**Table 1 – Major drug transporters expressed in the intestine and kidney**

| Transporter | Major pharmacokinetic/ physiological roles | Tissue distribution | Regulation/ diseases | Transcription factors | Reference |
|---|---|---|---|---|---|
| PEPT1 | Intestinal absorption, renal reabsorption | Small intestine, kidney | Fasting, diurnal rhythm | Sp1 | [35] |
| | | | | Cdx2 | [36] |
| | | | | PPARα | [37] |
| OCT1 | Hepatic uptake | Liver | Intrahepatic cholestasis | HNF-4α | [81] |
| OCT2 | Renal excretion | Kidney | Gender | Androgen receptor (Rat) | [75] |
| OCTN2 | Intestinal absorption, renal reabsorption | Intestine, kidney | Intestinal bowel diseases | Heat-shock transcription factor | [56] |
| OAT1 | Renal excretion | Kidney | Renal failure | Not identified | |
| OAT2 | Hepatic uptake | Liver | Gender | HNF-4α | [82] |
| OAT3 | Renal excretion | Kidney | Renal failure | HNF-1α/β | [79] |
| | | | | CREB-1 | [80] |
| | | | | ATF-1 | [80] |

### 2.1.2. *Regulation of PEPT1*

Adibi [13] summarized the recent progress in the study of the regulation of intestinal PEPT1. He classified the regulation of intestinal PEPT1 as physiological, pathological, or pharmacological regulation. As physiological factors, oligopeptides [14], various hormones (thyroid hormone, insulin, leptin, etc.) [15–17], cytokines (interferon-γ and tumor necrosis factor-α) [18,19], development [20,21], and diurnal rhythm [22–24] regulated the expression of intestinal PEPT1. These regulatory mechanisms vary as follows. In the case of oligopeptides [14], thyroid hormone [17], diurnal rhythm [22–24], and development [20,21], parallel changes in gene expression are brought about by alterations of the transcription and/or stability of PEPT1 mRNA. In contrast, treatment with insulin [15] and leptin [16] did not induce any change in *PEPT1* gene expression, and the mechanism of increased protein expression appears to be increased trafficking from a preformed cytoplasmic pool to the apical membranes. Interferon-γ does not affect the expression of PEPT1 at the mRNA or protein level, but enhances the $H^+$-electrochemical gradient across the apical plasma membrane in model intestinal epithelial cells [18].

In rats under various nutritional and metabolic conditions (high-protein diet [25,26], fasting [23,25], diabetes [27]), the expression of Pept1 in the intestine was mainly regulated at the transcriptional level. Under the chronic renal failure, intestinal Pept1 expression was regulated at protein level [28]. In patients with intestinal diseases including ulcerative colitis [29], Crohn's disease [29], and short-bowel syndrome [30], PEPT1 expression is induced in the colon. Pharmacological studies have shown that the mRNA expression of PEPT1 can be up-regulated by agents such as pentazocine [31] and 5-fluorouracil [32,33].

### 2.1.3. *Transcriptional regulatory mechanisms of PEPT1*

Shiraga et al. [26] have revealed that the rat Pept1 promoter was transcriptionally regulated by certain amino acids *via* an amino acid-responsive element. In the mouse Pept1 promoter, essential promoter/enhancer sites were shown to be present within 1140 bp upstream of the transcription start site [34]. Nevertheless, the *cis*-elements and/or transcription factors critical for basal transcriptional regulation have not been identified. To address these issues, we have recently cloned the human PEPT1 promoter region and examined its promoter activity using a human intestinal cell line, Caco-2 [35]. Deletion analysis of the human PEPT1 promoter suggested that the region spanning −172 to −35 bp was essential for basal transcriptional activity. This region lacked a TATA-box but contained some GC-rich sites which supposedly bind with the transcription factor Sp1. Electrophoretic mobility shift assay (EMSA), mutational analysis, inhibition analysis, and over-expression analysis have demonstrated a significant role for Sp1 in the basal transcriptional regulation of PEPT1 [35].

Because Sp1 is ubiquitously expressed, it cannot be the factor responsible for the intestine-specific expression of PEPT1. We then clarified the mechanisms behind the intestinal expression of the *PEPT1* gene [36]. Among the transcription factors investigated, only caudal-related homeobox protein 2 (Cdx2) markedly trans-activated the PEPT1 promoter, although the promoter region responsible for this effect lacked a typical Cdx2-binding sequence, but possessed some Sp1-binding sites. Cdx2 was suggested to have a novel mode of action, namely binding with Sp1. This hypothesis was confirmed by the results of a reporter assay, an immunoprecipitation assay, and a chromatin immunoprecipitation assay. The significance of CDX2 *in vivo* for *PEPT1* regulation was shown by the determination of mRNA levels of CDX2 and PEPT1 in human tissue. In gastric samples, some with intestinal metaplasia, the levels of PEPT1 and CDX2 mRNA were highly correlated (Fig. 2A). These findings collectively suggest that Cdx2 plays a key role in the transcriptional regulation of the intestine-specific expression of PEPT1 through interaction with Sp1 [36] (Fig. 2B).

Starvation markedly increased the amount of mRNA and protein of PEPT1 in rats, leading to altered pharmacokinetics of the PEPT1 substrates [23,25], but the mechanisms underlying this augmentation have not been clarified. We recently examined the role of peroxisome proliferator-activated receptor α (PPARα) in the augmentation of PEPT1 expression by fasting [37], because PPARα plays a pivotal role in the adaptive response to fasting in the liver and other tissues. In 48-h fasted rats, the expression level of PPARα mRNA in the small intestine markedly increased, accompanied by an elevation in serum level of free fatty acids, which are endogenous PPARα ligands. Oral administration of a synthetic

PPARα ligand WY-14643 to fed rats increased the mRNA level of intestinal PEPT1. In the small intestine of PPARα null mice, augmentation of PEPT1 mRNA expression during fasting was completely abolished. To test the possibility that PPARα directly regulates the human PEPT1 promoter, we searched for the potential PPAR responsive element (PPRE) in the promoter region up to 10 kb upstream of the transcription start site and found several candidates. However, none of these sites enhanced basal promoter activity in response to WY-14643 treatment in Caco-2 cells. The functional PPRE and/or some other regulatory region related to PPARα may be located in more distal regions or intronic regions. Taken together, these results indicate that PPARα plays critical roles in fasting-induced intestinal PEPT1 expression [37].

## 2.2. PEPT2 (SLC15A2)

### 2.2.1. *General function and pharmacokinetic roles*

PEPT2 (SLC15A2), mainly expressed in the kidney, shows about 50% amino acid identity with PEPT1, and has higher affinity for substrates than PEPT1 [8]. PEPT2 is also expressed in a variety of tissues such as lung, mammary gland and choroids plexus [8], and recent study revealed that PEPT2 function as an uptake system for peptides fragments from neuropeptide metabolism in the enteric nervous system [38]. This transporter mediates the renal reabsorption of di- and tripeptides and peptide-like drugs through glomerular filtration. Pept2$^{-/-}$ mice were viable and without obvious abnormalities of the kidney [39,40], and detailed *in vivo* analyses using these mice demonstrated that Pept2 is the predominant oligopeptide transporter in the kidney [41]. When Pept2$^{-/-}$ mice were fed diets of different protein contents, Pept2$^{-/-}$ mice adapted food intake to dietary protein content with higher consumption rates on low protein and reduced food intake rates on the high-protein diet [42].

### 2.2.2. *Regulation of PEPT2*

Little data is available on the regulation of PEPT2 compared with PEPT1. Using a cell culture model, it was found that Pept2 was regulated by intracellular Ca [43] and epidermal growth factor [44]. Hypothyroidism [45] and thyroidectomy [46] of rats resulted in an increased level of renal PEPT2 expression, suggesting that amino acid homeostasis and drug pharmacokinetics are regulated in states of altered thyroid function. Using 5/6 nephrectomized rats, we found that the renal expression of Pept2, not Pept1, was selectively up-regulated 2 weeks after surgery [47], but levels of both Pept1 and Pept2 were markedly decreased 16 weeks post-surgery [48]. As described above, rat intestinal Pept1 expression showed a

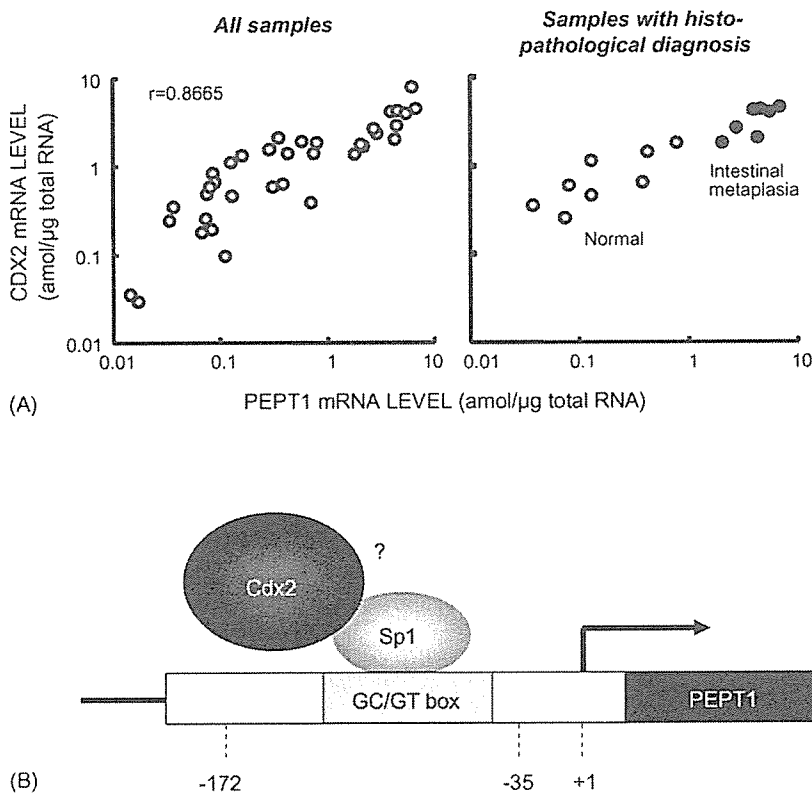


**Fig. 2 – Transcriptional regulation of intestinal PEPT1. (A) Correlation between PEPT1 and CDX2 mRNA levels in human gastric tissue samples. The mRNA levels of PEPT1 and CDX2 were quantified with the real-time PCR method in the gastric mucosal samples. Some of these tissue samples were examined by a pathologist who made a diagnosis of intestinal metaplasia. All samples were plotted (left panel). The samples examined by the pathologist were plotted (right panel). Open and closed symbols indicate the normal samples and the samples proved to be case of intestinal metaplasia, respectively [36]. (B) PEPT1 promoter activity is regulated by Cdx2 and Sp1. A reporter assay, immunoprecipitation assay, and chromatin immunoprecipitation assay demonstrated that Cdx2 plays a key role in the transcriptional regulation of the intestine-specific expression of PEPT1 through interaction with Sp1 [35,36].**

diurnal rhythm, but renal Pept2 and renal Pept1 expression did not [22]. These findings suggest that the regulatory mechanisms for peptide transporters differ between isoforms and tissues (intestine and kidney).

