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Chiral Recognition of Amethopterin Enantiomers by the Reduced Folate Carrier in Caco-2 Cells

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Summary: Stereoselectivity of the human reduced folate carrier (RFC1) in Caco-2 cells was examined using methotrexate (L-amethopterin, L-MTX) and its antipode (D-amethopterin, D-MTX) as model substrates. The initial uptake rate of L-MTX into Caco-2 cells followed Michaelis-Menten kinetics with a $K_m$ value of approximately 1 $\mu$M. The Eadie-Hofstee plot of the RFC1-mediated L-MTX uptake showed that it was mediated by a single transport system, RFC1. Dixon plots revealed that L-MTX uptake was inhibited competitively by folic acid (FA), L-MTX and D-MTX, with $K_i$ values of approximately 0.8, 1.5 and 180 $\mu$M, respectively. The results showed that the affinities of FA and L-MTX to RFC1 were approximately 120-fold greater than that of D-MTX. The uptake of L- and D-MTX into Caco-2 cells was also measured using LC-MS/MS analysis, which revealed that the L-MTX uptake was at least 7-fold greater than that of D-MTX. The present study revealed significant stereoselectivity of RFC1 toward amethopterin enantiomers with the L-isomer being much more favored.

Key words: methotrexate; amethopterin; stereoselectivity; reduced folate carrier; RFC1; Caco-2 cells

Introduction

Folates play significant roles in the synthesis of nucleotides and the metabolism of amino acids, and are essential nutrients for cell growth. Reduced folate carrier (RFC1, SLC19A1) is responsible for the transport of folates and their analogs across biological membranes in mammalian cells.1) RFC1 cDNAs of mouse, hamster and human have been cloned.2–8) In humans, RFC1 is expressed in various tissues, including the small intestine, placenta, brain, liver and kidney.2,9–11) In the small intestine, the carrier exists at not only the brush border membrane but also at the basolateral membrane of the intestinal epithelial cells.12–15) RFC1 located at the brush border membrane is reported to transport folates using a $\text{H}^+$ gradient as a driving force.13,14,16)

Methotrexate (L-amethopterin, L-MTX) is used clinically as an antineoplastic and antirheumatic drug. Because of the similarity in molecular structure to folic acid, orally administered L-MTX is absorbed by RFC1 in the small intestine. Indeed, human RFC1 from the small intestine has been cloned and shown to transport L-MTX using a $\text{H}^+$ gradient as a driving force.2,17)

D-Amethopterin (D-MTX) is the optical isomer of L-MTX, and the absorption of D-MTX from the human small intestine is significantly lower than that of L-MTX. It is reported that the area under the plasma concentration-time curve (AUC) following oral administration of L-MTX is approximately 40-fold greater than that following D-MTX administration.18) In our previous study using brush border membrane vesicles (BBMV) prepared from the small intestines of rabbits, the affinity of L-MTX to rabbit RFC1 was approximately 60-fold greater than that of D-MTX.19) The present study was conducted to examine the stereoselectivity of human RFC1 using Caco-2 cells. Since RFC1 is expressed in Caco-2 cells, this cell line is suitable for studying the stereoselectivity of human RFC1.20)

In our previous report, the stereoselectivity of RFC1 in Caco-2 cells was studied using folic acid (FA) as a model substrate.21) It is also reported by other groups that FA is transported by RFC1 with a $K_m$ value being similar to that of L-MTX.2,14,22,23) However, there are reports which show that the affinity of FA is much smaller than that of reduced folates and that the transport rate of FA is much smaller than that of L-MTX.24,25)
In the present study, the stereoselectivity of RFC1 was examined using L-MTX, one of the reduced folates. Moreover, the differences between MTX enantiomers were investigated not only for the affinity to but also for the transport by RFC1 with the use of LC-MS/MS analysis.

