Mechanism of the Pharmacokinetic Interaction between Methotrexate and Benzimidazoles: Potential Role for Breast Cancer Resistance Protein in Clinical Drug-Drug Interactions

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ABSTRACT

The antifolate drug methotrexate (MTX) is transported by breast cancer resistance protein (BCRP; ABCG2) and multidrug resistance-associated protein 1–4 (MRP1–4; ABCC1–4). In cancer patients, coadministration of benzimidazoles and MTX can result in profound MTX-induced toxicity coinciding with an increase in the serum concentrations of MTX and its main metabolite 7-hydroxymethotrexate. We hypothesized that benzimidazoles interfere with the clearance of MTX and/or 7-hydroxymethotrexate by inhibition of the ATP-binding cassette drug transporters BCRP and/or MRP2, two transporters known to transport MTX and located in apical membranes of epithelia involved in drug disposition. First, we investigated the mechanism of interaction between benzimidazoles (pantoprazole and omeprazole) and MTX in vitro in membrane vesicles from S9 cells infected with a baculovirus containing human BCRP or human MRP2 cDNA. In S9-BCRP vesicles, pantoprazole and omeprazole inhibited MTX transport (IC50 13 μM and 36 μM, respectively). In S9-MRP2 vesicles, pantoprazole did not inhibit MTX transport and at high concentrations (1 mM), it even stimulated MTX transport 1.6-fold. Secondly, we studied the transport of pantoprazole in MDCKII monolayers transfected with mouse Bcrp1 or human MRP2. Pantoprazole was actively transported by Bcrp1 but not by MRP2. Finally, the mechanism of the interaction was studied in vivo using Bcrp1−/− mice and wild-type mice. Both in wild-type mice pretreated with pantoprazole to inhibit Bcrp1 and in Bcrp1−/− mice that lack Bcrp1, the clearance of i.v. MTX was decreased significantly 1.8- to 1.9-fold compared with the clearance of i.v. MTX in wild-type mice. The conclusion is as follows: benzimidazoles differentially affect transport of MTX mediated by BCRP and MRP2. Competition for BCRP may explain the clinical interaction between MTX and benzimidazoles.

INTRODUCTION

Methotrexate (MTX) is an antifolate drug, which is frequently used in the treatment of, e.g., childhood acute leukemia and rheumatoid arthritis. The clinical application of MTX is hampered by the development of drug resistance and the existence of potentially toxic drug-drug interactions with many commonly used drugs. Resistance to MTX occurs via several mechanisms and can involve amplification or mutation of the target enzyme dihydrofolate reductase, as well as reduced drug uptake by a mutated reduced folate carrier, or alterations in the enzymes involved in MTX polyglutamylation (1–4). In addition, members of the multidrug resistance-associated protein subfamily of ATP-binding cassette-transporters (MRP1–4; ABCC1–4) and breast cancer resistance protein (BCRP; ABCG2) have been shown recently to play a role in MTX resistance and transport in vivo (5–13).

Clinically important pharmacokinetic drug-drug interactions exist between (intermittent high-dose or chronic low-dose) MTX and, e.g., nonsteroidal anti-inflammatory drugs, salicylic acid, and probenecid. These interactions may result in bone marrow suppression and acute renal failure (14–17). Takeda et al. (18) speculated that hOAT1, hOAT3, hOAT4, and possibly other transporters are involved in these interactions. Furthermore, in cancer patients, interactions have been reported between MTX, its main metabolite 7-hydroxymethotrexate, and the benzimidazoles omeprazole and pantoprazole, resulting in long-lasting extensive myelosuppression associated with systemic infections and severe mucositis (19–21). Omeprazole and pantoprazole inhibit the gastric hydrogen-potassium adenosine triphosphatase (H+ K+ -ATPase) and are frequently used in the treatment of peptic ulcers, pyrosis, and gastroesophageal reflux disease. Omeprazole was shown to inhibit MTX clearance, resulting in sustained, highly toxic MTX levels (20, 21). Coadministration with pantoprazole resulted in a 70% increase in the serum concentration of the metabolite 7-hydroxymethotrexate (19).

