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

MICHIKO TAKEDA, SUPARAT KHAMDANG, SHINICHI NARIKAWA, HIROAKI KIMURA, MAKOTO HOSOYAMADA, SEOK HO CHA, TAKASHI SEKINE, and HITOSHI ENDOU

Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan.

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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 Kₐ values for hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake were 553.8 ± 666.3, 21.1 ± 17.8, 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 Kᵢ values for the effects of salicylate, phenylbutazone, indomethacin, and probenecid on hOAT3-mediated methotrexate uptake were comparable with therapeutically relevant plasma concentrations of drugs. In addition, in the presence of human serum albumin, the Kᵢ values were comparable with therapeutically relevant plasma concentrations of drugs. 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. Among these possible causes, the renal tubular secretion of methotrexate has been thought to be a major site for drug interaction.

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; Basin 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.

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...
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) (Jacquemin 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° – 7 culture medium from Iwaki Co. (Tokyo, Japan), and TX-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 transiently transfecting S2 cells with pcDNA3.1-hOAT1, pcDNA3.1-hOAT3, and pcDNA3.1-hOAT4 coupled with pSV2neo, a neomycin-resistant gene, using TIT-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 and under 5% CO2 using RITC 80° 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 × 10⁶ 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, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO₃, 0.5 mM EDTA, and 5 mM HEPES, pH 7.2) 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 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:

\[ Kᵢ = \text{concentration of inhibitor} / (Kᵢ \text{of methotrexate with inhibitor}) - 1 \]

**Statistical Analysis.** Data are expressed as means ± S.E. Statistical differences were determined using one-way ANOVA with Dunnnett’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
containing 1 μM methotrexate (hOAT1) or 100 nM methotrexate (hOAT3 and hOAT4) for various periods at 37°C. As shown in Fig. 1, when the amount of methotrexate uptake in mock was subtracted from those in S2 hOAT1, S2 hOAT3, and S2 hOAT4, the specific uptake in S2 hOAT1 (Fig. 1A) and S2 hOAT4 (Fig. 1C) increased in a time-dependent manner and reached steady state, whereas that in S2 hOAT3 (Fig. 1B) decreased after reaching peak. The latter may be due to the observation that nonspecific methotrexate efflux overwhelmed hOAT3-mediated methotrexate uptake depending on the nature of monoclonal cells into which hOAT3 cDNA was transfected.

The kinetics of methotrexate uptake was examined to evaluate the pharmacological characteristics of hOAT1, hOAT3, and hOAT4 on methotrexate transport. The concentration-dependent uptake of methotrexate was observed in S2 hOAT1, S2 hOAT3, and S2 hOAT4 after subtraction of uptake by mock (data not shown). As shown in Fig. 2, Eadie-Hofstee plot analysis of hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake gave a single straight line. The estimated K_m values of methotrexate uptake by hOAT1, hOAT3, and hOAT4 were 553.8 ± 43.2 μM, 21.1 ± 2.8 μM, and 17.8 ± 1.6 μM, respectively (from three determinations in one typical experiment of two separate experiments). These results suggest that hOAT1, hOAT3, and hOAT4 mediate transport of methotrexate.

**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.

**Kinetic Analysis of Inhibition.** To further elucidate the inhibitory effects of NSAIDs, probenecid, and penicillin G on hOAT3-mediated methotrexate uptake, we have examined the inhibitory effects of these drugs at different concentrations of methotrexate. Typical results are shown in Fig. 3. Analysis of a Lineweaver-Burk plot of the effects of salicylate (Fig. 3A), phenylbutazone (Fig. 3B), indomethacin (Fig. 3C), and probenecid (Fig. 3D) on the hOAT3-mediated methotrexate uptake demonstrated that these drugs inhibited the methotrexate uptake in a competitive manner. The inhibitory effects of ibuprofen, ketoprofen, piroxicam, and penicillin G were also competitive (data not shown). The K_i values

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**TABLE 1**

<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>82.0 ± 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>53.1 ± 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>

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\* P < 0.001, ** P < 0.01, and *** P < 0.05 versus control.
concentrations of \([3H]\) methotrexate in the presence or absence of various 
eral side of the S2 segment of the proximal tubule (Hosoy-
eral specificity and localization: hOAT1 at the basolat-
transport of organic anions from the interstitium to the cells 
for each drug tested are shown in Table 2. In addition, we 
performed a kinetic analysis of the inhibitory effects of salicy-
ate, 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 (Hosoy-
amada 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 basolat-
eral side of the S2 segment of the proximal tubule (Hosoy-
amada et al., 1999) versus hOAT3 at the first, second, and 
third segments (S1, S2, and S3) 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 local-
ized 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 basolat-
eral 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 (Bes-
seghir 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 addi-
tion, hOAT2, which was recently identified to be localized to 
the basolateral side of the proximal tubule, showed no sig-
ificant uptake activity of methotrexate (M. Takeda, unpub-
lished 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 con-
tribution of hOAT4 and MRP2 to apical methotrexate transport 
should be clarified. In addition to human transporters, ro-
dent 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; Ma-
suda et al., 1999a,b), and the \(K_i\) value for OAT-K1-mediated 
methotrexate transport was determined to be 1.0 \(\mu\)M (Ma-
suda 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 methotrex-
ate 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 con-
centrations 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 \(K_i\) values with therapeu-
tically relevant concentrations of unbound drugs in the 
plasma, those for salicylate, phenylbutazone, indomethacin,
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 may be due to the species difference, i.e., between rats and humans. The discrepancy may be due to the fact that the $K_m$ 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 methotrexate as a result of inhibition of prostaglandin 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.

### References


