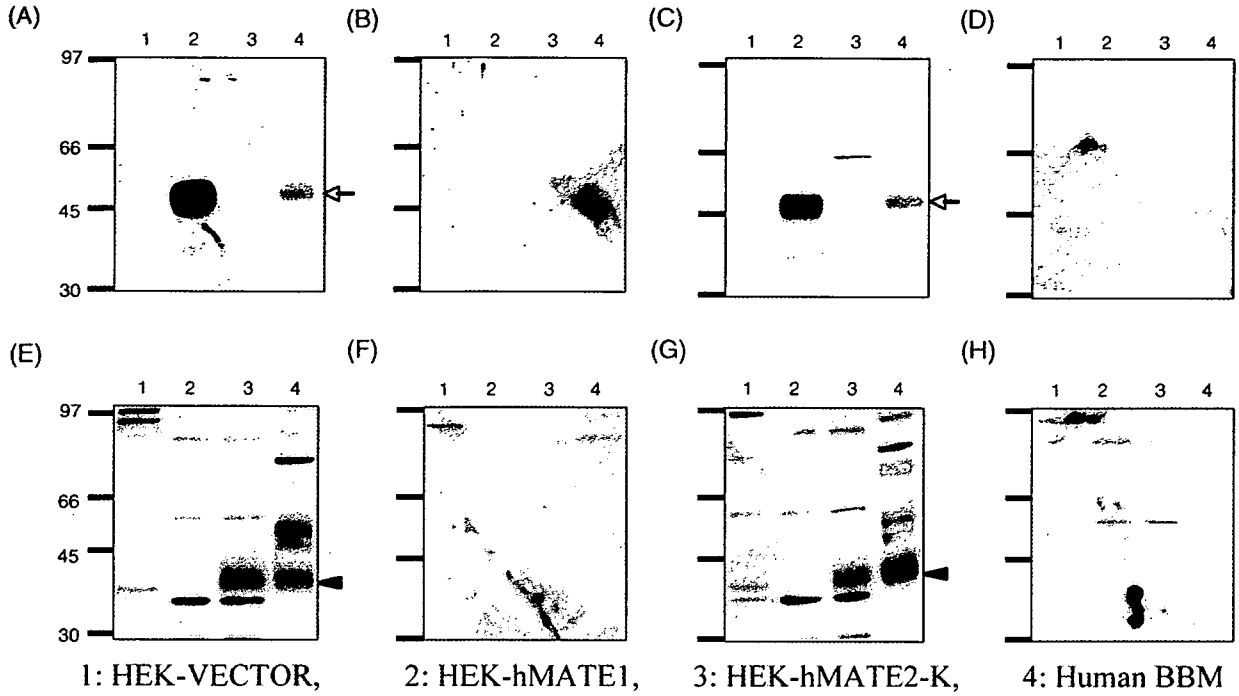

Fig. 1 – Western blot analyses of hMATE1 and hMATE2-K in stably expressing HEK293 cells and human brush-border membrane. Twenty micrograms of each membrane was separated by SDS-PAGE under nonreducing (A, B, E and F) or reducing (C, D, G and H) conditions. (A and C) Antiserum specific for hMATE1 was used as primary antibody. (B and D) Antiserum preabsorbed with the antigen peptide (20 μg/mL) of hMATE1 was used. (E and G) Antiserum specific for hMATE2-K was used as primary antibody. (F and H) Antiserum preabsorbed with the antigen peptide (20 μg/mL) of hMATE2-K was used. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies, and strips of blots were visualized by chemiluminescence on X-ray film. The arrow and arrowhead indicate the position of hMATE1 and hMATE2-K, respectively. BBM, brush-border membrane.

transport of guanidine, procainamide, and acyclovir were significantly smaller than those for hMATE2-K. The same results were obtained using a transient expression system (data not shown). The uptake of creatinine by hMATE1 and hMATE2-K was almost linear and not saturated at a concentration up to 10 mM.

Because quinolone antibiotics have been shown to be potent inhibitors of the apical H^+/organic cation antiport

Table 3 – Apparent K_m values of the uptake of various organic ions by hMATE1 and hMATE2-K at extracellular pH 7.4 after pretreatment with ammonium chloride

Compounds	K_m (mM)		V_{max} (nmol/mg protein/2 min)	
	hMATE1	hMATE2-K	hMATE1	hMATE2-K
TEA	0.38 ± 0.07	0.76 ± 0.18	2.37 ± 0.23	1.76 ± 0.25
MPP	0.10 ± 0.02	0.11 ± 0.01	1.47 ± 0.13	1.15 ± 0.09
Cimetidine	0.17 ± 0.02	0.12 ± 0.04	0.27 ± 0.03	0.23 ± 0.05
Metformin	0.78 ± 0.10	1.98 ± 0.48	4.46 ± 0.59	1.69 ± 0.34
Guanidine	2.10 ± 0.31	4.20 ± 0.05*	1.78 ± 0.19	1.16 ± 0.11
Procainamide	1.23 ± 0.03	1.58 ± 0.04**	7.56 ± 1.70	6.77 ± 0.94
Topotecan	0.07 ± 0.02	0.06 ± 0.01	0.42 ± 0.13	0.26 ± 0.02
Estrone sulfate	0.47 ± 0.02	0.85 ± 0.17	0.53 ± 0.06	0.85 ± 0.14
Acyclovir	2.64 ± 0.40	4.32 ± 0.44*	1.24 ± 0.24	1.89 ± 0.15
Ganciclovir	5.12 ± 0.27	4.28 ± 0.61	2.12 ± 0.25	1.61 ± 0.27

Experimental conditions are described in the legend for Fig. 6. The apparent K_m and V_{max} values were determined from Eadie-Hofstee plots of each compound's uptake after the correction for nonsaturable components. Nonsaturable components were estimated in the presence of 10 mM unlabeled compound. Data are shown as means ± S.E. of three independent experiments using three monolayers.

* $P < 0.05$ significantly different from the K_m value for hMATE1.

** $P < 0.01$ significantly different from the K_m value for hMATE1.

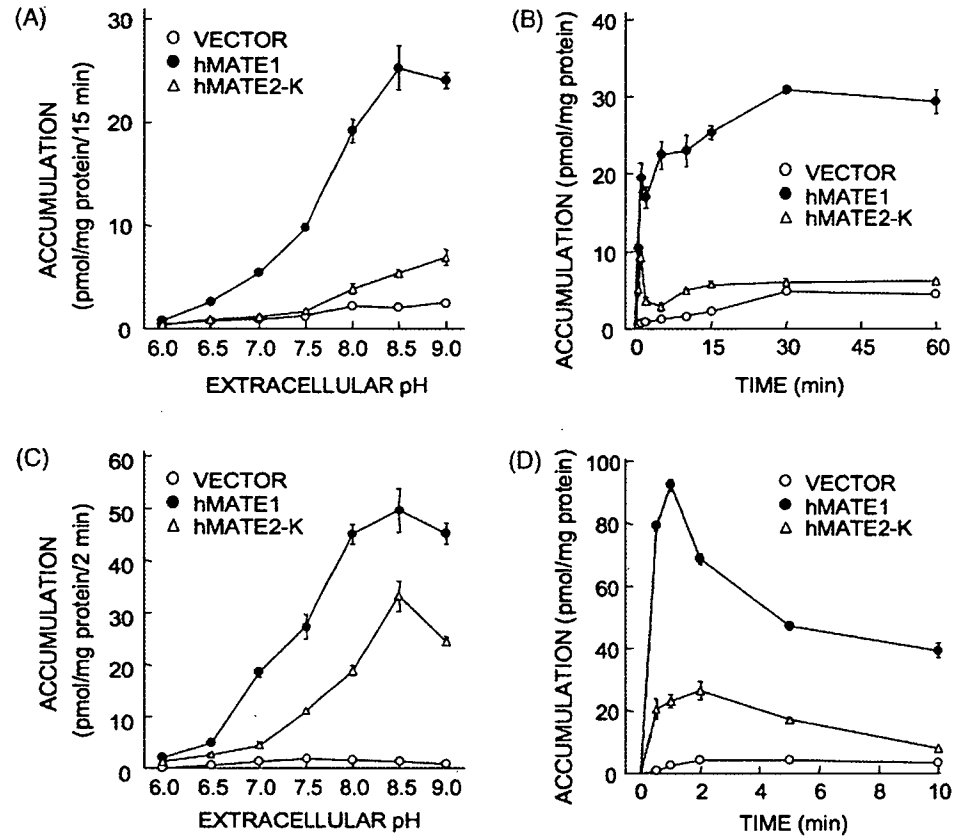

Fig. 2 – Oppositely directed H^+ gradient-dependence (A and C) and time course (B and D) of [^{14}C]TEA uptake by hMATE1 and hMATE2-K in the stably transfected HEK293 cells. (A) HEK293 cells stably expressing the empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle) were incubated for 15 min at 37 °C with incubation medium of various pH containing 5 μM of [^{14}C]TEA. (B) Time course of [^{14}C]TEA uptake by hMATE1 and hMATE2-K. HEK293 cells stably expressing the empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle) were incubated with 5 μM [^{14}C]TEA (10.36 kBq/mL, pH8.4) at 37 °C. (C) HEK293 cells stably expressing the empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle) were preincubated with incubation medium (pH 7.4) in the presence of 30 mM ammonium chloride for 20 min. Then, the preincubation medium was removed, and the cells were incubated for 15 min at 37 °C with incubation medium of various pH containing 5 μM of [^{14}C]TEA. (D) Time course of [^{14}C]TEA uptake by hMATE1 and hMATE2-K. HEK293 cells stably expressing the empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle) were preincubated with incubation medium (pH 7.4) in the presence of 30 mM ammonium chloride for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM [^{14}C]TEA (10.36 kBq/mL, pH 7.4) at 37 °C. Each point represents the mean ± S.E. of three monolayers from a typical experiment in at least three separate experiments.

system rather than basolateral transport system [16,17], quinolone antibiotics were considered to be potent inhibitors for MATEs. As shown in Fig. 3, the presence of levofloxacin and ciprofloxacin reduced the uptake of TEA at extracellular pH 7.4 after pretreatment with ammonium chloride by HEK-hMATE1 and HEK-hMATE2-K in a dose-dependent manner. The IC_{50} values were estimated by the method of Urakami et al. [18]. hMATE1 showed a moderately higher affinity for levofloxacin than hMATE2-K (IC_{50} values, 38.2 ± 11.8 μM for hMATE1 versus 81.7 ± 23.1 μM for hMATE2-K, mean ± S.E. of three separate experiments using three monolayers). However, ciprofloxacin inhibited the hMATE2-K-mediated uptake of TEA over a relatively lower concentration range than the hMATE1-mediated uptake (IC_{50} values, 231 ± 57.3 μM for hMATE1 versus 98.7 ± 14.1 μM for hMATE2-K, mean ± S.E. of three separate experiments using three monolayers).

