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Functional analysis of rat renal organic anion transporter OAT-K1: bidirectional methotrexate transport in apical membrane

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Abstract Renal organic anion transporter OAT-K1 was stably transfected in MDCK cells and examined for its transport characteristics and membrane localization. OAT-K1 mediated both uptake and efflux of methotrexate in the apical membranes. Immunoblotting showed that the apparent molecular mass of the expressed OAT-K1 was 50 kDa, which was comparable to that found in the rat renal brush-border membranes. The OAT-K1-mediated methotrexate transport was significantly inhibited in the presence of several organic anions such as folate and sulfobromophthalein. These findings suggest that OAT-K1 mediates bidirectional methotrexate transport across the apical membranes, and may be involved in the renal handling of methotrexate.

Key words: Organic anion transporter; Methotrexate; Brush-border membrane; Tubular secretion; MDCK cell; (Rat kidney)

1. Introduction

A wide variety of drugs and endogenous compounds are excreted into the urine by the renal tubular secretion systems [1,2]. Intensive use of the antifolate methotrexate has been demonstrated to be effective in the treatment of acute lymphocytic leukemia at high dosage [3,4], and of arthritis at relatively low doses [5]. Because urinary excretion represents the major route of elimination of this substrate from the body [6], methotrexate reaches high concentrations in the renal tubules, causing the conditions of nephrotoxicity.

We recently cloned a cDNA encoding a kidney-specific organic anion transporter, OAT-K1, and demonstrated basolateral uptake of methotrexate and folate in LLC-PK_1 cells stably transfected with OAT-K1 [7]. The OAT-K1 was expressed in the plasma membranes from the transfectant, showing an apparent molecular mass of about 70 kDa, similar to its predicted molecular weight [8]. By reverse transcription-coupled PCR analysis of OAT-K1 mRNA and Western blotting with anti-OAT-K1 antibody, we found that the OAT-K1 was localized in the brush-border membranes of the renal proximal straight tubules with an apparent molecular mass of about 40 kDa [9]. Studies on the membrane localization of the OAT-K1 and characterization of OAT-K1-mediated methotrexate transport would suggest its physiological role in renal handling of methotrexate. To obtain more information about OAT-K1, we have stably transfected the OAT-K1 cDNA into MDCK cells, and examined the transport characteristics of the transporter. The present results suggest that OAT-K1 mediates bidirectional transport of methotrexate in renal brush-border membranes.

2. Materials and methods

2.1. Materials

[3′,5′,7-^{3}H]Methotrexate sodium salt (359 GBq/mmol) was purchased from Amersham Int. (Buckinghamshire, UK), and [3′,5′,7,9-^{3}H]folate (1232 GBq/mmol) was from Moravek Biochemical, Inc. (Brea, CA, USA). [^{3}H]Taurocholate (128.39 GBq/mmol) and *p*-[glycyl-^{14}C]aminohippurate (1.5 GBq/mmol) were obtained from Du Pont-New England Nuclear Research Products (Boston, MA, USA). Unlabeled methotrexate and indomethacin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Unlabeled folate, sulfobromophthalein, taurocholate and *p*-aminohippurate were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used for the experiment were of the highest purity available.

2.2. Cell culture and transfection

The parental MDCK cells were cultured in complete culture medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD, USA) with 10% fetal calf serum (Microbiological Associates, Bethesda, MD, USA) in an atmosphere of 5% CO_2-95% air at 37°C. The whole OAT-K1 cDNA was subcloned into the *Sal*I- and *Not*I-cut mammalian expression vector pBK-CMV (Stratagene, La Jolla, CA, USA) [10]. MDCK cells were transfected with pBK-CMV/OAT-K1 or pBK-CMV using the calcium phosphate coprecipitation technique as described [7]. After incubation in 0.5 mg/ml G418 (Life Technologies) for 8–10 days, single colonies were picked up with cloning cylinders for subsequent screening. G418-resistant clonal cells were analyzed by Northern blotting for the expression of rat OAT-K1 mRNA. For the transport experiments, cells were seeded with the complete medium on 35 mm diameter culture dishes or microporous membrane filters (3 μm pores, 1 cm^2 growth area) inside a Transwell cell culture chamber (Costar, Cambridge, MA, USA) at a cell density of 2.0×10^5 cells/dish or 5×10^5 cells/filter, respectively.

