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Substrate specificity of MATE1 and MATE2-K, human multidrug and toxin extrusions/H^+-organic cation antiporters

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ABSTRACT

The substrate specificities of human (h) multidrug and toxin extrusion (MATE) 1 and hMATE2-K were examined to find functional differences between these two transporters by the transfection of the cDNA of hMATE1 and hMATE2-K into HEK293 cells. Western blotting revealed specific signals for hMATE1 and hMATE2-K consistent with a size of 50 and 40 kDa, respectively, in the transfectants as well as human renal brush-border membranes under reducing conditions. In the presence of oppositely directed H^+-gradient, the transport activities of various compounds such as tetraethylammonium, 1-methyl-4-phenylpyridinium, cimetidine, metformin, creatinine, guanidine, procainamide, and topotecan were stimulated in hMATE1- and hMATE2-K-expressing cells. In addition to cationic compounds, anionic estrone sulfate, acyclovir, and ganciclovir were also recognized as substrates of these transporters. Kinetic analyses demonstrated the Michaelis–Menten constants for the hMATE1-mediated transport of tetraethylammonium, 1-methyl-4-phenylpyridinium, cimetidine, metformin, guanidine, procainamide, topotecan, estrone sulfate, acycrovir, and ganciclovir to be (in mM) 0.38, 0.10, 0.17, 0.78, 2.10, 1.23, 0.07, 0.47, 2.64, and 5.12, respectively. Those for hMATE2-K were 0.76, 0.11, 0.12, 1.98, 4.20, 1.58, 0.06, 0.85, 4.32, and 4.28, respectively. Although their affinity for hMATE1 and hMATE2-K was similar, the zwitterionic cephalexin and cephradine were revealed to be specific substrates of hMATE1, but not of hMATE2-K. Levofloxacin and ciprofloxacin were not transported, but were demonstrated to be potent inhibitors of these transporters. These results suggest that hMATE1 and hMATE2-K function together as a detoxication system, by mediating the tubular secretion of intracellular ionic compounds across the brush-border membranes of the kidney.

1. Introduction

The oppositely directed H^+-gradient was demonstrated to be a driving force for the uptake of tetraethylammonium (TEA), a prototype substrate of the renal organic cation transport system, by rat renal brush-border membrane vesicles [1]. After that, various cationic drugs, toxins, and endogenous metabolites were revealed to be transported by the luminal H^+/

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Abbreviations: hOCT, human organic cation transporter; hMATE, human multidrug and toxin extrusion; MPP, 1-methyl-4-phenylpyridinium; TEA, tetraethylammonium

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organic cation antiport system [2,3]. However, the molecular identification of the luminal H^+/organic cation antiport system had not been successful for more than 20 years.

A human (h) orthologue of the bacterial multidrug and toxin extrusion (MATE) family, hMATE1, was identified as a H^+-coupled organic cation exporter [4]. Recently, we have isolated a kidney-specific homologue, hMATE2-K [5]. Functional analyses using mammalian cells transfected with hMATE1 cDNA suggested that hMATE1 transported cationic compounds, TEA and 1-methyl-4-phenylpyridinium (MPP). It was also demonstrated that the hMATE1-mediated transport of TEA was inhibited by other compounds such as cimetidine [4]. We reported that rat (r) MATE1 transported not only cationic compounds but also zwitterionic or anionic compounds [6], and hMATE2-K transported various cationic compounds [5]. Although we reported that the anticancer drug oxaliplatin was preferentially transported by hMATE2-K [7,8], little is known about the physiological and pharmacological roles of hMATE1 and hMATE2-K, especially regarding the recognition of substrates.

In the present study, we have simultaneously demonstrated the substrate specificity of hMATE1 and hMATE2-K, by measuring the activity to transport for various ionic compounds, and found some substrates to be specific for hMATE1.