#### 2.2.3. *Transcriptional regulatory mechanisms of PEPT2*

There is little information available about the transcriptional regulatory mechanisms of PEPT2 except for one report [49]. The mouse *Pept2* gene possesses a TATA-less promoter, and its core promoter was located between 432 and 286 bp upstream from the translation start site.

### 2.3. *Organic ion transporters (OCT/OCTN/OAT)*

#### 2.3.1. *General function and pharmacokinetic roles*

The organic ion transporter family (SLC22A) consists of organic cation transporters (OCTs), zwitterion/cation transporters (OCTNs) and organic anion transporters (OATs) [50]. This family plays important roles in the renal secretion and hepatic uptake of various compounds including drugs, toxins and endogenous metabolites (OCTs and OATs) [51,52], and in the intestinal and renal absorption of carnitine (OCTN2) [53]. Mutations of transporters for the SLC22 family are responsible for specific diseases such as "primary systemic carnitine deficiency" (OCTN2) [54], and are linked with rheumatoid arthritis (OCTN1) [55] and Crohn's disease (OCTN2) [56].

#### 2.3.2. *Regulation of organic ion transporters*

Not only the functional characteristics but also the regulational aspects of OCTs have been investigated. Ciarimboli and Schlatter [57] recently summarized how the transport of organic cations is regulated in the short and long term. The short-term regulation is mainly caused by the phosphorylation/dephosphorylation of OCT proteins, which induced a change in substrate affinity or membrane trafficking [57]. In the long-term, the regulation of development [58], hormones [59], chronic renal failure [60], and diabetes [61] were demonstrated to affect the expression of Octs in the kidney. The sex hormonal regulation of rat Oct2 in the kidney has been extensively investigated. Namely, the expression level of rat Oct2, but not Oct1 or Oct3, in the kidney was much higher in males than females [62]. The treatment of male and female rats with testosterone significantly increased rat Oct2 expression in the kidney [59,60], suggesting that testosterone plays a pivotal role in the transcriptional regulation of the rat *Oct2* gene. This gender difference in renal Oct2 expression was also observed in rabbits [63] and mice [64], but not clear in humans. In the liver, it was reported that rat hepatic Oct1, not renal Oct1, is down-regulated by obstructive cholestasis [65]. Mouse hepatic Oct1 is demonstrated to be regulated by peroxisome proliferator agonist receptor-$\alpha$ and -$\gamma$ [66].

The regulation of OATs, in addition to OCTs, has been well studied. Terlouw et al. [67] have summarized the regulation of renal organic anion transporters under various conditions. Using isolated proximal tubules of rabbit kidney and renal cultured cell lines such as OK, the regulation of Oats by various protein kinases and the signal molecules have been investigated, mainly focusing on the short-term regulation [68–70]. It was demonstrated that Oat1 and/or Oat3 in the rat kidney were regulated by development [58], hyperuricemia [71], bilateral ureteral obstruction [72] and acute biliary obstruction [73]. Human OAT1 is also down-regulated by renal failure [74].

#### 2.3.3. *Transcriptional regulatory mechanisms of organic ion transporters*

As described above, there are various reports about the regulation of OCTs and OATs, but most reports do not address the molecular mechanisms behind the alteration of mRNA expression. To understand the role of testosterone in the gender differences in the expression of renal Oct2 in rats, we performed functional reporter analyses of rat *Oct1–3* genes coexpressed with the androgen receptor [75]. It was found that a physiological concentration of testosterone (~10 nM) specifically enhanced transcription of the *Oct2* gene, but not of the *Oct1* or *Oct3* gene, and that androgen response element (ARE)-1 (−2975 to −2960) and ARE-3 (−1340 to −1325) in the rat Oct2 promoter region would play important roles in the enhanced transcription of *Oct2*.

Among the OCT and OAT families, OAT3 showed the most abundant expression in the human kidney [76], and plays important roles in the renal secretion of anionic cephalosporins [74,77,78]. Recently, Kikuchi et al. [79] and we [80] characterized the basal transcriptional activity of the human *OAT3* gene. The minimal promoter region of human *OAT3* was identified to be located approximately 300 bp upstream of the transcription start site, where there are a canonical TATA box (−32 to −27), a hepatocyte nuclear factor-1 (HNF-1)-binding site (−65 to −53), and a cAMP responsive element (CRE) (−87 to −80). Kikuchi et al. [79] demonstrated that HNF-1$\alpha$/1$\beta$ were involved in the basal expression of human OAT3 through the interaction of the HNF-1-binding site using intestinal Caco-2 and hepatic HepG2 cells. On the other hand, using the renal proximal tubular cell line OK, we demonstrated that CRE-binding protein (CREB)-1 and activating transcription factor (ATF)-1 were responsible for basal promoter activity by binding to CRE [80]. Furthermore, the activity of the OAT3 promoter was increased through the phosphorylation of CREB-1 and ATF-1 by treatment with 8-bromoadenosine 3′,5′-cyclic monophosphate, a protein kinase A (PKA) activator [80]. These findings indicate that HNF-1$\alpha$/1$\beta$, CREB-1, and ATF-1 function as constitutive regulators of the human OAT3 gene, and that PKA further stimulated OAT3 gene expression by the phosphorylation of CREB-1 and ATF-1 (Fig. 3).

In contrast to OAT3, other SLC22A members such as OCT1 and OAT2 were primarily expressed in the liver. Although the basal transcriptional mechanism of neither transporter has been clarified, liver-specific transcriptional mechanisms were recently demonstrated. Namely, the human *OCT1* [81] and human *OAT2* [82] genes in the liver are activated by a liver-enriched homodimeric nuclear receptor, hepatocyte nuclear factor-4$\alpha$ (HNF-4$\alpha$) and suppressed by a bile acid-inducible transcriptional repressor, a small heterodimer partner. The hepatic uptake of OCT1 and OAT2 substrates may be decreased in individuals with diseases associated with elevated intracellular levels of bile acids.

Pharmacogenomic analyses have also found the transcription factors involved in the gene expression of OCTN1 and OCTN2. Namely, single nucleotide polymorphisms (SNP)s in the promoter region (regulatory SNPs; rSNPs) of both transporters are suggested to be associated with chronic inflam-

matory diseases, and promoter regions including rSNPs were demonstrated to be functional binding sites of transcription factors, RUNX1 for OCTN1 [55] and heat-shock transcription factor for OCTN2 [56].

## 3. Expression profile of human intestinal and renal drug transporters

The development of quantitative real-time PCR techniques has meant that expression levels of drug transporters can be quantitatively determined using a very small amount of tissue sample. Recently, based on these techniques, expression profiles of various genes including those for human drug transporters have been determined using surplus tissue specimens collected during surgery or biopsy.

### 3.1. *Expression profile of intestinal drug transporters*

For SLC drug transporters, the expression profiles of PEPT1 and organic ion transporters along the human digestive tract were determined using normal portions of mucosal samples from cancer patients treated surgically [83]. PEPT1 mRNA was highly expressed in the small intestine (duodenum > jejunum > ileum) compared to other tissues, and some patients showed a significant level of expression in the stomach. The expressional pattern of PEPT1 in the stomach and histological diagnosis indicated that gastric PEPT1 originated from the intestinal metaplasia. This ectopic expression of PEPT1 is induced by the intestine-specific transcription factor CDX2 as described in Section 2.1.3. There was little expression of organic ion transporters except for OCTN2, which showed similar levels to PEPT1 [83]. Using Oct1 knockout mice, it was demonstrated that Oct1 plays important roles in the intestinal excretion of cationic drugs [84]. However, in the human intestine, there is little expression of OCT1.

Taipalensuu et al. [85] determined mRNA levels of 10 ABC drug transporters in the human jejunum, and found that multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) are more extensively expressed than multidrug resistance protein 1 (MDR1). In contrast, Zimmermann et al. [86] demonstrated that MRP3 among MRP1-5 and MDR1 was the most abundantly expressed in the duodenum and all segments of the colon, and that MDR1 showed the highest level of expression in the terminal ileum. The differences between these two reports may be due to the segment of the human intestine sampled. For the distribution of ABC transporters in the intestine, it was reported that MDR1 mRNA [83] and Pgp [87] levels gradually increased from the duodenum to ileum. MRP2 showed significant expression in the small intestine but hardly any expression in colonic segments [86]. BCRP mRNA expression was maximal in the duodenum and decreased continuously down to the rectum [88]. This expression profile contrasts to that of MDR1 mRNA, suggesting that BCRP and MDR1 complement the transport function of each other along the digestive tract as the substrate specificity of the two transporters overlaps.