We hypothesized that benzimidazoles interfere with the renal (and possibly hepatic) clearance of MTX and/or 7-hydroxymethotrexate by inhibition of the ATP-binding cassette-transporters BCRP and/or MRP2, two transporters known to transport MTX and located in apical membranes of epithelia involved in drug disposition. To test this hypothesis, we first investigated the mechanism of interaction between benzimidazoles and MTX in vitro in membrane vesicles from S9 cells infected with baculovirus containing human BCRP or MRP2 cDNA. Secondly, we studied in another drug transport model, i.e., MDCKII cells transfected with mouse Bcrp1 or human MRP2, whether pantoprazole itself is transported by Bcrp1 and/or MRP2. In addition, we studied in the MDCKII-Bcrp1 monolayers the effect of pantoprazole on the transport of the topoisomerase I inhibitors topotecan and SN38, which are good substrate drugs of BCRP (22, 23). Finally, we investigated the mechanism of the interaction between MTX and benzimidazoles in vivo by studying the plasma pharmacokinetics and fecal and urinary excretion of i.v.-administered MTX in Bcrp1 knockout and wild-type mice with or without coadministration of pantoprazole.

MATERIALS AND METHODS

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heim (Almere, the Netherlands), and OE67 filters were from Schleicher and Schuell (Dassel, Germany). All of the other chemicals and reagents were of analytical grade or better and were purchased from Sigma (St. Louis, MO).

Cell Lines. The MDCKII cells were cultured in DMEM supplemented with 10% FCS and 100 units penicillin/streptomycin/ml (24). Cells were grown at 37°C with 5% CO2 under humidifying conditions. Polarized MDCKII cells stably expressing human MRP2 (ABCC2) or murine Bcrp1 (ABCG2) cDNA have been described previously (7, 22, 25). Si9 insect cells in suspension were grown in SF-900 II serum-free medium in the absence of serum (Invitrogen). The MRP2 cDNA and BCRP cDNA containing baculoviruses were a kind gift from Balasz Sarkadi (National Institute of Hematology, Budapest, Hungary; Ref. 7).

Protein Analysis and Immunoblotting. Membrane vesicle preparations were diluted in buffer [10 mM KCl, 1.5 mM MgCl2, and 10 mM Tris-HCl (pH 7.4)] supplemented by a cocktail of protease inhibitors used at the concentration recommended by the manufacturer (Roche, Mannheim, Germany). The indicated amount of protein was size-fractionized on a 7.5% SDS polyacrylamide gel and subsequently blotted overnight in a tank blotting system. Multidrug resistance-associated protein 2 and BCRP were detected with the monoclonal antibodies M215 (1:250) and BXP-21 (1:150), respectively, as described previously (25, 26). Signals were visualized with chemiluminescence (enhanced chemiluminescence; Amersham, United Kingdom).

Preparation of Membrane Vesicles and Vesicular Transport Assays. Membrane vesicles from Si9 cells were obtained after infection with an MRP2 or BCRP cDNA containing baculovirus at a multiplicity of infection of 1 and were prepared as described previously (27). Vesicular transport assays were performed in buffer consisting of 100 mM KCl and 50 mM HEPES/KOH (pH 7.4) in the presence or absence of 4 mM ATP (28). The time- and concentration-dependent uptake of substrates into membrane vesicles were studied after the rapid filtration method as described previously (29). The ATP-dependent transport was calculated by the difference in transport in the absence or presence of ATP.