2.3. Uptake study in OAT-K1-expressing MDCK cells

Uptake measurements were performed as described previously [8] with some modifications. Cellular uptake of radioactive drugs was measured using monolayer cultures grown in the 35 mm diameter dishes. The incubation medium for uptake experiments was Dulbecco's phosphate-buffered saline (pH 7.4) [PBS buffer (in mM): 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$], containing 5 mM D-glucose (uptake buffer). For the efflux experiments, cell monolayers grown in the 35 mm diameter dishes were incubated with 0.8 ml of uptake buffer containing [^{3}H]methotrexate (1 and 10 μM) for 30 min. After the incubation, the cells were washed once with 2 ml of uptake buffer containing 1% bovine serum albumin (BSA) (pH 7.4) followed by three more washes with 2 ml of BSA-free uptake buffer (pH 7.4) at 4°C and then incubated with 0.8 ml of uptake buffer containing 1% BSA (pH 7.4) at 37°C for the specified time. At the end of incubation, cells were washed three times in BSA-free uptake buffer at 4°C. The cells were lysed in 0.5 N NaOH solution, and then the radioactivity in aliquots was determined in 5 ml of ACSII (Amersham). The protein content of the solubilized cells was

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Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction; oatp, organic anion transporting polypeptide

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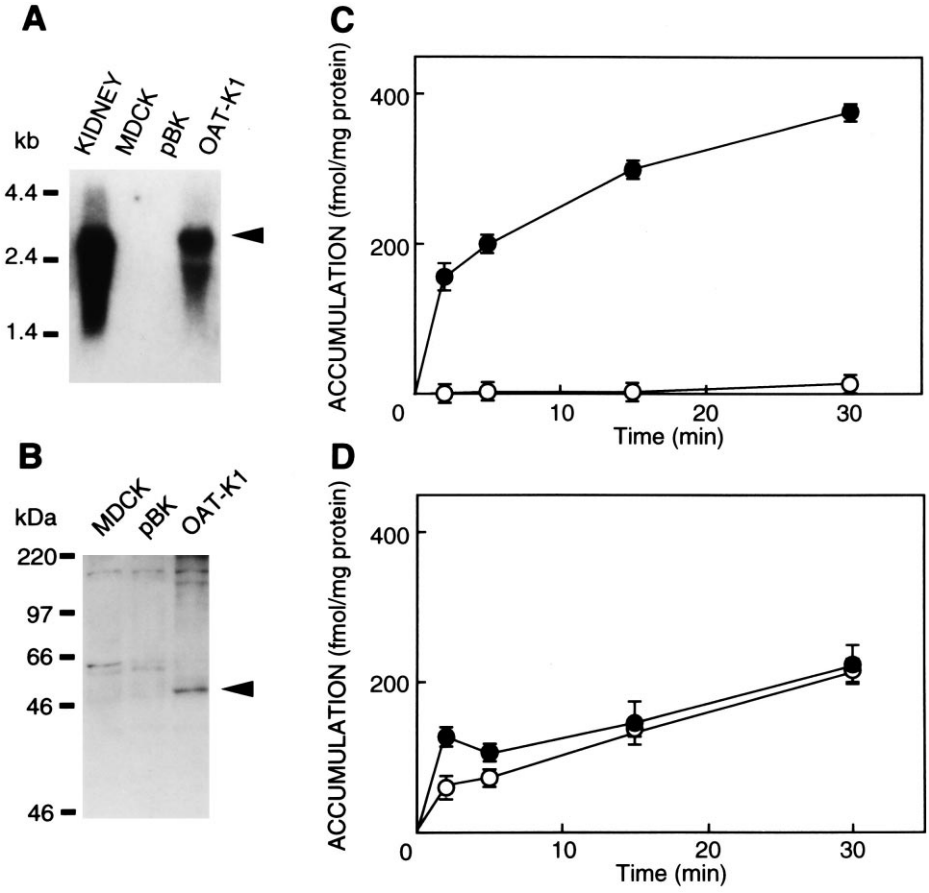

