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Dexamethasone reduces methotrexate biliary elimination and potentiates its hepatotoxicity in rats

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ABSTRACT

Increased hepatotoxicity of methotrexate has been reported during dexamethasone therapy in humans. Despite the observed inducing effect of dexamethasone on some methotrexate transporting proteins in the liver, the kinetic aspects of this interaction have not been studied yet. Thus, the aim of the present study was to evaluate the influence of dexamethasone on the hepatic and overall pharmacokinetics of methotrexate. Pharmacokinetics of methotrexate was evaluated in rats during an *in vivo* steady-state clearance study after either single intravenous dose of dexamethasone or its four-day oral administration in a dose optimized for transport proteins induction. Dexamethasone oral pretreatment reduced biliary clearance of methotrexate by 53%. Although liver tissue concentration of methotrexate increased only slightly in these animals, a significant increase in liver weights produced by dexamethasone pretreatment revealed a marked increase in liver content of the drug. An evaluation of plasma liver enzyme activities measured before and after methotrexate administration demonstrated a potentiation of corticosteroid hepatotoxicity by the cytostatic. Analysis of methotrexate transporter expression in the liver showed up-regulation of Mrp2, Oatp1a4, and Oat2, and down-regulation of Mrp3. These observations comply with increased biliary excretion and reduced plasma concentrations of their endogenous substrate, conjugated bilirubin. In contrast, single intravenous bolus of dexamethasone did not influence any pharmacokinetic parameter of methotrexate. In conclusion, these results indicate that hepatocellular impairment associated with reduced biliary elimination of methotrexate, and its raised liver content may contribute to increased hepatotoxicity of the drug when co-administered with dexamethasone. Moreover, an influence of dexamethasone on protein expression of anionic drugs transporters in the liver and kidney was demonstrated.

1. Introduction

Methotrexate (MTX), a folate antimetabolite, is an anionic cytostatic drug commonly used in the therapy of many malignancies such as acute lymphoblastic leukemia, osteosarcoma, and head and neck tumors (Sterba et al., 2009; van Dalen and de Camargo, 2009). In these indications, the drug is applied in high-dose regimen, which may be associated with severe toxic reactions, particularly in those patients who attain higher plasma concentrations for prolonged period (Hansen et al., 1971; Jolivet et al., 1983). Administered via a long-term intravenous infusion, high-dose MTX steady-state plasma concentrations are primary dependent on the function of excretory pathways. In humans, the main route of MTX elimination is urinary excretion which takes 70–90% of the applied dose. The remaining part is mostly excreted into bile with some minor metabolism to 7-hydroxy MTX. The driving processes of MTX elimination are glomerular filtration in the kidneys and several active transport processes in hepatocytes and renal tubular cells (Grim et al., 2003).

The most important transporters for methotrexate kidney/liver elimination are the organic anion transporters (Oat1–3), organic anion transporting polypeptide 2 (Oatp2, Oatp1a4), members of the multidrug resistance-associated protein subfamily (Mrp2–4; Abcc2–4) and breast cancer resistance protein (Bcrp; Abcg2) (Badagnani et al., 2006; Borst et al., 2007; Masuda et al., 1997; Takeda et al., 2002; Takeuchi et al., 2001; Vlaming et al., 2009). The function of these transporters can be altered by various drugs, and serious drug–drug interactions may result from impairment of MTX elimination. Bone marrow suppression and acute renal failure have been described as a consequence of raised MTX plasma

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concentration during co-administration with inhibitors of renal and/or liver organic anion transporting proteins such as nonsteroidal anti-inflammatory drugs or probenecid (McLeod, 1998). Logically, induction of the transport pathways may enhance elimination of the drug and thus reduce the incidence of adverse events. However, co-administration of MTX with a potent inducer of some of these active transport pathways in the liver and kidneys, dexamethasone (Demeule et al., 1999; Luttringer et al., 2002; Micuda et al., 2008), did not alleviate the adverse events, but even increased the hepatotoxicity of the cytostatic (English et al., 1987; van Outryve et al., 2002; Wolff et al., 1998). The mechanism of this interaction is currently unknown.

