Trans-Stimulation Effects of Folic Acid Derivatives on Methotrexate Transport by Rat Renal Organic Anion Transporter, OAT-K1

AYAKO TAKEUCHI, SATOHIRO MASUDA, HIDEYUKI SAITO, YUKIYA HASHIMOTO, and KEN-ICHI INUI

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan

Accepted for publication February 29, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

We examined the pharmacological role of the renal organic anion transporter OAT-K1, which localizes predominantly in the brush-border membranes of proximal straight tubules, in the urinary excretion of methotrexate and the possibility of its contribution to “folic acid rescue.” With Madin-Darby canine kidney (MDCK) cells stably transfected with OAT-K1 cDNA, OAT-K1-mediated methotrexate accumulation was inhibited in the presence of various folic acid derivatives. These derivatives included aminopterin, 5-methyltetrahydrofolic acid, unlabeled methotrexate, folic acid (citrovorum factor, leucovorin), and folic acid with apparent inhibition constant values of 0.5, 1.2, 1.8, 8.2, and 14.1 μM, respectively. In contrast, 10 μM taurocholic acid and sulfobromophthalein did not inhibit OAT-K1-mediated methotrexate accumulation. In addition, methotrexate efflux was stimulated in the presence of inwardly directed gradients of aminopterin, 5-methyltetrahydrofolic acid, unlabeled methotrexate, folic acid, and folic acid, but not of uric acid, taurocholic acid, and glutathione, indicating that OAT-K1-mediated methotrexate efflux is stimulated by a folic acid derivatives exchange. In conclusion, OAT-K1 was suggested to enhance the apical efflux of highly accumulated methotrexate in tubular epithelial cells and contribute at least in part to folic acid rescue by exchanging intracellular methotrexate for extracellular folic acid.

High-dose methotrexate with “folic acid rescue” is used to treat several kinds of malignancies (Bleyer, 1978; Frei et al., 1980; Twelves, 1986; Kepka et al., 1998). Methotrexate is a potent inhibitor of dihydrofolinic acid reductase, causing a depletion of intracellular tetrahydrofolatic acid pools and thus a decline of de novo DNA synthesis (Jackson, 1984). Folic acid serves as a source of reduced folic acid to replenish the cellular folinic acid. In humans, methotrexate is eliminated entirely as the unchanged form in urine, through glomerular filtration, tubular reabsorption, and secretion (Bourke et al., 1975; He et al., 1991). It is distributed concentrically to the kidney (Bischoff et al., 1971; Scheufler et al., 1981) and often causes nephrotoxicity, a severe problem associated with methotrexate therapy (Bleyer, 1978).

In humans, methotrexate is eliminated entirely as the unchanged form in urine, through glomerular filtration, tubular reabsorption, and secretion (Bourke et al., 1975; He et al., 1991). It is distributed concentrically to the kidney (Bischoff et al., 1971; Scheufler et al., 1981) and often causes nephrotoxicity, a severe problem associated with methotrexate therapy (Bleyer, 1978). Methotrexate is a potent inhibitor of dihydrofolinic acid reductase, causing a depletion of intracellular tetrahydrofolatic acid pools and thus a decline of de novo DNA synthesis (Jackson, 1984). Folic acid serves as a source of reduced folic acid to replenish the cellular folinic acid.

In humans, methotrexate is eliminated entirely as the unchanged form in urine, through glomerular filtration, tubular reabsorption, and secretion (Bourke et al., 1975; He et al., 1991). It is distributed concentrically to the kidney (Bischoff et al., 1971; Scheufler et al., 1981) and often causes nephrotoxicity, a severe problem associated with methotrexate therapy (Bleyer, 1978). Methotrexate is a potent inhibitor of dihydrofolinic acid reductase, causing a depletion of intracellular tetrahydrofolatic acid pools and thus a decline of de novo DNA synthesis (Jackson, 1984). Folic acid serves as a source of reduced folic acid to replenish the cellular folinic acid.