**Materials and Methods**

**Materials:** Methotrexate (L-amethopterin, L-MTX), D-amethopterin (D-MTX), aminopterin (AMT), probenecid and carbonyl cyanide p-(trifluoromethoxy)-phenyl hydrazone (FCCP) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Folic acid (FA), 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid (DIDS), HEPES (2-[4-(2-hydroxyethyl)-1-piperaziny]ethanesulfonic acid) and MES (2-morpholinoethanesulfonic acid) were purchased from Wako Pure Chemicals Co. (Osaka, Japan). 3H-labeled L-MTX (specific activity 33.5 Ci/mmmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, U.S.A.) at passage 18. The cells were cultured in six-well plates containing 5 mM D-glucose and 5 mM HEPES (for pH 7.4) was used as the rinse medium.

**Cell culture:** Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) at passage 18. The cells were cultured in six-well plates (9.6 cm²/well; Falcon®, Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) for between 21 and 28 days before being used in the uptake studies. Culture conditions were the same as those reported previously. Cells between the 30th and 40th passages were used in the present study.

**Uptake study:** Hank’s balanced salt solution (HBSS: 145 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂) containing 5 mM d-glucose and 5 mM MES (for pH 6.0) was used as the uptake medium. HBSS containing 5 mM d-glucose and 5 mM HEPES (for pH 7.4) was used as the rinse medium.

For the uptake study, Caco-2 cells in six-well plates were rinsed twice and pre-incubated with the rinse medium (pH 7.4) for 10 min at 37°C. Uptake was initiated by adding 1 mL of the pre-incubated drug solution. The drug solution was the uptake medium (pH 6.0) containing 3H-L-MTX, unlabeled L-MTX, or D-MTX. The drug solution was aspirated at the appropriate time in order to terminate the uptake. The cells were rinsed twice with ice-cold rinse medium (pH 7.4), then lysed with 1 mL of 0.1% Triton® X-100. To determine the uptake amount of unlabeled L-MTX and D-MTX, a 1 mL aliquot of the cell lysate was mixed with 1 mL of acetonitrile and then vortexed.

The mixture was then centrifuged at 825 g for 15 min and the supernatant filtered with a Millex-HV filter (0.45 μm; Millipore, Bedford, MA, U.S.A.). The filtrate was dried under a gentle stream of nitrogen gas at 80°C and the residue dissolved in 100 μL of the mobile phase (see below). A 20 μL aliquot was injected into an LC-MS/MS.

Adsorption of the drug to the cells was measured in the manner described above, but the drug solution was removed immediately after being added to the plate. The adsorbed amount was subtracted to obtain the net uptake. In the inhibition study, each inhibitor was added to the drug solution at an appropriate concentration, and uptake of the drug was measured in the manner described above. Drugs and inhibitors were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the drug solution was 5% or less.

**LC-MS/MS conditions:** A Waters Micromass tandem quadrupole Quattro micro mass spectrometer (Waters, Milford, MA, U.S.A.) was interfaced with the HPLC system via an electrospray ionization probe in the negative ion mode. The analytical column of HPLC was Inertsil® ODS-3 (5 μm in particle size, 2.1×150 mm; GL Sciences Inc., Tokyo, Japan). The mobile phase was solvent A (10 mM ammonium acetate, 0.05% formic acid and 1% isopropyl alcohol in water): solvent B (0.05% formic acid and 1% isopropyl alcohol in acetonitrile) = 9:1 (v/v). The flow rate was 0.2 mL/min, and an eluent between 6 and 8 min was introduced into the mass spectrometer. The ionization conditions were as follows: capillary voltage: 2.9 kV; cone voltage: 48 V; collision energy: 22 eV; source temperature: 150°C; desolvation temperature: 220°C; collision gas: argon. The sample was analyzed during the multiple reaction monitoring (MRM) mode of the mass spectrometer at a dwell time of 2 s per channel using m/z 453.08 > 324.08 as the transition.

**Protein assay:** For protein determination, Caco-2 cells were dissolved in 1 mL of 0.1% Triton® X-100 and the protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using bovine serum albumin as a standard.

**Data analysis:** To calculate kinetic parameters for L-MTX uptake, the data were fit to the following equation (Eq. 1) by a non-linear least-squares method.

