Proton Pump Inhibitors Inhibit Methotrexate Transport by Renal Basolateral Organic Anion Transporter hOAT3

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Abstract

The coadministration of methotrexate (MTX) and proton pump inhibitors (PPIs) can result in a pharmacokinetic interaction that delays MTX elimination and subsequently increases the MTX blood concentrations. Human organic anion transporters (hOATs) are responsible for the renal tubular secretion of MTX and are thought to be involved in this drug interaction. The aim of this study was to evaluate the inhibitory potencies of PPIs on hOAT1 and hOAT3, which are the two isoforms of OATs predominantly expressed in kidney proximal tubules. Using stably transfected cell systems that express the uptake transporters human embryonic kidney (HEK)-hOAT1 and HEK-hOAT3, we analyzed the inhibitory potencies of omeprazole, lansoprazole, and pantoprazole on OAT-mediated [3H]estrone sulfate (ES), [3H]p-aminomethylpropionic acid (PAH), and [3H]MTX uptake in vitro. hOAT3 is a high affinity transporter for MTX (Km = 21.17 ± 5.85 μM). Omeprazole, lansoprazole, and pantoprazole inhibited [3H]MTX uptake in HEK-hOAT3 cells with an IC50 of 6.8 ± 1.16, 1.14 ± 0.26, and 4.45 ± 1.62 μM, respectively, and inhibited the [3H]ES uptake in HEK-hOAT3 cells with an IC50 of 20.59 ± 4.07, 3.96 ± 0.96, and 7.89 ± 2.31 μM, respectively. Furthermore, omeprazole, lansoprazole, and pantoprazole exhibited inhibited PAH uptake on hOAT1 in a concentration-dependent manner (IC50 = 4.32 ± 1.26, 7.58 ± 1.06, and 63.21 ± 4.74 μM, respectively). These in vitro results suggest that PPIs inhibit [3H]MTX transport via hOAT3 inhibition, which most likely explains the drug-drug interactions between MTX and PPIs and should be considered for other OATs substrates.

Introduction

Methotrexate (MTX), an antifolate drug, is used in a wide range of doses for the treatment of certain neoplastic diseases, severe psoriasis, and rheumatoid arthritis (Jolivet et al., 1983; Tugwell et al., 1987). High-dose MTX is widely accepted as the first line treatment of lymphoid malignancy, osteogenic sarcoma, and acute leukemia, with intravenous doses ranging from 300 mg/m2 to 12 g/m2. MTX is a highly toxic drug with a low therapeutic index. The therapeutic drug monitoring of MTX is essential to prevent toxicity from high plasma MTX concentrations, because delayed elimination can result in serious and potentially life-threatening toxicities.

Renal excretion is the primary route of MTX elimination. In humans 80 to 90% of the intravenous administered dose is excreted unchanged in the urine within 24 hours (Shen and Azarnoff, 1978). Renal excretion occurs via glomerular filtration and active tubular secretion mediated in proximal tubular cells uptake, followed by active efflux in tubular lumen. Organic anion transporters are responsible for the passage from the blood to proximal tubules (uptake). Many transporters of organic anionic drugs have been identified on the apical side of the human kidney epithelium, including multidrug-resistance–related protein (MRP2, ABCB2; MRP4, ABCC4) and breast cancer resistance protein (BCRP, ABCG2), which are responsible for the secretion into the urine (Takeda et al., 2002a; Burckhardt and Burckhardt, 2003; Launay-Vacher et al., 2006; Nozaki et al., 2007; VanWert and Sweet, 2008).

Among human organic anion transporters (hOATs), hOAT1 and hOAT3 localize to the basolateral membrane of proximal tubular epithelial cells and have been shown to transport MTX (Uwai et al., 1998; Nozaki et al., 2007; Rizwan and Burckhardt, 2007). Members of the OAT family transport a variety of endogenous substances and drugs, including antineoplastic agents, antiviral agents, β-lactam–antibiotics, diuretics, and angiotensin-converting enzyme inhibitors (Takeda et al., 2002b; Uwai et al., 2007; Vallon et al., 2008; Vanwert et al., 2008).