### 3.2. *Expression profile of renal drug transporters*

We also reported expression levels of organic ion transporters in normal sections of renal tissue obtained from seven surgically nephrectomized patients with renal cell carcinoma

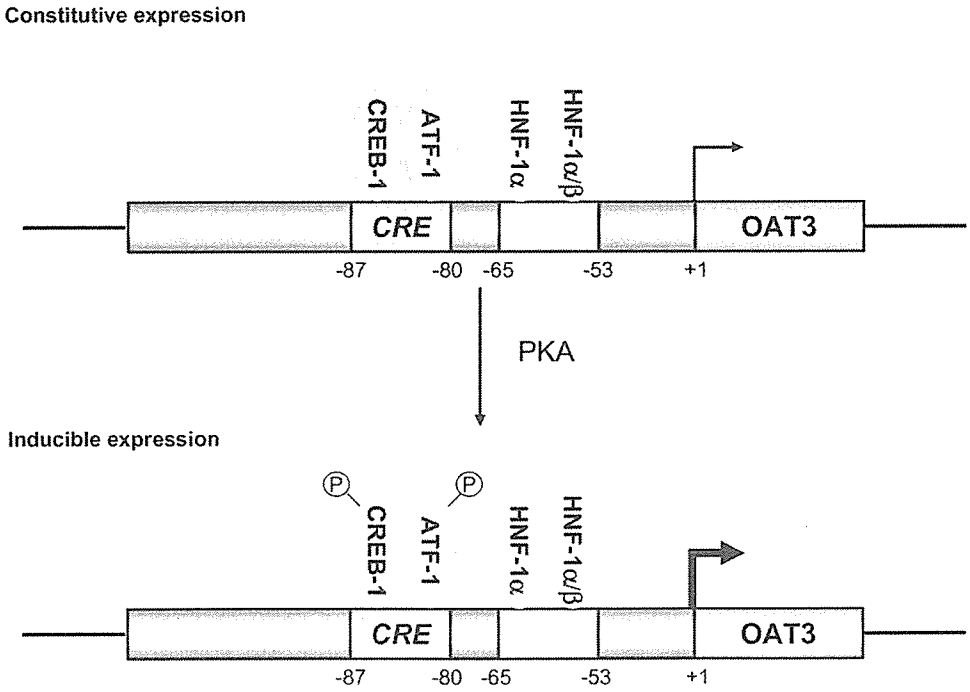


**Fig. 3 – Schematic model of transcriptional regulation of the human OAT3 gene. CREB-1 and ATF-1 bind to CRE, and HNF1α/β bind to the HNF1-binding site, and these transcription factors activate the transcription of the OAT3 gene (constitutive expression) [79,80]. PKA further stimulates the transcriptional activity of the OAT3 gene through phosphorylation of both CREB-1 and ATF-1 (inducible expression) [80].**

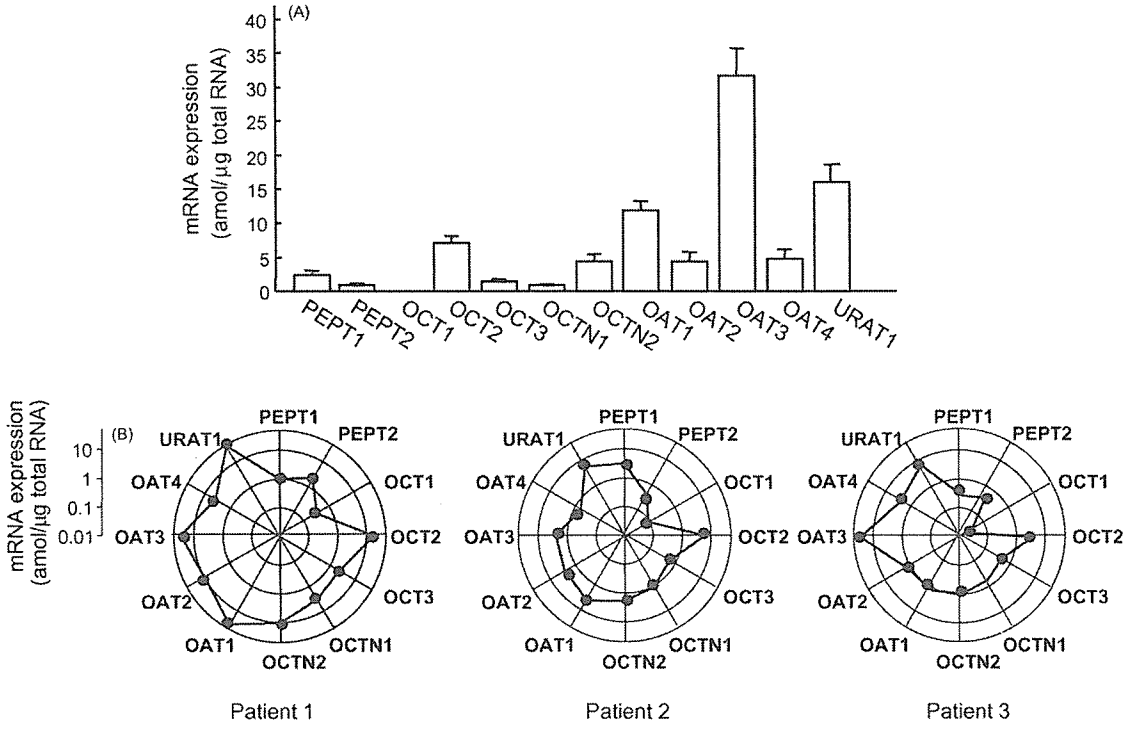


**Fig. 4 – Expression profiles of organic ion transporters and peptide transporters in the human kidney cortex. (A) Average expression levels. Each transporter's expression level was determined by the real-time PCR method using a portion of normal human kidney cortex from nephrectomized patients (*N* = 82). (B) Individual patients' expression profiles (typical patients). Patient 1 shows high levels of OAT1-4 and URAT1, whereas patient 2 exhibits low levels of these transporters, although the expression level of OCT2 was comparable between the two. Patient 3 shows a high level of OAT3, but low levels of other transporters.**

in 2002 [76]. Since then, the number of patients has increased to 82, and PEPT1, PEPT2, and an urate transporter (URAT1) have been included in current analyses (Fig. 4A). The mRNA expression level of OAT3 was the highest among the OAT family, followed by that of URAT1, OAT1. The OCT2 mRNA level was the highest in the OCT family, and OCT1 mRNA was rarely expressed. Among the OCTN family, OCTN2 showed the highest expression, but its level was lower than those of OAT1, OAT3, and OCT2. The expression levels of PEPT1 and PEPT2 were not so high as compared to levels of organic ion transporters, and PEPT1 expression was higher than PEPT2 expression. Fig. 4B shows the expression profile of drug transporters for typical individual patients. The expression pattern of drug transporters varied among patients. For example, patient 1 showed high levels of OAT1-4 and URAT1, whereas patient 2 exhibited low levels of these transporters. Regarding the expression of peptide transporters, PEPT1 < PEPT2 for patient 1, PEPT1 > PEPT2 for patient 2, and PEPT1 ≈ PEPT2 for patient 3. Patient 3 showed a high level of OAT3, but low expression levels of other transporters.

## 4. Conclusions and future perspectives

In these research updates, we addressed recent advances in the study of the gene regulation and expression of drug transporters in the intestine and kidney. Among drug transporters, MDR1 has been well studied in terms of its gene regulation, and many transcription factors for *MDR1* gene have been identified [3,4]. On the other hand, the history of gene regulation for intestinal and renal drug transporters is very short. For example, the transcription factors Sp1, Cdx2 and PPARα were just proven to be responsible for the expression and regulation of intestinal PEPT1, but other transcriptional regulatory mechanisms triggered by various stimuli have not been clarified yet. Information about transcription factors specific to the kidney, especially the proximal tubular cells, is also limited, perhaps due to the absence of an appropriate model of human renal proximal tubular cell lines. Some drug transporters show species differences in renal expression. For example, rat Oct1, but not human OCT1, is expressed in the kidney. The identification of kidney-specific transcription factors should help us to understand not only the molecular mechanisms of such species differences but also the gene regulation of renal drug transporters in various physiological and pathophysiological conditions.

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# Oppositely directed $H^+$ gradient functions as a driving force of rat $H^+$/organic cation antiporter MATE1

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**Tsuda M, Terada T, Asaka J-i, Ueba M, Katsura T, Inui K-i.** Oppositely directed $H^+$ gradient functions as a driving force of rat $H^+$/organic cation antiporter MATE1. *Am J Physiol Renal Physiol* 292: F593–F598, 2007. First published October 17, 2006; doi:10.1152/ajprenal.00312.2006.—Recently, we have isolated the rat (r) $H^+$/organic cation antiporter multidrug and toxin extrusion 1 (MATE1) and reported its tissue distribution and transport characteristics. Functional characterization suggested that an oppositely directed $H^+$ gradient serves as a driving force for the transport of a prototypical organic cation, tetraethylammonium, by MATE1, but there is no direct evidence to prove this. In the present study, therefore, we elucidated the driving force of tetraethylammonium transport via rMATE1 using plasma membrane vesicles isolated from HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells). A 70-kDa rMATE1 protein was confirmed to exist in HEK-rMATE1 cells, and the transport of various organic cations including [$^{14}$C]tetraethylammonium was stimulated in intracellular acidified HEK-rMATE1 cells but not mock cells. The transport of [$^{14}$C]tetraethylammonium in membrane vesicles from HEK-rMATE1 cells exhibited the overshoot phenomenon only when there was an outwardly directed $H^+$ gradient, as observed in rat renal brush-border membrane vesicles. The overshoot phenomenon was not observed in the vesicles from mock cells. The stimulated [$^{14}$C]tetraethylammonium uptake by an $H^+$ gradient [intravesicular $H^+$ concentration ($[H^+]_{in}$) > extravesicular $H^+$ concentration ($[H^+]_{out}$)] was significantly reduced in the presence of a protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). [$^{14}$C]tetraethylammonium uptake was not changed in the presence of valinomycin-induced membrane potential. These findings definitively indicate that an oppositely directed $H^+$ gradient serves as a driving force of tetraethylammonium transport via rMATE1, and this is the first demonstration to identify the driving force of the MATE family. The present experimental strategy is very useful in identifying the driving force of cloned transporters whose driving force has not been evaluated.

multidrug and toxin extrusion 1; transporter; tetraethylammonium; renal secretion; membrane vesicles

THE SECRETION OF DRUGS AND xenobiotics is an important physiological function of the renal proximal tubules. Cationic drugs are secreted from blood to urine by cooperative functions of two distinct classes of organic cation transporters: one driven by the transmembrane potential difference in the basolateral membranes and the other driven by the transmembrane $H^+$ gradient in the brush-border membranes (7, 16). So far, several membrane potential-dependent organic cation transporters (OCT1–3) have been identified, and their physiological and pharmacokinetic roles have been evaluated (2, 5, 10). However, the molecular nature of $H^+$/organic cation antiport systems has remained to be elucidated.