Transport Across MDCKII Monolayer. Cells were seeded on microporous polycarbonate membrane filters (Transwell 3414; Costar, Corning, NY) at a density of 1 × 106 cells/well in 2 ml of complete medium. Cells were grown for 3 days with medium replacements every day. Two hours before the start of the experiment, medium on both the apical and the basal sides of the monolayer was replaced with 2 ml of Optimem medium (Life Technologies, Inc. Ltd., Paisley, Scotland) containing the appropriate concentration of transport modulator and also 5 μM PSC833 to inhibit endogenous P-glycoprotein (P-gp) levels or 1 μM GF120918 (elacridar) to inhibit endogenous P-gp and Bcrp1 levels (22). The experiment was started by replacing the preincubation medium on either the apical or the basal side of the cell layer with 2 ml of the similar medium also containing the appropriate concentration of radioisotopically labeled drug or radiolabeled imatinib. Imatinib was added to check for the intactness of the monolayer. The cells were incubated at 37°C in 5% CO2, and 50 μl of aliquots were taken every hour up to 4 h. The radioactivity in these aliquots was measured by the addition of 4 ml of scintillation fluid (Ultima Gold; Packard, Meriden, CT) and subsequent liquid scintillation counting (Tri-Carb 2100 CA Liquid Scintillation analyzer; Canberra Packard, Groningen, the Netherlands). Retention times and peak areas were analyzed with Waters Millenium integration software. The mobile phase consisted of a mixture of formic acid 1% (v/v) and acetonitril 17.5% (v/v) adjusted to pH 6.65 with 1 M HCl. The flow rate was 1.0 ml/min, and all of the separations were carried out at 35°C. The excitation and emission settings were 376 and 534 nm, respectively. Preparation of standard solutions of SN38 has been described previously (30). Calibration standards were prepared by dilution of the stock solutions with Optimem medium. The calibration curve for SN38 ranged from 0.01 to 1 μM. Samples were acidified with 0.5 M HCl to maintain SN38 in its lactone form. The calibration curve for SN38 ranged from 0.01 to 1 μM. Samples were acidified with 0.5 M HCl to maintain SN38 in its lactone form.

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were male Bcrp1-/- (Bcrp1 knockout; Ref. 31) and wild-type mice of a comparable genetic background (FVB or mixed 129/Ola and FVB) between 9 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet (AM-II; Hope Farms, Woerden, the Netherlands) and acidified water ad libitum.

Drug Preparation, Administration, and Plasma Analysis. The clinical formulation of MTX (Emmethe) was diluted with NaCl 0.9% to a final concentration of 12.5 mg/ml. A tracer quantity of [3H]MTX was added to the MTX solution. A vial of pantoprazole (Pantozol) was diluted with NaCl 0.9% to a final concentration of 8 mg/ml.

In patients, interactions have been reported between high-dose methotrexate and standard-dose omeprazole, resulting in sustained, highly toxic MTX levels (20, 21). To mimic this interaction in wild-type and Bcrp1 knockout mice, we calculated the dosage of methotrexate and pantoprazole to be used in mice, as described previously by Freireich et al. (32). For MTX, we chose a clinical intermediate high dose of ~250 mg/m2. For pantoprazole, we also related the standard dose administered to patients (p.o. or i.v. 40–80 mg/day) to the dosage per body surface (i.e., 26–52 mg/m2). The applied dose in mice (i.e., 120 mg/m2) was 2- to 4-fold higher than the clinically applied dose of pantoprazole.

The [3H]MTX was administered i.v. in the tail vein at a dose of 85 mg/kg body weight, which is equivalent to a dose of 255 mg/m2 MTX (32). Three min before i.v. [3H]MTX, the mice received either i.p. pantoprazole at a dose of 40 mg/kg body weight (i.e., 120 mg/m2) or i.v. NaCl 0.9% (control) in an equal volume.

Blood samples (30 μl) were taken from the tail vein at 1.5, 3, 10, 30, 60, and 90 min after MTX administration. One hundred twenty minutes after MTX administration, animals were anesthetized with methoxyflurane, their blood was collected by cardiac puncture, and they were sacrificed by cervical dislocation. Coagulation of blood was prevented by use of heparinized capillaries (at time point 1–6) and needle (at last time point) for blood sampling. The plasma fraction of the blood samples was collected after centrifugation at 3000 g for 5 min. Radioactivity in plasma samples was determined by liquid scintillation counting (Tri-Carb 2100 CA Liquid Scintillation analyzer; Canberra Packard, Groningen, the Netherlands). Any radioactivity crossing the monolayer and appearing in the opposite compartment was noted as the fraction of total radioactivity added at the beginning of the experiment. Paracellular luminal flux was tolerated up to 2% of the total radioactivity over 4 h.

