Regular Article

Transport of Aminopterin by Human Organic Anion Transporters hOAT1 and hOAT3: Comparison with Methotrexate

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Summary: The transport of antifolate aminopterin by human organic anion transporters hOAT1 (SLC22A6) and hOAT3 (SLC22A8) was characterized using Xenopus laevis oocytes and was compared with that of methotrexate. Although hOAT1 and hOAT3 transported both aminopterin and methotrexate, uptake of methotrexate was greater in hOAT3-expressing oocytes than in hOAT1-expressing oocytes, and aminopterin was transported by hOAT1 more efficiently. The apparent 50% inhibitory concentration (IC₅₀) of aminopterin for p-aminohippurate uptake by hOAT1 was lower than that of methotrexate (methotrexate: 998 µM, aminopterin: 160 µM). On the other hand, IC₅₀ values of these antifolates for estrone sulfate transport by hOAT3 were comparable (methotrexate: 61.5 µM, aminopterin: 59.2 µM). The Michaelis-Menten constant and maximum velocity of aminopterin transport by hOAT1 were calculated to be 226 µM and 72.5 pmol/oocyte/2 hr, respectively. Probenecid and non-steroidal anti-inflammatory drugs strongly inhibited the transport. These findings show that both aminopterin and methotrexate are substrates of hOAT1 and hOAT3, and that there are differences between the antifolates in terms of their transport characteristics.

Keywords: hOAT1; hOAT3; methotrexate; aminopterin; transport

Introduction

Antifolate methotrexate has a 50-year history as a medicine for malignancies, and 20 years have passed since its application for rheumatoid arthritis was recognized. At present, other antifolates, including aminopterin, are being clinically tested or used. Aminopterin is structurally related to methotrexate (Fig. 1) and was the first antifolate to demonstrate efficacy for acute leukemia. Soon afterward, aminopterin was replaced by methotrexate because the toxicity of aminopterin was felt to be unpredictable, and methotrexate had a better therapeutic index in mice. However, in vitro experiments showed that aminopterin has superior characteristics to methotrexate, such as rapid uptake into L1210 leukemia cells, a long dissociation time from purified dihydrofolate reductase (which is the target enzyme of methotrexate), and great affinity for folylpolyglutamate synthetase, which enhances the cellular retention of these antifolates. Recently, Kamen’s group reported that aminopterin was effective against refractory and newly diagnosed acute lymphoblastic leukemia in children in clinical trials. In addition, they showed that the oral
bioavailability of aminopterin was nearly complete, and is greater than that of methotrexate. Accordingly, by elucidating the pharmacokinetics as well as the toxic mechanism of aminopterin in detail, the advantages of aminopterin can be identified.

It has been reported that methotrexate often induces severe adverse effects, such as bone marrow depression, hepatitis or acute renal failure, through an increase in blood methotrexate levels when non-steroidal anti-inflammatory drugs (NSAIDs) are simultaneously administered. Because NSAIDs are commonly prescribed, it is important to elucidate the elimination route of methotrexate and the mechanisms of drug interactions at the molecular level. Administered methotrexate is mostly excreted in the urine in an unchanged form, and organic anion transporters, expressed in the renal proximal tubules, are responsible for its tubular secretion. Human organic anion transporters hOAT1 (SLC22A6) and hOAT3 (SLC22A8), mainly expressed in the basolateral membrane of the renal proximal tubules, have been shown to transport methotrexate and to be inhibited by NSAIDs. This suggests that the interaction is caused by inhibition of the renal tubular uptake of methotrexate via hOAT1 and hOAT3 by NSAIDs.

Elucidation of the substrate specificity of drug transporters and identification of the target enzyme of the drug interaction described above are thought to be necessary for personalized drug therapy. Although hOAT1 and hOAT3 have been extensively investigated in terms of the transport characteristics of many classes of drugs, only methotrexate has been investigated among the antifolates. In this study, we examined the transport and interactions of aminopterin with hOAT1 and hOAT3 in comparison to those of methotrexate by performing uptake experiments using Xenopus laevis oocytes.