Furthermore, we examined whether hMATE1 and hMATE2-K transport some cephalosporin antibiotics, cephalexin, cephradine, and cefazolin. Because cephalexin and cephradine were zwitterionic ions, we performed the uptake experiments at pH 7.4 without or with the pretreatment of ammonium chloride. The uptake of cephalexin and cephradine by hMATE1 was markedly higher than that by control cells, and was stimulated after the pretreatment with ammonium chloride (Fig. 4). On the other hand, hMATE2-K did not transport cephalexin or cephradine under any conditions (Fig. 4). The anionic cephalosporin cefazolin was not transported by hMATE1 or hMATE2-K under any conditions either. Because cellular accumulation of cefazolin was not detected in these three cells (analytical limitation of HPLC was 0.1 μM), the data were not shown. In Fig. 4, cellular acidification had a modest effect on the uptake of cephalexin

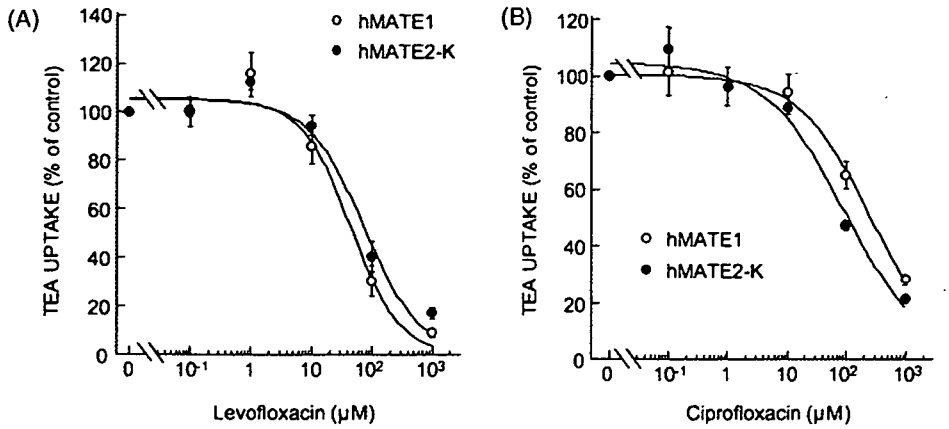

Fig. 3 – Effects of levofloxacin (A) and ciprofloxacin (B) on the uptake of [^{14}C]TEA by HEK293 cells stably expressing hMATE1 and hMATE2-K, respectively. HEK293 cells stably expressing hMATE1 (open circle) and hMATE2-K (closed circle) cDNA were incubated with 5 μM [^{14}C]TEA for 2 min at pH 7.4 at 37 °C in the presence of various concentrations of levofloxacin (A) or ciprofloxacin (B). For the kinetic analyses, ammonium chloride (30 mM, pH 7.4, 20 min) was used to achieve intracellular acidification. Each point represents the mean ± S.E. of three monolayers from a typical experiment in at least three separate experiments.

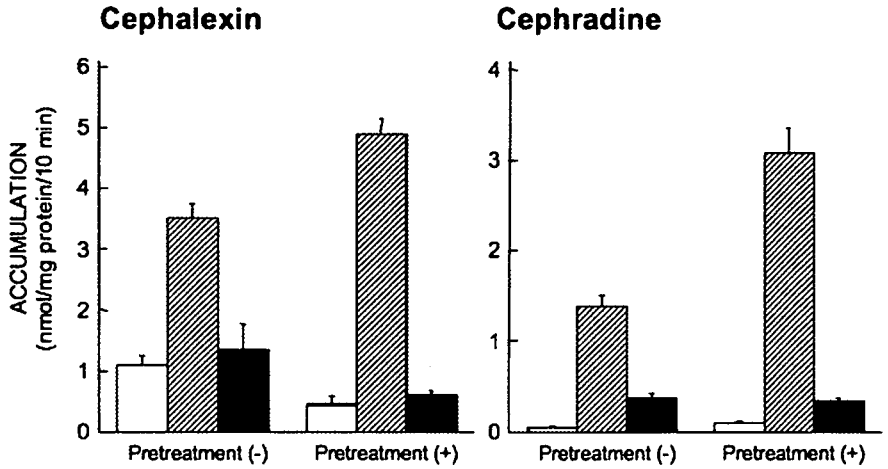

Fig. 4 – Uptake of cephalexin and cephradine by HEK293 cells expressing hMATE1 and hMATE2-K. HEK293 cells transfected with the empty vector (open column), hMATE1 cDNA (hatched column) or hMATE2-K cDNA (closed column) were preincubated with incubation medium (pH 7.4) in the presence or absence of 30 mM ammonium chloride for 20 min, and incubated with 1 mM cephalexin or 1 mM cephradine for 10 min at 37 °C and pH 7.4. Each point represents the mean ± S.E. of three monolayers from a typical experiment in at least three separate experiments.

or cephradine by hMATE1. Because of the analytical limitation of HPLC for cephalexin and cephradine, we could not examine the experiments at earlier than 10 min. Therefore, it is likely that the effects of the H^+ gradient might be lesser in incubation time at 10 min than 2 min. To determine the affinity of cephalosporins for hMATE1 and hMATE2-K, we examined the uptake of [^{14}C]TEA by HEK-hMATE1 and HEK-hMATE2-K in the presence of cephalexin, cephradine and cefazolin. As shown in Fig. 5A and B, cephalexin and cephradine inhibited the transport of TEA by hMATE1 with IC_{50} values (mean ± S.E. of three separate experiments using three monolayers) of 6.50 ± 1.34 mM and 4.04 ± 0.88 mM, respectively. Moreover, cephradine also inhibited TEA's transport by hMATE2-K in a dose-dependent manner (IC_{50}, 10.4 ± 0.65 mM), but cephalexin had no inhibitory effect on the hMATE2-K-mediated uptake of TEA. Cefazolin did not have any effect on the transport of TEA by hMATE1 and hMATE2-K (Fig. 5C).

4. Discussion

In the present study, we have screened the substrates of hMATE1 and hMATE2-K in transfected cells. Both hMATE1 and hMATE2-K transported endogenous organic cations and cationic drugs (Tables 1 and 2). These results indicate that both luminal hMATE1 and hMATE2-K share some of the

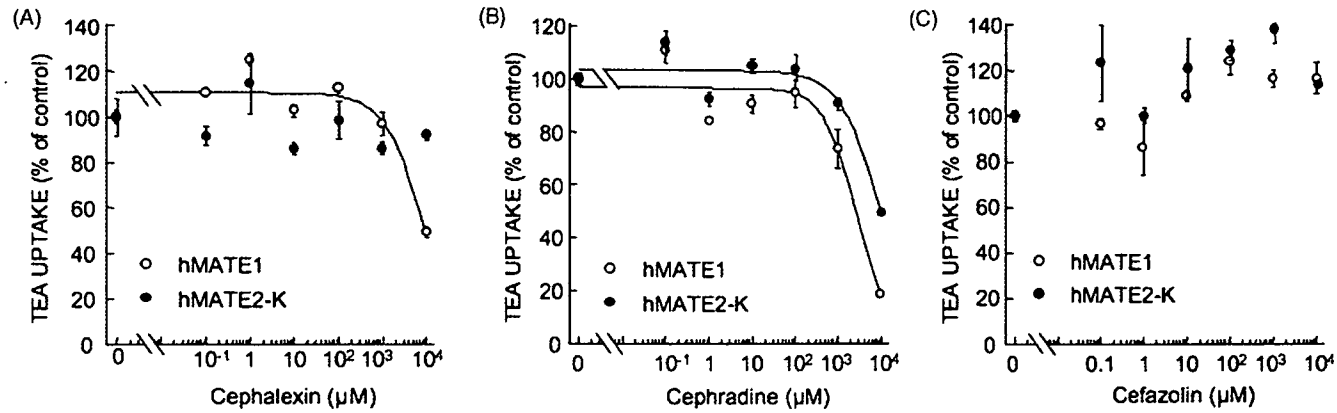

Fig. 5 – Effects of cephalosporin antibiotics on the uptake of [^{14}C]TEA by HEK293 cells stably expressing hMATE1 and hMATE2-K. HEK293 cells stably expressing hMATE1 (open circle) and hMATE2-K (closed circle) cDNA were incubated with 5 μM [^{14}C]TEA for 2 min at pH 7.4 and 37 °C in the presence of various concentrations of cephalexin (A), cephradine (B), or cefazolin (C). For the kinetic analyses, ammonium chloride (30 mM, pH 7.4, 20 min) was used to achieve intracellular acidification. Each point represents the mean ± S.E. of three monolayers from a typical experiment in at least three separate experiments.

substrates of basolateral hOCT1 and hOCT2 [19]. Therefore, cationic compounds accumulated in the liver by hOCT1 or in the kidney by hOCT2 would be secreted into bile by hMATE1 or into urine by both hMATE1 and hMATE2-K. If a double transfectant, composed of basolateral hOCT1 and luminal hMATE1, hOCT2 and hMATE1, or hOCT2 and hMATE2-K, was to become available, the vectorial transcellular transport of these substrates may be revealed.

In the transfectants, specific signals corresponding to hMATE1 and hMATE2-K were detected, and molecular masses were similar to those in the human renal brush-border membranes. Interestingly, the molecular masses of hMATE1 and hMATE2-K were smaller than the predicted values, 62 kDa for hMATE1 and hMATE2-K (Fig. 1). It was reported that some post-translational processing was required for localization to brush-border membranes in the rat organic anion transporting polypeptide oatp1/Slco1a1 and the kidney-specific organic anion transporter OAT-K1/Slco1a3 in the rat kidneys [20–22]. Taking into consideration these phenomena, some limited proteolysis might have occurred prior to the luminal localization of hMATE1 and hMATE2-K in the human kidney as well as HEK293 cells. Further study is needed to elucidate the molecular mechanism(s) of the post-translational cleavage site(s) and membrane localization of hMATE transporters.

Interestingly, hMATE1 and hMATE2-K also recognized some anionic compounds, acyclovir, ganciclovir and estrone sulfate, which are substrates of the human organic anion transporter family (hOAT) (Tables 1 and 2 and Fig. 6) [23,24]. The affinity for hMATE1 and hMATE2-K differed significantly among guanidine, procainamide, and acyclovir (Fig. 6, Table 3). Comparable with our report [10], zwitterionic amino-beta-lactam antibiotics, cephalexin and cephradine, have been found to be substrates of hMATE1 rather than hMATE2-K. However, it was revealed that a new platinum anticancer agent, oxaliplatin, is a good substrate for hMATE2-K rather than hMATE1 [7,8]. Furthermore, we recently identified the histidine and cysteine residues essential for the transport activity of MATE family [25]. Taken together, some structural determinants for substrate specificity of hMATE1 and hMATE2-K would be clarified in future studies. Thus, hMATE1 and hMATE2-K may have complementary roles as a detoxicating system mediating the tubular secretion of substrates specific for each transporter (Table 4). In addition, hMATE1 and hMATE2-K also recognize some anionic and zwitterionic

Table 4 – Substrate specificity of hMATE1 and hMATE2-K

	Substrate
Transported by hMATE1 and hMATE2-K	Tetraethylammonium, 1-methyl-4-phenylpyridinium, cimetidine, metformin, creatinine, guanidine, procainamide, thiamine, topotecan, estrone sulfate, acyclovir, ganciclovir
hMATE1 > hMATE2-K	Cephalexin, cephradine
hMATE2-K > hMATE1	Oxaliplatin[a]
Not transported by hMATE1 or hMATE2-K	Choline, quinidine, quinine, carnitine, nicotine, captopril, verapamil, levofloxacin, tetracycline, para-aminohippuric acid, ochratoxin A, dehydroepiandrosterone sulfate, uric acid, salicylic acid, indomethacin, prostaglandin F_2 alpha, valproic acid, adefovir, cidofovir, tenofovir, glycylsarcosine

[a] Reference from Yonezawa et al. [7] and Yokoo et al. [8].