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} + k_d [S] \]  

Eq.1

where \( v \) is the initial uptake rate, \( V_{\text{max}} \) is the maximum uptake rate, \( K_m \) is the Michaelis constant, \( k_d \) is the uptake clearance by passive diffusion, and \( [S] \) is the initial concentration of the drug.

**Statistical analysis:** Statistical analysis was conduct-
Time course of L-MTX uptake into Caco-2 cells at pH 6.0. Each point represents the mean ± S.D. of six determinations. (mean ± S.D., n = 6).

The initial uptake of 3H-L-MTX was measured at concentrations ranging from 0.5 to 200 μM in the presence of a H⁺ gradient. The uptake by passive diffusion was calculated by multiplying the above k_d value (0.270 μL/min/mg protein) by the L-MTX concentration, and the RFC1-mediated uptake was calculated by subtracting the uptake from passive diffusion from the total uptake. An Eadie-Hofstee plot of the RFC1-mediated uptake (Fig. 3) indicated that the saturable uptake of L-MTX into Caco-2 cells was mediated by a single transport system.

Inhibition studies: The inhibitory effects of 1 mM probenecid, 1 mM DIDS, 50 μM FCCP and 10 μM AMT on 0.02 μM 3H-L-MTX uptake were studied. The results are summarized in Table 1. L-MTX uptake was inhibited by probenecid and DIDS, which are inhibitors of organic anion transport systems such as OATs and OATPs, and AMT, which is a folate analog. FCCP, a protonophore, also inhibited L-MTX uptake, thus confirming that the proton gradient was the driving force for the uptake of L-MTX into Caco-2 cells.

The inhibitory effects of folic acid (FA), unlabeled L-MTX and D-MTX on 3H-L-MTX uptake were also studied (Table 1). L-MTX uptake was significantly inhibited by 2 and 100 μM of FA. The uptake was also significantly inhibited by 2 and 100 μM of unlabeled L-MTX. In contrast, 2 μM of D-MTX did not affect L-MTX uptake, and the inhibitory effect was obvious when 100 μM or 1 mM of D-MTX was added.

The initial uptake rates of 0.1 and 2 μM of 3H-L-MTX were measured in the presence of 0, 0.5, 1 and 2 μM of FA, and the data were analyzed using a Dixon plot (Fig. 4-A). FA competitively inhibited L-MTX uptake with a K_i value of 0.80 μM. The initial uptake rates for 0.1 and 2 μM of 3H-L-MTX were also measured in the presence of 0, 1, and 2 μM of unlabeled L-MTX. The Dixon plot showed that unlabeled L-MTX competitively

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**Results**

**Time course of L-MTX uptake:** The time course of 0.02 μM 3H-L-MTX uptake in the presence of a H⁺ gradient (pH 6.0) is shown in Fig. 1. Since the amount taken up in the cells increased linearly for up to 15 min, the initial uptake rate was calculated from the uptake amount at 5 min in the following studies.

**Concentration dependence of L-MTX uptake:** The initial uptake of 3H-L-MTX at various concentrations (0.02–20 μM) were measured in the presence of a H⁺ gradient (pH 6.0). The results are shown in Fig. 2. The uptake parameters calculated according to Eq.1 were as follows: K_m = 1.31 ± 0.26 μM, V_max = 1.55 ± 0.28 pmol/min/mg protein, k_d = 0.270 ± 0.027 μL/min/mg protein.
Table 1. Inhibitory effects of various compounds on the uptake of 0.02 μM L-MTX

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Uptake (% of control)</th>
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<tbody>
<tr>
<td>1 mM probenecid</td>
<td>36.0 ± 2.1*</td>
</tr>
<tr>
<td>1 mM DIDS</td>
<td>20.9 ± 3.2*</td>
</tr>
<tr>
<td>50 μM FCCP</td>
<td>50.0 ± 6.6*</td>
</tr>
<tr>
<td>10 μM AMT</td>
<td>28.1 ± 2.3*</td>
</tr>
<tr>
<td>2 μM FA</td>
<td>34.7 ± 2.4*</td>
</tr>
<tr>
<td>100 μM FA</td>
<td>4.6 ± 0.9*</td>
</tr>
<tr>
<td>2 μM L-MTX</td>
<td>62.1 ± 3.3*</td>
</tr>
<tr>
<td>100 μM L-MTX</td>
<td>9.6 ± 1.1*</td>
</tr>
<tr>
<td>2 μM D-MTX</td>
<td>94.2 ± 7.7</td>
</tr>
<tr>
<td>100 μM D-MTX</td>
<td>62.0 ± 2.8*</td>
</tr>
<tr>
<td>1 mM D-MTX</td>
<td>24.0 ± 1.1*</td>
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*: Significantly different from the control (p<0.005).
Each value represents the mean ± S.D. of six determinations.