2. Materials and methods

2.1. Materials

[^{14}C]TEA (2.035 GBq/mmol), [^{14}C]creatinine (2.035 GBq/mmol), [^{14}C]procainamide (2.035 GBq/mmol), [methyl-^{14}C]-choline (2.035 GBq/mmol), [9-^{3}H]quinidine (740 GBq/mmol), [^{3}H]quinine (740 GBq/mmol), L-[*N*-methyl-^{3}H]carnitine (3.145 TBq/mmol), [*N*-methyl-^{14}C]nicotine (2.035 TBq/mmol), [*N*-methyl-^{3}H]verapamil (2.96 TBq/mmol), and [7-^{3}H (N)]tetracycline (185 GBq/mmol) were obtained from American Radiolabeled Chemicals Inc (St. Louis, MO). [^{14}C]Metformin (962 MBq/mmol), [^{14}C]guanidine hydrochloride (1.961 GBq/mmol), [^{3}H]ochratoxin A (666 GBq/mmol), [^{14}C]uric acid (1.961 GBq/mmol), [^{14}C]glysylsarcosine (4.07 GBq/mmol), [^{3}H]valproic acid (2.035 TBq/mmol), [^{3}H]acyclovir (407 GBq/mmol), [^{3}H]ganciclovir (370 GBq/mmol), [^{3}H]9-(2-phosphonylmethoxyethyl)-adenine (adefovir; 333 GBq/mmol), [^{3}H](S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]-cytosine (cidofovir; 555 GBq/mmol), and [^{3}H]9-(2-phosphonylmethoxypropyl)-adenine (tenofovir; 370 GBq/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA). [^{3}H]MPP (2.7 TBq/mmol), [^{14}C]para-aminohippuric acid (1.9 GBq/mmol), [^{3}H]dehydroepiandrosterone sulfate (2.22 TBq/mmol), [^{14}C]salicylic acid (1.7 GBq/mmol), [^{14}C]indomethacin (740 MBq/mmol), [^{3}H]prostaglandin F_2 alpha (6.66 TBq/mmol), and [^{3}H]estrone sulfate (2.12 TBq/mmol) were from PerkinElmer Life Analytical Sciences (Boston, MA). [*N*-Methyl-^{3}H]Cimetidine (451 GBq/mmol) was from Amersham Biosciences (Uppsala, Sweden). Acyclovir was obtained from Sigma (St. Louis, MO). [^{14}C]Levofloxacin (1.07 GBq/mmol, Daiichi Pharmaceutical Co., Tokyo, Japan), [^{14}C]captopril (0.115 GBq/mmol, Sankyo Co., Tokyo, Japan), cephalexin (Shionogi, Osaka, Japan), cefazolin (Astellas Pharma Inc., Tokyo, Japan), cephradine (Sankyo Co., Tokyo, Japan), ciprofloxacin hydrochloride (Bayer AG, Leverkusen, Germany), [^{14}C]SK&F 104864 (topotecan; 1.78 GBq/mmol, Nippon Kayaku Co., Tokyo, Japan), and unlabeled SK&F 104864 (topotecan; Nippon Kayaku Co., Tokyo, Japan) were kindly provided by the respective suppliers. All other chemicals used were of the highest purity available.

2.2. Cell culture and transfection

HEK293 cells (American Type Culture Collection CRL-1573, 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 (Invitrogen, Carlsbad, CA) in an atmosphere of 5% CO_2/95% air at 37 °C, and used as host cells. hMATE1 and hMATE2-K cDNAs were subcloned into the pcDNA3.1/Hygro (+) plasmid vector (Invitrogen) as previously described [7]. HEK293 cells were transfected with hMATE1 cDNA, hMATE2-K cDNA, or pcDNA3.1 (+) empty vector using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, the cells were used for uptake experiments. To construct transfectants stably expressing hMATE1 or hMATE2-K, hygromycin B (0.2 mg/mL, Invitrogen) -resistant single colonies were picked out. Cells expressing hMATE1 (HEK-hMATE1) or hMATE2-K (HEK-hMATE2-K) were selected by measuring [^{14}C]TEA uptake. The stable transfectant of pcDNA3.1 (+) empty vector (HEK-VECTOR) was established as previously described [9]. The cell monolayers at day 3 of culture were used for uptake experiments.

2.3. Antibodies and Western blot analysis

Rabbit anti-hMATE1 and hMATE2-K antibodies were prepared as described previously [5]. For the Western blot analysis, crude plasma membranes (20 μg) were obtained from HEK293 cells stably transfected with vector alone (pcDNA3.1 (+)), hMATE1, and hMATE2-K, and were prepared as described previously with some modifications [9]. The brush-border membrane fractions were obtained from human renal cortex as described previously [10,11]. The membrane fractions were suspended in buffer (100 mM mannitol and 10 mM HEPES-KOH, pH 7.5) and solubilized in NuPAGE® LDS sample buffer (Invitrogen) with or without 50 mM dithiothreitol and heated at 70 °C for 10 min. The samples were separated by SDS-polyacrylamide gel electrophoresis (4–12% NuPAGE® Novex Bis–Tris Gel; Invitrogen) and transferred to polyvinylidene difluoride membranes (PVDF membrane 0.2 μm; Invitrogen) using an XCell II® Blot Module (Invitrogen) according to the manufacturer's instructions. The blots were incubated with antisera specific for hMATE1 (1:1000 dilution) and hMATE2-K (1:2000), respectively. The bound antibody was detected on X-ray film by enhanced chemiluminescence with horseradish peroxidase-conjugated anti-rabbit IgG antibody and cyclic diacylhydrazides (GE Healthcare Bio-Science Corp., Piscataway, NJ). To confirm the specificity of polyclonal antibodies, each antibody was absorbed with an excess amount of antigen peptide (20 μg/mL) used as an immunogen and processed similarly.