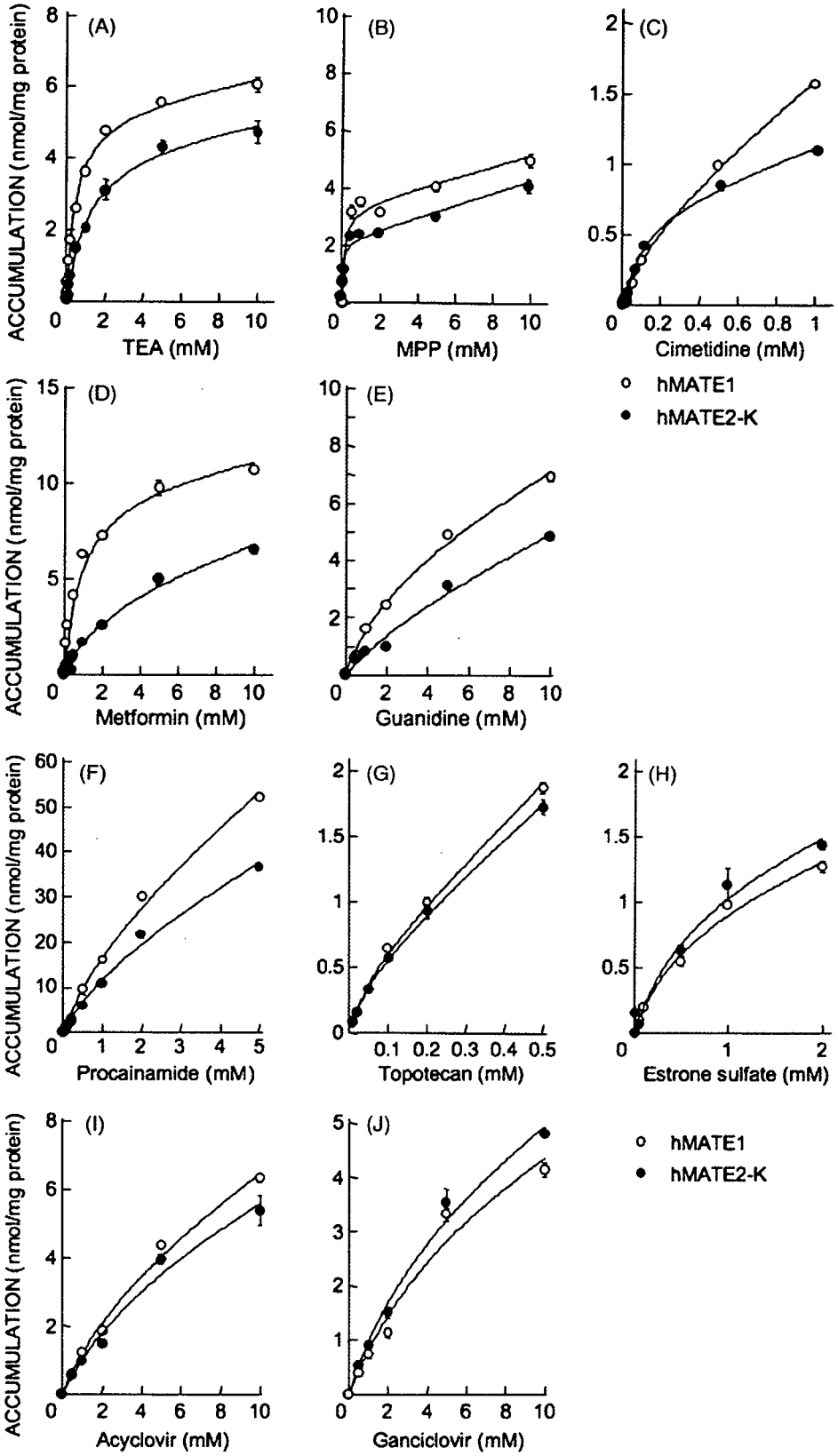

Fig. 6 – Concentration-dependence of substrates for hMATE1 and hMATE2-K using stable transfectants. HEK293 cells stably expressing hMATE1 (open circle) or hMATE2-K (closed circle) were incubated with various concentrations of [^{14}C]TEA (A), [^{3}H]MPP (B), [^{3}H]cimetidine (C), [^{14}C]metformin (D), [^{14}C]guanidine (E), [^{14}C]procainamide (F), [^{14}C]topotecan (G), [^{3}H]estrone sulfate (H), [^{3}H]acyclovir (I), and [^{3}H]ganciclovir (J) for 2 min at pH 7.4. For the kinetic analyses, ammonium chloride (30 mM, pH 7.4, 20 min) was used to achieve intracellular acidification. Each point represents the mean ± S.E. of three monolayers from a typical experiment in at least three separate experiments.

compounds as substrates, although the chemical charge of substrates of the hMATE family is generally positive. These results suggest that some unexpected drug–drug interaction may occur based on the substrate specificities of several drug transporters. Therefore, functional cooperation, such as between basolateral organic anion transporters and the apical hMATE family, should be clarified to explain the potential drug interactions between organic cations and organic anions.

Coadministration of ofloxacin significantly decreased the renal clearance of procainamide, but not its active metabolite N-acetyl procainamide [26]. Cotreatment with levofloxacin and ciprofloxacin decreased the renal clearance of procainamide and its metabolite [27]. In the present study, calculated IC_{50} values were comparable to the urinary concentration of these fluoroquinolones. Some drug-drug interaction may have potentially occurred such as decreased renal clearance and elevated plasma concentrations of cationic drugs after the coadministration of fluoroquinolone antibiotics.

In conclusion, the present study demonstrates the difference in the transport substrates between hMATE1 and hMATE2-K by use of transfectants. In addition, it is indicated that hMATE1 and hMATE2-K act as a detoxicating system together by mediating the tubular secretion of intracellularly accumulated cationic compounds across the brush-border membrane in the kidney. The difference in substrate specificity between two transporters should help to elucidate the molecular mechanisms behind the recognition and translocation of ionic compounds by these transporters.

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Differential contribution of organic cation transporters, OCT2 and MATE1, in platinum agent-induced nephrotoxicity

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ABSTRACT

The mechanism of severe nephrotoxicity caused by cisplatin, but not carboplatin, oxaliplatin, and nedaplatin, is not fully understood. The renal accumulation and subsequent nephrotoxicity of platinum agents were examined in rats. Among these four drugs, only cisplatin induced nephrotoxicity at 2 days after its intraperitoneal administration. The urinary activity of *N*-acetyl-β-D-glucosaminidase and expression of kidney injury molecule-1 mRNA and osteopontin were markedly enhanced in the cisplatin-treated rats. Although some markers were affected in the rats administered nedaplatin, only minor histological change was observed. The renal accumulation of cisplatin was much greater than that of the other drugs. In the *in vitro* study, the cellular accumulation of cisplatin and oxaliplatin was stimulated by the expression of rat (r) OCT2. Oxaliplatin was also transported by rOCT3. A luminal H^+/organic cation antiporter, rMATE1 (multidrug and toxin extrusion) as well as human (h) MATE1 and hMATE2-K, stimulated the H^+-gradient-dependent antiport of oxaliplatin, but not of cisplatin. Carboplatin and nedaplatin were not transported by these transporters. In conclusion, the nephrotoxicity of platinum agents was closely associated with their renal accumulation, which is determined by the substrate specificity of the OCT and MATE families.

1. Introduction

Cis-diamminedichloroplatinum II (cisplatin) is widely used against solid tumors of the prostate gland, bladder, colon, lung, testis, and brain. Despite the effectiveness of cisplatin, severe nephrotoxicity limits its clinical application. Other platinum agents, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum II (carboplatin), *trans*-L-1,2-diaminocyclohexaneoxalatoplatinum II (oxaliplatin) and *cis*-diammineglycolatoplatinum (nedaplatin), are less nephrotoxic than cisplatin [1]. However, there was no report that the influence of these four agents on renal function was simultaneously examined *in vivo*, and it is not clear why only cisplatin is nephrotoxic. The chemical structure of each of these agents is shown in Fig. 1.

The mechanisms of cellular uptake and efflux of platinum agents are not fully understood, although the cellular uptake of cisplatin was suggested to be mediated by specific transporter(s) in the renal epithelial cells [2]. We reported that rat organic cation transporter 2 (rOCT2/Slc22a2) transported cisplatin, and was responsible for cisplatin-induced nephrotoxicity [3]. Human (h)OCT2 was also found to transport cisplatin [4]. Recently, we have found that some platinum agents were transported by human organic cation transporters such as the OCT (SLC22A) family and MATE (multidrug

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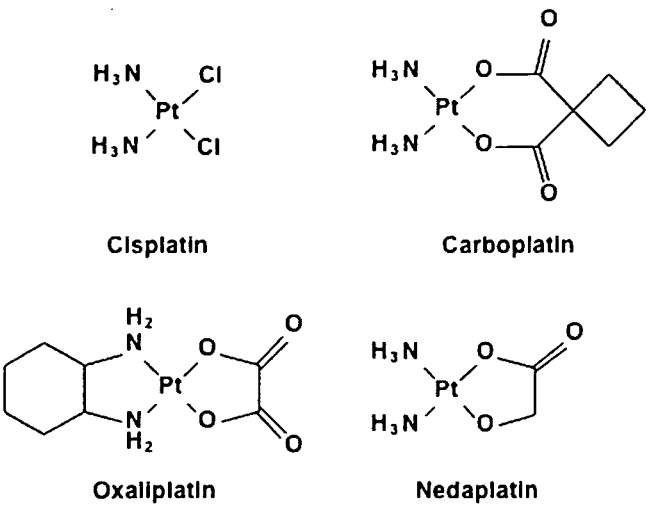

Fig. 1 – Chemical structures of four platinum agents.

and toxin extrusion) family [5]. These reports raise the possibility that substrate specificity of organic cation transporters determines the renal handling of platinum agents and the frequency/degree of nephrotoxicity.

The organic cation transporters transport drugs, toxins, and endogenous metabolites [6,7]. In the rat, rOCT1 (Slc22a1) is expressed in the liver and kidney [8], rOCT2 (Slc22a2) is expressed preferentially in the kidney [9], and rOCT3 (Slc22a3) is expressed predominantly in the placenta and weakly in the intestine, heart, brain, and kidney [10]. rOCT1 and rOCT2 are expressed in the basolateral membranes of proximal tubules and mediate the accumulation of various cationic drugs in proximal tubular epithelial cells from the circulation [6,7,11]. The membrane localization and physiological role of rOCT3 are not clearly understood. Rat multidrug and toxin extrusion 1 (rMATE1) was recently cloned and characterized in our laboratory [12]. It is considered to be expressed in the brush-border membrane of proximal tubules and to mediate tubular secretion of cationic drugs with an oppositely H^+-gradient as a driving force. These transporters are thought to play an important role in the renal handling of cationic drugs.

Based on these findings, we hypothesized that these tubular organic cation transporters were determinants of the extent to which platinum agents cause nephropathy. In this study, we compared the renal function and renal accumulation of platinum after the administration of platinum agents in rats. In addition, the substrate specificity of rOCT1-3, rMATE1, hMATE1, and hMATE2-K for platinum agents was also examined in the *in vitro* expression system.

2. Materials and methods

2.1. Animals

Male Wistar/ST rats (8 weeks) were purchased from SLC Animal Research Laboratories (Shizuoka, Japan). The rats were fed normal pellet food ad libitum, and given water freely. They were administered intraperitoneally with 2 mg/kg or 10 mg/kg of cisplatin (Randa®; Nippon Kayaku Co., Ltd., Tokyo, Japan), carboplatin (Paraplatin®; Bristol-Myers Squibb Co., Tokyo, Japan), oxaliplatin (Elplat®; Yakult Co., Ltd., Tokyo, Japan), or nedaplatin (Aqupla®; Shionogi & Co., Ltd., Osaka, Japan). These drug solutions were prepared at the concentration of 0.5 mg/ml. Control rats were administered with the same volume of saline. The day after the administration, rats were maintained in metabolic cages for 24 h to determine the urinary levels of creatinine and albumin, and urine output. Forty-eight hours after the administration of platinum agents, plasma, bladder urine, and kidneys were collected. The animal experiments were performed in accordance with the "Guidelines for Animal Experiments of Kyoto University." All protocols were previously approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

2.2. Renal functional and histological studies

For the measurement of creatinine, blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, we used commercial kits (Wako Pure Chemical Industries, Osaka, Japan). The concentration of urinary albumin was measured with an enzyme-linked immunosorbent assay (ELISA) kit (NEPHRAT II®; Exocell Inc., Philadelphia, PA). *N*-Acetyl-β-D-glucosaminidase (NAG) activity in bladder urine was measured using commercial kits (Shionogi). Kidneys were fixed in ethyl Carnoy's solution and stained with periodic acid–Schiff (PAS) reagent by Sapporo General Pathology Laboratory Co., Ltd. (Hokkaido, Japan).