High-Performance Liquid Chromatography (HPLC) Analysis of Pantoprazole. To determine whether pantoprazole is transported by MRP2 and/or Bcrp1, concentrations of pantoprazole in Optimem medium were measured by use of HPLC analysis. The HPLC system consisted of a P1000 pump and an AS3000 automatic Autosampler (Thermo Separation Products, Breda, the Netherlands). Separation was performed using a Zorbax SB-C8 (5 μm; 150 × 4.6 mm) analytical column (Atas, Zoetermeer, the Netherlands) and a Phenomenex C8 (Octyl, MOL; 4 × 2 mm) guard column (Bester, Amstelveen, the Netherlands). UV detection was performed using a UV-1000 detector (Thermo Separation Products). Retention times and peak areas were analyzed with ChromQuest 2.51 software (Thermo Quest, Breda, the Netherlands). The mobile phase consisted of a mixture of phosphate buffer 5 mM (70% v/v) adjusted to pH 7.0 with 2 M HCl and acetonitril (30% v/v). The flow rate was 1.0 ml/min, and the absorbance was measured at a wavelength of 280 nm. The stock solutions of pantoprazole (Pantozol i.v.) were prepared according to the manufacturer’s instructions. Standard samples for calibration were prepared by diluting the stock solution of pantoprazole with Optimem. For the calibration curve, a weighing factor of 1/(concentration)² was used. The calibration curve ranged from 0.5 to 300 μM. All of the samples were injected without additional preparation onto the chromatographic system.

HPLC Analysis of SN38. SN38 concentrations in Optimem medium were determined by HPLC analysis. The chromatographic system consisted of a Waters 616 dual piston pump, a Waters 717 plus autosampler, and a thermostated column compartment. Isocratic elutions were performed using a Chromsep ODS-2 glass column (5 μm; 100 × 3 mm) and a guard column (Chromsep RP; 5 μm; 10 × 2 mm). Detection was performed fluorimetrically using a Waters 474 scanning fluorescence detector. Retention times and peak areas were analyzed with Waters Millenium integration software. The mobile phase consisted of a mixture of formic acid 1% (v/v) and acetonitril 17.5% (v/v) adjusted to pH 6.65 with 1 M HCl. The flow rate was 1.0 ml/min, and all of the separations were carried out at 35°C. The excitation and emission settings were 376 and 534 nm, respectively. Preparation of standard solutions of SN38 has been described previously (30). Calibration standards were prepared by dilution of the stock solutions with Optimem medium. The calibration curve for SN38 ranged from 0.01 to 1 μM. Samples were acidified with 0.5 M HCl to maintain SN38 in its lactone form.

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Differences were considered to be statistically significant when \( P < 0.05 \). Pharmacokinetic parameters after i.v. administration of MTX were calculated according to a two-compartment open model, using the software package MW/Pharm (MEDWARE, version 3.02). The area under the concentration-time curve was calculated using the blood samples collected from the tail vein, i.e., from 0 to 90 min. The area under the concentration-time curve was calculated by the following formula: area under the plasma concentration-time curve = \( C_0/\alpha + C_\beta/\beta \), in which \( \alpha \) denotes the exponential rate constant of the initial phase, and \( \beta \) denotes the exponential rate constant of the terminal phase (33). The clearance was calculated by the following formula: clearance = dose/area under the plasma concentration-time curve (AUC). The half-lives of the initial and terminal phase were calculated by the following formula: \( t_{1/2} (\alpha) = \ln 2/\alpha \) and \( t_{1/2} (\beta) = \ln 2/\beta \), respectively (33).

Statistical analyses were performed by ANOVA using Bonferroni post-hoc test for multiple comparisons. Results are presented as means ± SD. Differences were considered to be statistically significant when \( P < 0.05 \).

**RESULTS**

**Transport of MTX by BCRP (ABCG2-R482).** Membrane vesicles were prepared from Sf9 insect cells transfected with a BCRP baculovirus coding for ABCG2-R482 (BCRP) or with a wild-type baculovirus. Detection with the monoclonal antibody BXP-21 (26) showed that high levels of ABCG2 protein were present in the membranes prepared from transfected Sf9 cells and not in the parental vector-transfected control cells, as indicated by the immunoreactive band of M\(_r\) ~ 70,000 (Fig. 1, inset). MTX is transported by BCRP as shown in Fig. 1, as also demonstrated by Chen et al. (12) and Volk et al. (13). The initial ATP-dependent uptake rate of 1 \( \mu \)M MTX over the first 2 min was ~3.5 pmol/mg/min in the Sf9-BCRP membrane vesicles and was negligible in the Sf9 wild-type vesicles.