2.4. Uptake experiments

Cellular uptake of ionic compounds was measured with cultures of HEK293 cells grown on poly-D-lysine-coated 24-well plates [9,12]. Typically, the cells were preincubated with 0.2 mL of incubation medium (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) for 10 min at 37 °C. The medium was then removed, and 0.2 mL of incubation medium containing radiolabeled substrates was added. The medium was aspirated off after the defined time for incubation, and the monolayers were rapidly rinsed three times with 1 mL of ice-cold incubation medium. The cells were solubilized in 0.5 mL of 0.5N NaOH, and then the radioactivity in aliquots was measured by liquid scintillation counting. The cellular uptake of cephalexin, cephradine, and cefazolin was performed as described previously [5,13]. To manipulate the intracellular pH, intracellular acidification was performed by pretreatment in the incubation medium with ammonium chloride (30 mM, 20 min at 37 °C, pH 7.4) [14,15]. When extracellular pH was varied between 6.0 and 9.0, buffering agents were MES (2-(N-morpholino)ethanesulfonic acid) for pH 6.0, 6.5 and 7.0, and HEPES for pH 7.5, 8.0, 8.5 and 9.0. The protein concentration of the solubilized cells was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine gamma-globulin as a standard.

2.5. Statistical analysis

Data are expressed as the mean ± S.E. Data were analyzed statistically using an unpaired t test. Significance was set at $P < 0.05$. In all figures, when error bars are not shown, they are smaller than the symbols.

3. Results

3.1. Substrate screening of hMATE1 and hMATE2-K

The uptake of various organic ions was examined in HEK293 cells transiently transfected with the pcDNA3.1 (+) empty vector, hMATE1 cDNA, and hMATE2-K cDNA, respectively. The uptake experiment at the extracellular pH 8.4 showed the good substrates of rMATE1 and hMATE2-K, and the relatively weak substrates for both transporters were confirmed by the uptake experiment after pretreatment of ammonium chloride generating the intracellular acidification [5,6]. Therefore, we employed these two conditions to demonstrate the substrate specificities of hMATE1 and hMATE2-K. The transfection of hMATE1 or hMATE2-K cDNA stimulated the oppositely-directed H^+-gradient-dependent transport of structurally diverse organic cations such as [^{14}C]TEA, [^{3}H]MPP, [^{3}H]cimetidine, [^{14}C]metformin, [^{14}C]creatinine, [^{14}C]guanidine, [^{14}C]procainamide, and [^{14}C]topotecan at the extracellular pH 8.4 (Table 1). The intracellular acidification resulted in marked stimulation of uptake compared to the change of extracellular pH (Table 2). The uptake of an anionic compound, [^{3}H]estrone sulfate, was also enhanced in the cells transfected with the hMATE1- or hMATE2-K-cDNA in both conditions. The uptake of [^{3}H]acyclovir was markedly increased in the hMATE1- and hMATE2-K-expressing cells after the pretreatment with ammonium chloride (Tables 1 and 2). The uptake of

Table 1 – Uptake of various organic ions in HEK293 cells expressing hMATE1 and hMATE2-K at extracellular pH 8.4

Compounds	Concentration (μM)	Uptake (μl/mg protein/15 min)		
		Vector	hMATE1	hMATE2-K
[^{14}C]TEA	5.0	0.33 ± 0.09	40.8 ± 3.00*** 123†	10.1 ± 0.67*** 31†
[^{3}H]MPP	0.004	11.0 ± 0.59	96.3 ± 4.96*** 8.7	22.5 ± 1.51** 2.0
[^{3}H]cimetidine	0.023	1.13 ± 0.10	6.97 ± 0.60*** 6.2	2.87 ± 0.20** 2.5
[^{14}C]metformin	9.9	0.82 ± 0.09	63.7 ± 1.22*** 78	9.48 ± 0.23*** 12
[^{14}C]creatinine	5.0	1.33 ± 0.04	2.93 ± 0.27** 2.2	1.75 ± 0.14* 1.3
[^{14}C]guanidine	5.3	9.02 ± 0.53	14.9 ± 0.95** 1.7	6.85 ± 0.27* 0.8
[^{14}C]procainamide	5.0	13.2 ± 0.57	47.6 ± 3.20*** 3.6	26.5 ± 1.53** 2.0
[^{14}C]choline	5.0	40.3 ± 2.79	46.3 ± 2.12 1.2	43.2 ± 0.90 1.1
[^{3}H]quinidine	0.014	309 ± 8.06	305 ± 32.9 1.0	320 ± 7.29 1.0
[^{3}H]quinine	0.014	160 ± 5.78	187 ± 2.23* 1.2	199 ± 15.1 1.2
[^{3}H]thiamine	0.028	13.4 ± 0.47	27.8 ± 0.85*** 2.1	18.1 ± 0.96* 1.4

Table 1 (*Continued*)