2.3. Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA from rat kidney was isolated from specimens using a MagNA Pure LC RNA Isolation Kit-High Performance (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions, and the concentrations of total RNA were measured by spectrophotometry.

Total RNA was reverse-transcribed with random hexamers using Superscript II reverse-transcriptase (Invitrogen Life Technology Co., Carlsbad, CA), followed by digestion with RNase H (Invitrogen). These single-stranded DNA fragments were amplified according to the following profile immediately after an initial 4-min denaturation step at 95 °C: 94 °C for 15 s, 63 °C for 15 s, 72 °C for 30 s, 30 cycles for kidney injury molecule-1 (Kim-1), or 94 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min, 24 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specific primer sets for Kim-1 and GAPDH are shown in Table 1. The amplified PCR products were separated in a 2% agarose gel for Kim-1 or a 1.2% of agarose gel for GAPDH and stained with ethidium bromide.

2.4. Western blot analysis

Crude membrane fractions were prepared from rat kidneys as described previously [3]. The crude membrane fractions (25 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Immobilon-P®, Millipore, Bedford, MA) by semi-dry electroblotting. The blots were blocked and incubated overnight at 4 °C with primary antibody

Table 1 – Oligonucleotide sequences of PCR primers used for the determination of Kim-1 and GAPDH by RT-PCR

Primer	Sequence	Position
Kim-1 (BC061820)		
Forward	5′-ACTCCTGCAGACTGGAATGG-3′	641–660
Reverse	5′-CAAAGCTCAGAGAGCCCATC-3′	835–854
GAPDH (M17701)		
Forward	5′-CCTTCATTGACCTCAACTAC-3′	131–150
Reverse	5′-GGAAGGCCATGCCAGTGAGC-3′	705–724

PCR was performed as described in Section 2. Each position is from the sequence in the rat GenBank database and each number indicates the accession number in the Genbank database.

specific for osteopontin (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), or the Na^+/K^+-ATPase α1 subunit (Upstate Biotechnology Inc., Lake Placid, NY). The bound antibody was detected on X-ray film using enhanced chemiluminescence (ECL) with horseradish peroxidase-conjugated secondary antibodies and cyclic diacylhydrazides (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.5. *Platinum accumulation in kidney*

The excised kidneys were gently washed, weighed, and homogenized in 10 volumes of buffer containing 250 mM sucrose and 5 mM HEPES. The amount of platinum was determined using inductively coupled plasma-mass spectrometry (ICP-MS) by the Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan).

2.6. *Cell culture and transfection*

Human embryonic kidney (HEK) 293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Whittaker Bioproducts Inc., St. Louis, MO) in an atmosphere of 5% CO_2–95% air at 37 °C.

For a transient expression system, pBK-CMV plasmid vector DNA (Stratagene, La Jolla, CA) or pcDNA3.1(+) plasmid vector DNA (Invitrogen), containing rOCT1, rOCT2, rOCT3, rMATE1, hMATE1, or hMATE2-K was purified using Midi-V100™ Ultrapure Plasmid Extraction Systems (Viogene-Biotek Corporation, Sunnyvale, CA). The day before transfection, HEK293 cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cells were transfected as previously described [5]. Forty-eight hours after the transfection, the cells were used for the experiments.

2.7. *Uptake experiment*

Cellular uptake of [^{14}C]tetraethylammonium bromide (TEA) (2.035 GBq/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO) and [^{3}H]1-methyl-4-phenylpyridium acetate (MPP) (2.7 TBq/mmol, Perkin-Elmer Life Analytical Science, Boston, MA) was measured with monolayer cultures grown on poly-D-lysine-coated 24-well plates. The composition of the incubation buffer 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 adjusted with NaOH). As previously reported, experiments on the uptake by rOCT1-3 [13] and by rMATE1 [12], hMATE1 and hMATE2-K [14] were performed.

For measurement of the cellular accumulation of platinum agents, HEK293 cells transiently expressing transporters were seeded on poly-D-lysine-coated 24-well plates. Cells expressing rOCT1, rOCT2, or rOCT3 were incubated with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and cisplatin (Sigma), carboplatin (Sigma), oxaliplatin (a gift from Yakult), and nedaplatin (LKT Laboratories Inc., St. Paul, MN) for 1 h. Cells expressing rMATE1, hMATE1, or hMATE2-K were incubated with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and platinum agents for 2 min after pretreatment with 30 mM ammonium chloride for 20 min. After this incubation, the monolayers were rapidly washed twice with ice-cold incubation buffer containing 3% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) and

Table 2 – Biochemical parameters in rats treated with platinum agents (2 mg/kg)

	Control	Cisplatin	Carboplatin	Oxaliplatin	Nedaplatin
Body weight (g)	275 ± 4	274 ± 4	282 ± 2	274 ± 2	274 ± 2
Urinary volume (ml/24 h)	17.3 ± 5.4	18.0 ± 2.8	11.6 ± 0.76	13.9 ± 0.6	14.8 ± 1.5
Pcr (mg/dl)	0.45 ± 0.02	0.49 ± 0.04	0.42 ± 0.02	0.49 ± 0.03	0.41 ± 0.02
Ccr (ml/min)	1.65 ± 0.08	1.70 ± 0.13	2.09 ± 0.18	1.72 ± 0.12	1.97 ± 0.16
BUN (mg/dl)	13.1 ± 0.8	13.2 ± 0.5	12.2 ± 0.5	11.1 ± 0.6	11.2 ± 0.6
Urinary albumin (mg/day)	0.45 ± 0.12	1.12 ± 0.20	0.64 ± 0.20	0.65 ± 0.34	0.81 ± 0.19
AST (IU/l)	59.8 ± 5.3	66.4 ± 3.6	66.5 ± 3.3	60.8 ± 2.0	75.7 ± 3.7*
ALT (IU/l)	23.3 ± 1.0	22.1 ± 1.6	20.8 ± 0.8	20.3 ± 0.8	21.3 ± 1.6

Values represent means ± S.E.M. of nine rats. Pcr, plasma creatinine; Ccr, creatinine clearance; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase. *$P < 0.05$, significantly different from control.

Table 3 – Biochemical parameters in rats treated with platinum agents (10 mg/kg)

	Control	Cisplatin	Carboplatin	Oxaliplatin	Nedaplatin
Body weight (g)	289 ± 5	254 ± 6**	279 ± 3	274 ± 2*	277 ± 4
Urinary volume (ml/24 h)	12.9 ± 1.8	16.6 ± 2.5	15.0 ± 1.2	19.3 ± 5.5	19.9 ± 3.6
Pcr (mg/dl)	0.53 ± 0.06	0.90 ± 0.08*	0.43 ± 0.09	0.46 ± 0.02	0.44 ± 0.02
Ccr (ml/min)	1.40 ± 0.20	0.82 ± 0.17*	1.66 ± 0.43	1.53 ± 0.13	1.74 ± 0.10
BUN (mg/dl)	14.8 ± 1.0	40.3 ± 3.7	12.1 ± 0.8	13.0 ± 0.6	13.3 ± 0.7
Urinary albumin (mg/day)	0.48 ± 0.08	5.83 ± 1.09*	0.38 ± 0.10	0.44 ± 0.11	0.51 ± 0.09
AST (IU/l)	71.6 ± 2.9	78.9 ± 3.5	72.3 ± 5.1	109.9 ± 4.0**	85.9 ± 4.7
ALT (IU/l)	22.8 ± 0.9	29.3 ± 2.4**	23.4 ± 1.1	26.3 ± 0.7*	25.4 ± 0.9

Values represent means ± S.E.M. of nine rats. Pcr, plasma creatinine; Ccr, creatinine clearance; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase. *$P < 0.05$; **$P < 0.01$, significantly different from control.

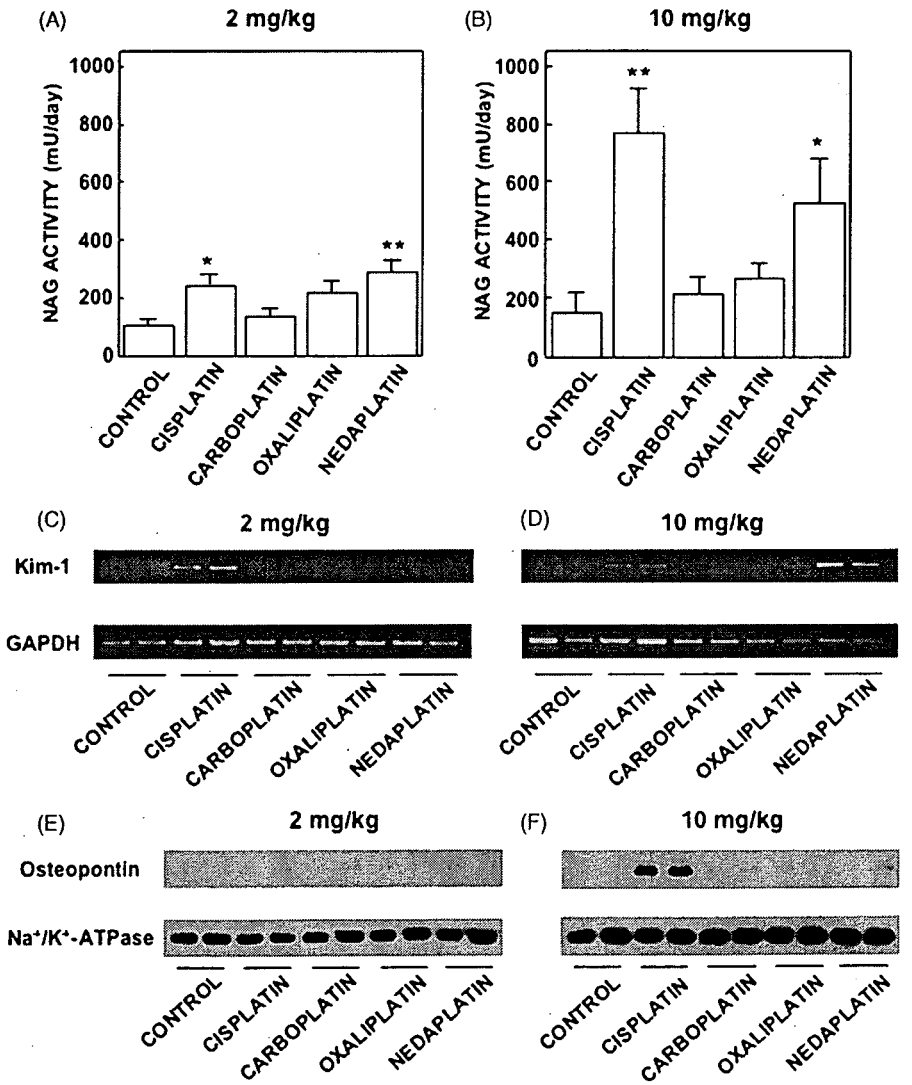

Fig. 2 – Tubular toxicity in rats treated with platinum agents. (A and B) The NAG activity in bladder urine at 2 days after the administration of platinum agents was measured. Control rats were administered the same volume of saline. Each bar represents the mean ± S.E.M. of nine rats. *$P < 0.05$; **$P < 0.01$, significantly different from control rats. (C and D) Total RNA from the kidney was reverse-transcribed, and the synthesized cDNA was amplified using a set of primers specific for Kim-1 or GAPDH. The PCR products were separated by electrophoresis through agarose gels, and stained with ethidium bromide. Representative photographs of RT-PCR are shown. (E and F) Protein expression of osteopontin and Na^+/K^+-ATPase α1 subunit in the kidney was examined by Western blotting. Representative photographs of Western blots are shown.

then washed three times with ice-cold incubation buffer. The cells were solubilized in 0.5N NaOH, and the amount of platinum was determined using ICP-MS.