Fig. 4. Dixon plots for the inhibitory effects of FA (A), unlabeled L-MTX (B) and D-MTX (C) on the RFC1-mediated uptake of 3H-labelled L-MTX into Caco-2 cells. 3H-labeled L-MTX uptake (pH 6.0, 5 min) was measured at 0.1 (●) and 2 (■) μM in the presence of 0, 0.5, 1 and 2 μM FA (A) or B, 1 and 2 μM L-MTX (B). 3H-labeled L-MTX uptake (pH 6.0, 5 min) was measured at 0.5 (●), 1 (■) and 5 (▲) μM in the presence of 0, 100 and 200 μM D-MTX (C). RFC1-mediated initial uptake rate (v) of L-MTX was calculated as the total uptake minus the uptake by passive diffusion. Each point represents the mean ± S.D. of six determinations.

Fig. 5. Time course of 0.5 μM unlabeled L-MTX (●) and D-MTX (○) uptake into Caco-2 cells at pH 6.0. L- and D-MTX in Caco-2 cells were assayed with LC-MS/MS. Each point represents the mean ± S.D. of six determinations.

inhibited 3H-L-MTX uptake with a $K_i$ value of 1.51 μM (Fig. 4-B). Furthermore, the initial uptake rates for 0.5, 1, and 5 μM of L-MTX were measured in the presence of 0, 100, and 200 μM of D-MTX. According to the Dixon plot (Fig. 4-C), D-MTX also inhibited L-MTX uptake competitively with a $K_i$ value of 185 μM.

**Uptake of L- and D-MTX:** Uptake amounts of unlabeled L- and D-MTX at 0.5 μM into Caco-2 cells in the presence of a H+ gradient (pH 6.0) were measured using LC-MS/MS analysis. The results are shown in Fig. 5. The uptake of L-MTX was significantly greater than that of D-MTX at all time points. The uptake rates were calculated from the slope, and divided by the initial concentration (0.5 μM) to obtain the uptake clearance. The obtained uptake clearance values were 0.903 and 0.130 μL/min/mg protein for L- and D-MTX, respectively, which revealed an approximately 7-fold difference in uptake rate between the enantiomers.

**Discussion**

Caco-2 cells are used widely as an *in vitro* model to estimate intestinal absorption of drugs. Most transporters expressed in human enterocytes are expressed in Caco-2 cells, although the expression levels appear to differ from those in intestinal epithelial cells *in vivo*, depending on the transporter. Since RFC1 is expressed in Caco-2 cells, this cell line was used in the present study to examine transport characteristics of human RFC1.
In the present study, we defined the carrier-mediated uptake of L-MTX into Caco-2 cells “the RFC1-mediated uptake”, which may be inappropriate because it is possible that the uptake is not mediated by RFC1. However, we believe the present uptake into Caco-2 cells is mediated by RFC1 for the following reasons. Firstly, there are numerous reports that L-MTX is a substrate of RFC1. Secondly, the carrier-mediated uptake of L-MTX into Caco-2 cells consisted of a single component (Fig. 3) with the $K_m$ value being very similar to the reported $K_m$ values to RFC1 (see below). Thirdly, L-MTX uptake into Caco-2 cells was correlated with RFC1 mRNA levels. In our preliminary study, L-MTX uptake and the RFC1 mRNA levels were measured using Caco-2 cells with various culture days. The results revealed a correlation between L-MTX uptake and RFC1 mRNA levels (data not shown). Based on these observations, the carrier-mediated uptake of L-MTX was defined “the RFC1-mediated uptake” in the present study.