Fig. 1. Expression of OAT-K1 mRNA (A) and protein (B) in MDCK-OAT-K1 cells, and accumulation of [^{3}H]methotrexate by MDCK-OAT-K1 or MDCK-pBK monolayers from the apical (C) or basolateral (D) side. A: Total RNA (10 μg) from rat kidney, MDCK, MDCK-pBK and MDCK-OAT-K1 cells was electrophoresed, blotted, and hybridized with the whole OAT-K1 cDNA as a probe at high stringency. B: Plasma membranes (50 μg) from MDCK, MDCK-pBK and MDCK-OAT-K1 cells were electrophoresed, blotted, and hybridized with the purified anti-OAT-K1 antiserum as primary antibody. C and D: Monolayers of MDCK-OAT-K1 (●) or MDCK-pBK (○) were incubated for the specified period at 37°C with 100 nM [^{3}H]methotrexate added to either the apical (C; pH 7.4) or basolateral (D; pH 7.4) side. Unlabeled incubation medium was added to the opposite side (pH 7.4). After incubation, the radioactivity of solubilized cells was counted. Each point represents the mean ± S.E.M. of three monolayers.

determined by the method of Bradford [11], using Bio-Rad Protein Assay kit (Bio-Rad) with bovine γ-globulin as a standard.

2.4. Northern blot analysis

Total RNA was extracted from rat kidney and MDCK transfectants using the RNeasy spin column (Qiagen, Hilden, Germany). For Northern blot analysis, 10 μg of total RNA from rat kidney and MDCK transfectant was resolved by electrophoresis in 1% agarose gels containing formaldehyde and transferred onto nylon membranes. After transfer, blots were hybridized at high stringency (50% formamide, 5×SSPE, 5×Denhardt's solution, 0.2% SDS, 10 μg/ml salmon sperm DNA at 42°C) with a whole OAT-K1 cDNA labeled with [α-^{32}P]dCTP as described [7].

2.5. Western blot analysis

Rabbit anti-OAT-K1 antibodies raised against the synthetic peptide corresponding to the 15 COOH-terminal amino acids of OAT-K1 [9] were used for Western blot analysis after affinity purification [12]. Crude plasma membrane fractions of either the stably transfected MDCK cells expressing OAT-K1 (MDCK-OAT-K1), mock-transfected cells (MDCK-pBK) or host MDCK cells were prepared. The samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Hybond-PVDF; Amersham) by semi-dry electroblotting for 30 min. Blots were blocked, washed and incubated with the purified antiserum preabsorbed with the synthetic antigen peptide (0.5 μg/ml) or the primary purified antibody (1:50) overnight at 4°C. The blots were washed and the bound antibody was detected on X-ray film.

2.6. Functional expression in Xenopus oocytes

An open reading frame of the cDNA encoding rat OAT-K1 was amplified by PCR with a set of specific primers, blunted with Klenow fragment and ligated into the blunt-ended dephosphorylation site of plasmid XβG vector (provided by Dr. P. Agre), which was constructed from pBluescript containing the 5′ and 3′ non-coding regions of *Xenopus* β-globin [13,14]. The recombinants were confirmed by enzymatic nucleotide sequencing. Five nanograms of capped complementary RNA (cRNA) transcribed in vitro was injected into *Xenopus* oocytes. Injected oocytes were maintained in modified Barth's medium at 18°C for 2–3 days. In general, functional expression of rat OAT-K1 was analyzed by measuring uptake of [^{3}H]methotrexate in groups of oocytes injected with 50 nl of water or cRNA as described [15]. Oocytes were incubated for 60 min at 25°C in an uptake solution (pH 7.4) (in mM: 100 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Tris) containing 500 nM [^{3}H]methotrexate. After the enhanced methotrexate uptake was confirmed in the OAT-K1 cRNA-injected oocytes, efflux of [^{3}H]methotrexate was measured. The oocytes were loaded with 50 nl of [^{3}H]methotrexate (0.6 pmol/oocyte), and efflux was immediately determined [16]. At the end of incubation, oocytes were washed three times in 1.5 ml of ice-cold uptake solution (pH 7.4), solubilized in 10% SDS solution and then the radioactivity was determined in 5 ml of ACSII (Amersham) by liquid scintillation counting.

