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Amiodarone Modulates Pharmacokinetics of Low-dose Methotrexate in Rats

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ABSTRACT: Clinical studies of low-dose methotrexate (LDMTX) pharmacokinetics document increased plasma concentrations of MTX after co-administration of the drug with amiodarone or macrolide antibiotics. As drug–drug interactions may increase the toxicity of LDMTX, a rat model was used to follow renal and biliary elimination of MTX during its constant-rate i.v. infusion and concomitant single bolus i.v. injections of amiodarone or azithromycin. The mean steady-state plasma concentration of $1.7 \pm 0.1\,\mu mol/l$ was reached and the total clearance achieved $17.7 \pm 1.0\,ml/min/kg$. Administration of amiodarone decreased the biliary clearance of MTX to 73% of the control values ($p < 0.05$). Correspondingly, the total clearance decreased to 72% and plasma MTX concentrations were augmented to $2.5 \pm 0.4\,\mu mol/l$ ($p < 0.05$). Amiodarone-treated rats exhibited a 3.3-fold decrease in the renal clearance ($p < 0.05$) of conjugated bilirubin, which was associated with its increased plasma concentration. In contrast, azithromycin did not alter any of the MTX pharmacokinetic parameters. In conclusion, this is the first report describing the impairment of MTX hepatic elimination during co-administration with amiodarone. This study also provides new insight into acute amiodarone-induced hyperbilirubinaemia, where increased bilirubin production and decreased renal clearance may contribute to this effect. Importantly, azithromycin seems to be a safe co-medication during LDMTX therapy. Copyright © 2008 John Wiley & Sons, Ltd.

Key words: methotrexate; amiodarone; azithromycin; biliary excretion; interaction

Introduction

Low-dose methotrexate (LDMTX) therapy has become effective in the treatment of autoimmune and lymphoproliferative diseases [1]. In these disorders, the therapeutic outcome of MTX is related to its systemic or tissue-specific concentrations [2]. Unfortunately, the pharmacokinetics of LDMTX is individually highly variable, resulting in a different systemic exposure to the drug and unpredictable therapeutic/toxic effects in patients. Among the main causes of this variability is the inhibition of transporter-mediated MTX excretion in the liver and kidney [1] as seen in drug–drug interactions between LDMTX and, for example, nonsteroidal anti-inflammatory drugs, salicylic acid and probenecid. These interactions may result in bone marrow suppression and acute renal failure [3]. In addition, clinical data suggest that other commonly used drugs such as amiodarone and macrolides are suspected of producing these

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interactions with MTX. However, no detailed interaction study quantitatively describing changes in MTX pharmacokinetics after administration of both suspected chemical structures is available yet.

Amiodarone, a benzofuranic acid derivative, is a potent drug used in the treatment of paroxysmal supraventricular tachycardia, malignant ventricular tachyarrhythmias, atrial flutter and fibrillation [4]. As a highly lipophilic molecule, AMD is widely bound in the tissues with a huge distribution volume and a correspondingly long serum elimination half-life of 40–60 days. The main route of elimination is the hepatic metabolism to active desethylamiodarone and subsequent excretion to bile [5]. Clinical usage of AMD is hampered by a wide spectrum of drug–drug interactions based on inhibition of several important cytochrome P450 isoforms, such as CYP3A4, CYP1A2 and CYP2C9 [6,7]. In addition, amiodarone may affect the excretion of drugs that are either poorly metabolized (e.g. digoxin) [8] or the metabolism is not a rate-limiting step for their elimination (e.g. anthracyclines and vinca alkaloids) [9]. Studies with *in vitro* cellular models identified that these interactions occur via the inhibition of the hepatic P-glycoprotein (P-gp) membrane transporter [10,11]. Moreover, inhibition of another transporter, Oatp2, has been described for amiodarone [12]. Although the involvement of both transporters in MTX pharmacokinetics seems to be minor, serious interaction between AMD and MTX has been reported in patients with psoriasis [13]. Authors have suggested pharmacokinetic mechanisms but the proof is still missing.