Materials and Methods

Materials: [3H]Methotrexate (27.7 Ci/mmol) and [3H]Aminopterin (35.1 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). [3H]p-Aminohippurate (4.53 Ci/mmol) and [3H]estrone sulfate (54.3 Ci/mmol) were obtained from PerkinElmer Life Science (Boston, MA, USA). Unlabeled methotrexate, etodolac, indomethacin, loxoprofen sodium salt dihydrate, piroxicam, salicylic acid and probenecid were from Wako Pure Chemical Industries (Osaka, Japan). Unlabeled aminopterin and diclofenac sodium salt were from Sigma-Aldrich (St. Louis, MO, USA). Flurbiprofen was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals used were of the highest purity available.

Uptake experiment using Xenopus laevis oocytes expressing hOAT1 or hOAT3: pBK-CMV plasmid vectors containing hOAT1 or hOAT3 were a kind gift from Prof. Ken-ichi Inui (Kyoto University Hospital, Kyoto, Japan). An uptake experiment using Xenopus laevis oocytes was performed as previously reported with slight modification. Briefly, capped RNA encoding hOAT1 or hOAT3 was transcribed from Xba I-linearized pBK-CMV containing hOAT1 or hOAT3, respectively, with T3 RNA polymerase. After 50 nl water or cRNA (25 ng) was injected into defolliculated oocytes, the oocytes were maintained in modified Barth’s medium (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.4 mM CaCl2, 0.8 mM MgSO4, 2.4 mM NaHCO3, and 5 mM HEPES) containing 50 mg/l gentamicin at 18°C. Two or three days after injection, the uptake reaction was initiated by incubating the oocytes in 500 µl uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES; pH 7.0) with each radiolabeled compound at room temperature in the presence or absence of an inhibitor for the indicated periods. The uptake reaction was terminated by adding 2 ml ice-cold uptake buffer to each well, and the oocytes were washed three times with 2 ml ice-cold buffer. After washing, each oocyte was transferred to a scintillation counting vial and solubilized in 200 µl of 10% sodium lauryl sulfate. Two milliliters of Clear-sol II (Nacalai Tesque, Kyoto, Japan) was added to each solubilized oocyte, and radioactivity was determined in a liquid scintillation counter.

Kinetic analysis: IC50 values of antifolates for hOAT1 and hOAT3 were estimated by non-linear least squares regression analysis of the competition curve with a one-compartment model according to the following equation: 

\[ V = \frac{100 \times IC50 \cdot [I]}{IC50 + [I]} + A \]

where V is the uptake amount of p-aminohippurate or estrone sulfate (percentage of control), [I] is the concentration of each antifolate, and A is the non-specific organic anion uptake (percentage of control).

The kinetic parameters of aminopterin transport by hOAT1 were calculated using non-linear least squares regression analysis from the following Michaelis-Menten equation:

\[ V = \frac{V_{max} \cdot [S]}{K_m + [S]} \]

where V is the transport rate (pmol/oocyte/2 hr), \( V_{max} \) is the maximum velocity by the saturable process (pmol/oocyte/2 hr), [S] is the concentration of aminopterin (µM), \( K_m \) is the Michaelis-Menten constant (µM).

Statistical analysis: Data were analyzed by an unpaired t-test or one-way analysis of variance followed by Dunnett’s test using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at P < 0.05.

Results

Transport of methotrexate and aminopterin by hOAT1 and hOAT3: To investigate whether hOAT1 and hOAT3 mediate the transport of aminopterin, and to make comparisons with the transport of methotrexate, we measured the accumulation of methotrexate and aminopterin in oocytes. Table 1 presents the uptake
amounts of p-aminohippurate and the antifolates in oocytes injected with water or hOAT1 cRNA. The injection of hOAT1 cRNA greatly stimulated the uptake of p-aminohippurate, a typical substrate of hOAT1, indicating the functional expression of hOAT1 in oocytes. The accumulation of methotrexate and aminopterin in hOAT1-expressing oocytes was approximately two-fold that of control oocytes, and the differences were statistically significant.

The results of hOAT3 are summarized in Table 2. The uptake of estrone sulfate, a representative substrate of hOAT3, in hOAT3-expressing oocytes was 28-fold that of the control. hOAT3-stimulated uptake of methotrexate and aminopterin was recognized. Although the ratio of aminopterin uptake by hOAT3-expressing oocytes to that by control oocytes was estimated to be 1.2, the differences were significant. These results mean that hOAT1 and hOAT3 transport both aminopterin and methotrexate.