2.7. Statistical analysis

Data were analyzed statistically using one-way analysis of variance followed by Fisher's *t*-test.

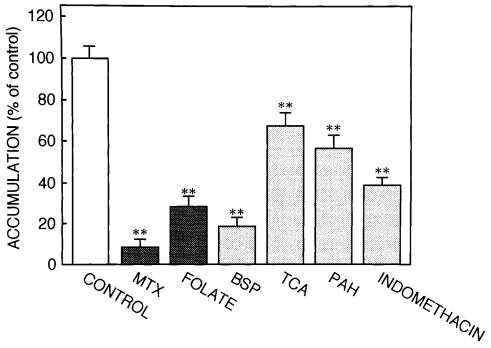

Fig. 2. Effect of various anionic drugs on [^{3}H]methotrexate accumulation by MDCK-OAT-K1 cells. [^{3}H]Methotrexate accumulation by MDCK-OAT-K1 cells (100 nM; pH 7.4) cultured in 35 mm dishes was measured for 15 min at 37°C in the absence (control) and presence of indicated drugs at a concentration of 100 μM. After incubation, the radioactivity of solubilized cells was determined. MTX, unlabeled methotrexate; BSP, sulfobromophthalein; TCA, taurocholate; PAH, *p*-aminohippurate. Data are expressed as % of the control value (318.6 ± 15.23 fmol/mg protein/15 min). Each column represents the mean ± S.E.M. of three monolayers. $^{**}P < 0.001$, significantly different from control.

3. Results

As illustrated in Fig. 1A, OAT-K1 mRNA was expressed in MDCK cells stably transfected with the rat OAT-K1 cDNA, MDCK-OAT-K1, which was similar in size to that in rat kidney, but not in the host MDCK and mock-transfected MDCK-pBK cells. The immunoreactive protein with an apparent molecular mass of 50 kDa was detected with the anti-OAT-K1 antiserum in the plasma membranes from MDCK-OAT-K1 cells, but not from MDCK and MDCK-pBK cells (Fig. 1B). Fig. 1C and D show the intracellular accumulation of methotrexate in MDCK-OAT-K1 and MDCK-pBK cells grown on membrane filters. The accumulation from the apical side was much higher in the MDCK-OAT-K1 than in the MDCK-pBK monolayers (Fig. 1C). In contrast, the accumulation from the basolateral side in MDCK-OAT-K1 monolayers was comparable to that in the MDCK-pBK monolayers (Fig. 1D). Therefore, the rat OAT-K1 was suggested to be expressed functionally in the apical membranes of the transfectant with an apparent molecular mass of 50 kDa, corresponding to OAT-K1 expression in rat kidney [9]. Because the OAT-K1 was localized in the apical membranes of the MDCK-OAT-K1 monolayers, further transport studies were done using the cells grown in the 35 mm culture dishes.

Table 1
Kinetic parameters of methotrexate transport determined by MDCK-OAT-K1 and LLC-OAT-K1 cells

Cells	[^{3}H]Methotrexate accumulation	
	K_m (μM)	V_{max} (pmol/mg protein/15 min)
MDCK-OAT-K1	2.1	7.2
LLC-OAT-K1[a]	1.0	31.7

MDCK-OAT-K1 cells were grown to confluence in 35 mm culture dishes. [^{3}H]Methotrexate accumulation was measured at various concentrations (0.1–30 μM) for 15 min at 37°C in the absence and presence of 1 mM unlabeled methotrexate. After incubation, the radioactivity of solubilized cells was determined. The specific component of the uptake was evaluated kinetically using non-linear least-squares regression analysis as described previously [7]. Transport experiments for each concentration were performed with three monolayers.
[a]Values are from [7].