Several drugs, including nonsteroidal anti-inflammatory drugs (Nozaki et al., 2007; Uwai et al., 2004; Maeda et al., 2008), penicillin G (Takeda et al., 2002b), and probenecid (Aherne et al., 1978), are known to inhibit the elimination of MTX. The molecular mechanism underlying these interactions partially relies on the blockade of the renal secretion of antifolate via the basal uptake transporters hOAT3 and hOAT1 (Giacomini et al., 2010).
Materials and Methods

**Radiolabeled.** $[^3]$Hestrone sulfate ($[^3]$HES; 250 µCi; 9.25 MBq; 54.26 Ci/mmol, 2.00762 TBq/mmol); 99.5% purity; $[^3]$H-probenecid ($[^3]$HPAH; 1 mCi/ml; 37 mBq; 4.53 Ci/mmol, 167.61 GBq/mmol); 99% purity, were purchased from Perkin Elmer (Waltham, MA). $[^3]$Hmethotrexate ($[^3]$HMTX; 250 µCi; 9.25 MBq; 32.3 Ci/mmol); 99% purity, was purchased from Moravek Biochemicals (Brea, CA).

Unlabeled. $[^3]$Hestrone sulfate and estrone sulfate uptake, which are both well-established substrates of hOAT1 and hOAT3, respectively (Burckhardt, 2012), were purchased from Sigma-Aldrich (St. Louis, MO). Methotrexate ($[^3]$HMTX) was purchased from Sigma-Aldrich. All unlabeled solid compounds were dissolved in dimethyl sulfoxide (DMSO).

Scintillation fluid, Ultima-Gold, was from Perkin Elmer (Waltham, MA).

**Cell Culture and Transfection.** Stably transfected human embryonic kidney (HEK) cell lines were established by using the Flp-In expression system (In-\textit{vitrochem}, Carlsbad, CA) according to the manufacturer’s protocol. HEK-293 cells were routinely grown in DMEM containing 10% fetal calf serum and 1% streptomycin/penicillin in a humidified incubator at 37°C and 5% CO2. Briefly, in separate reactions, the cDNAs, including the open reading frames of hOAT1 or hOAT3, were subcloned into the Flp-In expression vector pcDNA5/FRT, which contained a FRT site linked to a hygromycin resistance gene. The constructs pcDNA5/FRT-hOAT1 and pcDNA5/FRT-hOAT3 constructs were then cotransfected with the Flp recombinase expression vector pOG44 into Flp-In HEK-293 cells. Cells stably expressing the transporters were selected in hygromycin (100 µg/ml) according to the manufacturer’s protocol. The cells were grown in flasks cultured in DMEM supplemented with 10% fetal bovine serum and hygromycin (100 µg/ml). The membranes were washed with TTBS and incubated with secondary antibodies diluted at 1:1000 for 1 hour at 20°C. The membranes were washed again (5 times for 10 minutes) with TTBS and probed with the Western Lightning Chemiluminescence Reagent (Perkin Elmer).

**Quantitative Real-Time Polymerase Chain Reaction.** Quantitative real-time polymerase chain reaction (PCR) was performed for hOAT1 and hOAT3. RNA prepared from HEK mock, HEK-hOAT1, and HEK-hOAT3 was purified on RNeasy columns (Qiagen, Valencia, CA) and then reverse transcribed using Transcripter First Strand cDNA Synthesis Kit (Roche Applied Science, Nutley, NJ) using oligo-dT as a primer. Each cDNA sample was subjected to duplicate real-time PCR reactions using a CFX96 (Bio-Rad, Hercules, CA) thermal cycler with the following conditions: initial denaturation (95°C for 30 seconds) followed by 44 cycles of denaturation (95°C for 2 seconds), hybridization-extension (60°C for 5 seconds).