Azithromycin, a 15-ring member macrolide antibiotic, is widely used in the therapy of community-acquired but also hospital infections [14]. In comparison to other macrolide antibiotics, AZT possesses unique pharmacokinetic characteristics with a longer half-life, greater tissue distribution and higher intracellular concentration than others known [15]. It is mainly eliminated in unchanged form in the faeces via biliary excretion and intestinal secretion, whereas urinary excretion is the minor elimination route in humans [15,16]. Recently, Sugie *et al*. [17] have demonstrated that the active excretion of azithromycin is mediated via two ATP-dependent membrane transporters, P-glycoprotein and Mrp2. At the same time, azithromycin has been shown to produce inhibition of the hepatobiliary excretion of drugs that are substrates for Mrp2, the main transporter for biliary excretion of MTX [18,19]. Regarding co-administration with MTX, the interaction between MTX and another macrolide antibiotic has already been described [20]. Moreover, an inhibitory effect of azithromycin on the renal and biliary excretion of MTX given in a high-dose regimen was demonstrated recently [21]. Therefore, the question of whether AZT could also affect LDMTX pharmacokinetics arises.

The present study aimed to investigate whether amiodarone or azithromycin influences either the hepatobiliary or renal excretion of LDMTX in rats. At the beginning, the pharmacokinetic profile of both potential inhibitors was described in rats including their renal and biliary excretion. Thereafter, an *in vivo* clearance study was performed in rats where the influence of either amiodarone or azithromycin on the pharmacokinetics of MTX was examined during steady-state MTX plasma concentrations. In addition, the kinetics of another organic anion, endogenous conjugated bilirubin, was monitored in the same animals to obtain further information on the potential inhibitory influence of both compounds on this excretory pathway.

Methods

Chemicals

Amiodarone was purchased from Sigma Chemical (St Louis, MO). Azithromycin and clarithromycin were kindly donated by Zentiva (Praha, Czech Republic). Amiodarone, methotrexate and azithromycin for injection were obtained from EBEWE Pharma (Unterach, Austria) and Pliva d.d. (Zagreb, Croatia), respectively. All other reagents are commercially available and were of analytical grade. All reagents were used without further purification.

Animals

Male Wistar rats (280–320 g) were obtained from BioTest Ltd (Konarovice, Czech Republic). The

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rats were housed under controlled environmental conditions (temperature of 22 ± 1°C and humidity of 55% ± 5%) with a commercial food diet and water available *ad libitum*. All rats received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (revised 1996; http://www.nap.edu/books/0309053773/html/81.html). The study protocol was approved by the Animal Welfare Committee of Charles University in Prague, Faculty of Medicine in Hradec Kralove.

Amiodarone and azithromycin pharmacokinetics in rats

Rats (n=3) under anaesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) were fixed in a supine position and cannulated into the right jugular vein, left carotid artery, bile duct and urinary bladder for drug administration, blood sampling, bile collection and urine collection, respectively. After surgical preparations, the rats received an intravenous injection of either azithromycin (40 mg/kg) or amiodarone (25 mg/kg). The dose was selected on the basis of experience from previous studies [17,22] to allow comparison of the pharmacokinetic data. In addition, 4% mannitol solution was infused at a rate of 2 ml/h throughout the study to maintain a constant urine flow rate. Blood samples (≈ 0.3 ml) were taken at designated time intervals (4, 10, 20, 40, 70, 90 and 120 min) after injection of the drug. Plasma was obtained from the blood samples by centrifugation at 3000 × g for 5 min at 4°C. Simultaneously, bile and urine samples were collected in preweighed tubes at 20 min intervals. All specimens were stored at −80°C until analysis. The body temperature of the animals was maintained at 37°C by the placement of the animals on a heating platform.

Effect of azithromycin and amiodarone on the biliary and renal clearance of MTX in rats