Table 1 summarizes the kinetic parameters for methotrexate accumulation by MDCK-OAT-K1 cells comparing the values in LLC-PK$_1$ cells stably transfected with OAT-K1 (LLC-OAT-K1) [7]. The K_m value for methotrexate in the MDCK-OAT-K1 cells was comparable to that in LLC-OAT-K1 cells, whereas the V_{max} value in the MDCK-OAT-K1 cells was lower than that in LLC-OAT-K1 cells. Next, we examined the substrate specificity of the MDCK-OAT-K1 cells. Under *cis* inhibition conditions, all drugs had significant inhibitory effects on the [^{3}H]methotrexate accumulation by the transfectant (Fig. 2).

To investigate transport function of the rat OAT-K1 in the brush-border membranes, we examined the efflux of [^{3}H]methotrexate in MDCK-OAT-K1 cells. Fig. 3 shows the efflux of methotrexate from the MDCK-OAT-K1 cells. After methotrexate was preloaded in the monolayers, the efflux of methotrexate in the MDCK-OAT-K1 cells was much greater than that in the MDCK-pBK cells, suggesting that OAT-K1 mediates bidirectional methotrexate transport across the apical membranes. The efflux of methotrexate in MDCK-OAT-K1 cells was not different from that in MDCK-pBK when preloaded with methotrexate at a concentration of 10 μM. This may be due to the OAT-K1-mediated efflux of methotrexate being saturated at high intracellular concentrations of the substrate.

Furthermore, OAT-K1-mediated methotrexate transport was examined in *Xenopus* oocytes. When expressed in oocytes, OAT-K1 stimulated the uptake of [^{3}H]methotrexate and [^{3}H]folate, but not [^{3}H]taurocholate and [^{14}C]*p*-aminohippurate (Fig. 4A). We reported that OAT-K1 specifically transports methotrexate and folate, but not taurocholate and *p*-aminohippurate in LLC-OAT-K1 monolayers [7]. After the OAT-K1-mediated methotrexate uptake was confirmed in oocytes, we used the same batch of oocytes for efflux study. Following injection of [^{3}H]methotrexate into water- or OAT-K1 cRNA-injected oocytes, the oocytes were washed

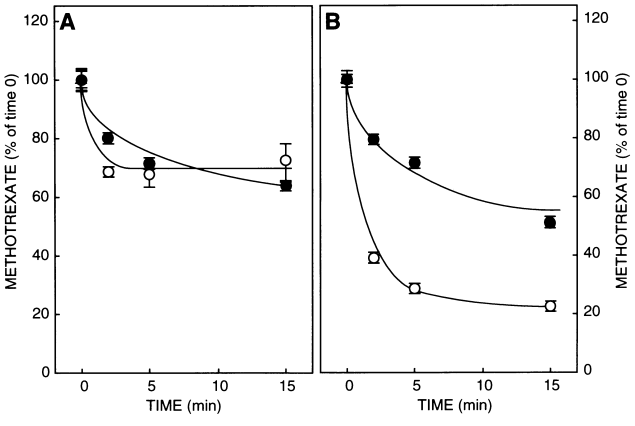

Fig. 3. Time course of methotrexate efflux by MDCK-pBK (A) and MDCK-OAT-K1 (B) cells. After [^{3}H]methotrexate accumulation at a concentration of 1 μM (○) or 10 μM (●) for 15 min at 37°C (pH 7.4), cells were washed and incubated in efflux buffer for the specified period at 37°C (pH 7.4). The [^{3}H]methotrexate remaining in MDCK-pBK (A) or MDCK-OAT-K1 (B) cells was measured. Data are expressed as % of the time 0 value. Each column represents the mean ± S.E.M. of three monolayers.

