Characterization of Methotrexate Transport and Its Drug Interactions with Human Organic Anion Transporters

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ABSTRACT

Life-threatening drug interactions are known to occur between methotrexate and nonsteroidal anti-inflammatory drugs (NSAIDs), probenecid, and penicillin G. The purpose of this study was to characterize methotrexate transport, as well as to determine the site and the mechanism of drug interactions in the proximal tubule. Mouse proximal tubule cells stably expressing basolateral human organic anion transporters (hOAT1 and hOAT3) and apical hOAT (hOAT4) were established. The \( K_{m} \) values for hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake were 553.8 \( \mu \)M, 21.1 \( \mu \)M, and 17.8 \( \mu \)M, respectively. NSAIDs (salicylate, ibuprofen, ketoprofen, phenylbutazone, piroxicam, and indomethacin), probenecid, and penicillin G dose dependently inhibited methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4. Kinetic analysis of inhibitory effects of these drugs on hOAT3-mediated methotrexate uptake revealed that these inhibitions were competitive. The \( K_{i} \) values for the effects of salicylate, phenylbutazone, indomethacin, and probenecid on hOAT3-mediated methotrexate uptake were comparable with therapeutically relevant plasma concentrations of unbound drugs. In addition, in the presence of human serum albumin, the \( K_{i} \) values were comparable with therapeutically relevant total plasma concentrations of drugs. In conclusion, these results suggest that methotrexate is taken up via hOAT3 and hOAT1 at the basolateral side of the proximal tubule and effluxed or taken up at the apical side via hOAT4. In addition, hOAT1, hOAT3, and hOAT4 are the sites of drug interactions between methotrexate and NSAIDs, probenecid, and penicillin G. Furthermore, it was predicted that hOAT3 is the site of drug interactions between methotrexate and salicylate, phenylbutazone, indomethacin, and probenecid in vivo.

Methotrexate is widely used at high dosages in the treatment of malignancies, whereas it is used at low dosages in rheumatoid arthritis. Methotrexate is eliminated almost entirely in an unchanged form in urine, which involves glomerular filtration and active tubular secretion (Shen and Azarnoff, 1978). Therefore, renal insufficiency or drug interactions, which reduce the clearance of methotrexate, are potentially toxic events.

Interactions between methotrexate and drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), probenecid, and penicillin G have been reported by several groups of investigators (Ellison and Servi, 1985; Thyss et al., 1986; Basen et al., 1991; Frenia and Long, 1992). The interactions may have been caused by protein binding displacement, inhibitory effects on the renal secretion of methotrexate, and a decline in glomerular filtration as a result of inhibition of prostaglandin synthesis (Tracy et al., 1992; Kremer and Hamilton, 1995). Among these possible causes, the renal tubular secretion of methotrexate has been thought to be a major site for drug interaction (Frenia and Long, 1992).

Two different types of human multispecific OATs (hOAT1 and hOAT3) were recently isolated (Reid et al., 1998; Hosoyamada et al., 1999; Cha et al., 2001). In the kidney, hOAT1 and hOAT3 are localized at the basolateral membrane of the proximal tubule (Hosoyamada et al., 1999; Cha et al., 2001). Since hOAT1 and hOAT3 mediate the transport of various drugs, endogenous substances, and xenobiotics, these transporters are considered to be responsible for the basolateral

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; OAT, organic anion transporter; hOAT, human organic anion transporter; OAT-K1, OAT-K2, renal organic anion transporter 1 and 2; oatp1, oatp2, organic anion-transporting polypeptide 1 and 2; MRP2, multidrug resistance protein 2; NPT1, human type I sodium-dependent inorganic phosphate transporter; \( S_{i} \), \( S_{o} \), \( S_{n} \), the first, second, and third segment of proximal tubule; D-FBS, Dulbecco’s modified phosphate-buffered saline; hPAHT, human kidney \( \rho \)-aminohippurate transporter.
uptake of organic anions in renal epithelial cells. On the other hand, OAT-K1 (Saito et al., 1996; Masuda et al., 1999b), OAT-K2 (Masuda et al., 1999a), hOAT4 (Cha et al., 2000), organic anion-transporting peptide 1 ( oatp1) (Jacquier-min et al., 1994), oatp3 (Abe et al., 1998), multidrug-resistance protein 2 (MRP2) (Leier et al., 2000), and human type I sodium-dependent inorganic phosphate transporter (NPT1) (Uchino et al., 2000) were isolated and identified as transporters of anionic drugs and substances at the apical membrane of the proximal tubule.