Recently, Moriyama and co-workers (3, 15) have identified human (h) and mouse MATE1 and MATE2, which are orthologs of the multidrug and toxin extrusion (MATE) family of bacteria. They demonstrated that MATE1 was predominantly expressed at the luminal membranes of the urinary tubules and bile canaliculi and transported tetraethylammonium, a prototypical organic cation, in a pH-dependent manner (3, 15). We also isolated cDNAs for rat (r) MATE1 (20) and the human kidney-specific isoform MATE2-K (13). rMATE1 was significantly expressed in the kidney and placenta, but not in the liver, and real-time PCR analyses of microdissected nephron segments showed that rMATE1 was expressed in the proximal convoluted and straight tubules (20). On the other hand, hMATE2-K was only expressed in the kidney and was located at the brush-border membranes of renal proximal tubular cells (13). By conducting functional analyses, we showed that rMATE1 and hMATE2-K can transport a wide variety of organic cations including tetraethylammonium, $N^1$-methylnicotinamide, and metformin (13, 20). These characteristics of MATE1 are similar to those of the $H^+$/organic cation antiport system revealed by renal brush-border membrane vesicle studies (4, 14, 18, 19, 23).

MATE1 exhibited pH-dependent transport of tetraethylammonium in cellular uptake and efflux studies, and intracellular acidification by $NH_4Cl$ pretreatment stimulated tetraethylammonium transport (3, 13, 15, 20), suggesting that MATE1 utilized an oppositely directed $H^+$ gradient as a driving force. However, these analyses are not enough to prove the $H^+$/tetraethylammonium antiport mechanism of MATE1, because it is possible that the pH-dependent transport of tetraethylammonium by MATE1 is regulated not by an $H^+$ gradient but by pH itself. Accordingly, in addition to the data obtained using the cell culture model, we need more direct evidence that an $H^+$ gradient is the driving force for MATE1.

In the present study, we developed HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells) and elucidated the driving force of rMATE1 by uptake studies using plasma membrane vesicles from HEK-rMATE1 cells for the first time.

## MATERIALS AND METHODS

*Materials.* [$^{14}$C]levofloxacin (1.07 GBq/mmol) was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan). [$^{14}$C]tetraethylammonium bromide (2.035 GBq/mmol), [$^{14}$C]creatinine (2.035 GBq/mmol), [$^{14}$C]procainamide (2.035 GBq/mmol), [$^3$H]quinidine (740 GBq/mmol), [$^3$H]quinine (740 GBq/mmol), L-[*N*-methyl-$^3$H]carnitine (3.145 TBq/mmol), and [*N*-methyl-$^{14}$C]nicotine (2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals (St. Louis,

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MO). [$^{14}$C]metformin (962 MBq/mmol), [$^{14}$C]guanidine hydrochloride (1.961 GBq/mmol), [8-$^{3}$H]acyclovir (110 GBq/mmol), and [8-$^{3}$H]ganciclovir (370 GBq/mmol) were purchased from Moravec Biochemicals (Brea, CA). [$^{3}$H]1-methyl-4-phenylpyridinium acetate (2.7 TBq/mmol), [$^{3}$H]estrone sulfate ammonium salt (2.1 TBq/mmol), and [$^{14}$C]*p*-aminohippurate (1.9 GBq/mmol) were purchased PerkinElmer Life Analytical Sciences (Boton, MA). [*N*-methyl-$^{3}$H]cimetidine (451 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). All other chemicals used were of the highest purity available.

*Cell culture and transfection.* HEK293 cells (American Type Culture Collection CRL-1573) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% $CO_2$-95% air at 37°C. pcDNA 3.1 (+) containing cDNA encoding rMATE1 or empty vector was transfected into HEK293 cells using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, the cells were split in complete medium containing G418 (0.5 mg/ml, Nacalai Tesque, Kyoto, Japan) at a dilution of 1:200. Fifteen days after transfection, single colonies were picked out. Cells expressing rMATE1 (HEK-rMATE1 cells) were selected by measuring [$^{14}$C]tetraethylammonium uptake. Cells transfected with empty vector (HEK-pcDNA cells) were used as controls. These transfectants were maintained in complete medium with G418 (0.5 mg/ml).

*Uptake experiments by HEK-rMATE1 cells.* The cellular uptake of [$^{14}$C]tetraethylammonium was measured by using monolayers grown on poly-D-lysine-coated 24-well plates as reported previously with some modifications (13, 20, 22). Briefly, the cells were preincubated with 0.2 ml of incubation medium, pH 7.4 (in mM: 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES) containing 30 mM $NH_4Cl$ for 20 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium (pH 7.4) containing each radiolabeled compound was added. After an appropriate period of incubation, the medium was aspirated, and the monolayers were rapidly washed twice with 1 ml of ice-cold incubation medium (pH 7.4). The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford (1) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as a standard.

*Preparation of membrane vesicles from HEK-rMATE1 cells.* Plasma membrane vesicles were prepared according to previous reports (6, 9). HEK-rMATE1 or HEK-pcDNA cells were seeded on 100-mm plastic dishes ($4 \times 10^6$ cells/dish), and 20 or 40 dishes were used to prepare membrane vesicles in a single preparation. All procedures were performed at 4°C. At the third day after seeding, HEK-rMATE1 or HEK-pcDNA cells were washed with PBS and scraped with a rubber policeman into PBS. The cell suspension was centrifuged at 200 *g* for 10 min, suspended in 20 ml of PBS, and recentrifuged at 200 *g* for 10 min. The packed cell pellet was resuspended in 20 vol of 250 mM mannitol/10 mM HEPES-Tris (pH 7.5)/0.5 mM $MgCl_2$ (*buffer A*), and the cells were gently suspended with five strokes of a loose-fitting Dounce homogenizer. The washed cell suspension was placed in a nitrogen cavitation bomb (Parr Instrument) at 700 lb/in.$^2$ for 15 min. After the homogenate was collected, $K_2EDTA$ (pH 7.5) was added to a final concentration of 1 mM. The homogenate was centrifuged at 750 *g* for 15 min, and the supernatant was centrifuged at 20,000 *g* for 15 min. The supernatant was centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in 100 mM mannitol/10 mM MES-KOH (pH 6.0; experimental buffer) or 100 mM mannitol/10 mM HEPES-KOH (pH 7.5; experimental buffer) and centrifuged again at 100,000 *g* for 60 min. The pellet was suspended in the same experimental buffer (pH 6.0 or 7.5) by sucking the suspension 10 times through a fine needle (~4–10 mg protein/ml). KCl (pH 6.0 or 7.5) was added to a final concentration of 100 mM.

*Transport experiments by membrane vesicles.* The uptake of [$^{14}$C]tetraethylammonium by membrane vesicles was measured by a rapid filtration technique with a slight modification (8, 19). In the regular assays, the reaction was initiated rapidly by adding 80 μl of buffer, containing 31.25 μM [$^{14}$C]tetraethylammonium, to 20 μl of membrane vesicle suspension at 25°C. After specified periods, the incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold stop solution containing (in mM) 150 KCl, 20 HEPES-Tris (pH 7.5), 0.1 $HgCl_2$, and 1 tetraethylammonium. The mixture was poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5 cm in diameter), and the filters were washed with 5 ml of ice-cold stop solution. The radioactivity of [$^{14}$C]tetraethylammonium trapped in membrane vesicles was determined using an ACS II (Amersham Biosciences) by liquid scintillation counting. The protein content was determined by the method of Bradford (1) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with bovine γ-globulin as a standard.

*Western blot analysis.* Polyclonal antibody was raised against a synthetic peptide corresponding to the intracellular domain of rMATE1 (CQQAQVHANLKVN, no. 465–477) (13). Brush-border membrane vesicles from rat kidney cortex were prepared as described previously (12). Membrane fractions were separated by SDS-PAGE and analyzed by Western blotting as described previously (17, 21).

*Data analysis.* Data were analyzed statistically with a one-way analysis of variance followed by Scheffé's test and are expressed as means ± SE.

## RESULTS

*Generation of HEK-rMATE1 cells.* First, we generated and characterized HEK293 cells stably expressing rMATE1. As shown in Fig. 1, an immunoreactive protein with a molecular weight of ~70 kDa was detected in HEK-rMATE1 cells and rat renal brush-border membranes but not in HEK-pcDNA cells. The functional expression of rMATE1 was assessed by measuring the

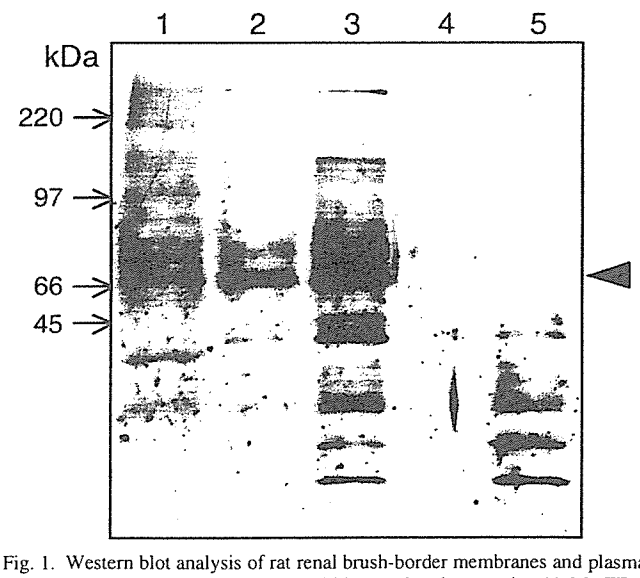


Fig. 1. Western blot analysis of rat renal brush-border membranes and plasma membranes obtained from HEK-rat multidrug and toxin extrusion 1( rMATE1) and HEK-pcDNA cells. Renal brush-border membranes (20 μg) and plasma membranes (5 or 20 μg) obtained from HEK-rMATE1 and HEK-pcDNA cells were separated by SDS-PAGE (10%) and blotted onto polyvinylidene difluoride membranes. The antiserum for rMATE1 (1:1,000) was used as a primary antibody. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies, and the strips of blots were visualized by chemiluminescence on X-ray film. The arrowhead indicates the position of rMATE1. Lanes were as follows: *lane 1*, rat renal brush-border membranes; *lane 2*, HEK-rMATE1 (5 μg); *lane 3*, HEK-rMATE1 (20 μg); *lane 4*, HEK-pcDNA (5 μg); and *lane 5*, HEK-pcDNA (20 μg).