2.8. Statistical analysis

Data are expressed as means ± S.E.M. Data were analyzed statistically using the unpaired Student's *t* test or multiple comparisons with Dunnett's two-tailed test after a one-way ANOVA. Probability values of less than 0.05 were considered statistically significant.

3. Results

3.1. Biochemical parameters

Biochemical parameters 2 days after the intraperitoneal administration of cisplatin, carboplatin, oxaliplatin, and nedaplatin at a dose of 2 mg/kg or 10 mg/kg are shown in Tables 2 and 3, respectively. Administration of 2 mg/kg of cisplatin tended to increase the urinary albumin level. In rats administered 10 mg/kg of cisplatin, the plasma creatinine level and urinary albumin level were significantly increased, and body weight and creatinine clearance were significantly decreased. There was not significant but potent increase in BUN level in rats administered 10 mg/kg of cisplatin. These parameters were not influenced by the administration of 2 mg/kg or 10 mg/kg of the other platinum agents. Statistically significant increase was observed in AST and ALT. However, the extent of increase of this study was assumed not to reflect hepatic dysfunction.

3.2. Proximal tubular dysfunction

As positive markers for proximal tubular injury, NAG activity in the bladder urine, and the mRNA expression of Kim-1 and the protein expression of osteopontin in the kidney were examined. The NAG activity was significantly increased in rats administered 2 mg/kg and 10 mg/kg of cisplatin and nedaplatin, but not carboplatin or oxaliplatin (Figs. 2A and B). By RT-PCR, we amplified the mRNA coding for Kim-1 in the kidney. When rats were administered with 2 mg/kg of cisplatin, the PCR product with the expected size of 214 bp for Kim-1 was detected (Fig. 2C). On treatment with 2 mg/kg of the other platinum agents, Kim-1 mRNA was not detected. Kim-1 mRNA was also observed in rat

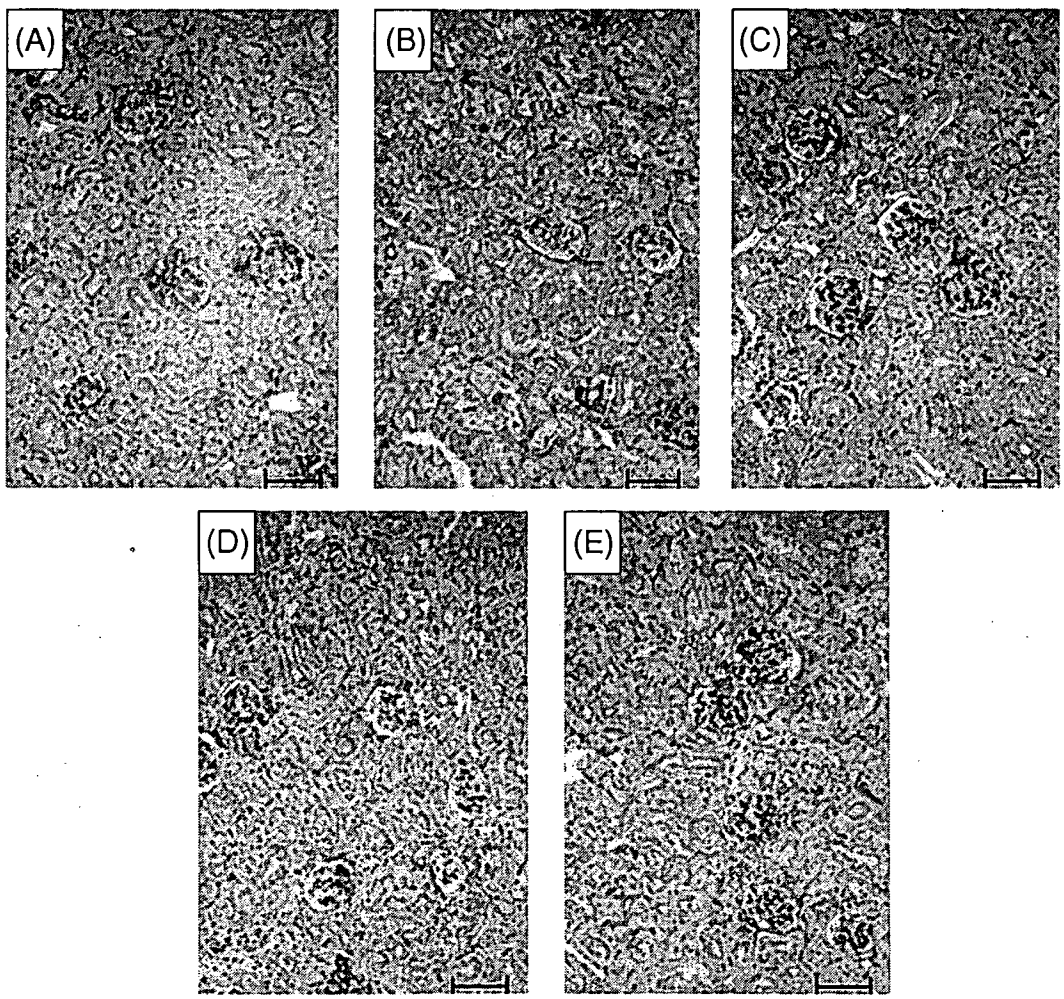

Fig. 3 – Histology of rat kidney. Kidney was obtained 2 days after the administration of 2 mg/kg of cisplatin (B), carboplatin (C), oxaliplatin (D), or nedaplatin (E). Control rats were administered the same volume of saline (A). PAS 200×. Scale bar: 100 μm. Only milder tubular cell vacuolation is seen in the kidney treated with 2 mg/kg of cisplatin (B).

kidney treated with 10 mg/kg of cisplatin and nedaplatin (Fig. 2D). Carboplatin and oxaliplatin did not affect the expression of Kim-1 mRNA. Western blotting was performed to detect osteopontin protein in the kidney (Figs. 2E and F). The expression of osteopontin was visualized in rats treated with cisplatin (10 mg/kg) compared to control rats. Except on administration of cisplatin (10 mg/kg), no osteopontin was found including in the control rats. No significant change was observed in the expression of the Na^+/K^+-ATPase α1 subunit among these kidneys.

3.3. *Renal histology*

Figs. 3 and 4 show paraffin-embedded sections of rat kidney with PAS staining. The pathology of the rat kidney treated with platinum agents was examined in the tubular cells. The degeneration of tubular cells including tubular dilatation, tubular cell vacuolation, tubular cell detachment from basement membrane and brush-border detachment, was most prominent in rats treated with 10 mg/kg of cisplatin (Fig. 4B). These changes were seen in a less degree in the rat kidney treated with 2 mg/kg of cisplatin (Fig. 3B). Similar changes were only focally seen in nedaplatin (10 mg/kg)-treated rat kidney (Figs. 4E and F), however, they were scarce or absent in the kidney treated with other agents.

3.4. *Accumulation of platinum in the kidney*

The renal accumulation of platinum was measured 2 days after the intraperitoneal administration of 2 mg/kg or 10 mg/kg of the agents (Fig. 5). When rats were administered with 2 mg/kg, the renal accumulation of cisplatin was markedly greater than that of any other drug (Fig. 5A). Moreover, at 10 mg/kg, the renal accumulation of cisplatin was also much greater than that of carboplatin, oxaliplatin or nedaplatin (Fig. 5B).

3.5. *Establishment of HEK293 cells transiently expressing organic cation transporters*

HEK293 cells were transfected with cDNA encoding rOCT1, rOCT2, rOCT3, rMATE1, hMATE1 or hMATE2-K. To check the expression of the transfected rOCT1, rOCT2, and rOCT3, the cellular uptake of typical substrates, 5 μM [^{14}C]TEA and

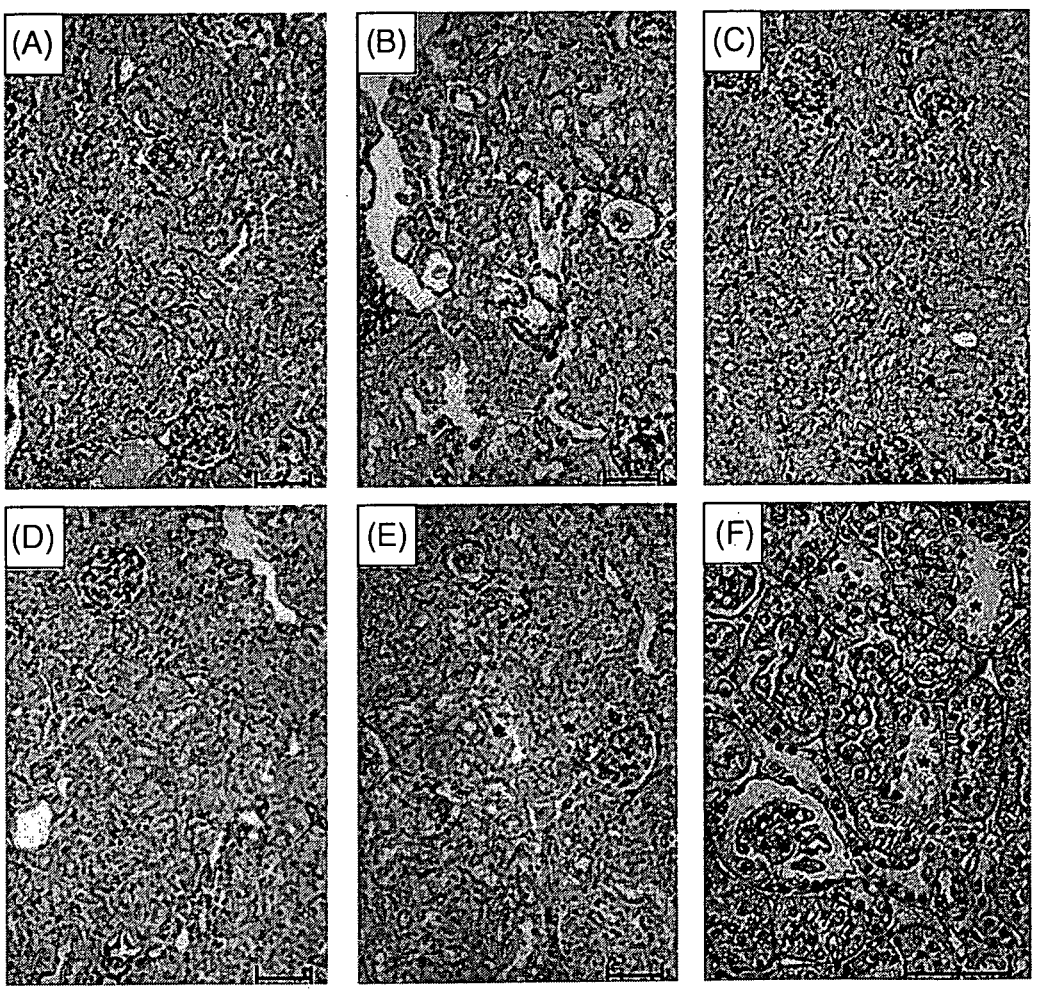

Fig. 4 – Histology of rat kidney. Kidney was obtained 2 days after the administration of 10 mg/kg of cisplatin (B), carboplatin (C), oxaliplatin (D), or nedaplatin (E and F). Control rats were administered the same volume of saline (A). (A–E) PAS 200×. (F) PAS 400×. Scale bar: 100 μm. Kidney treated with 10 mg/kg of cisplatin showed tubular degeneration (B), which is only focally seen in the kidney treated with 10 mg/kg of nedaplatin (E and F, asterisks).