Abnormal methotrexate transport via OAT-K1 has been reported in the apical membranes of the proximal tubules (Masuda et al., 1999b). Most recently, the bidirectional methotrexate transport via OAT-K1 has been reported in the apical membranes (Masuda et al., 1999b). However, the direction and the driving force of methotrexate transport via OAT-K1 under physiological conditions and clinical case remain to be elucidated. A rat liver organic anion-transporting polypeptide 1 ( oatp1), a homolog of OAT-K1, mediates the bidirectional transport of its substrate (Shi et al., 1995; Chan et al., 1998). Moreover, oatp1-mediated taurocholic acid uptake was stimulated in the presence of intracellular glutamate.
thione, and oatp1 was suggested to be an organic anion-glutathione exchanger (Li et al., 1998). Therefore, we have hypothesized that OAT-K1 is also an organic anion exchanger and probably participates in the tubular detoxification and secretion of anionic xenobiotics, especially methotrexate.

We report herein the characteristics of methotrexate efflux by OAT-K1. Functional analyses suggested that OAT-K1-mediated methotrexate transport is coupled to the exchange of organic anions, particularly folic acid derivatives, e.g., folinic acid, and contributes to folinic acid rescue.

### Experimental Procedures

**Materials.** [3',5',7'-3H(N)]Methotrexate, disodium salt (555 GBq/mmol) and [3',5',7',9'-3H]folic acid, diammonium salt (1.23 GBq/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). Unlabeled methotrexate and folinic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Aminopterin and 5-methyltetrahydrofolic acid were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity available. Figure 1 shows the chemical structures of methotrexate and various folic acid derivatives.

**Materials.**

**METHOTREXATE**

- NH₂
- CH₃
- CONHCH₂
- COOH

**FOLIC ACID**

- O
- CH₂NH₂
- CONHCH₂
- COOH

**FOLINIC ACID**

- O
- CHO
- CONHCH₂
- COOH

**5-METHYLTERAHYDROFOLIC ACID**

- O
- CH₂NH₂
- CONHCH₂
- COOH

**AMINOPTERIN**

- NH₂
- CH₂NH₂
- CONHCH₂
- COOH

**Fig. 1.** Chemical structures of folic acid derivatives.

### Results

**Effects of Various Anionic Drugs on Methotrexate Uptake by MDCK-OAT-K1 Cells.** First, we examined the inhibitory effects of various anionic compounds on the accumulation of [3H]methotrexate by MDCK-OAT-K1 cells under the cis-inhibition condition. All drugs were used at a concentration of 10 μM. As shown in Fig. 2, the accumulation of [3H]methotrexate via OAT-K1 was markedly inhibited in the presence of unlabeled methotrexate and folic acid. However, taurocholic acid and sulfobromophthalein did not inhibit OAT-K1-mediated [3H]methotrexate accumulation. Therefore, we further examined the inhibitory effects of other folic acid derivatives on the [3H]methotrexate uptake via OAT-K1, and the inhibition constant (Ki) values for the competitors were estimated by nonlinear regression analysis of the competition curves with one component and summarized in Table 1. The Ki values suggested that in terms of the affinity of OAT-K1, the folic acid derivatives ranked in the following order: aminopterin > 5-methyltetrahydrofolic acid > unlabeled methotrexate > folinic acid > folic acid.