Gene expression values were normalized to that of GAPDH in the corresponding cDNA samples.
\[
V = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]

Where \(v\) is the uptake velocity of the substrate (pmoles per milligram of protein per minute), \(S\) is the substrate concentration of the medium (micromolar), \(K_m\) is the Michaelis constant (micromolar), \(V_{\text{max}}\) is the maximal uptake velocity (pmoles per milligram of protein per minute).

**Statistics.** The uptake experiments were performed in triplicate, where the values are expressed as the mean of these replicates with error bars representing the standard error. All experiments were performed at least three times over three independent experiments in triplicate. Statistical significance was calculated by using unpaired Student’s t-test. Differences were considered statistically significant if \(P < 0.05\).

**Results**

**Characterization of hOAT1- and hOAT3-Expressing HEK Cells.** To test the inhibitory potencies of PPIs in vitro, we stably transfected HEK cells with cDNAs encoding human OAT1, the SLC22A6 gene, or human OAT3, the SLC22A8 gene. We validated these models by examining the presence of the proteins and the function of HEK-hOAT1– or HEK-hOAT3–transfected cells. The gene and protein expression levels of hOAT1 and hOAT3 were evaluated with quantitative real-time PCR and Western blot analysis. The respective recombinant OAT proteins were detected in the membrane fractions from OAT-expressing HEK cells but not in the HEK-mock control cells, at molecular masses of 60 kDa in the membrane fractions from hOAT1-expressing HEK cells, and 62 kDa in the membrane fractions obtained from hOAT3-expressing HEK cells (Supplemental Data).

**MTX Uptake.** To evaluate the uptake of MTX in HEK-hOAT1 and HEK-hOAT3, the cells were incubated in a solution containing 0.5 \(\mu\text{M}\) MTX (for HEK-hOAT1) (Fig. 2A) or 25 nM MTX (for HEK-hOAT3; Fig. 2B). The affinity of MTX for hOAT3 was higher than that for hOAT1 and hOAT3.
hOAT1 (Fig. 2A). The $K_m$ values of MTX uptake by hOAT3 was 21.17 ± 5.65 mM (Fig. 2C). The higher concentration tested on HEK-hOAT1 was 0.5 mM, with an accumulation of MTX in HEK-hOAT1, which was approximately twofold higher than that in the control cells. The $K_m$ was not determined for HEK-hOAT1, because the difference in the accumulation of MTX was too low (data not shown). The transporter-mediated uptake of [3H]MTX over time in HEK-hOAT3 is presented in Fig. 2B and was linear up to 10 minutes.

Inhibition of hOAT1- and hOAT3-Mediated Transport by PPIs.

The inhibition of hOAT1 and hOAT3 uptake of their specific substrate by PPIs was measured within the linear uptake phase. Regarding the inhibition of [3H]PAH uptake by hOAT1, omeprazole, lansoprazole, and pantoprazole inhibited the transport of PAH in HEK-hOAT1 in a concentration-dependent manner, with IC$_{50}$ values of 4.32 ± 1.26, 7.58 ± 1.06, and 63.21 ± 4.74 mM, respectively (Fig. 3).

Each tested PPI significantly inhibited hOAT3-mediated [3H]ES transport in a concentration-dependent manner (Fig. 4). The calculated half-maximal inhibitory concentration values were in the micromolar range. We obtained an IC$_{50}$ of 20.59 ± 4.07 mM for omeprazole, an IC$_{50}$ of 3.96 ± 0.96 mM for lansoprazole, and an IC$_{50}$ of 7.89 ± 2.31 mM for pantoprazole. Likewise, omeprazole, lansoprazole, and pantoprazole inhibited the transport of [3H]MTX in HEK-hOAT3 cells, with IC$_{50}$ values of 6.8 ± 1.16, 1.14 ± 0.26, and 4.45 ± 1.62 mM, respectively (Fig. 4).