The initial uptake rate of L-MTX was concentration dependent with a $K_m$ value of 1.31 $\mu$M (Fig. 2). This $K_m$ value is similar to those previously reported, which are 1.54 $\mu$M in rat intestinal BBMV,21) 2.65 $\mu$M in mouse L1210 cells,29) 1.65–3.15 $\mu$M in K562 cells transfected with human RFC1 cDNA,30–32) Since the Eadie-Hofstee plot of the saturable uptake of L-MTX showed a straight line ($K_m$ $=$ 
\begin{equation}
\frac{V_{max}}{[S]}
\end{equation}
), L-MTX uptake into Caco-2 cells is apparently mediated by a single transport system, RFC1, in the concentration range used in this study.

In the inhibition studies, L-MTX uptake decreased to 36% and 21% of the control in the presence of 1 mM probenecid and DIDS, respectively (Table 1). These results agree with those of previous reports. RFC1-mediated transport decreased to 30% and 10% in the presence of 1 mM probenecid and DIDS, respectively, in murine L1210 leukemia and RFC1 expressed cells.35,36) Approximately 70% inhibition of L-MTX uptake by 10 $\mu$M AMT is also in agreement with the $K_i$ value (5.4 $\mu$M) of AMT to RFC1.34) Assuming competitive inhibition, 10 $\mu$M of AMT with a $K_i$ value of 5.4 $\mu$M should inhibit 65% of RFC1-mediated transport. Moreover, the inhibition by FCCP indicates that the driving force of L-MTX uptake is a $H^+$ gradient. These results support the idea that L-MTX uptake into Caco-2 cells in the presence of a $H^+$ gradient is mediated by RFC1.

FCCP works as a mitochondrial uncoupler35,36) as well as a protonophore.37,38) When FCCP is used as a mitochondrial uncoupler, the cells are usually incubated with FCCP for 45–90 min. In contrast, if FCCP is used as a protonophore, FCCP is added to the cells together with a substrate. Under the present experimental conditions, it is most likely that FCCP works as a protonophore, not as a mitochondrial uncoupler. In order to further confirm the $H^+$ gradient as a driving force, membrane vesicles that are prepared from Caco-2 cells may have to be used.

It is known that L-MTX is a substrate of MRP2 and BCRP, and that these efflux transporters are present in Caco-2 cells. Since probenecid is an inhibitor of both RFC1 and MRP2, probenecid may inhibit both the uptake by RFC1 and the efflux by MRP2. When probenecid is used as an inhibitor of MRP, however, the cells are incubated with a MRP substrate and probenecid (an inhibitor) for 60–90 min, and then the accumulation of a substrate in the cells is measured.39,40) In the present inhibition study, L-MTX and probenecid were added to Caco-2 cells simultaneously, and the uptake of L-MTX in 5 min was measured. Therefore, it is unlikely that probenecid inhibited the efflux of L-MTX under the present experimental conditions.

L-MTX uptake decreased significantly in the presence of 2 and 100 $\mu$M of FA or unlabeled L-MTX. In contrast, L-MTX uptake was not influenced by 2 $\mu$M of D-MTX, but decreased significantly in the presence of 100 $\mu$M and 1 $\mu$M of D-MTX (Table 1). The results indicate that L-MTX is capable of binding to RFC1 with an affinity much lower than that of L-MTX.