The purpose of this study was to characterize methotrexate transport in the proximal tubule, as well as to determine the site and mechanism of interactions between methotrexate and NSAIDs, probenecid, and penicillin G using the second portion of the proximal tubule (S₂) cells stably expressing hOAT1, hOAT3, and hOAT4 (S₂ hOAT1, S₂ hOAT3, and S₂ hOAT4, respectively).

**Experimental Procedures**

**Materials.** [3H]Methotrexate (547.6 GBq/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Methotrexate, various NSAIDs, probenecid, penicillin G, transferrin, and human serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Other materials used included fetal bovine serum, trypsin and geneticin from Invitrogen (Carlsbad, CA), recombinant epidermal growth factor from Wakunaga (Hiroshima, Japan), insulin from Shimizu (Shizuoka, Japan), RITC 80 medium from Iwaki Co. (Tokyo, Japan), and TTX-50 from Promega (Madison, WI).

**Cell Culture and Establishment of S₂ hOAT1, S₂ hOAT3, and S₂ hOAT4.** S₂ cells, derived from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene, were established as described previously by us (Takeda et al., 1999). S₂ is the segment of the proximal tubule in which hOAT1, hOAT3, and hOAT4 were localized (Hosoyamada et al., 1999; Cha et al., 2001; Babu et al., 2002). The full-length cDNAs of hOAT1, hOAT3, and hOAT4 were subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA), a mammalian expression vector. S₂ hOAT1, S₂ hOAT3, and S₂ hOAT4 were obtained by transfecting S₂ cells with pcDNA3.1-hOAT1, pcDNA3.1-hOAT3, and pcDNA3.1-hOAT4 coupled with pSV2neo, a neomycin-resistant gene, using TTX-50 according to the manufacturer's instructions. S₂ cells transfected with pcDNA3.1 lacking an insert, and pSV2neo were designated as S₂ pcDNA 3.1 and used as a control (mock). These cells were grown in a humidified incubator at 33°C, which is commonly used for evaluating transporter activities. There was no difference in the amount of uptake between 33°C and 37°C (data not shown). Based on these results, the cells were cultured at 33°C, whereas uptake experiments were performed at 37°C. The S₂ cells were seeded in 24-well tissue culture plates at a cell density of 1 × 10⁵ cells/well. After the cells were cultured for 2 days, the cells were washed three times with Dulbecco’s modified phosphate-buffered saline (D-PBS) solution (containing 137 mM NaCl, 3 mM KCl, 1 mM KH₂PO₄, 1 mM NaHPO₄, 0.5 mM MgCl₂, 1 mM CaCl₂, and 0.5 mM MgCl₂, pH 7.4), and then preincubated in the same solution for 10 min in a water bath at 37°C. The cells were then incubated in D-PBS containing [³H]methotrexate at various concentrations as indicated in each experiment. The uptake was stopped by the addition of ice-cold D-PBS, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide and 2.5 ml of Aquasol-2, and radioactivity was determined using a β-scintillation counter (Aloka, Tokyo, Japan; LSC-3100).

**Inhibition Study.** To evaluate the inhibitory effects of various NSAIDs, probenecid, and penicillin G on methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4, S₂ hOAT1, S₂ hOAT3, and S₂ hOAT4 were incubated in a solution containing [³H]methotrexate in the absence or presence of various drugs at 37°C. Considering the Kᵢ values for hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake, hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake was estimated using a methotrexate concentration of 1 μM (hOAT1) or 100 nM (hOAT3 and hOAT4). In addition, based on the results of time course experiments (Fig. 1), hOAT3-mediated methotrexate uptake was evaluated during the 2-min incubation. On the other hand, since the specific methotrexate uptake activity, represented as the amount of hOAT1- and hOAT4-mediated methotrexate uptake subtracted by that by mock, was about two times larger during the 15-min incubation than that during the 2-min incubation, we chose 15 min as the incubation time for the inhibition experiments. Salicylate, probenecid, and penicillin G were dissolved in distilled water. Ibuprofen, ketoprofen, phenylbutazone, piroxicam, and indomethacin were dissolved in dimethyl sulfoxide and diluted with the incubation medium. The final concentration of dimethyl sulfoxide in the incubation medium was adjusted to less than 1%.