Discussion

MTX is currently used in a wide range of doses, and high-dose MTX schedules are associated with an incidence of nephrotoxicity of 1.8% and a fatality rate of almost 0.1%, despite therapeutic drug monitoring and supportive therapy (Widemann and Adamson, 2006). Although drug interactions between MTX and PPIs have been described in the clinic, the specific mechanism for this drug-drug interaction remains unknown.

Our major finding indicates that hOAT3, an uptake transporter expressed at the basolateral side of renal proximal tubular cells, selectively mediates the uptake of MTX, and this transporter is dramatically inhibited in the presence of PPIs. Different studies have suggested the involvement of multiple drug transporters in the elimination of MTX (Breedveld et al., 2004; Suzuki et al., 2009), but the uptake transporters have been well established to be the first limiting step of MTX elimination (VanWert and Sweet, 2008). Among the OATs, OAT1 and OAT3 localize to the basolateral membrane of proximal tubular cells and have been shown to play a central role in the renal uptake of anionic drugs, namely MTX.

Our study confirmed that hOAT3 is a high-affinity type transporter of MTX. In our study, the estimated $K_m$ value for hOAT3 was 21.17 ± 5.65 μM, which was consistent with the $K_m$ values of MTX uptake (10.9 and 21.1 μM) previously described by Cha et al. (2001) and Takeda et al. (2002a), respectively. Because this $K_m$ value determined in human kidney sections was similar to that observed for hOAT3 in this study rather than that observed for hOAT1 (553.8 ± 43.2 μM) by Takeda et al. (2002a) in transfected S2 cells, OAT3 likely more
significantly contributes to the net uptake process involved in MTX elimination. We failed to detect MTX transport in HEK-hOAT1 below a concentration of 50 nM; because the uptake experiment required the use of 0.5 μM MTX according to a study described by El-Sheikh et al. (2013), we could observe an uptake transport by incubating HEK-OAT1 with only 0.5 μM MTX. Unfortunately the difference from the mock cells was not sufficient to evaluate the drug-drug interaction. Moreover, we believe that a MTX concentration above 100 μM is not clinically relevant for therapeutic drug monitoring, because a slow elimination of MTX was defined as plasma concentrations exceeding 15 μM at 24 hours (Santucci et al., 2010). These concentrations are much higher than the human plasma concentrations of MTX and seem unlikely clinical practice. Our current results were also consistent with the findings of Lu et al. (1999), who cloned hPAHT (p-aminohippurate transporter, the first name of hOAT1), which exhibited no significant MTX uptake activity. Uwai et al. (2004) determined the $K_m$ value for hOAT1-mediated MTX uptake using a Xenopus laevis oocytes expression system to be 724 μM. In fact, this higher value of $K_m$ for hOAT1 supported our result, i.e., this concentration was not clinically relevant (Uwai et al., 2004). More recently Kurata et al. (2014) confirmed the same result with HEK-hOAT1. Nozaki et al. (2007) also examined MTX uptake using human tissue sections and estimated $K_m$ values within the same range (48.9 ± 17.3 μM) we observed for hOAT3 (Nozaki et al., 2007). As mentioned previously by various authors, the discrepancy may be due to species differences in the transport activity between rat and human OAT1 or differences in the expression system (Takeda et al., 2002a; Uwai et al., 2004; Uwai and Iwamoto, 2010).

The most striking result of our study was the potent inhibition of MTX uptake transport by all 3 PPIs in HEK-hOAT3 cells. The observed PPI IC$_{50}$ values for MTX uptake were in the micromolar range (6.80, 1.14, and 4.45 μM for omeprazole, lansoprazole, and pantoprazole, respectively). Interestingly, the IC$_{50}$ values for the three PPIs of the MTX uptake transport of by hOAT3 were higher. The observed PPI IC$_{50}$ values were higher for MTX than ES but were within the same concentration range as the plasma circulating concentrations. Moreover, the IC$_{50}$ values observed for each PPI were compared with the plasma concentrations of PPIs according to the CYP2C19 genotype (Ishizaki and Horai, 1999) (see Table 1). Indeed, PPIs are mainly metabolized by CYP2C19, and because the impact of CYP2C19 polymorphism on drug concentrations has been well established, different concentrations should be considered (Goldstein, 2001; Simon et al., 2011). A previous group described the maximum concentration of carriers of a loss of function allele in the plasma for omeprazole (3.1 μM), lansoprazole (4.9 μM), and pantoprazole (11.5 μM) according to the CYP2C19 “poor metabolizer” phenotype (Regardh et al., 1990; Pue et al., 1993; Yasuda et al., 1995; Ieiri et al., 2001; Freston et al., 2003). The plasma concentrations were lower in carriers of the normal allele with an “extensive metabolizer” phenotype, 1.6, 2.4, and 5.4 μM for omeprazole, lansoprazole, and pantoprazole, respectively.