**Inhibition of BCRP-Mediated MTX Transport by Sf9 Membrane Vesicles.** Using these Sf9-BCRP and Sf9-wild-type membrane vesicles, we studied the effect of different concentrations of pantoprazole and omeprazole on the transport of 1 \( \mu \)M MTX. The ATP-dependent transport of MTX by BCRP was inhibited by the benzimidazoles in a concentration-dependent manner, as shown in Fig. 2. A and B. IC\(_{50}\) values were 13 \( \mu \)M and 36 \( \mu \)M for pantoprazole and omeprazole, respectively.

**Stimulation of MRP2 Mediated Transport of MTX in Sf9 Membrane Vesicles.** The benzimidazoles did not inhibit the MRP2-mediated transport of MTX in Sf9-MRP2 membrane vesicles. Pantoprazole even stimulated MTX transport in a concentration-dependent manner. At high concentrations of pantoprazole (1 \( \mu \)M), transport of 1 and 100 \( \mu \)M MTX by MRP2 was stimulated 1.6-fold (Fig. 2C). This modest stimulation is not unexpected, because we have previously found substantial stimulation of MRP2-mediated transport of E\(_2\)17\( \beta \)G by benzimidazoles (27, 34).

**Transport of Benzimidazoles by Bcrp1 and not by MRP2 in MDCKII Monolayer.** Benzimidazoles have weakly basic properties. At pH 7.4, benzimidazoles (pKa ~ 4) are in an unprotonated form and diffuse readily across intact cells. To determine whether benzimidazoles interfere with drug transport by competition for BCRP, we studied whether pantoprazole is transported by Bcrp1 in MDCKII transfected cells. We also studied whether pantoprazole is transported by MRP2. To exclude any contribution of P-gp, the effective P-gp inhibitors PSC833 (5 \( \mu \)M) and GF120918 (1 \( \mu \)M) were added, respectively, in the MDCKII-Bcrp1 and MDCKII-MRP2 cell lines (22, 27, 35). We found transport of pantoprazole by Bcrp1 (Fig. 3) but not by MRP2 (data not shown). This Bcrp1-mediated transport of pantoprazole was saturable, as shown in Fig. 4. The pantoprazole concentration at which half-maximal transport (apparent K\(_{1/2}\) ) of pantoprazole occurred was 0.1–0.2 \( \mu \)M at 4 h transport, but varied from 0.2 to 0.4 \( \mu \)M at t = 2 h and t = 3 h transport of pantoprazole (data not shown).
Inhibition of Bcrp1-Mediated Transport of Topotecan and SN38 by Pantoprazole. To study the effect of benzimidazoles on the transport of substrate drugs by BCRP, we used 5 μM [14C]topotecan or 1 μM SN38. Both drugs are good model substrates in the MDCKII cell line for BCRP (22, 23). Because topotecan and SN38 are also transported at a low rate by P-gp, the P-gp inhibitor PSC833 (5 μM) was added in these assays to exclude any contribution of P-gp (22, 23, 36). Pantoprazole inhibited the Bcrp1-mediated transport of both drugs, as shown in Fig. 5 for topotecan. Complete inhibition of the Bcrp1-mediated transport of topotecan was reached at a concentration of 500 μM pantoprazole. Transport of SN38 in the Bcrp1-transfecant was inhibited 44% by 500 μM pantoprazole and 80% by 1 mM pantoprazole (results not shown). We did not detect transport of topotecan by MRP2, and for SN38, only minimal transport was observed in the MDCKII-MRP2 cell line (data not shown).

Role of Bcrp1 in the Clearance of MTX in Mice. To determine the role of Bcrp1 in the clearance of MTX in vivo, we administered i.v. [3H]MTX (85 mg/kg = 255 mg/m²) to Bcrp1+/+ (wild-type) and Bcrp1−/− (knockout) mice. We measured the MTX plasma concentration by total radioactivity at strategic time-points over a 90-min time period and calculated the pharmacokinetic parameters by a two-compartment pharmacokinetic analysis. The plasma concentration-time curves fitted the applied two-compartment model well ($r^2 \geq 0.99$), which is in line with the pharmacokinetics of MTX described in humans (37). As shown in Fig. 6 and listed in Table 1, the clearance of i.v. MTX was 1.9-fold decreased in Bcrp1 knockout mice compared with wild-type mice. Although this is highly significant ($P < 0.001$), the half-lives of the initial phase ($t_{1/2 \alpha}$) and the terminal phase ($t_{1/2 \beta}$) were not significantly different between wild-type and Bcrp1 knockout mice (Table 1). However, it should be noted that the main differences in plasma disposition of MTX between Bcrp1 knockout and wild-type mice originated already during the first 5–10 min (Fig. 6). Because the decline of the plasma concentration of MTX was very rapid during the first minutes, the determination of $t_{1/2 \alpha}$ was inaccurate as shown by the high variation coefficient in $t_{1/2 \alpha}$. This inaccuracy may mask the expected difference between Bcrp1 knockout and wild-type mice in $t_{1/2 \alpha}$. It is obvious, however, that Bcrp1 plays a significant role in the clearance of MTX in mice.