**Efflux Study in MDCK-OAT-K1 Cells.** We examined the efflux of [3H]methotrexate and [3H]folic acid in the MDCK-OAT-K1 cells. To prevent the reuptake of the released [3H]methotrexate or [3H]folic acid, 1% BSA was added to the efflux buffer. Figure 3A shows the efflux of [3H]methotrexate from the MDCK-OAT-K1 cells. After [3H]methotrexate was preloaded in the monolayers, the efflux of [3H]methotrexate from the MDCK-OAT-K1 cells was much greater than that from the MDCK-pBK cells. In addition, [3H]folic acid efflux from the MDCK-OAT-K1 cells was enhanced compared with that from the MDCK-pBK cells (Fig. 3B), suggesting that OAT-K1 mediates the bidirectional transport of methotrexate and folic acid across the apical membranes.
Trans-Stimulation Effects of Various Anions on Methotrexate Efflux from MDCK-OAT-K1 Cells. If the secretion of methotrexate via OAT-K1 is coupled with the uptake of counter anions, the OAT-K1-mediated efflux of methotrexate should be stimulated in the presence of the counterdirected transmembrane gradient of various anions. Next, we examined the time-dependent \([^{3}H]\)methotrexate efflux via OAT-K1 in the presence of inwardly directed gradients of several folic acid derivatives. To avoid the protein binding of the external organic anions, we used the BSA-free efflux buffer. Cells were preloaded with 1 \(\mu\)M \([^{3}H]\)methotrexate, and then incubated with 0 (control) or 10 \(\mu\)M test anion for 5, 10, and 30 min. As shown in Fig. 4, folic acid derivatives stimulated the \([^{3}H]\)methotrexate efflux throughout the experiments. Therefore, we examined the trans-stimulation effect of several folic acid derivatives and other organic anions on the OAT-K1-mediated 10-min methotrexate efflux. All folic acid derivatives examined stimulated \([^{3}H]\)methotrexate efflux (unlabeled methotrexate, 127.8 \pm 2.7; folic acid, 116.3 \pm 2.6; 5-methyltetrahydrofolic acid, 115.4 \pm 2.4; folic acid, 119.5 \pm 3.8; and aminopterin, 123.0 \pm 1.3\% of the control, respectively). However, external uric acid, taurocholic acid, and glutathione did not stimulate \([^{3}H]\)methotrexate efflux (uric acid, 97.5 \pm 3.8; taurocholic acid, 97.2 \pm 4.8; and glutathione, 100.9 \pm 4.0\% of the control, respectively). To obtain more information about the driving force of OAT-K1-mediated methotrexate transport, we examined the effect of extracellular pH on \([^{3}H]\)methotrexate efflux from MDCK-OAT-K1 cells. As summarized in Table 2, \([^{3}H]\)methotrexate efflux was significantly stimulated in the presence of inwardly directed gradients of unlabeled methotrexate at pH 6.5 and 7.4, and similar tendency also was observed at pH 8.0. In addition, changes in the extracellular pH did not have any additional trans-stimulation effects on the \([^{3}H]\)methotrexate efflux. Furthermore, when the extracellular Cl\(^{-}\) was depleted, there were no significant differences in the \([^{3}H]\)methotrexate efflux from the MDCK-OAT-K1 cells (control, 467.4 \pm 4.53 fmol/mg protein/5 min; and Cl\(^{-}\)-free, 424.5 \pm 48.35 fmol/mg protein/5 min).

**Discussion**

Although high-dose methotrexate with folinic acid rescue is used clinically to treat a variety of malignant diseases (Bleyer, 1978; Frei et al., 1980; Twelves, 1986; Kepka et al., 1998), the pharmacokinetic interactions between these drugs have not been clarified. Previous study demonstrated that folinic acid did not influence the glomerular filtration and tubular reabsorption of methotrexate, but folinic acid accelerated the renal excretion of methotrexate. The data indi-
cated that it plays an important role not only in rescuing normal cells but also in excreting methotrexate from the body (He et al., 1991). However, the molecular mechanisms of its actions remain to be elucidated.

Recently, a rat renal organic anion transporter, OAT-K1, was cloned and characterized (Saito et al., 1996). OAT-K1 was localized to the renal brush-border membranes (Masuda et al., 1997) and was revealed to transport methotrexate and folic acid (Saito et al., 1996; Masuda et al., 1999a,b). With MDCK cells stably transfected with OAT-K1, $^{[3]H}$methotrexate accumulation via OAT-K1 was markedly inhibited by folic acid derivatives, including folic acid (Fig. 2; Table 1). Therefore, the participation of folic acid in the OAT-K1-mediated methotrexate transport was implied. Moreover, the $K_i$ values showed that the affinities of OAT-K1 for the folic acid derivatives were relatively high compared with those for taurocholic acid and sulfobromophthalein (Fig. 2; Table 1). The $K_i$ values for taurocholic acid and sulfobromophthalein for the methotrexate transport by OAT-K1 suggested that both compounds had low affinities for OAT-K1. These findings suggest that folic acid derivatives are potential substrates for the methotrexate transport by OAT-K1, distinct from those for the other known transporters belonging to the oatp-gene family, such as oatp1 (Shi et al., 1995; Bergwerk et al., 1996), oatp3 (Abe et al., 1998), and prostaglandin transporter (Chan et al., 1998) in the kidney.