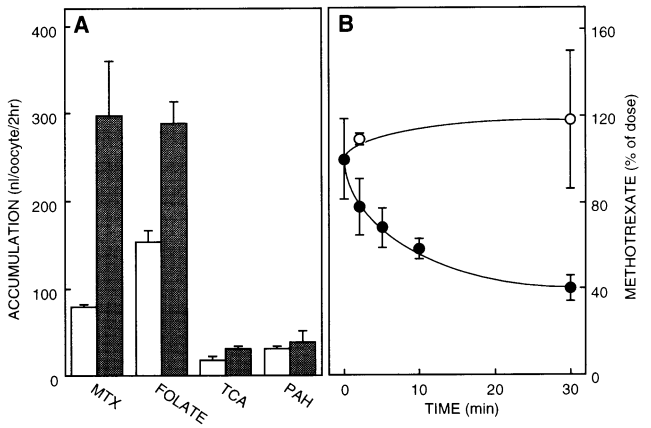

Fig. 4. Accumulation of anionic drugs (A) and efflux of [^{3}H]methotrexate (B) by *Xenopus* oocytes. A: Accumulation by oocytes was assayed for 2 h at 25°C in incubation buffer containing 500 nM [^{3}H]methotrexate (MTX), 150 nM [^{3}H]folate, 500 nM [^{3}H]taurocholate (TCA) and 15 μM of [^{14}C]*p*-aminohippurate (PAH), 2 days after injection of 50 nl of water (open column) or in vitro transcribed rat OAT-K1 cRNA (shaded column; 40 ng/oocyte). Accumulation of each drug is expressed as the uptake clearance. B: Efflux of [^{3}H]methotrexate by water (○)- or rat OAT-K1 cRNA (●)-injected oocytes was assayed for the specified period at 25°C in incubation buffer immediately after loading 50 nl of [^{3}H]methotrexate solution (1.2 μM). Each column or point represents the mean ± S.E.M. of three (A) or four (B) oocytes.

and then incubated in drug-free solution at 25°C. As shown in Fig. 4B, water-injected oocytes exhibited no efflux of [^{3}H]methotrexate. In contrast, rapid methotrexate efflux out of the OAT-K1-expressing oocytes was observed.

4. Discussion

In humans, an administered dose of methotrexate is recovered in mostly unchanged form in the urine, with the renal elimination involving glomerular filtration, active tubular secretion and reabsorption. Previous studies on clearance experiments and isolated renal tubules indicated that methotrexate secretion was affected by other anionic drugs such as *p*-aminohippurate [17] and probenecid [18]. A renal basolateral-type organic anion/dicarboxylate exchanger, OAT1, is suggested to mediate the basolateral uptake of various organic anions including methotrexate from the circulation into the proximal tubular epithelial cells [19]. However, the secretion mechanisms for methotrexate in the brush-border membranes have not been fully elucidated. We found that OAT-K1 was expressed predominantly in renal straight tubules and localized in the brush-border membranes [9]. Moreover, we demonstrated that OAT-K1 was involved in the potential interaction sites between methotrexate and non-steroidal antiinflammatory drugs in the kidney [8]. Therefore, OAT-K1-mediated transport of methotrexate in the brush-border membranes implies a significance for the pharmacological role of the transporter in the kidney.

