but the difference was not significant (Fig. 2A). The rate of urinary excretion of the conjugated bilirubin increased to 627% 1 d after BDO and recovered to control values 7 d after BDO. In the LPS group, the urinary excretion rate remained unchanged (Fig. 2B). The biliary excretion rate of the conjugated bilirubin increased to 677%, 733% and 467% in BDO1, BDO7 and LPS animals, respectively (Fig. 2C). Importantly, the ratios of conjugated bilirubin excretory parameters to serum concentrations revealed a profound drop in renal as well as biliary clearance values in all cholestatic groups except for biliary clearance in the LPS group (Figs. 2D, E). Changes in the liver function as shown by serum biochemical tests are summarized in Table 1. ALT and AST activities were significantly increased 1 and 7 d after BDO, but a non-significant increase was observed in LPS animals.

Characterization of Quality of Cholestatic Model Used by Measurement of mRNA Expression of Main Drug Transporters

The mRNA levels of selected molecules in the liver and kidneys as evaluated by qRT-PCR are shown in Fig. 3A (livers) and Fig. 4A (kidneys). In the liver, Mrp2 mRNA levels decreased to 86%, 66% and 62% compared with controls in BDO1, BDO7 and LPS rats, respectively. Unlike in BDO1 and BDO7 animals, where the liver Bcrp mRNA levels declined to 37% and 44%, respectively, mRNA levels increased to 491% in the LPS group. In contrast, liver Mrp3 expression increased in all cholestatic groups to 189%, 970% and 304% in BDO1, BDO7 and LPS rats, respectively. Liver Mrp4 mRNA levels increased to 172% in BDO1 group with no significant increase in BDO7 and LPS animals (116% and 115%, respectively). The mRNA expression of the liver Oatp1a1, Oatp1a4 and Oatp1b2 decreased to 44%,
78% and 63% in BDO1, 53%, 57% and 53% in BDO7 and 17%, 19% and 5% in LPS rats, respectively. Compared with control animals, kidney Mrp2 and Mrp3 mRNA levels increased to 172% and 156% in BDO1, 168% and 287% in BDO7, and 288% and 156% in LPS rats, respectively. Kidney Mrp4 mRNA levels increased to 209% in LPS group, decreased to 68% in BDO7 animals and remained unchanged in BDO1 group. Kidney Oatp1a1 mRNA levels decreased in all cholestatic groups to 80%, 80% and 37% in BDO1, BDO7 and LPS rats, respectively. No mRNA of Bcrp, Oatp1a4, and Oatp1b2 transporters was detected in the kidneys.

Characterization of Quality of Cholestatic Model Used by Measurement of Protein Expression of Main Drug Transporters Protein quantification was performed in total membrane fractions of the liver and kidneys from control (1 and 7 d sham-operated or pretreated with physiological saline), BDO1, BDO7, and LPS rats (Figs. 3B, 4B). The relative amount of protein was expressed as a percentage of the respective control animal values. In the liver, Mrp2 protein expression was unchanged 1 d after BDO, while a decrease to 49% and 84% of control values was observed 7 d after BDO and in LPS rats, respectively. The amount of liver Bcrp protein decreased to 56% and 70% in the BDO1 and BDO7 group, respectively, but remained unchanged in LPS animals. Expression of liver Mrp3 protein decreased to 71% and 75% 1 d after BDO and in the LPS group, respectively, but increased to 190% 7 d after BDO. Liver Mrp4 protein expression decreased in all cholestatic groups to 65%, 55% and 82% in BDO1, BDO7 and LPS animals, respectively. Oatp1a1 protein levels in liver increased to 125% and 157% in BDO1 and BDO7 rats, respectively. Liver Oatp1a4 protein levels significantly decreased to 74% and 28% after 1 and 7 d of BDO, respectively. LPS pretreatment had no influence on Oatp1a1 and Oatp1a4 levels. Liver Oatp1b2 protein levels decreased to 70% and 50% in BDO7 and LPS, but remained unchanged in BDO1 group. In the kidneys, the amount of Mrp2 protein increased to 386% and 661% of controls after 1 and 7 d of BDO, respectively. Conversely, LPS caused a decrease in kidney Mrp2 protein content to 87%. The level of Mrp3 protein in the kidney was unchanged in BDO1 group, decreased to 71% in LPS rats, and increased to 251% 7 d after BDO. Kidney Mrp4 and Oatp1a1 protein levels decreased in all cholestatic groups to 68% and 57% in BDO1, 35% and 46% in BDO7 and 73% and 64% in LPS rats, respectively. No Bcrp, Oatp1a4, and Oatp1b2 protein was detected in the kidneys.