The aim of the present study was to evaluate the effects of a single- or repeated-dose dexamethasone administration on methotrexate systemic, hepatic and renal kinetics in rats. As a reference inhibitor of organic anion transporters, probenecid was used to confirm the sensitivity of the *in vivo* model also to inhibition. Changes in the expression of the main methotrexate transporters (Mrp2–4, Bcrp, Oatp1a4, and Oat1–3) in the liver and kidney after dexamethasone pretreatment were evaluated using Western blot and qRT-PCR. In addition, kinetic parameters of a typical endogenous substrate of organic anion transporters, endogenous conjugated bilirubin, were evaluated in both control and dexamethasone-pretreated animals.

2. Materials and methods

2.1. Materials

Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lyophilized methotrexate for infusion was purchased from Ebewe Pharma, and the pure substance was a generous gift from Pliva-Lachema (Brno, Czech Republic). Mouse monoclonal antibody M_2III-5, directed to Mrp2 (170–190 kDa), and BXP-21 directed to Bcrp (70 kDa), were obtained from Signet Laboratories, Inc. (Dedham, MA, USA). Rabbit anti-Oatp1a4 (75 kDa) polyclonal antibody was obtained from Millipore Corporate Headquarters (Billerica, MA, USA). Mouse monoclonal antibody M_3II-21 directed to Mrp3 (180–190 kDa) was obtained from Alexis Corporation (Lausen, Switzerland). Anti-Mrp4 antibody was purchased from Abcam (Cambridge, UK). Oat1 and Oat2 monoclonal antibodies were purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA). As a loading control for Western blot, rabbit polyclonal β-actin antibody (42–45 kDa) was purchased from Sigma (Prague, Czech Republic). Horseradish peroxidase-linked sheep anti-mouse and donkey anti-rabbit immunoglobulin G were purchased from GE Healthcare (Prague, Czech Republic). All other reagents and supplies were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Bio-Rad (Hercules, CA, USA), respectively, and were of the highest purity available.

2.2. Animals and treatment

Male Wistar rats (Konarovice, Czech Republic) weighing 280–330 g were divided into four groups ($n = 6$ in each). One group received dexamethasone pretreatment (25 mg/kg daily) while the three remaining were applied with vehicle alone. Both regimens were applied for four days by stomach intubation. Dexamethasone dose used for pretreatments was selected as previously established optimum dose for hepatic transport protein induction and was within the range of dexamethasone doses used for this purpose (Chandra et al., 2005; Cherrington et al., 2002; Maher et al., 2005; Micuda et al., 2005). All animals were subjected to *in vivo* clearance studies 24 h after the last oral drug/vehicle application. Rats were housed under controlled environmental conditions (12-h light–dark cycle; temperature, 22 ± 1 °C) with a commercial food diet and freely available water. The study protocol was approved by the animal welfare committee of the Charles University in Prague, Faculty of Medicine in Hradec Kralove.

2.3. In vivo clearance study of methotrexate and bilirubin

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and cannulated with polyethylene tubes in the right jugular vein for drug administration, and the left carotid artery for blood sampling. The urinary bladder and bile duct were also cannulated for urine and bile collections. Body temperature of animals was maintained at 37 °C with a heat lamp. To evaluate the steady-state pharmacokinetic of methotrexate, all rats received a bolus intravenous injection of methotrexate at a loading dose of 8 μmol/kg followed by a constant-rate infusion (Perfusor Compact; Braun, Prague) of a 4% mannitol solution delivering dose of 10 μmol/kg of methotrexate/h at a rate of 2 ml/h until the end of the study. A 60-min infusion was found to result in a steady-state plasma concentration of methotrexate. The previously tested dosage was selected to obtain a methotrexate plasma concentration below reported K_m of basolateral (23 μM) and canalicular (300 μM) transport (Ueda et al., 2001). One untreated group of animals received a single intravenous bolus of dexamethasone (1 mg/kg – maximum daily dose in humans) 10 min before initiation of methotrexate application. Another group of untreated animals received, together with methotrexate, also an intravenous infusion of probenecid (200 μmol/kg/h) starting with a loading dose of 70 μmol/kg. Mannitol was always used as a constituent of the vehicle to obtain a constant and sufficient urine flow rate. After a 60-min infusion (attained steady-state), bile and urine were collected in preweighed tubes at 10-min intervals for 30 min. Blood samples were taken at the midpoint of the bile and urine collection periods. Plasma samples were obtained by centrifugation at 3000 × *g* for 10 min. The volume of bile and urine was measured gravimetrically, with specific gravity assumed to be 1.0. All plasma, bile, and urine samples were stored at −80 °C until analysis.