The Dixon plot showed that L-MTX uptake was inhibited competitively by FA with a $K_i$ value of 0.80 $\mu$M (Fig. 4-A). This $K_i$ value is almost equal to the previously obtained $K_m$ value of FA in Caco-2 cells ($K_m$ = 0.57 $\mu$M).21) These $K_m$ and $K_i$ values of FA are similar to other reported values ($K_m$ = 0.9–1.4 $\mu$M and $K_i$ = 0.5–2 $\mu$M),2,12,13,17,22,23,41) Unlabeled L-MTX and D-MTX competitively inhibited the uptake of $^3$H-labeled L-MTX with $K_i$ values of 1.51 and 185 $\mu$M, respectively (Figs. 4-B and C). The results clearly demonstrate that the affinity of L-MTX to RFC1 is comparable to that of FA, and that the affinity of D-MTX to RFC1 is approximately 120-fold smaller. These results are consistent with our previous observations using rabbit intestinal brush border membrane vesicles, which showed that the affinity of L-MTX to RFC1 was 60-fold greater than that of D-MTX.19)

The uptake clearance of each enantiomer was calculated from the slope of the uptake rate (Fig. 5), which were 0.903 and 0.130 $\mu$L/min/mg protein for L- and D-MTX, respectively. On the other hand, the uptake clearance values by RFC1 and passive diffusion at a concentration of 0.5 $\mu$M of L-MTX were calculated using the uptake parameters obtained in Fig. 2, which were 0.686 and 0.217 $\mu$L/min/mg protein, respectively. The results indicate that 76% of the total uptake is carrier-mediated at a concentration of 0.5 $\mu$M of L-MTX. When these values are compared with the uptake clearance of D-MTX at the same concentration, the uptake clearance of D-MTX (0.130 $\mu$L/min/mg protein) is similar or slightly smaller than that of
L-MTX by passive diffusion (0.217 μL/min/mg protein). The results suggest that D-MTX is taken up mainly by passive diffusion with very little or no contribution from RFC1. Although the total uptake clearance (RFC1 plus passive) of L-MTX is 7-fold greater than that of D-MTX (Fig. 5), RFC1-mediated uptake of L-MTX is expected to be much greater than that of D-MTX. Studies are underway to obtain uptake parameters for L- and D-MTX in order to reveal the stereoselectivity of RFC1 in more detail.

It may be due to the differences in MRP2-mediated efflux between L- and D-MTX that the uptake clearance of D-MTX (0.130 μL/min/mg protein) is smaller than that of L-MTX by passive diffusion (0.217 μL/min/mg protein). It is reported that both L- and D-MTX are transported by rat mrp2 and that the affinity of D-MTX to rat mrp2 is 3.5-fold greater than that of L-MTX.42) Since MRP2 is expressed in Caco-2 cells, it is possible that D-MTX is effluxed from the cells at a rate faster than that of L-MTX, which may result in a smaller uptake of D-MTX. The effect of MRP2 on the uptake of L- and D-MTX is not known, yet.

When an equal dose of each amethopterin enantiomer was orally administered to humans, L-MTX concentrations in plasma were markedly greater than those of D-MTX.18) The area under the plasma concentration-time curve (AUC) of L-MTX was approximately 40-fold greater than that of D-MTX. Since the elimination rate constant of D-MTX was only 1.7-fold greater than that of L-MTX, which may result in a smaller uptake of D-MTX. The effect of MRP2 on the uptake of L- and D-MTX is not known, yet.

Assuming that a normal dose of L-MTX (10 mg) is orally administered with 200 mL of water and completely dissolved in the stomach, the estimated maximum concentration of L-MTX in the GI tract will be approximately 100 μM. This concentration is much greater than the Km value of L-MTX to RFC1 (13.1 μM), suggesting that RFC1-mediated transport is saturated and that passive diffusion may predominate in the intestinal absorption. On the other hand, when the intestinal absorption of L-MTX is predicted based on the molecular polar surface area, L-MTX should be very poorly absorbed if it is absorbed only by passive diffusion.43,44) Moreover, it is reported that higher doses of L-MTX result in reduced absorption.45) These observations support the idea that L-MTX is absorbed in the small intestine mainly by a carrier-mediated process, i.e., RFC1, and not by passive diffusion.

The present study showed that the affinity of L-MTX to RFC1 is approximately 120-fold greater than that of D-MTX. It also showed that L-MTX is taken up into Caco-2 cells mainly by RFC1, whereas D-MTX uptake occurs mainly by passive diffusion with very little or no contribution of RFC1. It is very likely that the significant differences in the oral absorption of amethopterin enantiomers reflect the stereoselectivity of human RFC1.

References