**Kinetic Analysis.** After preincubation as described above, S₂ hOAT3 was incubated in D-PBS containing [³H]methotrexate at different concentrations in the absence or presence of various drugs for 2 min. For the kinetic analysis of inhibitory effects, double reciprocal plot analyses were performed based on methotrexate uptake under each condition (Apiwattanakul et al., 1999). When the inhibition was competitive, the Kᵢ values were calculated based on the following equation,

\[
Kᵢ = \frac{[I]_{50} \text{ of methotrexate with inhibitor} - [I]_{50} \text{ of methotrexate without inhibitor}}{1}
\]

**Statistical Analysis.** Data are expressed as means ± S.E. Statistical differences were determined using one-way ANOVA with Dunnett’s post hoc test. Differences were considered significant at P < 0.05.

**Results**

**Methotrexate Uptake.** To evaluate the time-dependent uptake of methotrexate in S₂ hOAT1, S₂ hOAT3 and S₂ hOAT4, S₂ cell monolayers were incubated in a solution...
Fig. 1. Time course of methotrexate uptake in S2 hOAT1 (A), S2 hOAT3 (B), and S2 hOAT4 (C). These cells were incubated in a solution containing 1 μM [3H]methotrexate (hOAT1) or 100 nM [3H]methotrexate (hOAT3 and hOAT4) for various periods at 37°C. The values for the specific uptake in S2 hOAT1, S2 hOAT3, and S2 hOAT4 (open squares) were obtained by subtracting that in mock (closed circles) from those in S2 hOAT1, S2 hOAT3, and S2 hOAT4 (open circles). Each value represents the mean ± S.E. of four determinations from one typical experiment of the two separate experiments.

Inhibitory Effects on Methotrexate Uptake. We examined the effects of various drugs on methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4. The drugs used were NSAIDs (salicylate, ibuprofen, ketoprofen, phenylbutazone, piroxicam, and indomethacin), probenecid, and penicillin G. These drugs inhibited methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4 in a dose-dependent manner, whereas penicillin G did not inhibit hOAT1-mediated methotrexate uptake and salicylate did not inhibit hOAT4-mediated methotrexate uptake (data not shown). The effects of these NSAIDs on methotrexate uptake by hOAT1, hOAT3, and hOAT4 are listed in Table 1.

TABLE 1
The effects of various NSAIDs on methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4. 

<table>
<thead>
<tr>
<th>Drugs</th>
<th>hOAT1</th>
<th>hOAT3</th>
<th>hOAT4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>84.7 ± 7.95***</td>
<td>41.5 ± 2.90*</td>
<td>94.8 ± 8.53</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>47.0 ± 3.05*</td>
<td>64.0 ± 3.30**</td>
<td>67.8 ± 5.23**</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>53.4 ± 5.43*</td>
<td>45.2 ± 3.45*</td>
<td>81.0 ± 1.78***</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>30.0 ± 1.13*</td>
<td>31.3 ± 2.43*</td>
<td>55.7 ± 7.20*</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>40.9 ± 10.7*</td>
<td>56.2 ± 3.82*</td>
<td>62.0 ± 8.34**</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>60.6 ± 7.28*</td>
<td>38.5 ± 3.11*</td>
<td>60.9 ± 5.74**</td>
</tr>
<tr>
<td>Probenecid</td>
<td>55.1 ± 4.29*</td>
<td>30.2 ± 4.32*</td>
<td>81.6 ± 6.26***</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>102.3 ± 6.18*</td>
<td>27.8 ± 2.56*</td>
<td>79.7 ± 4.88***</td>
</tr>
</tbody>
</table>

* P < 0.001, ** P < 0.01, and *** P < 0.05 versus control.
for each drug tested are shown in Table 2. In addition, we performed a kinetic analysis of the inhibitory effects of salicylate, phenylbutazone, indomethacin, and probenecid on hOAT3-mediated methotrexate uptake in the presence of 5% human serum albumin. The inhibitory effects of these drugs on hOAT3-mediated methotrexate uptake in the presence of 5% human serum albumin were also competitive, and the $K_i$ values are listed in Table 2.

**Discussion**

hOAT1 and hOAT3 were recently cloned and characterized as multispecific OATs, and were found to mediate active transport of organic anions from the interstitium to the cells in the basolateral membrane of the proximal tubule (Hosoyamada et al., 1999; Cha et al., 2001). Some differences in characteristics exist between hOAT1 and hOAT3, such as substrate specificity and localization: hOAT1 at the basolateral side of the S3 segment of the proximal tubule (Hosoyamada et al., 1999) versus hOAT3 at the first, second, and third segments ($S_1$, $S_2$, and $S_3$) of the proximal tubule (Cha et al., 2001). In addition, hOAT1, but not hOAT3, exhibits transport properties as an exchanger (Hosoyamada et al., 1999; Cha et al., 2001). On the other hand, hOAT4 is localized to the apical membrane of the proximal tubule (Babu et al., 2002), and hOAT4 exhibits a relatively narrow substrate specificity compared with hOAT1 and hOAT3 (Cha et al., 2000).