Because the efflux of $^{[3]H}$methotrexate and $^{[3]H}$folic acid from the MDCK-OAT-K1 cells was much greater than that from the MDCK-pBK cells (Fig. 4), OAT-K1 is suggested to mediate the bidirectional transport of folic acid derivatives across the apical membranes. Similar findings were reported for the homolog of OAT-K1, i.e., the multispecific organic anion transporter, OAT-K2, which has been cloned in our laboratory (Masuda et al., 1999a). In addition, with inducible HeLa-transfectants, bidirectional sulfobromophthalein transport via oatp1 transporter was reported (Shi et al., 1995), and with oocytes and HeLa-transfectants, bidirectional transport of prostanoid via oatp1 transporter was reported (Chan et al., 1998). Therefore, the bidirectional transport activity would be one of the features of oatp-related transporters.

OAT-K1-mediated $^{[3]H}$methotrexate efflux was stimulated in the presence of extracellular folic acid derivatives such as...
folic acid (Figs. 4 and 5), providing for the possibility of methotrexate/folic acid exchange. The stimulation of the anion exchange between extracellular folic acid and intracellular methotrexate is consistent with previous findings that extracellular folic acid stimulated the efflux of methotrexate from tumor cells (Goldman, 1971). Schilsky and Ratain (1990) reported that the peak plasma concentration of an active isomer (R)-folic acid was 59.1 ± 22 μM after the administration of 1000 mg of folic acid, and 46% of this appeared unchanged in form in the urine within 24 h. The concentration of folic acid in the primary urine would be high enough to stimulate the methotrexate efflux from the brush-border membranes. Therefore, the methotrexate/folic acid exchange via OAT-K1 may confer the beneficial effect of folic acid in inducing the cytoprotection caused by methotrexate at the molecular level.

In contrast to the report that oatp1 mediated glutathione/taurocholic acid exchange, [3H]methotrexate efflux via OAT-K1 was not stimulated in the presence of extracellular glutathione (Fig. 5). This result raised the possibility that there is a major difference between OAT-K1 and oatp1, in the coupling of counter anions, although anion exchange is a common mechanism for this family of transporters. Previous studies have shown that OAT-K2, a homolog of OAT-K1, also mediates the bidirectional transport of methotrexate (our unpublished data), so it is possible that OAT-K2 as well as OAT-K1 plays a role in excreting methotrexate into urine. Additional studies are needed to clarify the transport characteristics of OAT-K1 and OAT-K2 concerning the driving forces, and their physiological significance.

Moreover, there was no influence of the change in extracellular pH and depletion of extracellular Cl⁻ on the [3H]methotrexate efflux via OAT-K1 (Table 2). These data suggest that OAT-K1 is likely to be distinct from the anion exchanger at the brush-border membrane, which can exchange various organic anions and inorganic anions such as p-aminobiphruric acid, uric acid, Cl⁻, Br⁻, HCO₃⁻, and OH⁻ as substrate in rat kidney (Ohoka et al., 1993).

In conclusion, this study demonstrates that OAT-K1-mediated methotrexate efflux is accompanied by folic acid derivatives exchange. Therefore, OAT-K1 could serve to enhance the apical efflux of methotrexate accumulated in the tubular epithelial cells and contribute to folic acid rescue by exchanging intracellular methotrexate for extracellular folic acid. These findings suggest that OAT-K1 participates at least in the tubular detoxification of methotrexate and provide useful information regarding the appropriate use of these drugs.

References


Send reprint requests to: Ken-ichi Inui, Ph.D., Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: inui@kuhp.kyoto-u.ac.jp