Effect of Pantoprazole on the Bcrp1-Mediated Clearance of MTX in Mice. To investigate whether the interaction between MTX and benzimidazoles, which was observed in cancer patients, is (partly) mediated by BCRP, we determined whether pantoprazole inhibits the Bcrp1-mediated clearance of MTX in mice. We administered i.v. pantoprazole (40 mg/kg = 120 mg/m²) or i.v. NaCl 0.9% (control) to wild-type mice 3 min before i.v. MTX (85 mg/kg = 255 mg/m²) and performed plasma pharmacokinetic measurements and model fitting as described previously. The two-compartment model described the observed data well ($r^2 \geq 0.99$). As shown in Fig. 6 and listed in Table 1, coadministration of pantoprazole reduced the clearance of MTX in wild-type mice to the same extent, i.e., 1.8-fold ($P < 0.001$), as observed for the decreased clearance of MTX in the Bcrp1 knockout mice compared with wild-type mice (1.9-fold). Both the half-life of the initial phase ($t_{1/2 \alpha}$) and the half-life of the terminal phase ($t_{1/2 \beta}$) were not significantly changed by pantoprazole treatment (Table 1). Also in this situation, however, the variation coefficient in $t_{1/2 \alpha}$ was high, which may mask the difference between wild-type mice treated with or without pantoprazole.

To determine whether other drug transporters may also play a role in the interaction between benzimidazoles and MTX in vivo, we compared the effect of i.v. pantoprazole with the effect of i.v. NaCl 0.9% (control) on the clearance of MTX in Bcrp1 knockout mice (Fig. 5).
Table 1). The clearance was not significantly different ($P = 0.73$), which suggests that Bcrp1 is the major mediator of the interaction between benzimidazoles and MTX in mice at the dose level of MTX studied.

Fecal and Urinary Excretion of [$^3$H]MTX in Bcrp1 Knockout and Wild-Type Mice. To determine the contribution of Bcrp1 to fecal and urinary excretion of [$^3$H]MTX and the effect of pantoprazole on this, we administered i.v. [$^3$H]MTX ($100\text{ mg/kg} \approx 300\text{ mg/m}^2$) to Bcrp1 knockout and wild-type mice and measured fecal and urinary radioactivity. The mice were housed in metabolic cages. Most of the radioactivity in urine and feces was excreted during the first 24 h. In the 24–48 h urine and feces portions, radioactivity was low ($\approx 5\%$ additional excretion; data not shown). In wild-type mice, $57 \pm 4.4\%$ of the given radioactivity was recovered from feces and $28 \pm 2.5\%$ was recovered from urine over the 24 h time-period after administration of [$^3$H]MTX, indicating that fecal excretion is the main excretory pathway for [$^3$H]MTX in mice (Fig. 7). Fecal excretion diminished 1.2-fold in Bcrp1 knockout mice ($46 \pm 5.5\%$; $P < 0.05$), 1.5-fold in wild-type mice pretreated with pantoprazole ($37 \pm 10\%$; $P < 0.01$), and 1.6-fold in Bcrp1 knockout mice pretreated with pantoprazole ($36 \pm 3.0\%$; $P < 0.01$) compared with control wild-type mice. However, urinary excretion was not significantly different in Bcrp1 knockout mice ($29 \pm 3.7\%$), in wild-type mice pretreated with pantoprazole ($28 \pm 8.5\%$), and in Bcrp1 knockout mice pretreated with pantoprazole ($39 \pm 8.5\%$) compared with control wild-type mice. Together, the data suggest that absence of Bcrp1 or inhibition of Bcrp1 by pantoprazole mainly reduces the fecal excretion and, thus, most likely the hepatic clearance of [$^3$H]MTX in mice.