Until recently, most studies investigated the effects of PPIs on different in vivo or in vitro models and suggested some effect of PPIs on efflux transporters. The effect of PPIs on the uptake transporter was poorly understood. The present finding also confirms that PPIs potently interact with different uptake transporters (hOAT1 and hOAT3) and their well-established substrates. Among the three PPIs tested for the PAH uptake by HEK-hOAT1, two elicited a strong inhibitory effect (omeprazole IC$_{50}$ = 4.32 ± 1.26 μM and lansoprazole 7.58 ± 1.06 μM). In agreement with our results, Nies et al. (2011) recently published that PPIs inhibited hOCT-mediated metformin uptake in vitro. All five tested PPIs (omeprazole, pantoprazole, lansoprazole, rabeprazole, tenatoprazole) significantly inhibited metformin uptake by HEK-hOCT1, -hOCT2, and -hOCT3 in a concentration dependent manner. Consistent with our result, the IC$_{50}$ values of these PPIs were in the low micromolar range (3–36 μM) (Nies et al., 2011). In addition, the IC$_{50}$ values of potent OAT drug inhibitors, such as ibuprofen, ketoprofen, piroxican, indomethacin, and probenecid, described for adefovir uptake transport by hOAT1 were 8.0, 1.3, 20.5, 3.0, and 7.4 μM, respectively (Takeda et al., 2002a), which agrees with our results.

Although the clinical consequences are not easily predicted based on in vitro data, detailed advantages and limitations of various in vitro systems for evaluation of drugs as substrates, as inhibitors, or for their potential for drug-drug interactions have been delineated (Brouwer et al., 2011).
et al., 2013; Giacomini and Huang, 2013; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2013). According on the decision trees of the recommendations of the International Transporter Consortium (Giacomini et al., 2010), the values of the ration $[I]/IC_{50}$ of the three PPIs tested on the uptake of MTX on HEK hOAT3 are lower than 0.1 except for lansoprazole in poor metabolizers, for which the ratio is higher than 0.1, giving thought to a clinical interaction between lansoprazole and MTX. It would be very interesting to confirm if this in vitro drug-drug interaction would be relevant in humans in a prospective study. Some factors support such an assumption, because

**Fig. 4.** Inhibition of hOAT3-mediated $[^3]H$ES and $[^3]H$MTX uptake by PPIs. Inhibitory effect of omeprazole, lansoprazole, and pantoprazole on hOAT3-mediated $[^3]H$ES or $[^3]H$MTX uptake after 10-minute incubation. IC$_{50}$ values were calculated by fitting the data to a sigmoidal dose-response regression curve. Data points are the means ± S.E.M. of three independent experiments.