Rats (n=6, in each group) under light anaesthesia with sodium pentobarbital (50 mg/kg) were fixed in a supine position and cannulated into the right jugular vein, left carotid artery, bile duct and urinary bladder for drug administration, blood sampling, bile collection and urine collection, respectively. After surgical preparations, the rats received a bolus injection of MTX in a loading dose of 4 μmol/kg, followed by a constant-rate infusion (Perfusor Compact; Braun, Prague) of a saline solution containing 4% mannitol delivering 1.8 μmol/kg of MTX per h at a rate of 2 ml/h until the end of the study. The loading and maintenance doses of MTX were determined by preliminary biliary and renal clearance experiments. Mannitol solution was used to maintain a sufficient and constant urine flow rate. After a 60 min infusion when MTX C_{ss} was attained, bile and urine samples were collected at 20 min intervals for 40 min. After a 100 min infusion, a bolus of amiodarone (25 mg/kg) or azithromycin (40 mg/kg) or isotonic saline was administered intravenously. Bile and urine samples were thereafter collected in preweighed tubes at 20 min intervals from 160 to 220 min. Blood samples were collected at the midpoints of the bile collection periods (70, 90, 170, 190 and 210 min after the start of MTX infusion). Plasma samples were obtained by immediate centrifugation of blood samples and were kept frozen (−80°C). The volume of bile and urine samples was measured gravimetrically with specific gravity assumed to be 1.0. The body temperature of the animals was maintained at 37°C by the placement of the animals on a heating platform.

Drug analysis

The concentration of amiodarone, azithromycin and methotrexate in plasma, urine and bile were determined by high-performance liquid chromatography (HPLC) methods.

The concentrations of methotrexate were measured after deproteination of samples according to a previously described method [2] with the following minor modifications. 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 column Gemini C18, 110A, 4.6 × 150 mm and precolumn Gemini C18, 4 × 3 mm (Phenomenex, Torrance, USA). The mobile phase flowing at the rate of 0.6 ml/min consisted of ammonium acetate and acetonitrile (87:13, v/v).

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For amiodarone and desethylamiodarone analysis, plasma, bile and urine samples (0.1 ml) were diluted by using 0.4 ml of water. A solution of zinc sulfate 20 µl (10%) and acetonitrile (1 ml) was added. The samples were mixed and centrifuged for 10 min at 15000 × *g*. The injection volume of supernatant was 70 µl. Analysis was performed on a 2695 Waters Separations Module (Waters Corp., Milford, MA, USA) equipped with a 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). Empower Software (Waters Corp., Milford, MA, USA) was employed for the data acquisition and processing. The separation of amiodarone and desethylamiodarone was performed on the analytical column Symmetry C18 (Waters) 5 µm particle size (4.6 mm i.d × 150 mm). A Waters Symmetry C18 5 µm particle size Guard Column (3.9 mm i.d. × 20 mm) was used as the analytical precolumn. The isocratic flow rate of the mobile phase was set at 1.1 ml/min. The mobile phase consisted of acetonitrile (47%) and 50 mM phosphate buffer pH 3.1 (53%). UV spectra of all chromatographic peaks were recorded in the range 200–600 nm using a diode-array UV detector with a resolution at 1.2 nm.The wavelength of 242 nm was used for quantitation. The lower limit of detection was 0.10 µmol/l (amiodarone) and 0.2 µmol/l (metabolite), respectively. The inter- and intra-batch accuracies and precisions reached values of 92.6–104.7% (recovery) and 2.3–9.4% (RSD), respectively.

The HPLC method for azithromycin analysis was performed as follows. The plasma sample (150 µl) was mixed with the same volume of 0.05 M potassium carbonate, 5 µl of acetonitrile and 50 µl of internal standard (20 mg/l clarithromycin). After 5 s shaking, 1.2 ml of tert-butylmethylether was added and the mixture was vigorously vortexed for 30 s and centrifuged at 2200 × *g* for 10 min. The organic layer was transferred to an Eppendorf tube and evaporated to dryness. The remnant was dissolved in 100 µl of mobile phase and 50 µl was injected on a column. Samples of bile and urine were diluted with water and 50 µl of internal standard (20 mg/l clarithromycin) was added. The mixture was directly injected on an HPLC column. The chromatographic system consisting of HPLC pump LC-20AD, autoinjector SIL-10ADvp (Shimadzu, Japan), thermostated column compartment LCO102 (Ecom, Czech Republic) and coulochem detector with analytical cell model 5010 (ESA Inc., MA, USA) was used for all separations. Chromatographic data were captured and evaluated with Clarity Lite software (Prague, Czech Republic). Isocratic separation at a flow rate of 1.0 ml/min was carried out on a Gemini C18 reverse phase column (150 × 4.6 mm, 3 µm particle size), protected with a Gemini C18 4 × 3 mm guard column (Phenomenex, Torrance, CA, USA) at a temperature of 40°C. The mobile phase consisted of 0.05 M phosphate buffer (pH=8.0) and acetonitrile (60:40, v/v). The effluent was monitored at an electrode potential of 900 mV with a total sample run time of 20 min. The lower limit of detection was 0.156 µM. The inter- and intra-batch accuracies reached values of 2.2–19.7%.