DISCUSSION

In this study, we show that coadministration of benzimidazoles significantly inhibits BCRP-mediated transport of MTX in vitro and reduces the clearance of MTX in vivo. In vitro, inhibition of BCRP-mediated transport of MTX was reached at clinically relevant concentrations of benzimidazoles (Fig. 2, A and B). Concentrations of $10\mu M$ pantoprazole or omeprazole resulted in $46\%$ and $25\%$ inhibition of $1\mu M$ MTX, respectively. Standard doses of benzimidazoles in patients give plasma concentrations in the range of $5$–$10\mu M$ (38). Our in vitro results additionally reveal that benzimidazoles are actively transported by Bcrp1 but not by MRP2 (Fig. 3). The Bcrp1-mediated transport of benzimidazoles was saturable (Fig. 4). The observed...
interaction between benzimidazoles and MTX at the level of BCRP is compatible with competitive inhibition of transport. We hypothesized that this competitive inhibition for BCRP may (partly) explain the clinically documented interaction between MTX and benzimidazoles (19, 20, 21). Our in vivo data support this assumption. Pantoprazole significantly reduced the clearance of MTX in wild-type mice, and it reduced it to similar levels as in Bcrp1 knockout mice (Fig. 6). Moreover, in Bcrp1 knockout mice, the clearance of MTX was not reduced by pantoprazole. Thus, Bcrp1 appears to be the major mediator of the interaction between MTX and benzimidazoles in vivo at the dose level of MTX studied.

In humans, the major route of MTX elimination is renal excretion of unmetabolized MTX (39). In rats, 62% of i.v.-administered MTX was excreted into bile, whereas 27% of the dose was excreted into urine, as was shown by Masuda et al. (40). This biliary excretion was mediated by MRP2. Furthermore, it was shown in rats that 5.8% of MTX is metabolized by hepatic aldehyde-oxidase to 7-hydroxymethotrexate, which, in turn, is excreted predominantly into the bile as well (41). Henderson et al. (42) showed that in mice at a relatively low dose of 15 mg/kg, 60–80% of i.v. [3H]MTX is excreted into the urine, largely within 8 h after injection. However, when [3H]MTX was administered p.o., only 21% appeared in the urine during the 24 h after a 15 mg/kg dose and 37% within 24 h after a 0.5 mg/kg dose. At a dose of 100 mg/kg, we found that 57 ± 4.4% of the i.v.-administered MTX is excreted into feces and 28 ± 2.5% in urine in wild-type mice. When Bcrp1 was absent or inhibited by pantoprazole, the fecal excretion diminished significantly 1.2- to 1.6-fold. However, the urinary excretion was not significantly different compared with control wild-type mice. This suggests that absence of Bcrp1 or inhibition of Bcrp1 by pantoprazole predominantly affects hepatic clearance of [3H]MTX in mice. The 1.8- to 1.9-fold reduction in plasma clearance of MTX in Bcrp1 knockout mice and in wild-type mice treated with pantoprazole is most likely caused by reduced hepatobiliary excretion of MTX. In mice, the highest expression of Bcrp1 mRNA was found in kidney, whereas humans appear to have low renal BCRP expression (22, 43, 44). The immunohistochemical studies of Maliepaard et al. (26) showed that BCRP was present in the bile canalicular membrane of human liver hepatocytes. Therefore, it is of interest to explore whether in humans pantoprazole exerts its pharmacological effect by affecting the hepatobiliary elimination of (high-dose) MTX.

We find that the contribution of Bcrp1 to the elimination of MTX (Fig. 6; Table 1) occurs mainly during the first 5 to 10 min after i.v. administration of MTX, when the plasma concentrations of MTX decline rapidly. Thus, the interaction between MTX and pantoprazole seems to take place at high plasma concentrations of MTX. This is plausible because BCRP is a low-affinity, high-capacity transporter of MTX. At high concentrations of MTX, other high-affinity transporters, e.g., the reduced folate carrier by which MTX is actively transported across the proximal tubule via hOAT4. They demonstrated that these hOATs mediated drug interactions between MTX and nonsteroidal anti-inflammatory drugs, probenecid, and penicilllin G. Whether hOATs transport benzimidazoles and whether they are involved in the interaction between MTX and benzimidazoles remains to be investigated.
Also, it is not known whether benzimidazoles interfere with cellular uptake of MTX by the reduced folate carrier (45, 46).