DISCUSSION

In the present study, we investigated the influence of cholestasis on the pharmacokinetics of MTX in rats (Table 1, Fig. 1). Intrahepatic (LPS-induced) or obstructive cholestasis was induced in respective groups of animals as confirmed by changes in serum biochemical parameters and liver transporter expression. According to the type of cholestasis, administration of MTX was associated with the differential alteration of $\text{CL}_{\text{Bile}}$ and $\text{CL}_{\text{R}}$ of MTX. While acute or chronic bile duct obstruction was associated with a drop in $\text{CL}_{\text{Bile}}$ of MTX and a slight compensatory rise in its $\text{CL}_{\text{R}}$, pretreatment with LPS decreased the capacity of both excretory mechanisms with more pronounced impairment of MTX $\text{CL}_{\text{R}}$. Significant changes were seen in endogenous conjugated bilirubin kinetics (Fig. 2).

In humans, the biliary excretion is finally responsible for elimination of 10—30% of applied MTX dose. Nevertheless, the real contribution of hepatic transporters seems to be much higher because the median ratio of biliary/renal excre-
tion of MTX in humans was 0.94, which indicates extensive entero-hepatic cycling and active reabsorption of the drug from GIT. Indeed, oral administration of resin colestimide, which binds MTX effectively in vitro, markedly accelerated MTX elimination in patients undergoing high-dose intravenous MTX treatment. As a consequence, alteration of this pathway by chronic cholestasis might have much deeper impact on MTX accumulation than expected. Nevertheless, only a few kinetic studies have previously directly demonstrated that extrahepatic cholestasis is associated with marked impairment of biliary excretion of xenobiotics.

Dietrich et al. reported a significant reduction of biliary excretion of the food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine 1 and 6 weeks after bile duct obstruction in rats. In similar studies, Chen et al. and Rodriguez-Garay et al. showed an acute decrease of biliary excretion of

Fig. 3. mRNA (A) and Protein (B) Expression of Mrp2, Bcrp, Mrp3, Mrp4, Oatp1a1, Oatp1a4 and Oatp1b2 in Liver

mRNA and proteins were isolated from control and cholestatic rat livers and analyzed by RT-PCR and Western blot, respectively. mRNA data normalized to those of GAPDH and protein data after densitometric analysis are expressed as a percentage of controls, as described in Materials and Methods. Data were expressed as mean±S.E.M. (n=6, in each group). Sham1, Sham7 and LPS-C-control rats (1 and 7 d sham-operated or pretreated with physiological saline), BDO1 and BDO7—common bile duct obstruction lasting for 1 or 7 d, LPS—administration of LPS. *p<0.05, **p<0.01, ***p<0.001, compared with controls. For the Western blot images: top: densitometric analysis (controls=100%); bottom: representative Western blots. Equal loading of protein was confirmed by beta-actin.