The concentrations of creatinine and bilirubin (direct and total) were measured on Cobas Integra ® 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Pharmacokinetic analysis

Pharmacokinetic analyses were conducted with Kinetica (version 4.4.1). Non-compartmental analysis was used to describe the disposition of amiodarone and azithromycin during 120 min, i.e. the same period during which the drugs were present in the organism together with MTX in the subsequent steady-state interaction study. Maximum observed serum concentrations (C_{max}) of azithromycin and amiodarone were estimated for each animal directly from the serum concentration-time data. The time of the maximum concentration (T_{max}) was defined as the time of the first occurrence of C_{max} (i.e. coincident with the initial blood sample). Area under the plasma concentration-time curve (*AUC*) from time 0 to T_{last} was estimated according to the log-linear trapezoidal rule where T_{last} was the last quantified concentration. The biliary (CL_{Bile}) and renal (CL_R) clearance was calculated by Equations (1) and (2), where X_{bile} and X_{urine} were the amount of azithromycin or amiodarone excreted to bile and urine, respectively, during the evaluated

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period and T_{last} was 120 min [23]:

$$CL_{Bile} = X_{Bile}/AUC_{0\text{-}Tlast} \quad (1)$$

$$CL_R = X_{Urine}/AUC_{0\text{-}Tlast} \quad (2)$$

Steady-state pharmacokinetic parameters of MTX for the interaction study were calculated for each animal as the mean of three points in 160′–220′ of experiment. The total plasma clearance (CL_{Total}) of MTX was estimated by dividing the constant infusion rate of MTX by the steady-state concentration in plasma (C_{ss}). Biliary and renal clearance (CL_{Bile} and CL_R) of MTX during each collection period was calculated by dividing the respective excretion rate by C_{ss} determined for that collection period. Kinetic parameters of endogenous conjugated bilirubin, an Mrp2 substrate, were calculated by the same approach, on the basis of the assumption that bilirubin plasma concentrations were in steady-state with the exception of CL_{Bile} as this cannot be calculated due to the fact that liver is the organ that synthesizes conjugated bilirubin synthesis. The glomerular filtration rate (GFR) was evaluated as the clearance of endogenous creatinine (CL_{CR}). The renal clearance ratio of MTX and bilirubin was calculated as CL_R/CL_{CR}.

Statistical analysis

Interaction experiments were carried out in five animals per group. All experimental data are expressed as mean ± SEM. Statistical significance was examined by unpaired *t*-test using Graphpad Prism 4.0 software (Graphpad Software, San Diego, USA). A value of $p < 0.05$ was considered statistically significant.