Carrier-mediated transport of methotrexate in the basolateral side of the proximal tubule was suggested based on the results of the experiments using renal slices (Nierenberg, 1983; Williams et al., 1984) or isolated proximal tubule (Bessegir et al., 1989). The current results suggest that hOAT3 and hOAT1 mediate the transport of methotrexate in the basolateral side of the proximal tubule. Human OAT cloned by Lu was designated as human kidney p-aminohippurate transporter (hPAHT) (Lu et al., 1999), and there was 98.2% amino acid sequence homology between hPAHT and hOAT1 (data not shown). In contrast to hOAT1, hPAHT exhibited no significant methotrexate uptake activity. The discrepancy in methotrexate uptake activity between hOAT1 and hPAHT may be due to this small difference in amino acid sequence or the experimental conditions. To clarify this, a site-directed mutagenesis study should be performed. The current results were also consistent with the previous results that rat OAT1 mediates methotrexate uptake (Uwai et al., 2000). In addition, hOAT2, which was recently identified to be localized to the basolateral side of the proximal tubule, showed no significant uptake activity of methotrexate (M. Takeda, unpublished observation). This is in contrast to the recent report that cells expressing hOAT2 showed uptake of methotrexate (Sun et al., 2001). The reason for the discrepancy remains unknown, and further studies should be performed. It is also possible that unidentified OATs other than hOAT3 and hOAT1 are also involved in methotrexate transport in the basolateral membrane of the proximal tubule.

In addition to hOAT4, human transporters mediating the transport of organic anions in the apical membrane of the proximal tubule have been cloned, including MRP2 (Leier et al., 2000) and NPT1 (Uchino et al., 2000). Moreover, human MRP2 was shown to mediate the efflux of methotrexate using stable cells (Hooijberg et al., 1999). Thus, the relative contribution of hOAT4 and MRP2 to apical methotrexate transport should be clarified. In addition to human transporters, rodent transporters mediating the apical transport of organic anions have been cloned, including OAT-K1 (Saito et al., 1996; Masuda et al., 1999b), OAT-K2 (Masuda et al., 1999a), oatp1 (Jacquemin et al., 1994), and oatp3 (Abe et al., 1998). Among them, OAT-K1 and OAT-K2 were reported to mediate bidirectional methotrexate transport (Saito et al., 1996; Masuda et al., 1999a,b), and the $K_m$ value for OAT-K1-mediated methotrexate transport was determined to be 1.0 μM (Masuda et al., 1997). Thus, the cloning of human homologs of these rodent transporters and the functional analysis for the role of these transporters in apical methotrexate transport should be performed.

Since various drugs tested in this study dose dependently inhibited hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake, it was suggested that hOAT1, hOAT3, and hOAT4 are sites of drug interactions between methotrexate and these drugs. Among these interactions, it appears that those between methotrexate and salicylate, phenylbutazone, indomethacin, or probenecid at hOAT3 may have clinical significance, as follows. The total and unbound plasma concentrations of various drugs are as listed in the Table 2. Therapeutically relevant concentrations of unbound drugs in the plasma are thought to be within 5-fold of the maximum steady-state concentrations of unbound drugs in the plasma (Zhang et al., 2000). Comparing the $K_v$ values with therapeutically relevant concentrations of unbound drugs in the plasma, those for salicylate, phenylbutazone, indomethacin,
TABLE 2
The $K_i$ values of various drugs that inhibit methotrexate uptake in S$_2$ hOAT3
S$_2$ hOAT3 was incubated with solution containing various concentrations of $[^{3}H]$methotrexate in the absence or presence of various drugs. Experiments were performed in the absence or presence of 5% human serum albumin (HSA). The values of the uptake in mock were subtracted from those in S$_2$ hOAT3. The unbound plasma concentrations were calculated from the total plasma concentrations and unbound fractions of drugs.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>$K_i$ (HSA$^{(-)}$) $\mu M$</th>
<th>$K_i$ (HSA$^{(+)}$) $\mu M$</th>
<th>Concentration of Drugs $\mu M$</th>
<th>Total Plasma Concentration $\mu M$</th>
<th>Unbound Fraction</th>
<th>Unbound Plasma Concentration $\mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>1020</td>
<td>2440</td>
<td>2000</td>
<td>2000</td>
<td>2200$^a$</td>
<td>19.6$^a$</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1170</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
<td>48.5$^a$</td>
<td>1.00$^a$</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>1160</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
<td>1.20$^b$</td>
<td>0.80$^b$</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>34.7</td>
<td>350</td>
<td>50</td>
<td>750</td>
<td>320$^b$</td>
<td>3.90$^b$</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>1200</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
<td>24.0$^d$</td>
<td>0.02$^d$</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5.95</td>
<td>105</td>
<td>10</td>
<td>200</td>
<td>84.0$^b$</td>
<td>10.0$^b$</td>
</tr>
<tr>
<td>Probencid</td>
<td>29.8</td>
<td>110</td>
<td>50</td>
<td>200</td>
<td>170.0</td>
<td>11.0$^i$</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>97.6</td>
<td>100</td>
<td></td>
<td>9.60</td>
<td>49.1$^i$</td>
<td>4.71</td>
</tr>
</tbody>
</table>