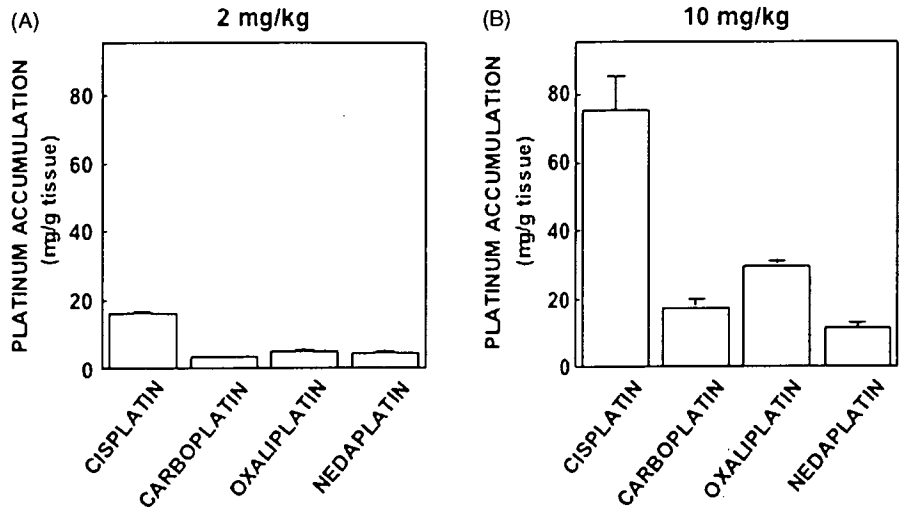

Fig. 5 – Platinum accumulation in the kidney. The kidney was excised 2 days after the administration of 2 mg/kg (A) or 10 mg/kg (B) of platinum agents, and homogenized in 10 volumes of buffer. Then, the renal accumulation of platinum was evaluated by ICP-MS. Each column represents the mean ± S.E.M. of nine rats.

13.7 nM [^{3}H]MPP, was measured (Fig. 6A and B). In the HEK293 cells expressing rMATE1, hMATE1, and hMATE2-K, intracellular acidification was achieved by pretreatment with ammonium chloride (Fig. 6C and D). The transport activity of the cells expressing these transporters was confirmed, and then, these expression systems were used in subsequent experiments on the cellular transport of platinum agents.

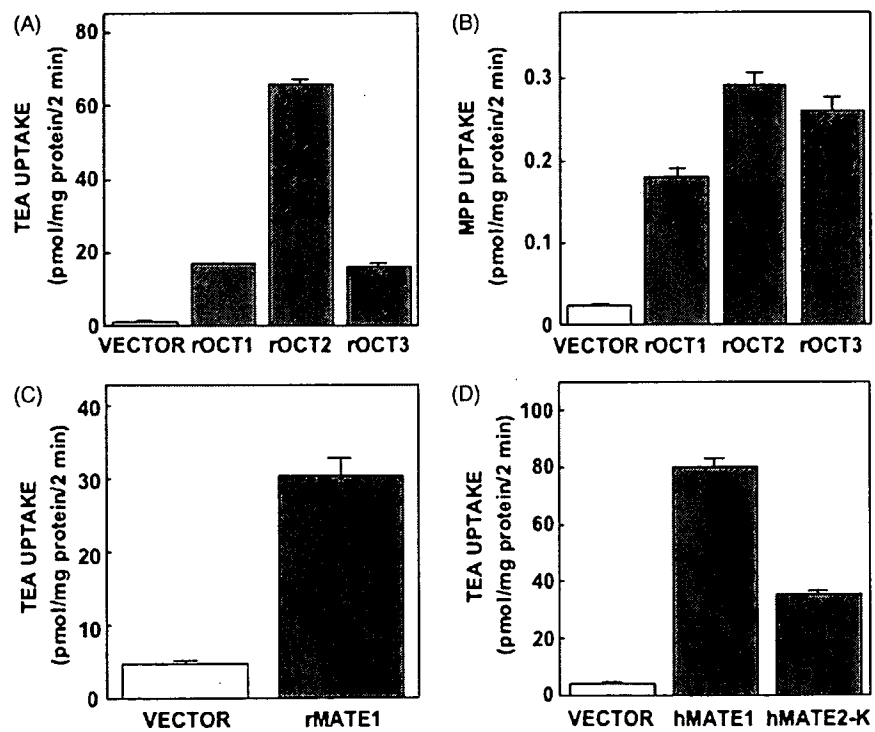

Fig. 6 – Uptake of [^{14}C]TEA or [^{3}H]MPP by rOCT1, rOCT2, rOCT3, or rMATE1 transiently expressed in HEK293 cells. (A and B) HEK293 cells transiently expressing rOCT1, rOCT2, or rOCT3 were preincubated with incubation buffer for 10 min. The buffer was removed, and then the cells were incubated with 5 μM [^{14}C]TEA (A) or 13.7 nM [^{3}H]MPP (B) for 2 min at 37 °C. (C and D) HEK293 cells transfected with rMATE1 (C), hMATE1, or hMATE2-K (D) were preincubated with 30 mM ammonium chloride for 20 min. After the removal of the preincubation buffer, the cells were incubated with 5 μM [^{14}C]TEA for 2 min at 37 °C. The amounts of substrates were determined by measuring the radioactivity of solubilized cells. Each column represents the mean ± S.E.M. of three or four wells.

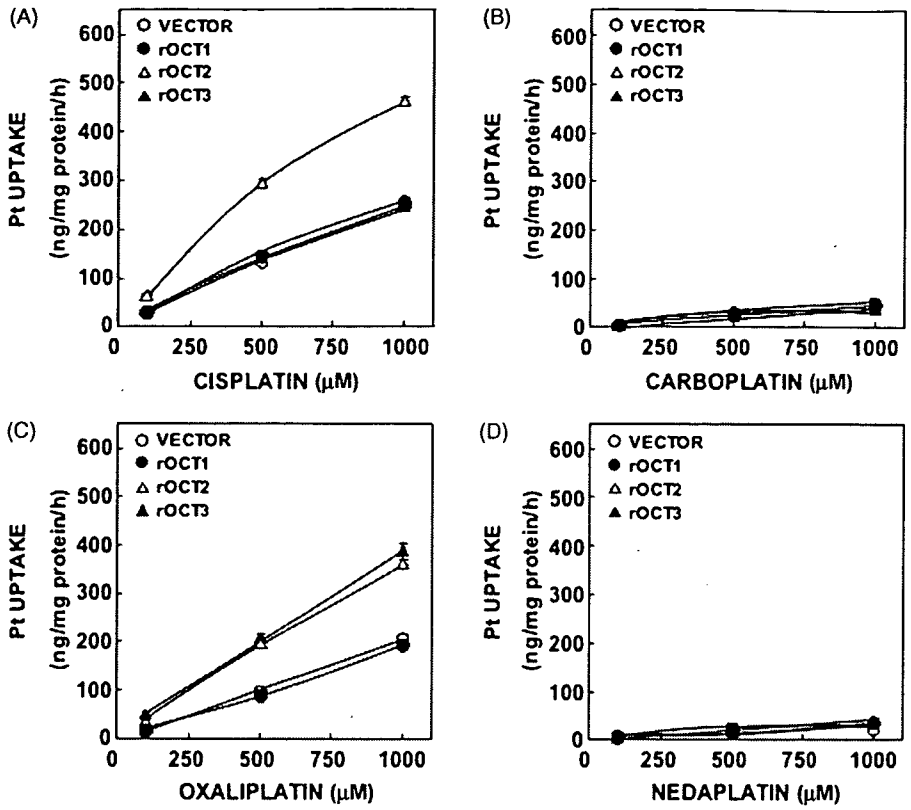

Fig. 7 – Uptake of platinum agents by HEK293 cells expressing rOCT1, rOCT2, or rOCT3. HEK293 cells were transfected with empty vector (open circle), rOCT1 (closed circle), rOCT2 (open triangle), or rOCT3 (closed triangle). The cells were treated with medium containing 100 μM, 500 μM and 1000 μM cisplatin (A), carboplatin (B), oxaliplatin (C), and nedaplatin (D) for 1 h. After washing, the cells were solubilized in 0.5N NaOH, and the amount of platinum was determined by ICP-MS. Each point represents the mean ± S.E.M. of four wells.

3.6. *Uptake of platinum agents by HEK293 cells transiently expressing organic cation transporters*

In vivo, renal toxicity of cisplatin, carboplatin, oxaliplatin, and nedaplatin were comparable with those accumulations in the kidney (Figs. 2–5, Tables 2 and 3). Therefore, the roles of organic cation transporters for cellular accumulation of platinum were examined in the HEK293 cells transiently expressing rOCT1, rOCT2, and rOCT3, which are basolateral type organic cation transporters, after incubation with medium containing platinum agents for 1 h (Fig. 7). The expression of rOCT2 stimulated the accumulation of platinum after the incubation with cisplatin and oxaliplatin (Fig. 7A and C). The accumulation of oxaliplatin was also enhanced in the HEK293 cells expressing rOCT3 (Fig. 7C). However, the expression of rOCT1 did not stimulate the accumulation of platinum after incubation with any platinum agents. In addition, no significant increase in platinum was observed in the cells expressing rOCT1, rOCT2, and rOCT3 after the treatment with carboplatin and nedaplatin (Fig. 7B and D).

To obtain more information on the tubular accumulation of platinum agents, we examined whether members of the MATE family mediate the cellular transport of platinum agents. Because the MATE transporters are activated by the oppositely generated H^+-gradient across the plasma membrane, the transporter-expressing cells were incubated with medium containing platinum agents for 2 min after pretreatment with ammonium chloride [12,14]. Among the four agents, only the uptake of oxaliplatin was significantly increased by the expression of rMATE1 (Fig. 8). The uptake of neither cisplatin nor nedaplatin was significantly increased by the rMATE1-expressing cells under the acidified intracellular conditions. The level of carboplatin accumulated in rMATE1-expressing cells was below the limit of detection. Because two isoforms against rMATE1 were identified in the human genome [14,15], we examined the transport of platinum agents in the HEK293 cells expressing hMATE1 and hMATE2-K. The expression of hMATE2-K markedly stimulated the H^+-gradient-dependent uptake of oxaliplatin-derived platinum, and hMATE1 significantly but weakly increased the intracellular accumulation of the drug (Fig. 9). There was no significant stimulation of the accumulation of platinum in the cells transfected with the hMATE1 and hMATE2-K cDNA after treatment with cisplatin, carboplatin, and nedaplatin, under conditions of ammonium chloride-generated intracellular acidification.