The protocol of pretreatment and *in vivo* study of bilirubin clearance was identical to that mentioned above, with the only exception that animals received an infusion of 4% mannitol only.

2.4. Immunoblot analysis

Crude membrane-containing homogenates were prepared from freshly harvested livers and kidneys as described previously (Micuda et al., 2007). Homogenates (50 μg) were incubated with sample buffer at room temperature for 30 min and separated on a 7.5% polyacrylamide gel. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), the membrane was blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS). The membrane was then incubated with primary antibodies (1:500) for 1 h, washed, and incubated for 1 h with a peroxidase-conjugated secondary antibody (1:1000). After washing five times with TTBS, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare, Prague, CZ) and subjected to autoluminography for 1–5 min. The immunoreactive bands on the exposed films were scanned with a densitometer ScanMaker i900 (UMAX, Prague, CZ) and semiquantified using the QuantityOne imaging software (Bio-Rad).

2.5. Examination of transporter gene expression by qRT-PCR

RNA was isolated from liver and kidney tissue samples using TRIzol reagent (Invitrogen, USA) and converted into cDNA via an iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, USA). Ten nanograms of cDNA were loaded into one reaction, performed in triplicate. The amplification was completed using the TaqMan® Fast Universal PCR Master Mix and pre-designed TaqMan® Gene Expression Assay kit for the following genes: Mrp2 (Abcc2, Rn00563231_m1), Mrp3 (Abcc3, Rn00589786_m1), Mrp4 (Abcc4, Rn01465702_m1), Bcrp (Abcg2, Rn00710585_m1), Oatp1a4 (Slco1a4, Rn00756233_m1), Oat1 (Slc22a6 Rn00568143_m1), Oat2 (Slc22a7, Rn00585513_m1), Oat3 (Slc22a8, Rn00580082_m1). Rat GAPDH (P/N 4308313) and Eukaryotic 18S rRNA (P/N4319413E) endogenous control kits were used as housekeeping genes. All pre-made assays were provided by Applied Biosystems (Foster City, USA). The time–temperature profile used in the "fast" mode was: 95 °C for 20 s; 40 times: 95 °C for 3 s, 60 °C for 30 s, and run on 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). The relative expression ratio (R) was then calculated according to Pfaffl (2001):

$$R = \frac{E_{\text{target}}^{\Delta C^{(\text{control}-\text{sample})}/t_{\text{target}}}}{E_{\text{housekeeping}}^{\Delta C^{(\text{control}-\text{sample})}/t_{\text{housekeeping}}}}$$

where E_{target} and $E_{\text{housekeeping}}$ are the effectivity values determined from the calibration curve slopes for each gene, $\Delta Ct_{\text{target}}$ and $\Delta Ct_{\text{housekeeping}}$ are the differences in threshold values (Ct) between control and pretreated (dexamethasone) sample for each of the genes, i.e. target and housekeeping.

2.6. Analytical procedures

The concentrations of methotrexate in the liver homogenate, plasma, urine and bile were determined by HPLC after deproteination of samples according to a previously described method (Fuksa et al., 2008). Briefly, the instrument was an Agilent 1100 series (Agilent, Palo Alto, USA) chromatograph provided with a fluorescence detector (excitation, 350 nm; emission 430 nm). Separation was achieved at 30 °C using a Gemini C18, 110A, 4.6 × 150 mm column and Gemini C18, 4 mm × 3 mm pre-column (Phenomenex, Torrance, USA). The mobile phase, flowing at a rate of 0.6 ml/min, consisted of ammonium acetate and acetonitrile (87:13, v/v). The concentrations of bilirubin and creatinine in plasma and urine were measured on a Cobas Integra® 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. This instrument was also used for determination of liver enzyme activities (ALT, AST), cholesterol and triglyceride concentration in plasma.

2.7. Protein binding experiments

The plasma protein binding of methotrexate was evaluated by ultrafiltration through Ultrafree-MC Centrifugal Filter Units (Millipore, Billerica, MA, USA). Four