Compounds	Concentration (μM)	Uptake (μl/mg protein/15 min)		
		Vector	hMATE1	hMATE2-K
[^{3}H]carnitine	0.003	44.5 ± 2.98	36.4 ± 2.29 0.8	29.3 ± 1.82* 0.7
[^{14}C]nicotine	5.0	5.51 ± 0.54	7.65 ± 1.04 1.4	7.77 ± 0.30* 1.4
[^{14}C]captopril	90	0.32 ± 0.07	0.54 ± 0.04 1.7	0.33 ± 0.07 1.0
[^{3}H]verapamil	0.004	49.9 ± 4.10	42.8 ± 0.54 0.9	60.1 ± 7.35 1.2
[^{14}C]levofloxacin	14	4.01 ± 0.20	4.95 ± 0.14* 1.2	3.98 ± 0.07 1.0
[^{3}H]tetracycline	0.056	7.03 ± 0.36	6.32 ± 0.93 0.9	8.86 ± 0.24* 1.3
[^{14}C]topotecan	5.8	6.33 ± 0.27	9.30 ± 0.13*** 1.5	6.73 ± 0.32* 1.1
[^{14}C]para-aminohippuric acid	5.6	4.68 ± 0.53	4.06 ± 0.07 0.9	3.13 ± 0.05* 0.7
[^{3}H]estrone sulfate	0.005	1.91 ± 0.11	3.85 ± 0.17*** 2.0	4.22 ± 0.16*** 2.2
[^{3}H]ochratoxin A	0.016	3.96 ± 0.55	2.80 ± 0.23 0.7	3.66 ± 0.29 0.9
[^{3}H]dehydroepiandrosterone sulfate	0.005	19.9 ± 1.73	19.8 ± 1.08 1.0	20.4 ± 1.26 1.0
[^{14}C]uric acid	5.3	0.75 ± 0.04	1.04 ± 0.26 1.4	1.06 ± 0.16 1.4
[^{14}C]salicylic acid	5.8	1.62 ± 0.16	2.20 ± 0.09* 1.4	2.00 ± 0.34 1.2
[^{14}C]indomethacin	13	9.96 ± 0.60	9.23 ± 0.60 0.9	12.4 ± 1.25 1.3
[^{3}H]prostaglandin F_2 alpha	0.002	1.45 ± 0.19	1.03 ± 0.08 0.7	2.04 ± 0.19 1.4
[^{3}H]valproic acid	0.006	0.50 ± 0.01	0.54 ± 0.04 1.1	0.57 ± 0.01 1.1
[^{3}H]acyclovir	0.092	1.74 ± 0.16	2.54 ± 0.26 1.5	1.53 ± 0.74 0.9
[^{3}H]ganciclovir	0.028	1.40 ± 0.12	1.48 ± 0.37 1.1	2.44 ± 0.18** 1.7
[^{3}H]adefovir	0.031	1.30 ± 0.09	1.07 ± 0.10 0.8	1.40 ± 0.08 1.1
[^{3}H]cidofovir	0.017	0.28 ± 0.04	0.34 ± 0.03 1.2	0.20 ± 0.03 0.7
[^{3}H]tenofovir	0.028	0.85 ± 0.11	1.26 ± 0.10* 1.5	1.10 ± 0.09 1.3
[^{14}C]glycylsarcosine	2.5	0.27 ± 0.06	0.23 ± 0.02 0.9	0.21 ± 0.02 0.8

HEK293 cells cultured in a 24-well plate were transfected with the empty vector (Vector), hMATE1 cDNA, or hMATE2-K cDNA, and incubated for 15 min at pH 8.4 in medium containing the 33 radiolabeled compounds indicated. The radioactivity of solubilized cells was then determined. Uptake was expressed as clearance, which was obtained by dividing the net uptake value by each concentration of substrate in the medium. Data represent the mean ± S.E. for three monolayers from a typical experiment in at least three separate experiments. †Below each uptake value is the uptake ratio compared to vector-transfected cells.

* $P < 0.05$ significantly different from vector-transfected cells.

** $P < 0.01$ significantly different from vector-transfected cells.

*** $P < 0.001$ significantly different from vector-transfected cells.

Table 2 – Uptake of various organic ions in HEK293 cells expressing hMATE1 and hMATE2-K at extracellular pH 7.4 after pretreatment with ammonium chloride