To assess the transport activity of hOAT1 and hOAT3 for methotrexate and aminopterin in detail, the time dependency of uptake of these antifolates was examined. As

### Table 1. Uptake of p-aminohippurate, methotrexate and aminopterin by hOAT1

<table>
<thead>
<tr>
<th>Uptake (nl/oocyte/hr)</th>
<th>Control</th>
<th>hOAT1</th>
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<tbody>
<tr>
<td>p-Aminohippurate</td>
<td>40.8 ± 3.7</td>
<td>3120 ± 994***</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>66.6 ± 2.7</td>
<td>131 ± 10***</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>197 ± 13</td>
<td>521 ± 46***</td>
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</tbody>
</table>

Water-injected (control) and hOAT1 cRNA-injected oocytes were incubated with 221 nM [3H]p-aminohippurate, 36.1 nM [3H]methotrexate or 28.5 nM [3H]aminopterin for 1 hr. The uptake amounts of the compounds in each oocyte were determined and divided by their concentrations in uptake buffer. The values represent the mean ± S.E.M. of 7 to 9 oocytes. **P < 0.01, significantly different from the control values. ***P < 0.001, significantly different from the control values.

### Table 2. Uptake of estrone sulfate, methotrexate and aminopterin by hOAT3

<table>
<thead>
<tr>
<th>Uptake (nl/oocyte/hr)</th>
<th>Control</th>
<th>hOAT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone sulfate</td>
<td>74.1 ± 7.9</td>
<td>2097 ± 290***</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>58.7 ± 1.8</td>
<td>128 ± 6***</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>233 ± 7</td>
<td>290 ± 9***</td>
</tr>
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</table>

Water-injected (control) and hOAT3 cRNA-injected oocytes were incubated with 18.4 nM [3H]estrone sulfate, 36.1 nM [3H]methotrexate or 28.5 nM [3H]aminopterin for 1 hr. The uptake amounts of the compounds in each oocyte were determined and divided by their concentrations in uptake buffer. The values represent the mean ± S.E.M. of 7 to 10 oocytes. ***P < 0.001, significantly different from the control values.

**Fig. 2. Time-dependent uptake of methotrexate and aminopterin by hOAT1 (A, C) and hOAT3 (B, D)**

(A, B) Oocytes injected with water (open circle) or expressing hOAT1 or hOAT3 (closed circle) were incubated with 36.1 nM [3H]methotrexate for the indicated periods. (C, D) Oocytes injected with water (open circle) or expressing hOAT1 or hOAT3 (closed circle) were incubated with 28.5 nM [3H]aminopterin for the indicated periods. The uptake amounts of [3H]methotrexate or [3H]aminopterin in each oocyte were determined. Each point represents the mean ± S.E.M. of 6 to 10 oocytes.
shown in Figure 2, the transport of methotrexate by hOAT1 and hOAT3 was observed. The ratio of methotrexate accumulation in hOAT1-expressing oocytes to that in water-injected oocytes did not exceed 2 for any incubation time up to 2 hr. With hOAT3, the uptake of methotrexate increased linearly, and the accumulation in hOAT3-expressing oocytes at 2-hr incubation was approximately 4 times that in water-injected oocytes. With aminopterin, the uptake by hOAT1 continued to increase up to 2 hr. Although the accumulation of aminopterin in hOAT3-expressing oocytes was higher than in control oocytes, it appeared comparable when shown using the same scale as that for hOAT1. These results imply that hOAT1 prefers aminopterin as a substrate, but methotrexate prefers hOAT3.

**Inhibitory effects of methotrexate and aminopterin on hOAT1 and hOAT3:** To examine the affinity of methotrexate and aminopterin for hOAT1 and hOAT3, dose-dependent inhibitory effects of these antifolates on the uptake of p-aminohippurate by hOAT1 and of estrone sulfate by hOAT3 were investigated. The findings are shown in Figure 3. The obtained IC₅₀ values are summarized in Table 3. It was demonstrated that the affinity of aminopterin for hOAT1 was higher than that of methotrexate, and that the inhibitory effects of aminopterin and methotrexate on hOAT3 were comparable.

**Kinetic parameters of aminopterin transport by hOAT1:** To estimate the kinetic parameters of aminopterin transport by hOAT1, the concentration dependency of its uptake was investigated. As shown in Figure 4, hOAT1 mediated the dose-dependent uptake of aminopterin, and saturation was observed. The apparent Kₘ value and Vₘₐₓ value were estimated to be 226 ± 43 μM and 72.5 ± 4.0 pmol/oocyte/2 hr, respectively, from 3 independent experiments (mean ± S.E.M.).