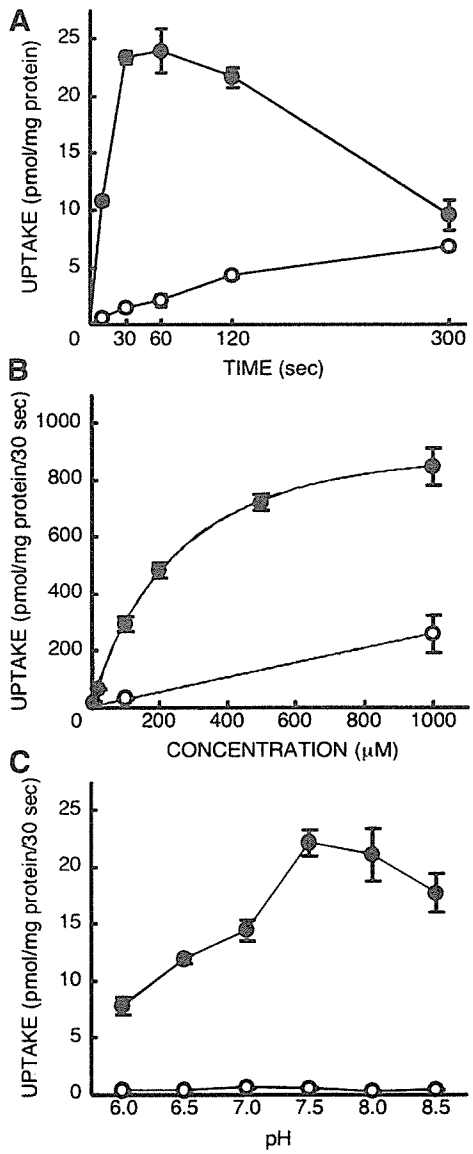


Fig. 2. Transport of [$^{14}$C]tetraethylammonium (TEA) by HEK-rMATE1 cells. *A*: time course of [$^{14}$C]TEA uptake by HEK-rMATE1 and HEK-pcDNA cells. HEK-rMATE1 cells (●) and HEK-pcDNA cells (○) were preincubated with 30 mM $NH_4Cl$ (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [$^{14}$C]TEA (pH 7.4) for indicated time at 37°C. Each point represents the mean ± SE of 3 monolayers. This figure is representative of 3 separate experiments. *B*: concentration dependence of [$^{14}$C]TEA uptake by HEK-rMATE1 cells. HEK-rMATE1 cells were preincubated with 30 mM $NH_4Cl$ (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with various concentration of [$^{14}$C]TEA (pH 7.4) in the absence (●) or presence (○) of 5 mM TEA for 30 s at 37°C. Each point represents the mean ± SE of 3 monolayers. *C*: effect of extracellular pH on [$^{14}$C]TEA uptake by HEK-rMATE1 and HEK-pcDNA cells. HEK-rMATE1 cells (●) and HEK-pcDNA cells (○) were preincubated with 30 mM $NH_4Cl$ (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [$^{14}$C]TEA (indicated pH) for 30 s at 37°C. Each point represents the mean ± SE of 3 monolayers. The figure is representative of 2 separate experiments.

uptake of [$^{14}$C]tetraethylammonium in the HEK-rMATE1 cells under the intracellular acidified conditions caused by $NH_4Cl$ pretreatment. A time- and concentration-dependent uptake of [$^{14}$C]tetraethylammonium by HEK-rMATE1 cells was observed (Fig. 2, *A* and *B*). [$^{14}$C]tetraethylammonium uptake by HEK-rMATE1 cells exhibited saturable kinetics, and an apparent $K_m$ value of 304 ± 80 μM was calculated from three separate experiments. When the extracellular pH was changed from 6.0 to 8.5, a bell-shaped pH profile of [$^{14}$C]tetraethylammonium uptake via rMATE1 was observed, and the uptake was greatest at pH 7.5 and lowest at pH 6.0 (Fig. 2*C*).

*Uptake of various compounds by HEK-rMATE1 cells.* We then examined the substrate specificity of rMATE1. As shown in Fig. 3, rMATE1 mediated the transport of various organic cations with different chemical structures such as [$^{14}$C]tetraethylammonium, [$^{3}$H]1-methyl-4-phenylpyridinium acetate, [$^{3}$H]cimetidine, and [$^{14}$C]metformin. The transport of other organic cations such as [$^{14}$C]procainamide, [$^{14}$C]creatinine, and [$^{14}$C]guanidine was greater in HEK-rMATE1 cells than in HEK-pcDNA cells, although the stimulation was not remarkable.

*Characteristics of [$^{14}$C]tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells.* Next, we performed transport experiments using plasma membrane vesicles isolated from HEK-rMATE1 cells and HEK-pcDNA cells. In the presence of an $H^+$ gradient [intravesicular $H^+$ concentration ($[H^+]_{in}$) > extravesicular $H^+$ concentration ($[H^+]_{out}$)], a marked

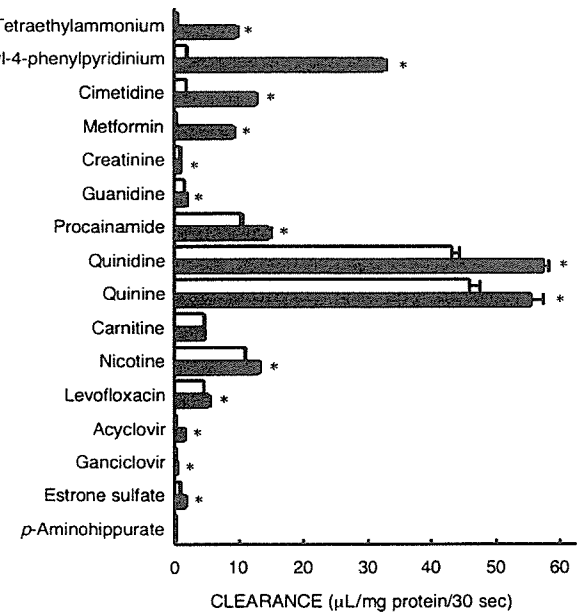


Fig. 3. Uptake of various compounds by HEK-rMATE1 cells. HEK-pcDNA cells (open bars) and HEK-rMATE1 cells (filled bars) were preincubated with 30 mM $NH_4Cl$ (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with [$^{14}$C]TEA (5 μM), [$^{3}$H]1-methyl-4-phenylpyridinium acetate (3.8 nM), [$^{3}$H]cimetidine (11.1 nM), [$^{14}$C]metformin (10 μM), [$^{14}$C]creatinine (5 μM), [$^{14}$C]guanidine hydrochloride (5 μM), [$^{14}$C]procainamide (5 μM), [$^{3}$H]quinidine (13.9 nM), [$^{3}$H]quinine (13.9 nM), [$^{3}$H]carnitine (3.3 nM), [$^{14}$C]nicotine (5 μM), [$^{14}$C]levofloxacin (14 μM), [$^{3}$H]acyclovir (92 nM), [$^{3}$H]ganciclovir (28 nM), [$^{3}$H]estrone sulfate (4.86 nM), or [$^{14}$C]*p*-aminohippurate (5 μM) for 30 s at 37°C. Each bar represents the mean ± SE of 3 monolayers. The figure is representative of 2 separate experiments. *$P < 0.05$ significantly different from HEK-pcDNA cells.

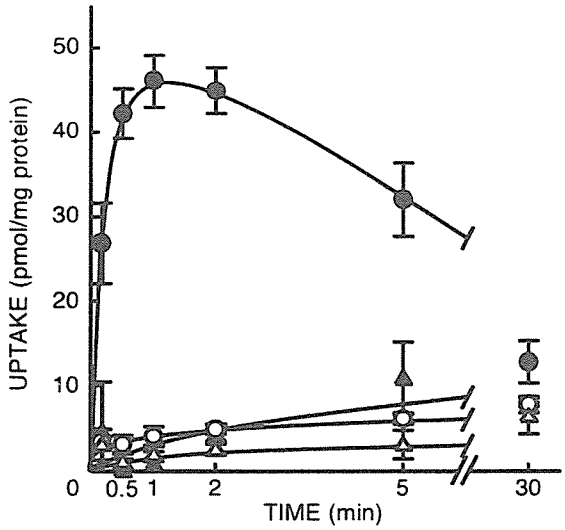


Fig. 4. Time course of [$^{14}$C]TEA uptake by membrane vesicles from HEK-pcDNA and HEK-rMATE1 cells. The uptake of [$^{14}$C]TEA by membrane vesicles from HEK-pcDNA cells (○, △) and HEK-rMATE1 cells (●, ▲) was examined in the absence (○, ●) or presence (△, ▲) of 10 mM TEA. Membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [$^{14}$C]TEA was examined in the experimental buffer containing 31.25 μM [$^{14}$C]TEA and 100 mM KCl at pH 7.5 in the absence or presence of 10 mM TEA. Each point represents the mean ± SE of 3 determinations.

stimulation of [$^{14}$C]tetraethylammonium uptake (overshoot phenomenon) was observed in membrane vesicles from HEK-rMATE1 cells, but not in those from HEK-pcDNA cells (Fig. 4). The overshoot phenomenon disappeared in the presence of an excess of cold tetraethylammonium.

*Driving force for [$^{14}$C]tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells.* To elucidate the driving force of tetraethylammonium transport by rMATE1, we performed [$^{14}$C]tetraethylammonium transport experiments using membrane vesicles from HEK-rMATE1 cells. As shown in Fig. 5, the presence of an $H^+$ gradient ($[H^+]_{in} > [H^+]_{out}$) induced a marked stimulation of [$^{14}$C]tetraethylammonium uptake against the concentration gradient. On the other hand, no stimulation of [$^{14}$C]tetraethylammonium uptake was observed in the absence of the gradient or in the presence of the reverse gradient ($[H^+]_{in} < [H^+]_{out}$). The final amount of [$^{14}$C]tetraethylammonium taken up in the presence of the $H^+$ gradient ($[H^+]_{in} > [H^+]_{out}$) was not so different from that attained in the absence of the gradient or in the presence of the reverse gradient ($[H^+]_{in} < [H^+]_{out}$).