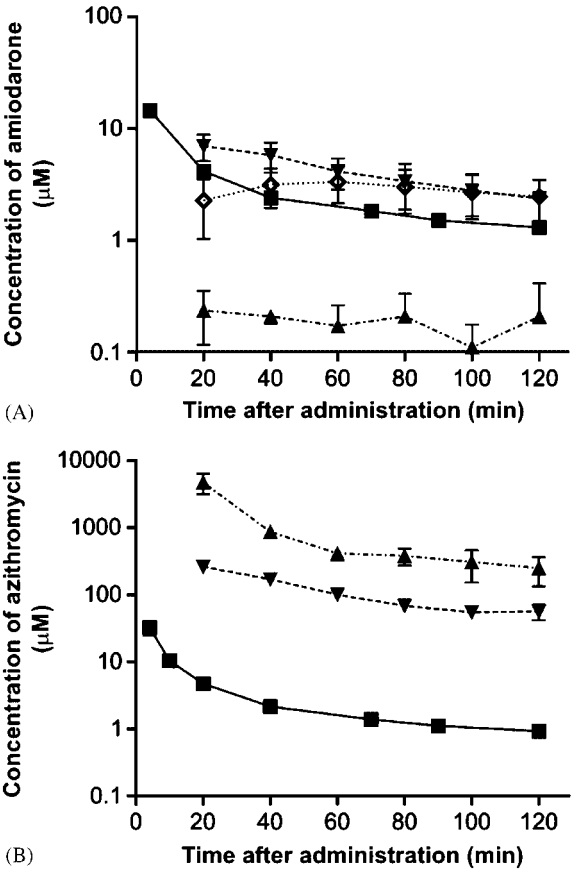

Figure 1. Semilogarithmic plots of amiodarone (A) and azithromycin (B) plasma, biliary and urinary concentration versus time curves measured in Wistar male rats after intravenous administration of a single bolus dose (25 mg/kg of amiodarone and 40 mg/kg of azithromycin). For amiodarone, the main metabolite, desethylamiodarone, was detected only in bile. Symbols: ■, drug concentrations in plasma; ▼ drug concentrations in bile; ▲, drug concentrations in urine; ◇, desethylamiodarone concentrations in bile. Values are the mean ± SEM (n=3)

Results

Plasma concentration-time curve of amiodarone and azithromycin

Semilogarithmic plots of plasma, biliary and urinary concentration-time data for amiodarone and azithromycin after a single intravenous injection are shown in Figure 1, respectively. Regarding amiodarone, the maximum concentrations of the compound in plasma (C_{max}), measured 4 min after administration, were 14.5 ± 1.9 μM. The main metabolite, desethylamiodarone, was detected only in bile. Concentrations of both amiodarone and desethylamiodarone in bile quickly exceeded amiodarone concentrations in plasma (Figure 1A). Within 120 min of administration, the rats excreted only 0.1% of the applied amiodarone dose, 97% of which appeared in bile and 3% in urine. The CL_{Bile} and CL_R of amiodarone were 5.9 ± 0.7 ml/h/kg and 0.2 ± 0.07 ml/h/kg, respectively. In the

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case of azithromycin, C_{max} measured within 4 min after administration was 32.5 ± 7.8 μM. Both bile and urine concentrations were well above those measured in plasma (Figure 1B). A total of 18% of the injected azithromycin dose was excreted in urine and bile over the evaluated period (120 min), 8% of which was into the bile and 92% into urine. The CL_{Bile} and CL_R of azithromycin were 90.4 ± 17.9 ml/h/kg and 1116 ± 377 ml/h/kg, respectively.

Steady-state pharmacokinetics of methotrexate in control animals

Pharmacokinetic data of MTX during steady-state of plasma concentrations in control rats are summarized in Table 1. A steady-state concentration of MTX was attained after 60 min from the start of its constant rate infusion. Concentrations of MTX in bile were 169-fold higher than in plasma, suggesting an important contribution of the active transport mechanism. Urinary concentrations were also 98-fold higher than in plasma, but a CL_R/CL_{CR} ratio below 1 suggests that glomerular filtration together with tubular reabsorption play main roles in methotrexate renal elimination. The sum of MTX biliary and urinary excretion rates accounted for 87% of the infusion rate in the absence of inhibitors. Thus, the metabolism of methotrexate under these conditions should be minor and any potential effect of an interaction involving metabolism should be minimal.

Effect of amiodarone and azithromycin on biliary and renal clearance of MTX

The effects of amiodarone (25 mg/kg) and azithromycin (40 mg/kg) on the biliary and renal excretion of MTX were investigated under steady-state conditions obtained by the continuous intravenous infusion. The pharmacokinetic parameters of MTX are summarized in Table 1. Administration of amiodarone significantly decreased the CL_{Bile} of MTX by 27% with a corresponding reduction of CL_{Tot} by 28%. The renal clearance of MTX remained unaffected after amiodarone; however, CL_{CR} was significantly reduced by 33%. In comparison, there was no change in any of the MTX pharmacokinetic parameters after azithromycin administration (Table 1).