Compounds	Concentration (μM)	Uptake (μl/mg protein/2 min)		
		Vector	hMATE1	hMATE2-K
[^{14}C]TEA	5.0	0.28 ± 0.02	20.7 ± 1.56*** 74†	6.47 ± 0.17*** 23†
[^{3}H]MPP	0.004	2.92 ± 0.35	51.0 ± 4.29*** 17	16.4 ± 0.95*** 5.6
[^{3}H]cimetidine	0.023	2.62 ± 0.22	30.3 ± 2.47*** 12	12.5 ± 0.57*** 4.8
[^{14}C]metformin	9.9	0.35 ± 0.04	29.0 ± 1.23*** 83	4.20 ± 0.12*** 12
[^{14}C]creatinine	5.0	0.41 ± 0.04	2.76 ± 0.11*** 6.7	0.69 ± 0.05* 1.7
[^{14}C]guanidine	5.3	2.60 ± 0.08	4.09 ± 0.16** 1.6	3.05 ± 0.03** 1.2
[^{14}C]procainamide	5.0	13.2 ± 0.77	23.7 ± 0.49*** 1.8	16.0 ± 0.61* 1.2
[^{14}C]choline	5.0	6.81 ± 0.18	7.55 ± 0.37 1.1	6.61 ± 0.38 1.0
[^{3}H]quinidine	0.014	87.6 ± 7.05	103 ± 5.12 1.2	84.3 ± 4.45 1.0
[^{3}H]quinine	0.014	65.9 ± 4.36	91.1 ± 4.81* 1.4	80.3 ± 7.02 1.2
[^{3}H]thiamine	0.028	3.73 ± 0.30	10.2 ± 0.30*** 2.8	5.67 ± 0.13** 1.5
[^{3}H]carnitine	0.003	8.98 ± 0.31	8.31 ± 0.37 0.9	8.56 ± 0.16 1.0
[^{14}C]nicotine	5.0	2.62 ± 0.34	3.12 ± 0.16 1.2	2.91 ± 0.16 1.1
[^{14}C]captopril	90	0.13 ± 0.04	0.31 ± 0.08 2.4	0.27 ± 0.09 2.1
[^{3}H]verapamil	0.004	29.3 ± 1.44	25.0 ± 0.92 0.9	31.3 ± 2.31 0.9
[^{14}C]levofloxacin	14	5.95 ± 0.11	7.31 ± 0.32* 1.2	5.90 ± 0.20 1.0
[^{3}H]tetracycline	0.056	2.37 ± 0.23	2.72 ± 0.24 1.2	2.49 ± 0.21 1.1
[^{14}C]topotecan	5.8	6.04 ± 0.20	8.71 ± 0.42** 1.4	8.78 ± 0.50** 1.5
[^{14}C]para-aminohippuric acid	5.6	1.01 ± 0.02	1.11 ± 0.26 1.1	1.15 ± 0.06 1.1
[^{3}H]estrone sulfate	0.005	1.23 ± 0.04	4.49 ± 0.43** 3.7	2.40 ± 0.16** 2.0
[^{3}H]ochratoxin A	0.016	4.08 ± 0.74	3.33 ± 0.34 0.8	2.45 ± 0.02 0.6
[^{3}H]dehydroepiandrosterone sulfate	0.005	10.3 ± 0.48	10.6 ± 0.61 1.0	10.7 ± 0.14 1.0
[^{14}C]uric acid	5.3	0.35 ± 0.05	0.36 ± 0.01 1.0	0.39 ± 0.01 1.1
[^{14}C]salicylic acid	5.8	0.49 ± 0.05	0.65 ± 0.08 1.3	0.45 ± 0.08 0.9
[^{14}C]indomethacin	13	5.04 ± 0.28	4.52 ± 0.21 0.9	5.61 ± 0.22 1.1

Table 2 (*Continued*)

Compounds	Concentration (μM)	Uptake (μl/mg protein/2 min)		
		Vector	hMATE1	hMATE2-K
[^{3}H]prostaglandin F_2 alpha	0.002	0.59 ± 0.11	0.77 ± 0.06 1.3	0.93 ± 0.02 1.6
[^{3}H]valproic acid	0.006	0.19 ± 0.09	0.14 ± 0.08 0.7	0.29 ± 0.13 1.5
[^{3}H]acyclovir	0.092	0.43 ± 0.05	1.28 ± 0.13** 3.0	1.03 ± 0.13* 2.4
[^{3}H]ganciclovir	0.028	0.41 ± 0.03	0.74 ± 0.07* 1.8	1.60 ± 0.12*** 3.9
[^{3}H]adefovir	0.031	0.29 ± 0.03	0.26 ± 0.08 0.9	0.29 ± 0.03 1.0
[^{3}H]cidofovir	0.017	0.09 ± 0.03	0.10 ± 0.03 1.1	0.09 ± 0.03 1.0
[^{3}H]tenofovir	0.028	0.18 ± 0.05	0.20 ± 0.04 1.1	0.17 ± 0.05 0.9
[^{14}C]glycylsarcosine	2.5	0.12 ± 0.03	0.12 ± 0.06 1.0	0.12 ± 0.02 1.0

HEK293 cells cultured in a 24-well plate to examine the uptake of radiolabeled compounds were transfected with the empty vector (Vector), hMATE1 cDNA or hMATE2-K cDNA, and incubated for 20 min with medium containing 30 mM ammonium chloride. After washing, 2 min uptake measurements at extracellular pH 7.4 were carried out as described in the legend for Table 1. Data represent the mean ± S.E. for three monolayers from a typical experiment in at least three separate experiments. †Below each uptake value is the uptake ratio compared to vector-transfected cells.

* $P < 0.05$ significantly different from vector-transfected cells.