To further evaluate the effect of an outwardly directed $H^+$ gradient on [$^{14}$C]tetraethylammonium uptake, the influence of a protonophore, FCCP, was examined. As shown in Fig. 6*A*, the initial rate of [$^{14}$C]tetraethylammonium uptake in the presence of an $H^+$ gradient ($[H^+]_{in} > [H^+]_{out}$) was markedly

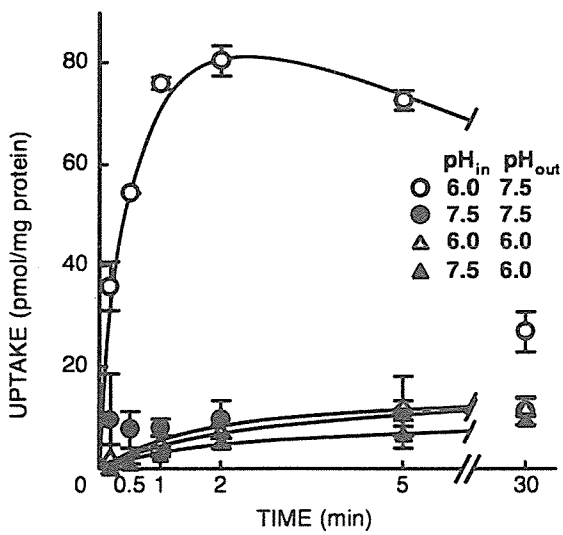


Fig. 5. Effect of $H^+$ gradient on [$^{14}$C]TEA uptake by membrane vesicles from HEK-rMATE1 cells. Membrane vesicles were prepared in the experimental buffer at pH 6.0 (○, △) or 7.5 (●, ▲). The uptake of [$^{14}$C]TEA was examined in the experimental buffer containing 31.25 μM [$^{14}$C]TEA and 100 mM KCl at pH 6.0 (△, ▲) or 7.5 (○, ●). Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments. $pH_{in}$, intravesicular pH; $pH_{out}$, extravesicular pH.

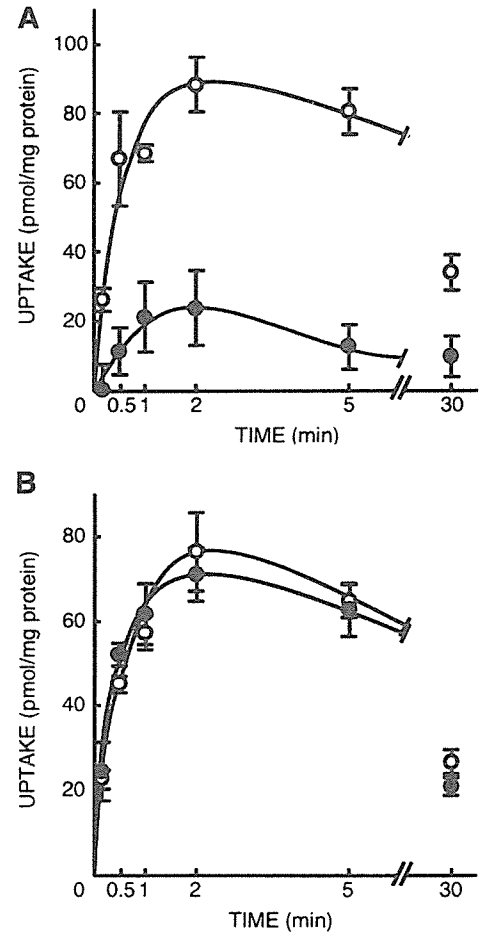


Fig. 6. Effect of FCCP (*A*) and valinomycin (*B*) on [$^{14}$C]TEA uptake in the presence of an outwardly directed $H^+$ gradient by membrane vesicles from HEK-rMATE1 cells. *A*: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [$^{14}$C]TEA was examined in the experimental buffer containing 31.25 μM [$^{14}$C]TEA and 100 mM KCl at pH 7.5 in the absence (○) or presence (●) of 40 μM FCCP. Each point represents the mean ± SE of 3 determinations. The figure is a representative of 2 separate experiments. *B*: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [$^{14}$C]TEA was examined in the experimental buffer containing 31.25 μM [$^{14}$C]TEA and 100 mM CsCl at pH 7.5 in the absence (○) or presence (●) of 8 μM valinomycin. Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments.

reduced by FCCP, although the values at 30 min were similar in the absence or presence of FCCP.

To determine whether [$^{14}$C]tetraethylammonium uptake depends on membrane potential, the effect of a $K^+$ diffusion potential generated by valinomycin on [$^{14}$C]tetraethylammonium uptake was examined. As shown in Fig. 6*B*, the $H^+$ gradient-stimulated [$^{14}$C]tetraethylammonium uptake was not altered by the presence of valinomycin. Furthermore, we also examined the effect of a $K^+$ diffusion potential generated by valinomycin on rMATE1-mediated [$^{14}$C]tetraethylammonium uptake in the absence of $H^+$ gradient ($[H^+]_{in} = [H^+]_{out}$, pH 7.5). [$^{14}$C]tetraethylammonium uptake was not significantly changed with or without valinomycin at 30 s (with valinomycin, 1.77 ± 0.21; without valinomycin, 1.84 ± 0.14 pmol·mg protein$^{-1}$·30 s$^{-1}$; $n = 3$) and at 1 min (with valinomycin, 3.10 ± 0.18; without valinomycin, 3.48 ± 0.68 pmol·mg protein$^{-1}$·min$^{-1}$; $n = 3$). These results indicate that the inside-negative membrane potential does not affect [$^{14}$C]tetraethylammonium uptake by rMATE1, suggesting the electroneutral antiport of $H^+$ and [$^{14}$C]tetraethylammonium.

## DISCUSSION

Transport studies in brush-border and basolateral membrane vesicles from renal epithelial cells have been successfully utilized to characterize a number of transport systems under well-defined in vitro conditions. The membrane vesicle studies are particularly useful for identifying the driving forces of secondary active transport systems, compared with other analyses. This is because the ionic composition inside or outside membrane vesicles is easily manipulated, and ion gradients and membrane potential can be provided artificially. In fact, it was clearly demonstrated that organic cation transport systems at the renal brush-border membranes are driven by an outwardly directed $H^+$ gradient (4, 19, 23). Recent cloning and functional studies of MATE1 from various species have suggested that an oppositely directed $H^+$ gradient was a driving force of tetraethylammonium transport by MATE1 (3, 13, 15, 20), but there had been no evidence of a direct coupling of organic cation transport to $H^+$.

In the present study, by using membrane vesicles from HEK-rMATE1 cells, we provide the first direct evidence that MATE1 mediates the $H^+$-coupled uphill transport of [$^{14}$C]tetraethylammonium. Furthermore, this stimulation disappeared in the presence of a protonophore, FCCP, indicating that MATE1 functions as the $H^+$/organic cation antiporter. The $K^+$ diffusion potential generated by valinomycin had no effect on [$^{14}$C]tetraethylammonium uptake by membrane vesicles from HEK-rMATE1 cells with or without an $H^+$ gradient. This is consistent with a report that the tetraethylammonium uptake by brush-border membrane vesicles was not enhanced by inside-negative membrane potential (19). Taken together, it is suggested that the antiport of $H^+$ and tetraethylammonium via rMATE1 is electroneutral and that the stoichiometry might be 1:1.

In our previous study (20), using rMATE1-transiently expressing cells without $NH_4Cl$ pretreatment, we assessed the time course of [$^{14}$C]tetraethylammonium uptake (pH 8.4), pH profile of [$^{14}$C]tetraethylammonium uptake, and substrate specificity at the pH 8.4. In the present study, using HEK-rMATE1 cells with $NH_4Cl$ pretreatment, the transport characteristics for rMATE1 were analyzed by [$^{14}$C]tetraethylammonium (pH 7.4) or various compounds (pH 7.4). It was reported that the intracellular pH of HEK293 cells is ~7.2 and transiently acidified to 6.0–6.5 by $NH_4Cl$ pretreatment (11). These distinct experimental conditions may have affected the different transport characteristics of rMATE1. For example, we previously reported that the intracellular accumulation of [$^{14}$C]tetraethylammonium via rMATE1 showed a time-dependent increase. In the present study, [$^{14}$C]tetraethylammonium intracellular accumulation by rMATE1 peaked at 30–60 s and then gradually decreased. This may be due to the consumption of the outward $H^+$ gradient within 30–60 s and subsequent back flux of [$^{14}$C]tetraethylammonium via MATE1. In addition, [$^3$H]1-methyl-4-phenylpyridinium acetate and [$^{14}$C]procainamide were transported by rMATE1 in the present study, but not in the previous study. This may be due to the lack of a strong enough driving force to transport [$^3$H]1-methyl-4-phenylpyridinium acetate and [$^{14}$C]procainamide in the previous conditions.

In conclusion, we generated HEK293 cells stably expressing rMATE1, and clearly demonstrated that the driving force of tetraethylammonium transport by rMATE1 is an oppositely directed $H^+$ gradient using membrane vesicles from this stable transfectant. These findings can provide important information about the renal tubular secretion of organic cations, and these experimental strategies may be useful for elucidating the mechanisms of action used by single transporters in heterologous expression systems.

## GRANTS

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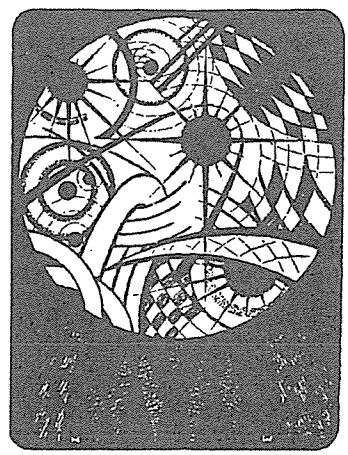

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*Short Communication*

# Renal Transport of Adefovir, Cidofovir, and Tenofovir by SLC22A Family Members (hOAT1, hOAT3, and hOCT2)

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***Purpose.*** The nephrotoxicity of the nucleotide antivirals adefovir, cidofovir and tenofovir is considered to depend on the renal tubular transport of them. Although it is known that the antivirals are substrates of the human renal organic anion transporter hOAT1 (SLC22A6), there is no information available on other organic ion transporters. The aim of the present study was to investigate whether the other renal organic anion transporter hOAT3 (SLC22A8) and organic cation transporter hOCT2 (SLC22A2) transport the antivirals.
***Materials and Methods.*** Uptake experiments were performed using HEK293 cells transfected with cDNA of the organic ion transporters.
***Results.*** The uptake of adefovir, cidofovir and tenofovir in monolayers stably expressing hOAT3 increased time-dependently, compared with control. Probenecid, a typical inhibitor of organic anion transporters, completely inhibited their transport. The amounts of the antivirals taken up by hOAT3 were much lower than those by hOAT1. The transient expression of hOCT2 did not increase uptake of the antivirals.
***Conclusion.*** These results indicate that adefovir, cidofovir and tenofovir are substrates of hOAT3 as well as hOAT1, but that quantitatively hOAT1 is the major renal transporter for these drugs.