**Inhibitory effects of probenecid and NSAIDs on aminopterin transport by hOAT1:** The effects of methotrexate and aminopterin for hOAT1 and hOAT3, dose-dependent inhibitory effects of these antifolates on the uptake of p-aminohippurate by hOAT1 and of estrone sulfate by hOAT3 were investigated. The findings are shown in Figure 3. The obtained IC₅₀ values are summarized in Table 3. It was demonstrated that the affinity of aminopterin for hOAT1 was higher than that of methotrexate, and that the inhibitory effects of aminopterin and methotrexate on hOAT3 were comparable.

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**Table 3.** IC₅₀ values of methotrexate and aminopterin for uptake of p-aminohippurate by hOAT1 and of estrone sulfate by hOAT3

<table>
<thead>
<tr>
<th>IC₅₀ value (μM)</th>
<th>Methotrexate</th>
<th>Aminopterin</th>
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<tbody>
<tr>
<td>hOAT1</td>
<td>998 ± 101</td>
<td>160 ± 58</td>
</tr>
<tr>
<td>hOAT3</td>
<td>61.5 ± 11.4</td>
<td>59.2 ± 14.9</td>
</tr>
</tbody>
</table>

IC₅₀ values of methotrexate and aminopterin for uptake of p-aminohippurate by hOAT1 and of estrone sulfate by hOAT3 were determined from the data in Figure 3 by non-linear least squares regression analysis. Values represent the mean ± S.E.M. of 3 independent experiments.
Fig. 5. Effects of probenecid and various NSAIDs on aminopterin uptake by hOAT1

hOAT1-expressing oocytes were incubated with 28.5 nM [3H]aminopterin in the absence (control) or presence of probenecid or various NSAIDs at 100 μM for 1 hr. The uptake amounts of [3H]aminopterin in each oocyte were determined. Each bar represents the mean ± S.E.M. in 6 to 10 oocytes. ***P < 0.001, significantly different from the control values.

Discussion

Methotrexate was shown to be a substrate of hOAT1 and hOAT3,14,15 but the transport characteristics of other antifolates by transporters are unclear. The purpose of the present study was to characterize the transport of aminopterin by hOAT1 and hOAT3, and to make comparisons with that of methotrexate. First, we examined the accumulation of these antifolates in oocytes expressing hOAT1 and hOAT3. As reported previously, methotrexate transport by hOAT1 and hOAT3 was recognized (Tables 1 and 2). The uptake of aminopterin was stimulated by the expression of hOAT1 (Table 1). In addition, its accumulation by hOAT3-expressing oocytes was slightly but significantly greater than that of control oocytes (Table 2). These results imply that not only methotrexate but also aminopterin are substrates of hOAT1 and hOAT3.

Of the results obtained in this study, the most interesting finding was thought to be that illustrated by Figure 2, i.e., that methotrexate was transported efficiently by hOAT3 but aminopterin was transported efficiently by hOAT1, which was unexpected. Many previous reports on the substrate specificity of hOAT1 and hOAT3 have been published, and they found that all tested acyclic nucleotide analogues, adenosine, cidofovir and tenofovir, were good substrates of hOAT1, but poor substrates of hOAT3.16 Ueo et al. showed that hOAT3 transported all cephalosporins tested much more efficiently than hOAT1.17 This evidence led to speculation that structural similarity would be recognized among substrates of hOAT1 and hOAT3, respectively, and that there might be structural differences in the classes of substrates of hOAT1 and hOAT3. On the other hand, although structural similarity is observed between methotrexate and aminopterin, the transport characteristics of these antifolates by hOAT1 and hOAT3 did not concur with this hypothesis. To our knowledge, this is the first report to show that a structurally small difference in compounds can reverse the transport characteristics of drugs by hOAT1 and hOAT3.

To assess the relationship between transport efficiency and the affinity of antifolates with hOAT1 and hOAT3, we examined the dose-dependent inhibitory effects of methotrexate and aminopterin on the transporters and estimated the IC50 values. As shown in Table 3, aminopterin had a lower IC50 value for hOAT1 than methotrexate. Accordingly, it is possible that the higher affinity of aminopterin for hOAT1 links to the fact that hOAT1 prefers aminopterin as a substrate. On the other hand, no significant difference was recognized between methotrexate and aminopterin in the IC50 value for hOAT3 (P = 0.9087). To elucidate the substrate specificity and drug transport mechanisms of hOAT1 and hOAT3, further study is necessary, and methotrexate and aminopterin may be key compounds.