4. Discussion

Until now, no investigation has simultaneously compared the extent to which four platinum agents cause nephropathy *in vivo*. In this report, the nephropathy caused by platinum

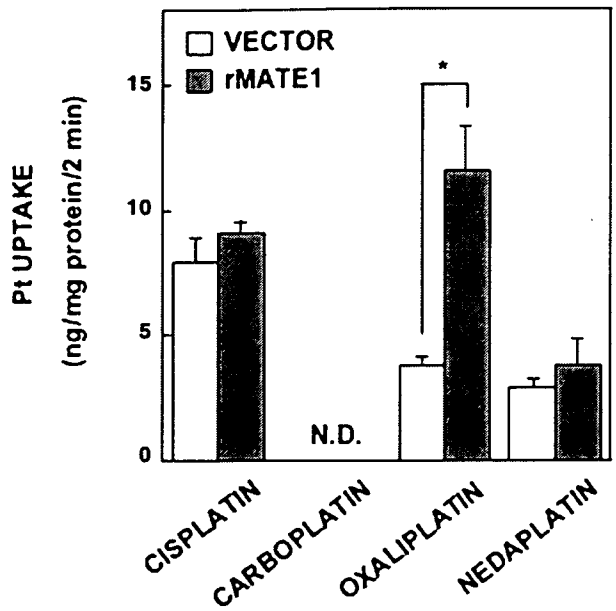

Fig. 8 – Uptake of platinum agents by HEK293 cells expressing rMATE1. HEK293 cells were transfected with empty vector (open column) or rMATE1 (gray column). The cells were treated with medium containing 500 μM platinum agents for 2 min after pretreatment with 30 mM of ammonium chloride for 20 min. The cells were washed three times, and solubilized in 0.5N NaOH. Then the amount of platinum was determined by ICP-MS. Each column represents the mean ± S.E.M. of four wells. N.D., not detected. *P < 0.05, significant differences.

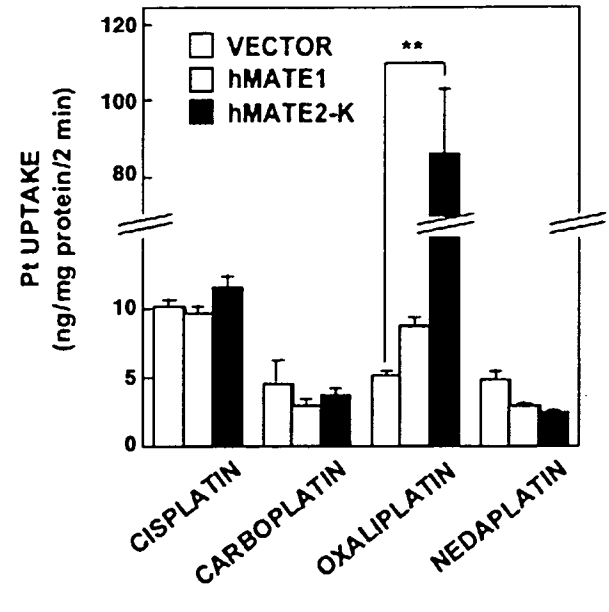

Fig. 9 – Uptake of platinum agents by HEK293 cells expressing hMATE1 or hMATE2-K. HEK293 cells were transfected with empty vector (open column), hMATE1 (gray column), or hMATE2-K (black column). The cells were treated with medium containing 500 μM platinum agents for 2 min after pretreatment with 30 mM of ammonium chloride for 20 min. The cells were washed three times, and solubilized in 0.5N NaOH. Then the amount of platinum was determined by ICP-MS. Each column represents the mean ± S.E.M. of four wells. **P < 0.01, significant differences.

agents, and the relationship between the nephrotoxicity and platinum accumulation in the kidney *in vivo* was examined. Based on the various biochemical parameters, carboplatin, oxaliplatin, and nedaplatin were shown to have lower nephrotoxicity than cisplatin, and as expected, the nephrotoxicity of the platinum agent was comparable with the renal accumulation of platinum (Figs. 2–5; Tables 2 and 3). Therefore, cisplatin was indicated to cause severe nephropathy, and its tendency to accumulate in the kidney would limit its dose escalation in the clinic. Previously, we found a role of rOCT2 in the renal distribution and tubular toxicity of cisplatin in rats, which was comparable with past finding *in vitro* [2,3,16]. Recently, oxaliplatin as well as cisplatin, but not carboplatin and nedaplatin, was found to be a substrate of tubular organic cation transporters, hOCT family and hMATE family [5]. The present results and our previous findings strongly suggest that renal accumulation via organic cation transporters determines the extent to which platinum agents cause nephropathy.

Cisplatin was transported by rOCT2, but not by rOCT1, rOCT3, or rMATE1 (Figs. 7A and 8). Therefore, the renal accumulation of cisplatin in the kidney was suggested to be mainly mediated by rOCT2. Moreover, it was presumed that the kidney-specific toxicity of cisplatin was a result of extensive accumulation by rOCT2 from the circulation and weak tubular secretion into the urine by rMATE1.

Oxaliplatin is a weakly nephrotoxic agent, whose spectrum of activity and mechanisms of action and resistance differ from those of cisplatin and carboplatin [17–19]. In this study, oxaliplatin was found to be a substrate of rOCT2 and rOCT3 (Fig. 7C). The extent to which cisplatin and oxaliplatin were transported by rOCT2 was similar, however, less oxaliplatin was accumulated in the kidney (Fig. 5). The luminal extrusion transporter rMATE1 mediated the oppositely generated H^+-gradient-dependent transport of oxaliplatin, suggesting extensive tubular secretion of oxaliplatin via rMATE1 at the brush-border membrane (Fig. 8). In addition, hMATE1 and hMATE2-K as well as hOCT2 mediated the transport of oxaliplatin (Fig. 9) [5]. Graham et al. [20] reported that the clearance of oxaliplatin was similar to or exceeded the average of the glomerular filtration rate (GFR) in humans, suggesting the tubular secretion of oxaliplatin. The vectorial transport of drugs across the proximal tubules is performed effectively by two distinct classes of transporters: one at the basolateral membranes mediating the cellular uptake of substrates from blood and the other at the brush-border membranes mediating the efflux of cellular substrates into the tubular lumen [21–23]. These findings and the background suggest that the basolateral OCT2 and luminal MATE play important roles in determining the pharmacokinetics of oxaliplatin and lowered renal accumulation of the drug both in rats and in humans. The roles of these transporters on platinum agent-induced nephrotoxicity would be confirmed in the future by using OCT2/MATE double transfected cells, or knockout mice of *OCT* or *MATE* gene.

In the present study, oxaliplatin was found to be a substrate of rOCT2 and rOCT3 (Fig. 7C) as well as hOCT2 and hOCT3 [5]. Because the membrane localization and pharmacokinetic role of rOCT3 are obscure, the pharmacological significance of the OCT3-mediated transport of oxaliplatin is not clear. However, the tissue distribution of OCT3 mRNA was broad compared to that of OCT1 and OCT2 [6,10]. Hayer-Zillgen et al. [24] reported that hOCT3, but not hOCT2, was expressed in some human tumor cell lines such as Ski-1 (human glioma), Caki-1 (human kidney carcinoma), and HepG-2 (human hepatoma). Although more pathophysiological studies are required, the hOCT3-expressing neoplasm may be a target of oxaliplatin.

In this study, the renal levels of carboplatin and nedaplatin were lower than that of cisplatin. In addition, the two agents were not transported by rOCT1-3 and rMATE1 (Figs. 7B, 7D and 8). It is indicated that neither is a substrate of tubular organic cation transporters, and therefore, neither is distributed into the proximal cells. Although there are a number of reports on the association between NAG activity, expression of Kim-1 or expression of osteopontin and tubular dysfunction [25–27], little is known with the superiority in platinum agent-induced nephropathy. Therefore, in this study, we simultaneously examined these three tubular toxicity markers to evaluate the platinum agent-induced tubulotoxicity. When rats were administered a low dose of cisplatin, these tubular markers were increased (Fig. 2) and showed milder degree of tubular injury (Fig. 3B) without a decrease in the level of GFR (Table 2). Considering the results after the administration of the higher dosage of cisplatin (Table 3 and Fig. 4B), cisplatin initially caused toxicity in the tubules, and thereafter a decrease of GFR. Administration of nedaplatin did not affect GFR (Tables 2 and 3), but increased some tubular toxicity markers (Fig. 2), which was in accordance with the pathological findings showing only focal tubular injury (Fig. 4E and F). It was reported that intravenous injection of 24 mg/kg of nedaplatin did not induce any morphological changes in the cortex [28]. Therefore, we speculate that nedaplatin did not induce renal failure despite the increases of NAG activity and mRNA expression of Kim-1, and the mechanism by which nedaplatin caused these phenomena may be different from that of cisplatin. It is not clear that the detailed mechanism of increase of NAG activity in rats treated with nedaplatin, and therefore, the transcriptome or proteome analyses would clarify some signal pathways.

In rats, only rMATE1 has been cloned and characterized as a H^+/organic cation antiporter in the brush-border membrane of proximal tubules. In humans, hMATE1 [15] and hMATE2-K [14] have been characterized and suggested to play a role in the secretion of cationic drugs from proximal tubules. The cellular accumulation of both cisplatin and oxaliplatin was weakly but significantly increased by the expression of hMATE1 and hMATE2-K [5]. In that study, the cells were incubated for 1 h in a culture medium containing platinum agents without an artificial H^+-gradient prior to the overnight culture in the cytotoxicity experiment. In the present study, we investigated the uptake of platinum by hMATE1- and hMATE2-K-expressing cells with an artificial H^+-gradient in the hope of finding more obvious differences. On pretreatment with ammonium chloride, remarkable transport of oxaliplatin by hMATE2-K and significant transport by hMATE1 were observed. We previously indicated that the inhibitory effect of oxaliplatin was much stronger on the uptake of [^{14}C]TEA by hMATE2-K than by hMATE1 [5]. It was indicated that oxaliplatin was a good substrate for hMATE2-K in humans. In addition, it was also transported by hMATE1. Moreover, cisplatin may be slightly transported by hMATE1 and hMATE2-K. It is suggested that not only in rats but also in humans, the MATE family is closely related to low nephrotoxicity with lowered renal accumulation of oxaliplatin.

In the present study, whether a platinum agent is a substrate of tubular organic cation transporters (the OCT and MATE families) was closely associated with the renal accumulation and subsequent nephrotoxicity of that agent. Despite that basolateral rOCT2 mediated the renal distribution of cisplatin and oxaliplatin, oxaliplatin was a superior substrate of the luminal rMATE1, and did not show nephrotoxicity in the rats compared to cisplatin. Carboplatin and nedaplatin were not substrates for these transporters. In conclusion, the nephrotoxicity of platinum agents depends on the amount of platinum accumulated in the kidneys, and organic cation transporters could play the predominant role in determining the extent of the nephropathy caused by these agents.

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Cl^--dependent upregulation of human organic anion transporters: different effects on transport kinetics between hOAT1 and hOAT3

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Ueo H, Motohashi H, Katsura T, Inui K. Cl^--dependent upregulation of human organic anion transporters: different effects on transport kinetics between hOAT1 and hOAT3. *Am J Physiol Renal Physiol* 293: F391–F397, 2007. First published April 11, 2007; doi:10.1152/ajprenal.00376.2006.—Chloride ion has a stimulatory effect on the transport of organic anions across renal basolateral membranes. However, the exact mechanisms at molecular levels have been unclear as of yet. Human organic anion transporters hOAT1 and hOAT3 play important roles in renal basolateral membranes. In this study, the effects of Cl^- on the activities of these transporters were evaluated by using HEK293 cells stably expressing hOAT1 or hOAT3 (HEK-hOAT1 or HEK-hOAT3). The uptake of *p*-[^{14}C]aminohippurate by HEK-hOAT1 and [^{3}H]estrone sulfate by HEK-hOAT3 was greater in the presence of Cl^- than in the presence of SO_4^{2-} or gluconate. Additionally, the uptake of various compounds by HEK-hOAT1 and HEK-hOAT3 was significantly higher in the Cl^--containing medium than the gluconate-containing medium, suggesting that the influences of Cl^- are not dependent on substrate and that Cl^- directly stimulates the functions of hOAT1 and hOAT3. The substitution of gluconate with Cl^- did not change the K_m value for the uptake of *p*-[^{14}C]aminohippurate by HEK-hOAT1 but caused an approximately threefold increase in the maximal uptake rate (V_{max}) value. On the other hand, replacement of gluconate with Cl^- decreased the K_m value for the uptake of [^{3}H]estrone sulfate and cefotiam by HEK-hOAT3 to about one-third, while it did not change the V_{max} value. In summary, Cl^- upregulates the activities of both hOAT1 and hOAT3, but its effects on transport kinetics differ between these transporters. It was suggested that Cl^- participates in the *trans*-location process for hOAT1, and the substrate recognition process for hOAT3.

renal secretion; basolateral membrane; SLC22A; HEK293

THE ORGANIC ANION TRANSPORT systems in renal proximal tubules play important physiological roles in the excretion of a wide variety of anionic compounds, including endogenous substances, xenobiotics, and their metabolites, into the urine. These systems are highly effective and mediate the tubular secretion of various drugs. Tubular secretion in the proximal epithelia consists of two processes, uptake at the basolateral membrane and efflux at the brush-border membrane. Numerous studies have been performed regarding these transport systems (2, 7, 15).