**KEY WORDS:** antivirals; nephrotoxicity; organic anion transporter; renal transport.

## INTRODUCTION

The SLC22A superfamily comprises organic anion transporters and organic cation transporters, and is responsible for the tissue distribution and disposition of various organic compounds including endogenous metabolites, toxins, xenobiotics and drugs (1,2). So far, cDNAs of the human organic anion transporters 1–4 (hOAT1-4), the urate transporter (URAT) and the human organic cation transporters 1–3 (hOCT1-3), hOCTN1 and hOCTN2 have been isolated and characterized in terms of function and expression. The SLC22A members are known to transport clinically important drugs such as cephalosporins, diuretics, non-steroidal anti-inflammatory drugs, $H_2$ receptor antagonists, antivirals and antitumor agents, and the clinical relevance of each transporter has been assessed (3,4). In the members, hOAT1 (SLC22A6), hOAT3 (SLC22A8) and hOCT2 (SLC22A2) are highly expressed at the basolateral membrane in the renal proximal tubules, indicating that they play important roles in the tubular uptake of organic compounds from circulation (5).

hOAT1, hOAT3 and hOCT2 are determinants of the renal toxicity of cephaloridine, ochratoxin A, cisplatin and so on (6,7). In other words, the basolateral uptake of these compounds via the organic ion transporters in the renal proximal tubules is the first step in their nephrotoxicity. Similarly, the acyclic nucleotide analogues adefovir, cidofovir and tenofovir, which are currently used to treat infections of hepatitis B virus, cytomegalovirus and human immunodeficiency virus, respectively, induce renal impairment triggered by their renal tubular transport (8). It is shown that they are substrates of hOAT1 (9) and that the expression of hOAT1 is required for the cytotoxicity of adefovir and cidofovir (10), although there is no report on the transport of the antivirals by other SLC22A members.

Recently, we showed that mRNA expression levels of hOAT3 in the kidney cortex were higher than those of hOAT1 (5), and that mRNA levels of hOAT3 but not of hOAT1 correlated with the elimination rates of cefazolin and phenolsulfonphthalein in patients with renal diseases (11,12). Accordingly, to understand the renal basolateral transport mechanisms for the antivirals in detail, information on hOAT3 is required. The purpose of this study is to investigate the transport of adefovir, cidofovir and tenofovir by hOAT3. In addition, the contribution of hOCT2 was also examined.

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**ABBREVIATIONS:** hOAT, human organic anion transporter; hOCT, human organic cation transporter; MRP, multidrug resistance protein.

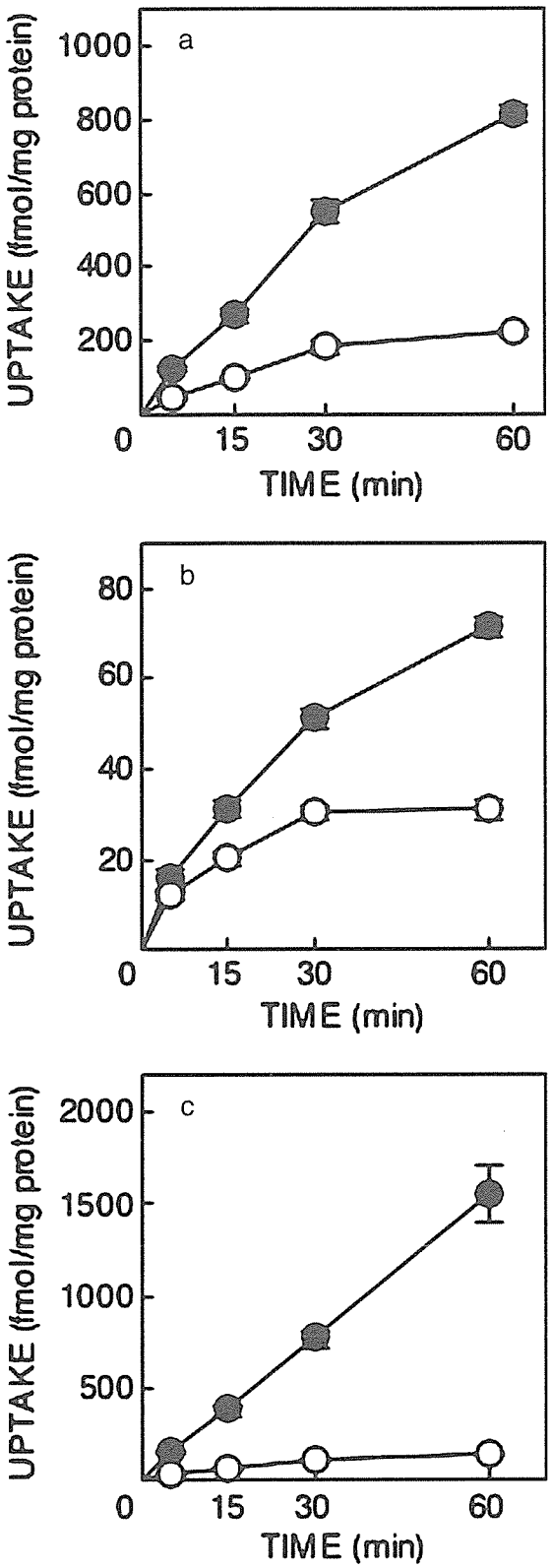


**Fig. 1.** Time-dependent uptake of adefovir (**a**), cidofovir (**b**) and tenofovir (**c**) by HEK-pBK and HEK-hOAT3. HEK-pBK (*open circle*) and HEK-hOAT3 (*closed circle*) were incubated with 111 nM [$^3$H]adefovir, 66.7 nM [$^3$H]cidofovir or 100 nM [$^3$H]tenofovir for the periods indicated. Each point represents the mean±S.E. of the uptake amounts of each antiviral in three monolayers. When the error bar is not shown, it is smaller than the symbol.

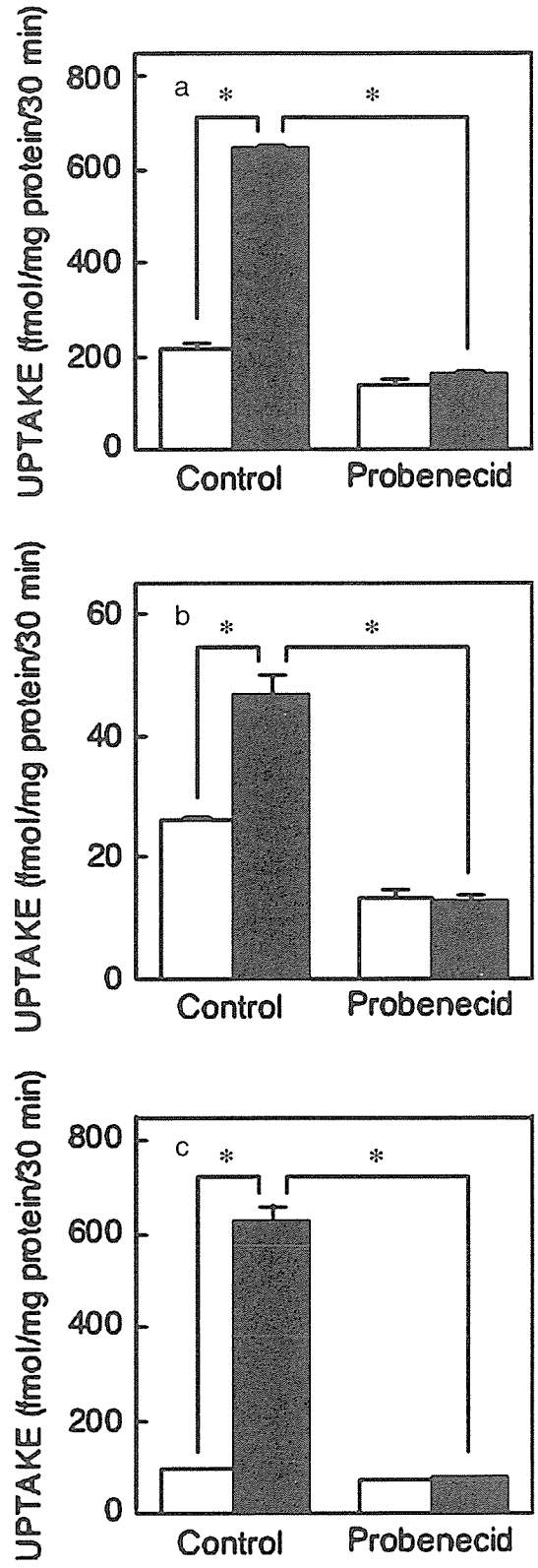


**Fig. 2.** Effect of probenecid on hOAT3-mediated uptake of adefovir (**a**), cidofovir (**b**) and tenofovir (**c**). HEK-pBK (*open column*) and HEK-hOAT3 (*closed column*) were incubated with 111 nM [$^3$H]adefovir, 66.7 nM [$^3$H]cidofovir or 100 nM [$^3$H]tenofovir in the absence (control) or presence of 1 mM probenecid for 30 min. Each column represents the mean±S. E. of the uptake amounts of each antiviral in three monolayers. *, $P<0.001$, significantly different.

**Table I.** Uptake of Adefovir, Cidofovir and Tenofovir by HEK293 Cells Stably Expressing hOAT1 or hOAT3

| Compounds | Control | hOAT1 | hOAT3 |
|---|---|---|---|
| | | μl/mg protein/5 min | |
| *p*-Aminohippurate | 2.19±0.08 | 24.9±0.6** | 3.38±0.17 |
| Estrone sulfate | 1.41±0.03 | 1.98±0.05* | 14.4±0.1** |
| Adefovir | 0.369±0.007 | 46.1±0.5** | 1.38±0.08 |
| Cidofovir | 0.135±0.010 | 22.5±0.4** | 0.295±0.024 |
| Tenofovir | 0.260±0.018 | 26.0±0.7** | 1.22±0.07 |

HEK-pBK (control), HEK-hOAT1 and HEK-hOAT3 were incubated with 5 μM *p*-[$^{14}$C]aminohippurate, 17.5 nM [$^{3}$H]estrone sulfate, 111 nM [$^{3}$H]adefovir, 66.7 nM [$^{3}$H]cidofovir or 100 nM [$^{3}$H]tenofovir for 5 min. Each value represents the mean ± S. E. of the uptake amounts of each compound in three monolayers.
**, $P<0.001$, significantly different from control.
*, $P<0.01$, significantly different from control.