** $P < 0.01$ significantly different from vector-transfected cells.

*** $P < 0.001$ significantly different from vector-transfected cells.

[^{3}H]ganciclovir was stimulated in the hMATE2-K-expressing cells but not in the hMATE1-expressing cells at the extracellular pH 8.4, and it was also stimulated in the hMATE1-expressing cells after pretreatment with ammonium chloride (Tables 1 and 2).

3.2. *Construction of HEK293 cells stably expressing hMATE1 and hMATE2-K*

To obtain quantitative information about the hMATE1- or hMATE2-K-mediated transport of ionic compounds, we constructed HEK 293 cells stably expressing hMATE1 (HEK-hMATE1) and hMATE2-K (HEK-hMATE2-K). The protein expression of hMATE1 in HEK-hMATE1 cells and hMATE2-K in HEK-hMATE2-K cells was confirmed by Western blotting with specific antibody for each transporter. In the nonreducing and reducing conditions, immunoreactive protein was detected in each stable transfectant with an apparent molecular mass of 50 kDa for anti-hMATE1 antibody (Fig. 1A and C), and 40 kDa for anti-hMATE2-K antibody (Fig. 1E and G), respectively. No signal was observed in another transfectant or the vector-transfected control. These positive bands disappeared when the antiserum was preabsorbed with each antigen peptide (Fig. 1B, D, F and H). Consistent with the protein expression in each transfectant, specific signals for hMATE1 (Fig. 1A and C) and hMATE2-K (Fig. 1E and G) were observed in the human renal brush-border membranes, both of which were abolished with the antigen-preabsorbed antibodies (Fig. 1B, D, F and H). The functional expression of hMATE1 and hMATE2-K was assessed by the uptake of [^{14}C]TEA by HEK-hMATE1 and HEK-hMATE2-K cells, respectively. Both the hMATE1- and hMATE2-K-mediated uptake of [^{14}C]TEA without pretreatment with ammonium chloride increased in accordance with the extracellular pH between 6.0 and 9.0 (Fig. 2A), and increased in a time-dependent manner (Fig. 2B). After the pretreatment with ammonium chloride, the uptake of [^{14}C]TEA in the HEK-hMATE1 and HEK-hMATE2-K cells also increased with extracellular pH between 6.0 and 9.0. The uptake was greatest and lowest at an extracellular pH of 8.5 and 6.0, respectively (Fig. 2C). Although the apparent linearity of the time course of the hMATE1- and hMATE2-K-mediated uptake of [^{14}C]TEA was less than 30 s in the condition of the cellular acidification (Fig. 2D), the transport characteristics of hMATE1 and hMATE2-K in the transfectants pretreated with ammonium chloride were examined at 2 min in subsequent experiments for reasons of technical limitations and reproducibility.

3.3. *Comparison of functional characteristics between hMATE1 and hMATE2-K*

The uptake of TEA, MPP, cimetidine, metformin, guanidine, procainamide, topotecan, estrone sulfate, acycrovir, and ganciclovir at extracellular pH 7.4 after pretreatment with ammonium chloride by hMATE1 and hMATE2-K exhibited saturable kinetics, following the Michaelis-Menten equation. The apparent K_m and maximal uptake velocity (V_{max}) values of TEA, MPP, cimetidine, metformin, guanidine, procainamide, topotecan, estrone sulfate, acycrovir, and ganciclovir are shown in Table 3. The K_m values for hMATE1-mediated