Effect of amiodarone and azithromycin on conjugated bilirubin (CB) excretion

To further investigate the influence of both potential inhibitors on the organic anion elimination pathways, the biliary and renal excretion of another MRP2 substrate, endogenous CB, was evaluated in the MTX-infused animals. The results of endogenous bilirubin kinetics in rats are presented in Table 2. Amiodarone produced a 5.9-fold increase in the biliary excretion of conjugated bilirubin. However, as the concentrations of CB in plasma rose 5.5-fold as well, the overall influence of amiodarone on CB biliary clearance remained insignificant. Despite the

Table 1. Effects of amiodarone and azithromycin on biliary and renal excretion of MTX in rats

	MTX		
	Control	AMD	AZT
Urine flow rate (μl/min)	11.8 ± 2.0	13.1 ± 3.1	13.8 ± 2.5
Bile flow rate (μl/min)	18.7 ± 2.1	18.7 ± 4.7	20.8 ± 0.7
Urinary excretion rate (nmol/kg/min)	7.3 ± 1.7	12.2 ± 3.3	8.1 ± 1.5
Biliary excretion rate (nmol/kg/min)	18.7 ± 1.5	18.7 ± 1.5	17.0 ± 1.9
C_{ss} (μM)	1.7 ± 0.1	2.5 ± 0.4^a	1.6 ± 0.1
CL_R (ml/kg/min)	4.3 ± 0.9	5.1 ± 1.4	5.6 ± 1.4
CL_{Bile} (ml/kg/min)	11.1 ± 1.2	7.9 ± 1.5^a	10.4 ± 0.5
CL_{Tot} (ml/kg/min)	17.7 ± 1.0	12.7 ± 1.8^a	19.7 ± 1.8
CL_{CR} (ml/kg/min)	7.7 ± 1.2	5.2 ± 0.6^a	7.6 ± 0.9
CL_R/CL_{CR}	0.6 ± 0.1	1.0 ± 0.2^a	0.7 ± 0.1

Values are mean ± SEM (n=5).
Significantly different from control values ($^a p < 0.05$).

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Table 2. Kinetics of endogenous bilirubin in rats (n=5 in each group) after administration of either amiodarone (25 mg/kg) or azithromycin (40 mg/kg)

	Control	AMD	AZT
Conjugated bilirubin			
Urinary excretion rate (nmol/kg/min)	0.12 ± 0.03	0.16 ± 0.04	0.13 ± 0.03
Biliary excretion rate (nmol/kg/min)	3.6 ± 1.2	21.4 ± 3.2[c]	3.6 ± 0.3
C_{ss} (μM)	0.2 ± 0.1	1.1 ± 0.2[b]	0.1 ± 0.002[a]
CL_R (ml/kg/min)	0.7 ± 0.2	0.2 ± 0.05[a]	1.3 ± 0.3
CL_R/CL_{CR}	0.1 ± 0.03	0.03 ± 0.008[a]	0.2 ± 0.03
Total bilirubin			
C_{ss} (μM)	4.1 ± 0.1	6.1 ± 0.4[**]	5.0 ± 0.5

Data on urine and bile flow rates are listed in Table 1—samples from the same animals were analysed. Pharmacokinetic analysis was performed on the basis of the assumption that bilirubin serum concentrations were in steady-state.
Values are mean ± SEM (n=5).
Significantly different from control values ([a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$).

administration of amiodarone not changing the CB urinary excretion rate, the renal clearance of CB was decreased. In contrast, administration of azithromycin induced a significant decrease in CB C_{ss}.

Discussion

This study initially describes in detail the biliary and urinary excretion of amiodarone and azithromycin during the first 120 min upon intravenous administration of a single bolus dose to male Wistar rats. Thereafter, the influence of both compounds on steady-state pharmacokinetics of MTX was monitored in separate groups of animals during the same period. A significant decrease of MTX biliary clearance was observed after administration of amiodarone, which was associated with increased MTX plasma concentrations and decreased total clearance of the drug. In contrast, no change in MTX pharmacokinetics was detected after administration of azithromycin. The evaluation of kinetics of another organic anion, endogenous conjugated bilirubin, showed a significant increase in its plasma concentrations and biliary excretions after administration of amiodarone, while a decrease of plasma CB concentrations was detected after application of azithromycin.