**TABLE 1**

IC$_{50}$ values for hOAT3 in MTX uptake and plasmatic concentrations of proton pump inhibitors according to CYP2C19 genetic polymorphism and plasma unbound fraction

PPI dosage and maximal total PPI concentration in the systemic circulation (C$_{inax}$) were obtained from the indicated references. $[I]$ = (C$_{inax}$ total) * (% plasma unbound fraction)/100.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Uptake MTX IC$_{50}$ hOAT3</th>
<th>Dose</th>
<th>Genotype CYP2C19</th>
<th>C$_{inax}$ Observed in Humans</th>
<th>$[I]$</th>
<th>$[I]/IC_{50}$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omeprazole</td>
<td>6.80 ± 1.16</td>
<td>20</td>
<td>EM</td>
<td>1.6 ± 1.0</td>
<td>0.05 ± 0.03</td>
<td>0.007</td>
<td>(Regardh et al., 1990; Yasuda et al., 1995)</td>
</tr>
<tr>
<td>Lansoprazole</td>
<td>1.14 ± 0.26</td>
<td>30</td>
<td>EM</td>
<td>2.44 ± 0.7</td>
<td>0.07 ± 0.02</td>
<td>0.061</td>
<td>(Ieiri et al., 2001; Freston et al., 2003)</td>
</tr>
<tr>
<td>Pantoprazole</td>
<td>4.45 ± 1.62</td>
<td>40</td>
<td>EM</td>
<td>5.4 ± 1.4</td>
<td>0.11 ± 0.03</td>
<td>0.025</td>
<td>(Regardh et al., 1990; Pue et al., 1993)</td>
</tr>
</tbody>
</table>
| PM, poor metabolizer phenotype; EM, extensive metabolizer phenotype. $[I]$, unbound inhibitor concentration.
plasma MTX is predictive of the risk of toxicity; therapeutic drug monitoring is often used for patients to evaluate the delayed elimination. Recently, the delayed elimination of MTX associated with serious side effects was described in three retrospective clinical studies of patients treated with high doses of MTX and PPIs (Joerger et al., 2006; Suzuki et al., 2009; Santucci et al., 2010; Leveque et al., 2011) and one prospective study of low dose MTX (Vakily et al., 2005). Although conflicting data were reported in some case reports for either omeprazole or pantoprazole (Whelan et al., 1999; Beorlegui et al., 2000; Troger et al., 2002; Bauters et al., 2008), recent clinical studies are in line with our results suggesting that PPIs might decrease MTX renal clearance via OAT3-mediated inhibition. In the first study, Joerger et al. (2006) described 76 patients who received high-dose MTX, 13 of whom received omeprazole or lansoprazole. Patients that received MTX and PPIs were associated with a 27% decrease in the clearance of MTX, which resulted in a significantly higher plasma concentration of MTX (Joerger et al., 2006). The second study is a retrospective non-interventional cohort study that included 79 French patients with cancer treated with high-doses of MTX. The coprescription of PPIs (pantoprazole, lansoprazole, omeprazole, or esomeprazole) was found in half of the cycles with delayed elimination and only in 15% of the cycles without delayed elimination (Santucci et al., 2010). The third study examined 74 Japanese patients with cancer. MTX was administered intravenously with a concomitant administration of omeprazole, lansoprazole, and rabeprazole. The MTX residual concentrations (311 measurements of plasma MTX) were analyzed in 171 cycles of high dose MTX. They found that PPI coadministration was still a significant risk factor for delayed elimination after adjustment for six variables (Suzuki et al., 2009). Interestingly, the delayed elimination of plasma MTX previously mentioned in these studies was not observed in all patients who received PPIs; based on our results, this finding may be due to higher concentrations of PPIs in carriers of CYP2C19 loss of function variant alleles (Ieiri et al., 2001; Simon et al., 2011).

Renal tubular secretion involves different uptake transporters. A recent study showed that the basolateral localization of mouse reduced folate carrier (RFC-1) in the kidney is responsible for the uptake of MTX (Nozaki et al., 2004). Other uptake transporters that are mainly expressed in the liver (OATP1B1) or intestine (OATP1A2) were found to transport MTX in vitro. These transporters were very recently found in vivo in transgenic mice that expressed liver-specific human (Suzuki et al., 2009). Reports on the drug-drug interaction between PPIs and renal transporters are lacking, and the role of these transporters should be clarified in subsequent studies. Additionally, reports on the drug-drug interaction between PPIs and other OAT3 substrates, such as antiviral drugs,