and probenecid were comparable. In addition, the $K_i$ values obtained in the presence of 5% human serum albumin were comparable with total plasma concentrations of salicylate, phenylbutazone, indomethacin, or probenecid. Based on these, it was predicted that these drugs could inhibit the hOAT3-mediated methotrexate uptake in vivo, leading to the increased plasma concentration of methotrexate.

Uwai et al. (2000) have already demonstrated that the IC$_{50}$ values of salicylate, ketoprofen, and indomethacin for rat OAT1-mediated methotrexate uptake were 1410, 0.5, and 2.7 $\mu M$, respectively. However, we found that the IC$_{50}$ values of salicylate, ketoprofen, and indomethacin for hOAT1-mediated methotrexate uptake were $>2000, 1200,$ and $>2000\mu M$, respectively (data not shown). This discrepancy in findings may be due to the species difference, i.e., between rats and humans. Compared with rat OAT1, the amino acid sequence of hOAT1 exhibited 86.0% homology (Sekine et al., 1997). On the other hand, the IC$_{50}$ values of ibuprofen, ketoprofen, piroxicam, indomethacin, and probenecid for adefovir uptake in Chinese hamster ovary cells stably expressing hOAT1 were 8.0, 1.3, 20.5, 3.0, and 7.4 $\mu M$, respectively (Mulato et al., 2000). The results were similar to those for rat OAT1-mediated methotrexate uptake (Uwai et al., 2000), but much lower than those for hOAT1-mediated methotrexate uptake. The discrepancy may be due to the fact that the $K_i$ value for hOAT1-mediated adefovir uptake was 23.8 $\mu M$ (Ho et al., 2000), whereas for hOAT1-mediated methotrexate uptake was 553.8 $\mu M$.

At present, the mechanism for the interaction between methotrexate and ibuprofen, ketoprofen, or piroxicam, or penicillin G remains unclear. It is possible that other transporters mediate methotrexate transport and are responsible for drug interactions. Since protein-binding displacement of methotrexate by NSAIDs except salicylate is generally thought to account for more than a small transient increase in free methotrexate concentration, protein binding changes would probably not be of major clinical significance (Taylor and Halprin, 1977). In addition, since prostaglandin synthesis is stimulated to maintain the glomerular filtration rate in the setting of prerenal volume contraction (Ciabattoni et al., 1984; Dunn, 1984), NSAIDs may decrease glomerular filtration of methotrexate as a result of inhibition of prostaglandin synthesis (Kremer and Hamilton, 1995). However, patients who do not have a condition that predisposes them to activation of renal prostaglandins would not have a decrease in renal function following treatment with NSAIDs (Kremer and Hamilton, 1995).

In conclusion, these results suggest that methotrexate is taken up in the basolateral membrane by hOAT3 and hOAT1, and taken up or effluxed in the apical membrane of the proximal tubule via hOAT4. In addition, hOAT1, hOAT3, and hOAT4 were sites of drug interactions between methotrexate and NSAIDs, probenecid, and penicillin G. Furthermore, it was predicted that hOAT3 is a site of drug interactions between methotrexate and salicylate, phenylbutazone, indomethacin, or probenecid in vivo. Stable cells established in this study provide a good opportunity for evaluating drug interaction of methotrexate and newly developed drugs, particularly NSAIDs, in the preclinical stage.


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