In humans, the major route of MTX elimination is renal excretion of unmetabolized drug, which accounts for approximately 60% to 90% of the total body clearance. In addition, about 3% to 4% of the applied dose is excreted in urine as 7-hydroxy-methotrexate [3]. The remaining 10–30% of methotrexate is eliminated by active biliary excretion, which is mediated mostly by MRP2 [24,25]. In rats, 62% of i.v.-administered MTX is excreted into bile, whereas 27% of the dose is excreted into urine, as shown by Masuda *et al*. [19]. Similarly to humans, biliary excretion is mediated by MRP2 as demonstrated by the almost complete abolition of methotrexate biliary recovery in Eisai hyperbilirubinaemic rats (EHBR), the strain deficient in MRP2 expression and function in the liver and intestine [26]. Consistent with previous reports, the present study demonstrated that biliary excretion represents the major route of MTX elimination in rats. Importantly, unlike these studies, a dosage regimen was used that maintained steady-state MTX concentrations equal to those measured after low-dose MTX administration [2]. The equilibrated rate of administration and the sum of urinary and biliary excretion confirmed the steady-state. An observed small discrepancy between the overall excretion and the infusion velocity could, at least partly, be explained by metabolism to 7-hydroxymethotrexate by hepatic aldehyde-oxidase, which may account for 6–10% of the administered dose [27]. Concerning renal elimination, steady-state concentrations of MTX used in our study were associated with a ratio of CL_R/CL_{CR} below 1, which suggests tubular reabsorption. A similar observation was reported previously in humans [1].

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Before the interactions study with MTX, a short study was performed to obtain information about the kinetics of amiodarone and azithromycin in rats during an appropriate time period. Complete pharmacokinetic parameters of the compounds could not be described due to the long half-life of both compounds [17,28]. Nevertheless, the study focused on actual concentrations in fluids and biliary and renal excretions during the period of planned co-administration with MTX. Regarding amiodarone, as expected [22], the principal route of the drug elimination was biliary excretion. A small proportion of the dose excreted during the evaluated periods corresponds well with the very long biological half-life of amiodarone in humans and rats [5,28]. Importantly for interactions, the measured plasma concentrations in our study comply with those observed in clinical settings where therapeutic concentrations are 1–2.5 mg/l, i.e. 1.5–3.7 μM [5]. Administration of azithromycin also yielded pharmacokinetic behaviour that complies with previously reported data [17]. The only difference was higher renal clearance of azithromycin in our study. This fact may be related to the continual infusion of fluids and the maintenance of constant urine flow throughout the study to provide the same conditions as in the case of the following interaction study. Similarly to amiodarone, plasma concentrations of azithromycin were within the range attainable in humans [29,30]. Thus, the basal condition for evaluation of MTX interactions was fulfilled for both potential inhibitors.

The study evaluated the effect of amiodarone on the steady-state biliary and renal excretion of methotrexate in rats. As reported by Reynolds *et al*. [13], administration of amiodarone to patients receiving oral LDMTX therapy induced serious skin necrosis which healed rapidly when methotrexate was discontinued. Despite no interaction study being available yet, the authors proposed that the interaction had a pharmacokinetic background. In agreement with this suggestion, our study showed, for the first time, that amiodarone significantly decreased the biliary clearance of methotrexate with a corresponding increase in its C_{ss}. Considering MTX pharmacokinetics, the interaction described in our study is clearly based on changes in activities of transport proteins in the liver. Nevertheless, we are aware that the methodology used in our study did not allow us to answer the question of which transporter is involved in the observed interaction. Studies with transfected cell lines showed the inhibitory effect of amiodarone on P-gp- and OATP2-mediated transport of digoxin and anthracycline [11,12]. No study reporting the involvement of P-gp in methotrexate biliary excretion is available. However, P-gp is a transporter of cationic compounds with large lipophilic molecules and indirect evidence of there being no influence of MTX on the biliary secretion of other P-gp substrates suggests that the contribution of P-gp to MTX biliary elimination is small [31]. In contrast, recent data demonstrated that both main members of the OATP family, human OATP1A2 and OATP1B1 and rat Oatp1a1 (Oatp1) and Oatp1a4 (Oatp2), which function similarly, may contribute to MTX transport [32–34]. Moreover, Ueda *et al*. [35] suggested that interactions of methotrexate with organic anions take place at the level of basolateral membrane, the location of OATPs in the liver. Taken together, the inhibition of Oatp2-mediated hepatic uptake of MTX seems to be the principal mechanism of increased plasma concentration of MTX during amiodarone administration. In kidneys, reduced CL_{CR} was observed in amiodarone-administered animals. This result complies with the described impairment of kidney function during acute amiodarone therapy [36]. Nevertheless, a decreased glomerular filtration rate after administration of amiodarone was not associated with reduction of MTX CL_R. The ratio of CL_{RMTX}/CL_{CR} was increased after amiodarone, suggesting a blockade of reabsorption when compared with control animals. Amiodarone's inhibitory influence on OATP1 in the kidney, where this protein is expressed on the apical membrane of the distal tubule and seems to play a role in active tubular reabsorption of MTX [33], requires further elucidation.