Benzimidazoles probably do not interfere with MTX at the level of drug metabolism. Although benzimidazoles are primarily metabolized by CYP2C19 and to a variable extent by CYP3A4 (38, 49), MTX is excreted mostly unchanged, and cytochrome P450 (CYP) enzymes are not involved in MTX metabolism. Approximately 10% of MTX is metabolized by hepatic aldehyde-oxidase to 7-hydroxymethotrexate (39, 41, 42), and whether benzimidazoles interfere with the oxidation of MTX by aldehyde-oxidase is not known. After i.v. administration of MTX, drug-drug interactions may occur because of protein binding displacement and decreased renal clearance of the drug (14–16). Because the plasma protein binding of MTX is only 50%, it seems unlikely that interactions at this level have clinical relevance (50).

In membrane vesicles, we found that pantoprazole inhibited the BCRP-mediated transport of MTX at clinically relevant concentrations (10 μM). Much higher concentrations of pantoprazole appeared to be needed in intact cells to inhibit the BCRP-mediated transport of topotecan and SN38 (Fig. 5; data not shown). Topotecan, SN38, and MTX differ in their affinity for BCRP/BCRP-1–topotecan and SN38 having a high affinity (22, 23), and MTX having a low affinity (12, 13). Hence, competition of pantoprazole with topotecan/SN38 for BCRP is probably ineffective, making a clinically relevant pharmacokinetic interaction between pantoprazole and these anticancer agents less likely.

Pauli-Magnus (35) et al. described recently that benzimidazoles are substrate drugs of P-gp and inhibit the P-gp-mediated transport of digoxin in Caco-2 cells (IC50 values of 17.7 and 17.9 μM) and inhibit the P-gp-mediated transport of systemic clearance of MTX. The bioavailability of MTX may be as much as 30% because pantoprazole effectively inhibits BCRP and P-gp-mediated drug transport. This approach could be explored for MTX despite the interaction between benzimidazoles and MTX at the level of the systemic clearance of MTX. The bioavailability of MTX may be as high as 20% when doses exceed 80 mg/m2, and these higher doses of MTX are routinely administered i.v. (39). We expect that coadministration of pantoprazole will improve the oral bioavailability of MTX and reduce interpatient variability in systemic exposure to MTX.

Identification of the role of ATP-binding cassette-transporter proteins and/or other transporter systems in drug-drug interactions may help to prevent these interactions and associated toxicities clinically. It is of interest to determine whether BCRP, multidrug resistance-associated protein2, and/or other ATP-binding cassette-transporter proteins are involved in other clinically important drug-drug interactions with MTX, e.g., nonsteroidal anti-inflammatory drugs, penicillins, ciprofloxacin, cyclosporin A, trimethoprim-sulfamethoxazole, or furosemide (14–18, 54–59). Benzimidazoles are used frequently in the treatment of peptic ulcers, pyrosis, and gastroesophageal reflux disease. This class of drugs is number one on the list of most prescribed drugs worldwide. It is of interest for the clinic, therefore, to explore whether BCRP, multidrug resistance-associated protein2, and/or P-gp are involved in other known drug-drug interactions with benzimidazoles, e.g., in combination with triazoles (itraconazole, ketoconazole, voriconazole), phenytoin, or diazepam (60, 61).

In conclusion, benzimidazoles represent a new class of drugs that differentially affect BCRP- and multidrug resistance-associated protein2-mediated transport of MTX. Competition for BCRP may explain the pharmacokinetic interaction between MTX and benzimidazoles observed in patients.

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REFERENCES

INTERACTION BETWEEN METHOTREXATE AND BENZIMIDAZOLES


35. Fahrig L, Brasch H, Iven H. Pharmacokinetics of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) in rats and evidence for the metabolism of MTX to 7-OH-MTX. Cancer Chemother Pharmacol 1989;23:156–60.


41. Fahrig L, Brasch H, Iven H. Pharmacokinetics of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) in rats and evidence for the metabolism of MTX to 7-OH-MTX. Cancer Chemother Pharmacol 1989;23:156–60.


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