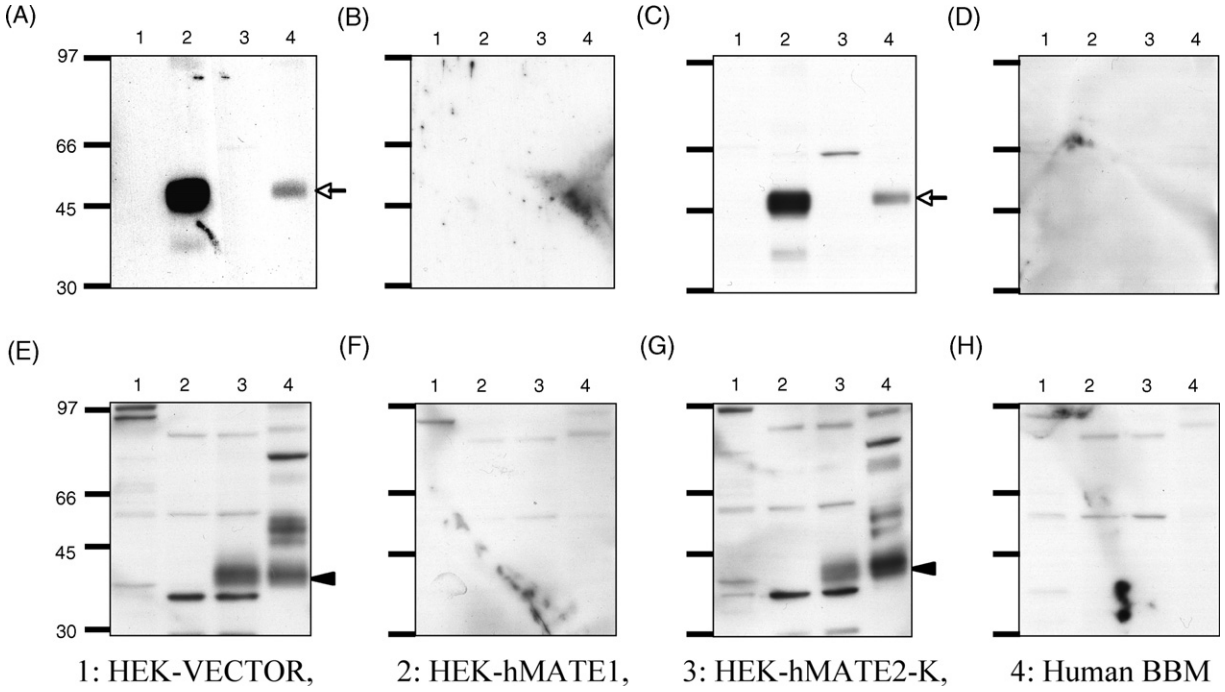

Fig. 1 – Western blot analyses of hMATE1 and hMATE2-K in stably expressing HEK293 cells and human brush-border membrane. Twenty micrograms of each membrane was separated by SDS-PAGE under nonreducing (A, B, E and F) or reducing (C, D, G and H) conditions. (A and C) Antiserum specific for hMATE1 was used 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.

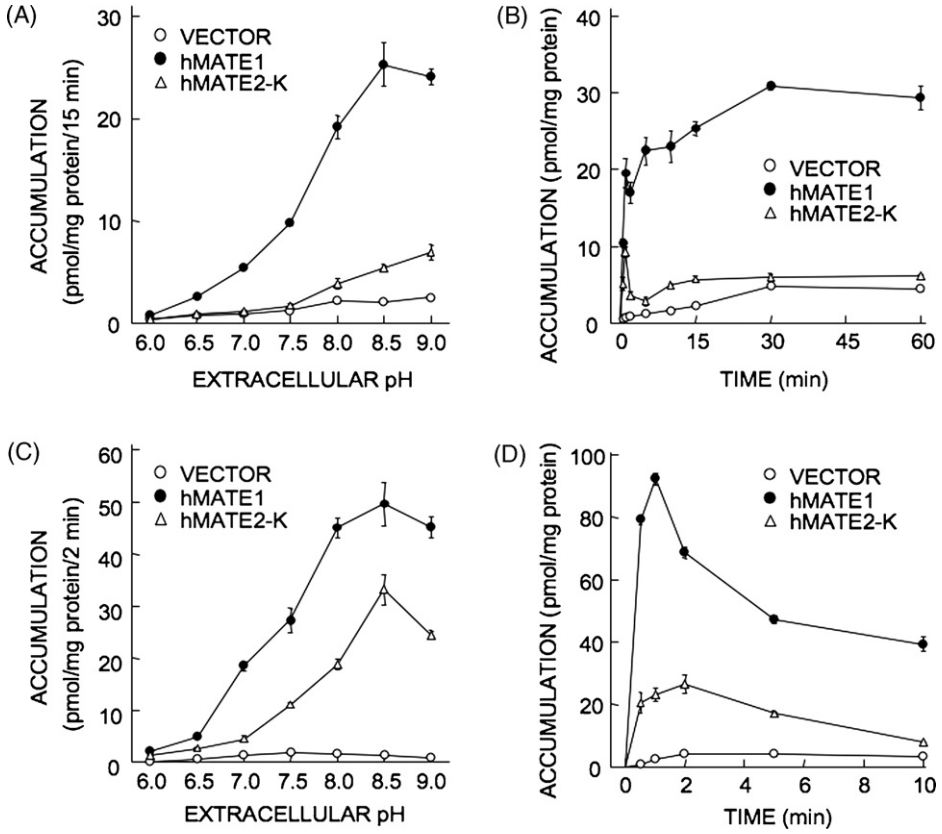

Fig. 2 – Oppositely directed H^+ gradient-dependence (A and C) and time course (B and D) of [^{14}C]TEA uptake by hMATE1 and hMATE2-K in the stably transfected HEK293 cells. (A) HEK293 cells stably expressing the empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle) were incubated 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