Fig. 4. mRNA (A) and Protein (B) Expression of Mrp2, Mrp3, Mrp4 and Oatp1a1 in Kidneys

mRNA and proteins were isolated from control and cholestatic rat kidney and analyzed by RT-PCR and Western blot, respectively. mRNA data normalized to those of GAPDH and protein data after densitometric analysis are expressed as a percentage of controls, as described in Materials and Methods. Data were expressed as mean±S.E.M. (n=6, in each group). Sham1, Sham7 and LPS-C-control rats (1 and 7 d sham-operated or pretreated with physiological saline), BDO1 and BDO7—common bile duct obstruction lasting for 1 or 7 d, LPS—administration of LPS. *p<0.05, **p<0.01, ***p<0.001, compared with controls. No expression of Bcrp, Oatp1b2 or Oatp1a4 protein was detected in the kidneys. For the Western blot images: top: densitometric analysis (controls=100%); bottom: representative Western blots.
sulfobromophthalein and ciprofloxacin after 1—7 d of obstruction of the bile duct.\textsuperscript{21,25} Our results with MTX comply with these observations. We noticed a marked decrease in $R_{\text{bile}}$ and $CL_{\text{bile}}$ of MTX, which produced a considerable reduction in its systemic clearance (Table 1). In comparison, we observed a less intensive reduction in $CL_{\text{Bile}}$ of MTX in endotoxin-induced cholestasis. Importantly, the extent of the reduction agrees with previous studies using LPS where authors indicated similar decreases in biliary excretion of other organic anions such as taurocholate, temocapril, and doxorubicin.\textsuperscript{25,26} Importantly, the changes in MTX hepatic kinetics are only in partial agreement with observed alterations of the main MTX transporting proteins, and the relationship is influenced by type of cholestasis (Fig. 3).

As described previously, MTX is taken from the blood into hepatocytes via the Oatps (mostly by Oatp1a4). The canalicular transport of MTX from hepatocytes into bile is accomplished by the Mrp2 and Bcrp transporters.\textsuperscript{13,15} Mrp3 and Mrp4 are efflux proteins expressed at the basolateral membrane of hepatocytes and mediate backward transport of MTX from hepatocytes to blood.\textsuperscript{27,28} Thus, spectrum of involved rat transporters closely resembles those mediating hepatic elimination of MTX in humans.\textsuperscript{3,15,29—31} The down- or up-regulation of these transporters during either intrahepatic or extrahepatic cholestasis has been suggested to be a compensatory mechanism protecting the liver against the damage produced by cumulating endobiotics.\textsuperscript{1,5} Regarding obstructive cholestasis, present data united the mRNA and protein expression of the evaluated transporters in one study. Observed changes in expression of selected transporters were similar to previous reports,\textsuperscript{21,34,35} confirming the correctness of the cholestatic model used, and supporting the detected alteration of MTX biliary kinetics. Expression of transporter protein corresponded in our study with mRNA levels suggesting transcriptional regulation of the expression during extrahepatic cholestasis (Fig. 3).

In contrast to obstructive cholestasis, administration of LPS produced changes in hepatic transporter expression mostly at the mRNA level, where the down- or up-regulation of respective molecules comply with the results of previous studies (Fig. 3).\textsuperscript{3,24} However, at the protein level, only Mrp3 and Oatp1b2 were affected. Regarding other protein expression, only a few data are available describing the influence of LPS on the level of transporter protein expression in addition to the mRNA level. Therein, authors observed, as in our study, decreased Oatp1a1, Oatp1a4 and Oatp1b2 mRNA but unchanged protein, except decreased Oatp1b2 protein level.\textsuperscript{4} Our findings regarding Bcrp and Mrp3 protein expression are new. The discrepancies between transporters mRNA, protein expression and the reduction of in vivo MTX $R_{\text{bile}}/CL_{\text{bile}}$ after LPS administration await elucidation (Table 1). Nevertheless recently demonstrated retrieval of Mrp2 from canalicular membrane of hepatocyte after administration of LPS may help to explain the observation.\textsuperscript{46} Formerly, the same effect was also described during extrahepatic cholestasis.\textsuperscript{37}