The idea for evaluation of potential azithromycin–MTX interaction originates from the fact that interaction of MTX with other macrolide-like antibiotics has already been described, and that azithromycin was shown as an inhibitor of the

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Mrp2 transporter, an important molecule for biliary excretion of MTX [19,26]. Nevertheless, our study showed that the administration of azithromycin did not change LDMTX pharmacokinetics. This also provides important information for clinical practice where these two compounds may be co-administered in situations such as chemically induced abortion [37]. In addition, the absence of interaction supports the significance of Oatps for the pharmacokinetics of LDMTX. First, azithromycin is not an inhibitor of Oatps, thus indicating that potency to inhibit methotrexate pharmacokinetics is only through Mrp2 [38]. Second, Mrp2 is a low-affinity high-capacity MTX transporter with K_m of 300 μM, thus its importance for pharmacokinetics of LDMTX is likely less prominent than for interactions with a high-dose regimen [19,35]. This information is supported by our recent finding that azithromycin produced a decrease in biliary and renal elimination of MTX when its C_{ss} approached an anticancer regimen [21].

To further extend information on the influence of amiodarone and azithromycin on the elimination pathways for organic anions the kinetics of endogenous conjugated bilirubin was evaluated in the same (MTX-infused) animals. It is known that Mrp2-mediated biliary excretion of conjugated bilirubin serves as the main rate-limiting step in the biliary elimination of the compound and that MRP2 deficiency is associated with hyperbilirubinaemia in rats and humans [39]. Because both evaluated compounds have been demonstrated previously to produce intrahepatic cholestasis during repeated long-term administration, we expected a decrease in the biliary excretion of the compound associated with its increased plasma concentrations [40,41]. Indeed, administration of amiodarone induced a marked increase of endogenous conjugated bilirubin plasma concentration (5.9-fold); however, the biliary excretion of CB was correspondingly increased (5.5-fold), too. Because CB is the metabolite which is formed from bilirubin intrahepatically, the decreased uptake to hepatocytes is unlikely to be the cause of increased C_{ss} of the compound after amiodarone injection. Therefore, the acute increase seems to be related to increased production of CB rather than to the cholestasis reported after chronic treatment. This finding is supported by mild haemolysis observed in serum and urine of amiodarone bolus administered animals (unpublished observation). Indeed, haemolytic anaemia due to impaired erythrocyte membrane function and *in vitro* photohaemolysis was described for amiodarone [42,43]. In addition, the observed decrease in CL_{CR} which was associated with decreased renal clearance of CB may point to another mechanism of amiodarone induced hyperbilirubinaemia. These data may bring new insight into the mechanism of hyperbilirubinaemia observed after acute high-dose amiodarone administration in humans [44]. Regarding azithromycin, similar kinetic behaviour of CB, i.e. decreased CB plasma concentration, was observed in mice lacking Mrp3, the basolateral transporter of conjugated bilirubin with overlapping substrate specificity with Mrp2 [45,46]. Possibly, azithromycin also inhibits Mrp3, thus preventing backward transport of CB to blood.

In conclusion, the present study suggests that amiodarone, an inhibitor of Oatp2, increases the plasma concentration of MTX by inhibiting Oatp2-mediated hepatic uptake of MTX in rats. Although the data obtained in the present study cannot be extrapolated directly to humans, the results provide useful information about the mechanism of interaction already described in clinical practice. Therefore, the co-administration of both compounds would better be avoided in humans or, if inevitable, then careful monitoring of MTX plasma concentrations with immediate correction of its dosage is obligatory. In addition, our data show that one of the most commonly used antibiotics, azithromycin, at clinically relevant plasma concentrations, failed to demonstrate any significant effect on the *in vivo* biliary or renal excretion of low-dose methotrexate in rats and thus suggests a safe combination for therapy with low-dose methotrexate.

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