In this study, the uptake amount of methotrexate via hOAT3 in Xenopus laevis oocytes was greater than that via hOAT1 (Figure 2). Similar phenomena were observed in other experiments using HEK293 cells17 or S2 cells derived from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene.18 In the kidney, mRNA levels of hOAT3 are about 3-times higher than those of hOAT1.18 Furthermore, Nozaki et al. demonstrated that the Km value of methotrexate uptake by human kidney slices corresponded to the value of methotrexate transport by hOAT3.19 Taking these findings together, it is suggested that hOAT3 might be an important transporter responsible for the tubular uptake of methotrexate.

Although it is unknown whether aminopterin will be accepted as a treatment in the future, clinical trials of aminopterin have been performed recently.8,9,20 To our knowledge, four reports have illustrated the pharmacokinetics of aminopterin, but its urinary excretion ratio in the unchanged form has not been described.8,9,20,21 Because Glode et al. demonstrated delayed aminopterin
elimination in nephrotoxic patients, it is possible that the kidney is responsible for its excretion. In the future, the contribution of the kidney to its elimination should be determined if aminopterin is to be administered to patients. Our study demonstrates that aminopterin is a better substrate for hOAT1 than hOAT3, suggesting that hOAT1 plays a role, at least in part, in its renal tubular uptake. The contribution of hOAT1 could be assessed by investigating effects of specific inhibitors for hOAT1 on aminopterin uptake using renal slices. Sakurai et al. reported that mRNA levels of hOAT1 in renal biopsy specimens from patients with kidney disease were significantly lower than those in normal kidney cortex from nephrectomized patients, and that a significant difference was not observed in hOAT3 mRNA levels. Accordingly, if hOAT1 and hOAT3 play important roles in renal tubular uptake of aminopterin and methotrexate, respectively, it is possible that the pharmacokinetics of aminopterin would be affected more in patients with kidney disease. Also, the K_m value of aminopterin transport by hOAT1 was estimated to be 226 μM (Fig. 4). This value is higher than the K_m value of methotrexate transport by hOAT3, and lower than methotrexate transport by hOAT1. Cole et al. reported that serum aminopterin concentrations at every time point were less than 1 μM when 2 mg/m^2 aminopterin was administered to patients. Accordingly, as the dosage is comparable to that used in this clinical trial, it is difficult to think that hOAT1 would be saturated by aminopterin. In addition, in consideration of Figure 3, aminopterin in this clinical range would not strongly inhibit hOAT3.

Simultaneous administration of anionic drugs, such as probenecid and NSAIDs, retards the elimination of methotrexate, and methotrexate toxicity would be induced. It was reported that probenecid and NSAIDs have inhibitory effects on methotrexate transport by hOAT1 and hOAT3, and it is accepted that drug interaction via transporters influences the disposition of methotrexate. The present study demonstrates that probenecid and NSAIDs potently inhibited hOAT1-mediated uptake of aminopterin (Fig. 5), and Nozaki et al. determined their inhibition constants (K_value) for hOAT1 and suggested that inhibition of hOATs by probenecid and several NSAIDs is clinically relevant. Accordingly, probenecid and NSAIDs might reduce the renal tubular uptake of aminopterin via hOAT1, leading to aminopterin toxicity via its accumulation in blood.

In conclusion, this study shows that aminopterin and methotrexate are substrates of both hOAT1 and hOAT3, and that hOAT1 prefers aminopterin as a substrate but hOAT3 prefers methotrexate. In addition, the affinity of methotrexate for hOAT1 was lower than that of aminopterin, and their affinities for hOAT3 were comparable. These findings imply a difference in the recognition of these antifolates by hOAT1 and hOAT3. Furthermore, it is suggested that hOAT1 is, at least in part, involved in the renal disposition of aminopterin, and that probenecid and NSAIDs may influence the pharmacokinetics of aminopterin.

Acknowledgments: We thank Prof. Ken-ichi Inui (Kyoto University Hospital, Kyoto, Japan) for kindly providing pBK-CMV plasmid vectors containing hOAT1 or hOAT3.

References
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Transport of Aminopterin by hOAT1 and hOAT3