We observed a rise in $R_g$ of MTX in BDO animals. Although these data comply with the previously suggested increase in urinary excretion of certain anionic compounds such as bilirubin, p-aminohippurate, pravastatin or temocapril during obstructive cholestasis,\textsuperscript{2,38,39} recalculation of this parameter of MTX to renal clearance using plasma concentrations, which are increased too, bring only insignificant increase in $CL_g$ of the drug (Table 1). This proves that increased renal expression of efflux transporters such as Mrp2 (Fig. 4) cannot compensate anion systemic accumulation during obstructive cholestasis. On the other hand, induction of efflux transporters such as Mrp2 may protect the kidney tubular cells from the accumulation of methotrexate under cholestatic condition, which may contribute to nephroprotective effect of short-term obstructive cholestasis observed previously.\textsuperscript{40} Decreased mRNA and protein expression of Mrp4, another efflux transporter localized at brush border membrane of proximal tubular cells, observed in our study was shown also previously.\textsuperscript{41} The increased expression of Mrp3, a basolateral transporter, seen in renal tubuli of our animals, may contribute to this effect. Although the functional consequence needs further elucidation, this finding is supported by a similar increase in renal Mrp3 detected in hyperbilirubinemic rats with inherited deficiency of Mrp2.\textsuperscript{42} Unlike in extrahepatic cholestasis, we observed reduced protein expression of Mrp2 and Mrp3 in the kidneys of LPS-administered animals, thus providing novel data that support previously described histological findings of impaired proximal tubular cells during endotoxemia.\textsuperscript{3,43} In addition, glomerular filtration was profoundly reduced in LPS administered animals similarly as described previously.\textsuperscript{7,8} As a consequence, the marked reduction of MTX renal clearance seen in our study may be attributable not only to altered transporter expression but especially to reduced kidney perfusion. This effect could be caused by induction of cytokine production in kidneys after LPS administration.\textsuperscript{45}

The kinetic parameters of conjugated bilirubin, an endogenous Mrp2 substrate, are commonly used to evaluate the severity of cholestasis (Fig. 2). In the present study, shown in Fig. 2C, we identified increased $R_{\text{bile}}$ of the compound in both types of cholestasis, which complies with previous report.\textsuperscript{5} However, when the measurements were corrected to the serum concentration of conjugated bilirubin, we observed a marked reduction of the parameter ($CL_{\text{bile}}$) in bile duct-obstructed rats while only an insignificant decrease was detected after LPS administration (Fig. 2E). This corroborates the reduced transporter expression, and suggests this mechanism as the main cause of increased plasma concentrations of conjugated bilirubin in obstructive cholestasis (Fig. 2A). Because no relevant kinetic data are available as yet, we could only compare them with our previous report, which demonstrated similar results.\textsuperscript{46} Regarding the $R_g$ of conjugated bilirubin, Tanaka et al. demonstrated an increase 24 h after bile duct obstruction, as also seen in the present study (Fig. 2B). However, again, correction of $R_g$ of conjugated bilirubin to its plasma concentrations revealed marked reduction of $CL_g$ in BDO animals (Fig. 2D). Although the exact mechanism of this effect is not known because studies of transporter expression do not reflect the situation, animal and human studies point to the harmful effect of increased bilirubin serum concentration on proximal tubular cells during cholestasis.\textsuperscript{45,46} In agreement with this, the absence of increased bilirubin concentration in plasma of LPS-administered animals (Fig. 2A) was associated with only a moderate decrease in the renal clearance of the compound (Fig. 2D), in which reduction of glomerular filtration may take place.\textsuperscript{7,47}

In conclusion, the results of the present study demonstrate
for the first time the marked alteration of methotrexate (MTX) pharmacokinetics during obstructive and LPS-induced cholestasis in rats. Moreover, detailed analysis of endogenous conjugated bilirubin kinetics in the study provides information about its excretory parameters in two widely used animal models of cholestasis. The reliability of cholestatic models was verified by serum biochemistry and study of expression of major drug transporting proteins, which provide also partial explanations for mechanism responsible for the observed functional changes. Despite the fact that some expression changes correlate with the kinetics, the existence of discrepancies in the quantity and character of the respective kinetic and expression results suggests the importance of kinetic data for assessment of the final effects of cholestasis on drug elimination. Finally, although we are aware of limits of our animal models for prediction of clinical behavior of the drug, based on results of kinetic studies presented herein, increased accumulation of MTX might be expected in patients with cholestasis.

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