## MATERIALS AND METHODS

### Materials

*p*-[$^{14}$C]Aminohippurate (1.9 GBq/mmol) was obtained from NEN™ Life Science Products Inc. (Boston, MA, USA). [$^{3}$H]Estrone sulfate, ammonium salt (2.1 TBq/mmol) and [$^{14}$C]tetraethylammonium bromide (88.8 MBq/mmol) were from Perkin-Elmer Life Sciences Inc. (Boston, MA, USA). [$^{3}$H]Adefovir (9 Ci/mmol), [$^{3}$H]cidofovir (15 Ci/mmol) and [$^{3}$H]tenofovir (10 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Probenecid was obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity available.

### Functional Analyses of hOAT1 and hOAT3

The functions of hOAT1 and hOAT3 were evaluated according to our former report (13), using HEK293 cells stably transfected with pBK-CMV vector containing hOAT1 cDNA, hOAT3 cDNA or no cDNA, named HEK-hOAT1, HEK-hOAT3 and HEK-pBK, respectively. In brief, 48 h after the cells were seeded on poly-D-lysine-coated 24-well plates at a density of $2\times10^5$ cells/well, the uptake of organic compounds by the cells was examined. The composition of the incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose and 5 mM HEPES (pH 7.4). After the preincubation of the cells with 0.2 ml of the incubation medium at 37°C for 10 min, the medium was replaced with 0.2 ml of the incubation medium containing test compounds. At the end of the incubation, the medium was aspirated, and then the cells were washed twice with 1 ml of ice-cold incubation medium. The cells were lysed in 250 μl of 0.5 N NaOH solution, and the radioactivity in aliquots was determined in 3 ml of ACSII (Amersham International, Buckingham shire, UK). The protein contents of the solubilized cells were determined by the method of Bradford using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine γ-globulin as a standard.

### Functional Analysis of hOCT2

As previously reported (14), the transient expression system with HEK293 cells was used to examine whether hOCT2 transports the antivirals. Briefly, 1 day after HEK293 cells were seeded on poly-D-lysine-coated 24-well plates at a density of $2\times10^5$ cells/well, the cells were transfected with plasmid cDNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after the transfection, uptake experiments were performed as above described.

### Statistical Analysis

Data were statistically analyzed with a one-way analysis of variance followed by Scheffe's test.

**Table II.** Uptake of Adefovir, Cidofovir and Tenofovir by HEK293 Cells Transiently Expressing hOCT2

| Compounds | Control | hOCT2 |
|---|---|---|
| | | μl/mg protein/30 min |
| Tetraethylammonium | 12.8±0.8 | 97.7±9.0* |
| Adefovir | 0.964±0.062 | 0.857±0.026 |
| Cidofovir | 0.189±0.023 | 0.161±0.007 |
| Tenofovir | 0.674±0.049 | 0.653±0.017 |

HEK293 cells transfected with empty pCMV6-XL4 (control) or hOCT2 cDNA were incubated with 5 μM [$^{14}$C]tetraethylammonium, 111 nM [$^{3}$H]adefovir, 66.7 nM [$^{3}$H]cidofovir or 100 nM [$^{3}$H]tenofovir for 30 min. Each value represents the mean±S. E. of the uptake amounts of each compound in three monolayers.
*, $P<0.001$, significantly different from control.

## RESULTS AND DISCUSSION

First, we investigated the time-dependent uptake of adefovir, cidofovir and tenofovir by HEK-pBK and HEK-hOAT3. As shown in Fig. 1, the expression of hOAT3 enhanced the amounts of these antivirals taken up into the cells, and their uptake by hOAT3 was increased time-dependently. Fig. 2 depicts the effect of probenecid, a typical inhibitor of renal organic anion transporters, on the transport of the antivirals by hOAT3. Probenecid significantly inhibited the hOAT3-mediated uptake of the antivirals. These results indicate that hOAT3 recognizes adefovir, cidofovir and tenofovir as substrates.

Table I represents the amounts of adefovir, cidofovir and tenofovir taken up by HEK-pBK, HEK-hOAT1 and

HEK-hOAT3, including those of *p*-aminohippurate and estrone sulfate, the representative substrates of hOAT1 and hOAT3, respectively. As previously reported (9), hOAT1-mediated transport of adefovir, cidofovir and tenofovir was observed. The uptake amounts of the antivirals via hOAT1 were much greater than those via hOAT3. It is important to compare the kinetic parameters of the hOAT1-mediated versus hOAT3-mediated transport of adefovir, cidofovir and tenofovir. However, we could not perform the experiments on the concentration-dependent uptake, because their unlabeled compounds were not commercially available. Information on the expression levels of hOAT1 and hOAT3 in HEK293 cells would make the data in Table I more significant. We previously quantified mRNA levels of each transporter in HEK-hOAT1 and HEK-hOAT3 to be 64.9 and 225.6 amol/μg total RNA, respectively (13). Because the transport activities of the typical substrates by each transfectant in the present study tended to be similar to those in our previous report (13), it was considered that the mRNA levels of hOAT3 in HEK-hOAT3 used in this study could be higher than those of hOAT1 in HEK-hOAT1. Taking these findings into account, it is suggested that hOAT1 is a potent transporter of adefovir, cidofovir and tenofovir, compared with hOAT3, under the conditions tested in this study. Furthermore, the facts that the blood levels of adefovir and tenofovir in patients are comparable to the concentrations examined in the present study could indicate that hOAT1 is a key transporter mediating the entry of adefovir and tenofovir into the renal tubules from blood.

As described in the Introduction, we recently quantified mRNA levels of drug transporters in normal parts of the kidney cortex from nephrectomized patients and in the renal biopsy specimens of patients with renal diseases. Two important findings were made. First, mRNA levels of hOAT3 were about 3-time higher than those of hOAT1 in the normal kidney cortex (5). Second, mRNA levels of only hOAT1 among hOAT1-4 were significantly lower in the biopsy specimens, compared in the normal segments of the kidney cortex, suggesting that hOAT1 expression is readily influenced by renal diseases (11). Therefore, there is a possibility that the contribution of hOAT3 to tubular uptake of the antivirals could be greater in patients with renal failure.

Now, multidrug resistance protein 4 (MRP4) is thought to be a candidate which transports these antivirals from the proximal epithelial cells into the lumen, because MRP4-mediated efflux of adefovir from cells was observed (15,16) and the functional expression of Mrp4 in the brush-border membrane of the renal proximal tubule was recognized using Mrp4-knockout mice (17). Our previous study using serial sections showed that the localization of hOAT1 and hOAT3 was not completely identical in the proximal tubules (5). An investigation of the exact distribution of MRP4 in the renal proximal tubules and a comparison with hOAT1 and hOAT3 would facilitate elucidation of the mechanisms behind the nephrotoxicity of adefovir, cidofovir and tenofovir.

The transport of adefovir, cidofovir and tenofovir by hOCT2 was also examined in this study. As represented in Table II, no uptake of these antivirals via hOCT2 was observed. Previously, we reported that cimetidine was transported by hOAT1 and hOAT3 as well as hOCT2, and that hOAT3 but not hOAT1 or hOCT2 transported famotidine (18). Because famotidine and cimetidine exist partly in the cationic forms at the experimental pH, the results of the study were surprising, and the substrate recognition of the SLC22A family might be complicated. Although our previous report indicated that preconceived ideas on the substrate recognition of SLC22A members should be reconsidered, the findings of the present study were consistent with the preconception that hOCT2 would not transport the anionic antivirals. hOCT2 is not likely to contribute to the elimination of adefovir, cidifovir and tenofovir.

In conclusion, this is the first report representing that adefovir, cidofovir and tenofovir are substrates of hOAT3 as well as hOAT1. Furthermore, it is suggested that hOAT1 rather than hOAT3 plays a crucial role in the basolateral uptake of the antivirals into the renal proximal tubules. These findings provide useful information for the elucidation of the molecular mechanisms of disposition and nephrotoxicity of adefovir, cidofovir and tenofovir.

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# Pharmacokinetic significance of luminal multidrug and toxin extrusion 1 in chronic renal failure rats

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ABSTRACT

Functional and expressional depression of the rat organic cation transporter rOCT2 after 5/6 nephrectomy (Nx) is accompanied by the decreased plasma level of testosterone in the male rats. Though vectorial transport across the tubular epithelial cells is important in the secretion of cationic compounds, there has been no imformation about the luminal organic cation transporter in disease state. In the present study, the role of luminal multidrug and toxin extrusion 1 (rMATE1) was examined using female rats with or without Nx, avoiding the influence of testosterone. The tubular secretion of cimetidine was markedly decreased in female Nx rats as well as male rats. Unlike in the male rats, the plasma level of testosterone and the expression of basolateral rOCT2 were unchanged in the female rats after Nx. On the other hand, the expression of rMATE1 was markedly decreased in both male and female Nx rats, and the level of rMATE1, but not of rOCT2, correlated well with the tubular secretion of cimetidine in the female rats ($r = 0.74$). Immunohistochemical analysis revealed that rMATE1 and $Na^+/H^+$ exchanger (NHE) 3 were localized at the brush-border membrane of proximal tubules. The level of NHE3 was also markedly depressed in both male and female Nx rats, suggesting that the expression level on the luminal rMATE1 in combination with NHE3 was indicated to be a crucial factor for the tubular secretion of cimetidine.



## 1. Introduction

The tubular transport of organic substances plays an essential role in the removal of *xenobiotics* including drugs and numerous chemicals contained in our environment and some metabolites. Basolateral types of organic anion and cation transporters, OAT1 (SLC22A6), OAT3 (SLC22A8), OCT1 (SLC22A1) and OCT2 (SLC22A2), have been cloned and characterized [1–4]. These transporters are indicated to mediate the basolateral entry of various anionic or cationic drugs into the proximal tubular epithelial cells [5]. Recently, a renal luminal type of $H^+$/organic cation antiporter, multidrug and toxin extrusion 1 (MATE1), has been cloned, and considered to be responsible for the final step in the excretion of organic cations [6–8]. However, the pharmacokinetic and pathophysiological role of the luminal MATE1 in combination with the basolateral OCT2 is not clear. Furthermore, there has been no information regarding the change in luminal MATE1 during chronic renal failure.

More gene product of rat (r) OCT2 is found in the male kidney than female kidney, and the expression of rOCT2 in the kidney is regulated by the plasma level of testosterone [9,10]. We previously found that the renal tubular secretion of *para*-aminohippuric acid and cimetidine were markedly decreased in male 5/6 nephrectomized (Nx) rats, the protein expression of rOCT2 but not of rOCT1, rOAT1, or rOAT3 was depressed in male Nx rats [11]. Furthermore, a lowered plasma level of testosterone was likely to be responsible for the depressed rOCT2 expression in chronic renal failure.

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