The protein with an apparent molecular mass of 50 kDa in the plasma membrane fractions of MDCK-OAT-K1 cells, but not of MDCK-pBK and MDCK cells (Fig. 1B), was smaller than the calculated molecular mass of 74 kDa of the OAT-K1 [7], but comparable to that found in rat renal brush-border membranes [9]. In this study, we have found that the OAT-K1-mediated methotrexate transport was enhanced from the apical side, but not from the basolateral side, in MDCK-OAT-K1 monolayers (Fig. 1C,D). Moreover, we reported that proteolytic cleavage of the translation product of the OAT-K1 mRNA could occur in the renal tubular cells, thereby the 40 kDa OAT-K1 protein might be localized in the brush-border membranes [9]. On the other hand, OAT-K1 protein was found to be localized in the basolateral membranes of the transfected LLC-PK$_1$ cells with an apparent molecular weight of about 70 kDa [7,8], suggesting a lack of proteolysis of the protein. Therefore, the proteolytic processing and/or excision of OAT-K1 in renal tubular cells would be retained in the MDCK cells, but not in the LLC-PK$_1$ cells. A similar event was reported in the localization of oatp1, a rat liver organic anion transporting polypeptide [20,21]. The oatp1 protein was detected in rat liver basolateral membranes and HeLa transfectant plasma membranes with an apparent molecular mass of about 80 kDa which was comparable to its calculated molecular mass of about 74 kDa [21,22]. Moreover, the oatp1 proteins were detected with apparent molecular masses of about 33 and 35 kDa in rat renal brush-border membranes under the reducing condition [21]. However, there is no evidence that the small type of oatp1 proteins can transport anionic compounds in rat kidney. This is the first report that the methotrexate transport activity is conserved with a small type of OAT-K1 molecule, suggesting the small type of OAT-K1 protein-mediated methotrexate transport in the renal brush-border membranes. The precise mechanisms of the membrane localization of the OAT-K1 and the proteolytic site in the transporter molecule should be further investigated.

The K_m value of methotrexate accumulation from the apical side in the MDCK-OAT-K1 cells was comparable with that from basolateral side in the LLC-OAT-K1 monolayers. However, the V_{max} value of the transport in the MDCK-OAT-K1 cells was lower than that in the LLC-OAT-K1 monolayers (Table 1). There would be a difference in the expression level of the transporter and/or the methotrexate transport velocity between the basolateral and apical OAT-K1. The inhibitory potency of the anionic drugs was in the order methotrexate > sulfobromophthalein > folate > indomethacin > *p*-aminohippurate ≅ taurocholate (Fig. 2). The order of the inhibitory potency for these drugs was comparable between LLC-OAT-K1 [7] and MDCK-OAT-K1 cells. Particularly, *p*-aminohippurate and taurocholate had inhibitory effects on the OAT-K1-mediated methotrexate transport (Fig. 2), but both compounds were not transported via OAT-K1 (Fig. 4). Both compounds may have binding affinity to OAT-K1 by anionic charge interaction, but are not translocated via OAT-K1 probably due to their unsuitable structural requirements. These results suggest that both basolateral and apical OAT-K1-expressing transfectants show a similar sensitivity against various anionic drugs. Therefore, the expression system of the OAT-K1 in the MDCK transfectants should be a useful in vitro model for studying the mechanisms involved in transport functions and membrane localization.

The OAT-K1-expressing MDCK transfectants and *Xenopus* oocytes have shown methotrexate efflux activity (Figs. 3B and 4B). Using inducible HeLa transfectants, bidirectional sulfobromophthalein transport via the oatp1 was reported [22]. In addition, a rat prostaglandin transporter PGT, which shows 35% amino acid identity to OAT-K1, mediated the bidirectional prostanoid transport in oocytes and HeLa transfectants [23]. The bidirectional transport activity would be one of the

features of this *oatp* gene family. Thereby, OAT-K1 may mediate the bidirectional transport of methotrexate across the apical membranes.

Methotrexate accumulates extensively in the kidney in vivo [17]. Considering membrane potential, cellular accumulated organic anions, including methotrexate, are likely to be transported into the lumen across the brush-border membrane, i.e. tubular secretion. OAT-K1 could facilitate methotrexate efflux when methotrexate was accumulated in the cytoplasm. Therefore, OAT-K1 may participate in the tubular secretion of methotrexate, preventing its potential toxicity.

In conclusion, we have extended the study on functional characteristics of OAT-K1 using the MDCK transfectant and *Xenopus* oocyte expression system. The findings suggest that intracellular proteolytic cleavage might be necessary for the apical localization of OAT-K1, and OAT-K1 mediates bidirectional methotrexate transport. These data imply that OAT-K1 may be involved in the renal tubular secretion of organic anions and play a role in detoxication of methotrexate from tubular epithelial cells into the urine.

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