Chloride ion is the most abundant anion in the blood and is involved in various physiological processes, including the regulation of cell volume, regulation of intracellular pH, and maintenance of blood osmolality. In our previous study (8), the effects of Cl^- on the basolateral transport of organic anions in the proximal tubules were investigated using rat renal basolateral membrane vesicles. The substitution of Cl^- on both sides of the vesicles with SCN^- or SO_4^{2-} decreased the uptake of *p*-aminohippurate into the vesicles, suggesting that Cl^- plays an important role in the organic anion transport systems in basolateral membranes.

Several organic anion transporters have been identified in renal basolateral membranes (3, 6). The mRNA levels of human organic anion transporter (hOAT)-1 and hOAT3 are much higher than those of other organic ion transporters in the human kidney cortex, and both transporters are located at the basolateral membranes (12). These findings indicated that hOAT1 and hOAT3 play important roles in the tubular uptake of various drugs from the circulation. However, little is known about the influences of Cl^- on hOAT1 and hOAT3 and their relation to renal drug secretion.

The purpose of this study is to clarify whether Cl^- affects hOAT1 and hOAT3 activities. Using cells expressing these transporters, we found differences in Cl^--dependent regulation between hOAT1 and hOAT3.

MATERIALS AND METHODS

Materials. *p*-[Glycyl-1-^{14}C]aminohippurate (1.9 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA). [6,7-^{3}H(*N*)]estrone sulfate, ammonium salt (2.1 TBq/mmol), was from PerkinElmer Life Sciences (Boston, MA). [*N*-methyl-^{3}H]cimetidine (451 GBq/mmol) was from Amersham Biosciences (Uppsala, Sweden). [3′,5′,7′-^{3}H(*N*)]methotrexate, disodium salt (851 GBq/mmol), and [^{3}H(G)]ochratoxin A (666 GBq/mmol) were from Moravek Biochemicals (Brea, CA). [^{14}C]captopril (115 MBq/mmol) was kindly provided by Sankyo (Tokyo, Japan). Cefotiam was from Takeda Chemical Industries (Osaka, Japan). Probenecid and aspartate aminotransferase were from Sigma Chemical (St. Louis, MO). Malate dehydrogenase was from Toyobo (Osaka, Japan). NADH was from Nacalai Tesque (Kyoto, Japan). 6-Methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) was from Biotium (Hayward, CA). All other chemicals used were of the highest purity available.

Uptake of radiolabeled compounds by HEK293 cells stably expressing hOAT1 or hOAT3. According to our previous report (21), uptake experiments were carried out using HEK293 cells stably expressing hOAT1 (HEK-hOAT1) or hOAT3 (HEK-hOAT3). Cells transfected with empty vector (HEK-pBK) were used as control cells. HEK-pBK, HEK-hOAT1, and HEK-hOAT3 were seeded on poly-D-lysine-coated 24-well plates at a density of 2×10^5 cells/well. At 48 h after seeding, the cells were used for the uptake experiments.

In the experiments on the effects of extracellular anions on the uptake of *p*-[^{14}C]aminohippurate and [^{3}H]estrone sulfate by HEK-hOAT1 and HEK-hOAT3, respectively, the composition of the incubation medium was as follows (in mM): 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, 5 HEPES, and the salt indicated [145 NaCl, 145 NaBr,

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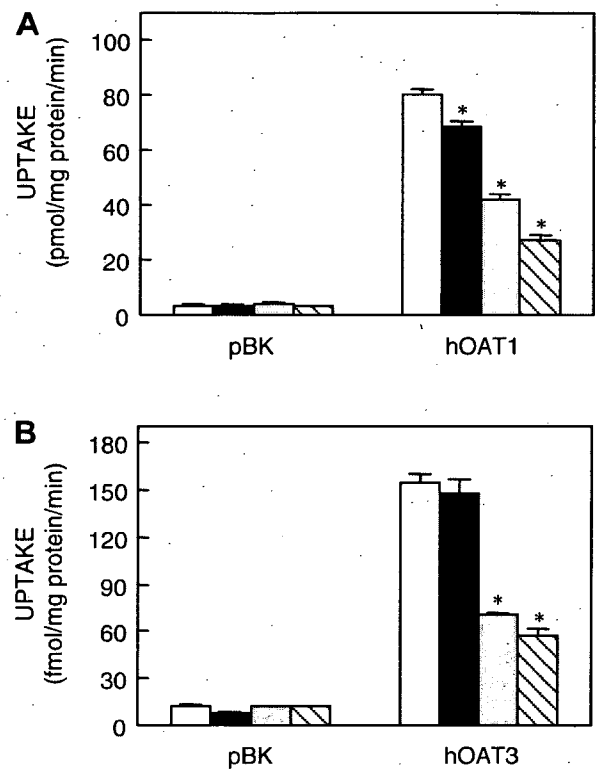

Fig. 1. Effects of extracellular anions on the uptake of *p*-[^{14}C]aminohippurate by HEK-hOAT1 (*A*) and [^{3}H]estrone sulfate by HEK-hOAT3 (*B*). HEK-pBK, HEK-hOAT1, and HEK-hOAT3 were incubated with 5 μM *p*-[^{14}C]aminohippurate (*A*) or 10 nM [^{3}H]estrone sulfate (*B*) in the presence of 145 mM Cl^- (open column), 145 mM Br^- (black column), 72.5 mM SO_4^{2-} (shaded column), or 145 mM gluconate (hatched column) at 37°C for 1 min. Each column represents the mean ± SE of 3 monolayers from a typical experiment. hOATs, human organic anion transporters; HEK-hOAT1, HEK293 cells stably expressing hOAT1; HEK-hOAT3, HEK293 cells stably expressing hOAT3; HEK-pBK, cells transfected with empty vector. *$P < 0.05$, significantly different from Cl^-.

72.5 Na_2SO_4, or 145 Na gluconate for each incubation medium (pH 7.4)]. In the experiments on the effects of extracellular Cl^-, the composition of Cl^--free [$Cl^-(-)$] incubation medium was as follows (in mM): 145 Na gluconate, 3 K gluconate, 1 Ca gluconate, 0.5 $MgSO_4$, 5 D-glucose, and 5 HEPES (pH 7.4). And the composition of Cl^--containing [$Cl^-(+)$] incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). After preincubation of the cells with 0.2 ml of $Cl^-(+)$ incubation medium at 37°C for 10 min, the medium was replaced with 0.2 ml of each incubation medium containing test compounds. At the end of the incubation, the medium was aspirated, and then cells were washed two times (3 times for the uptake of ochratoxin A) with 1 ml of ice-cold incubation medium.

In the experiments on the effects of Cl^- at various concentrations, for which intracellular and extracellular Cl^- were equilibrated, the composition of $Cl^-(-)$ high-K medium was as follows (in mM): 25 Na gluconate, 120 K gluconate, 1 Ca gluconate, 0.5 $MgSO_4$, 5 D-glucose, and 5 HEPES (pH 7.4). And the composition of $Cl^-(+)$ high-K medium was as follows (in mM): 25 NaCl, 120 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). The high-K media containing each concentration of Cl^- were made by mixture of these solutions. After preincubation of the cells with 0.2 ml of high-K medium containing each concentration of Cl^-, 5 μM nigericin, and 10 μM tributyltin at 37°C for 10 min, the cells were incubated with 0.2 ml of each medium containing 5 μM nigericin, 10 μM tributyltin, and test compound for 1 min. At the end of the incubation, the medium was aspirated, and then cells were washed two times with 1 ml of ice-cold $Cl^-(+)$ incubation medium.

After uptake was finished, the cells were lysed in 0.25 ml 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 (1) using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) with bovine γ-globulin as a standard.

Uptake of cefotiam by HEK293 cells stably expressing hOAT1 or hOAT3. The uptake of cefotiam was examined as described previously (21) with some modifications. Briefly, HEK-pBK, HEK-hOAT1, and HEK-hOAT3 were seeded on poly-D-lysine-coated 35-mm dishes at a density of 1×10^6 cells/dish. At 48 h after seeding, the cells were used for the uptake experiments. The composition of the incubation medium was as described above. After preincubation of the cells with 1 ml of $Cl^-(+)$ incubation medium at 37°C for 10 min, the medium

Table 1. *Effects of extracellular Cl^- on the uptake of various compounds by HEK-hOAT1 and HEK-hOAT3*

Compound	Medium	Uptake, μl/mg protein		
		HEK-pBK	HEK-hOAT1	HEK-hOAT3
p-Aminohippurate (5 μM, 15 min)	Cl^- (−)	1.38±0.02	3.54±0.11	2.98±0.04
	Cl^- (+)	3.04±0.11	18.25±1.05*	5.48±0.07*
Estrone sulfate (10 nM, 15 min)	Cl^- (−)	2.67±0.10	1.72±0.05	11.71±0.32
	Cl^- (+)	2.37±0.08	1.78±0.06	23.59±1.02*
Cimetidine (40 nM, 1 min)	Cl^- (−)	1.19±0.06	1.36±0.01	1.56±0.05
	Cl^- (+)	1.06±0.07	1.57±0.03*	3.94±0.08*
Methotrexate (20 nM, 1 min)	Cl^- (−)	1.32±0.04	2.03±0.01	1.96±0.13
	Cl^- (+)	0.63±0.03	4.10±0.24*	2.52±0.14*
Captopril (200 μM, 5 min)	Cl^- (−)	0.60±0.02	1.47±0.05	1.01±0.03
	Cl^- (+)	0.36±0.01	3.13±0.12*	1.15±0.02
Ochratoxin A (10 nM, 5 min)	Cl^- (−)	3.97±0.45	11.82±2.05	10.14±0.16
	Cl^- (+)	3.14±0.14	45.23±0.84*	16.72±0.61*
Cefotiam (500 μM, 30 min)	Cl^- (−)	0.066±0.014	0.071±0.012	0.354±0.034
	Cl^- (+)	0.026±0.001	0.028±0.003	0.537±0.009*

HEK-pBK, HEK-hOAT1, and HEK-hOAT3 were incubated in Cl^--free (−) or Cl^--containing (+) incubation medium with specified concentrations of various compounds at 37°C for specified periods. Each value represents the mean ± SE of 3 separate experiments. HEK-hOAT1 and HEK-hOAT3, HEK293 cells stably expressing human organic anion transporters-1 and -3, respectively; HEK-pBK, HEK293 cells transfected with empty vector. *$P < 0.05$, significantly higher than the uptake in Cl^--free incubation medium.