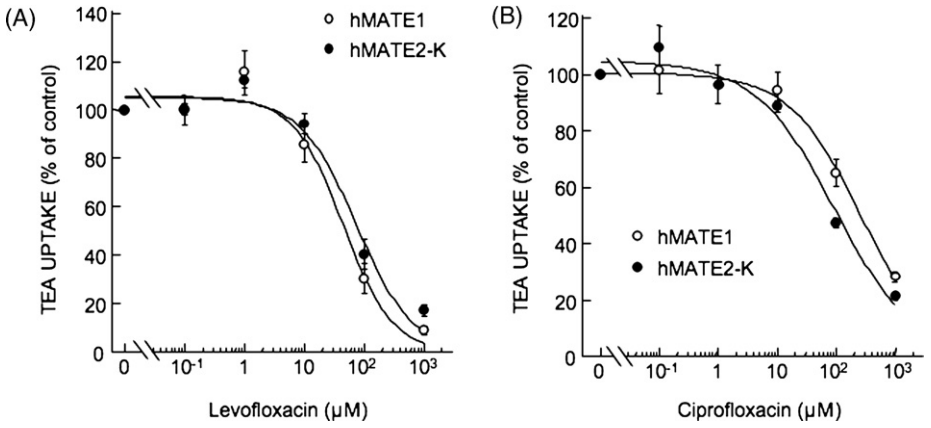

Fig. 3 – Effects of levofloxacin (A) and ciprofloxacin (B) on the uptake of [^{14}C]TEA by HEK293 cells stably expressing hMATE1 and hMATE2-K, respectively. HEK293 cells stably expressing hMATE1 (open circle) and hMATE2-K (closed circle) cDNA were incubated with 5 μM [^{14}C]TEA for 2 min at pH 7.4 at 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.

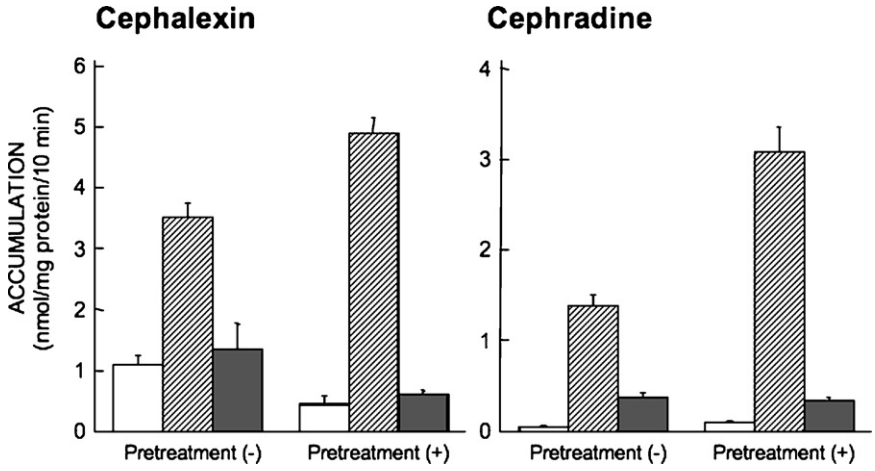

Fig. 4 – Uptake of cephalexin and cephradine by HEK293 cells expressing hMATE1 and hMATE2-K. HEK293 cells transfected with the empty vector (open column), hMATE1 cDNA (hatched column) or hMATE2-K cDNA (closed column) were preincubated with incubation medium (pH 7.4) in the presence or absence of 30 mM 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

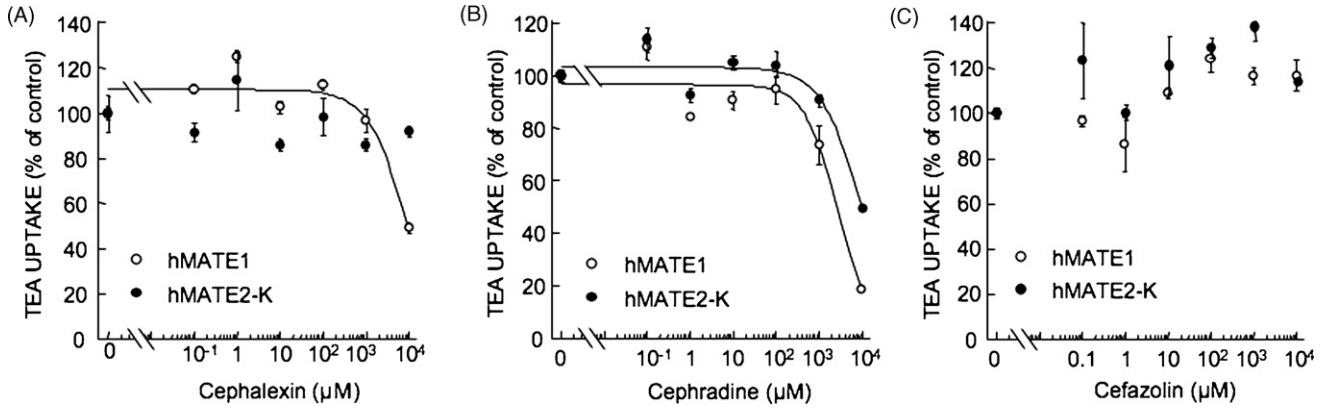

Fig. 5 – Effects of cephalosporin antibiotics on the uptake of [^{14}C]TEA by HEK293 cells stably expressing hMATE1 and hMATE2-K. HEK293 cells stably expressing hMATE1 (open circle) and hMATE2-K (closed circle) cDNA were incubated with 5 μM [^{14}C]TEA for 2 min at pH 7.4 and 37 °C in the presence of 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].

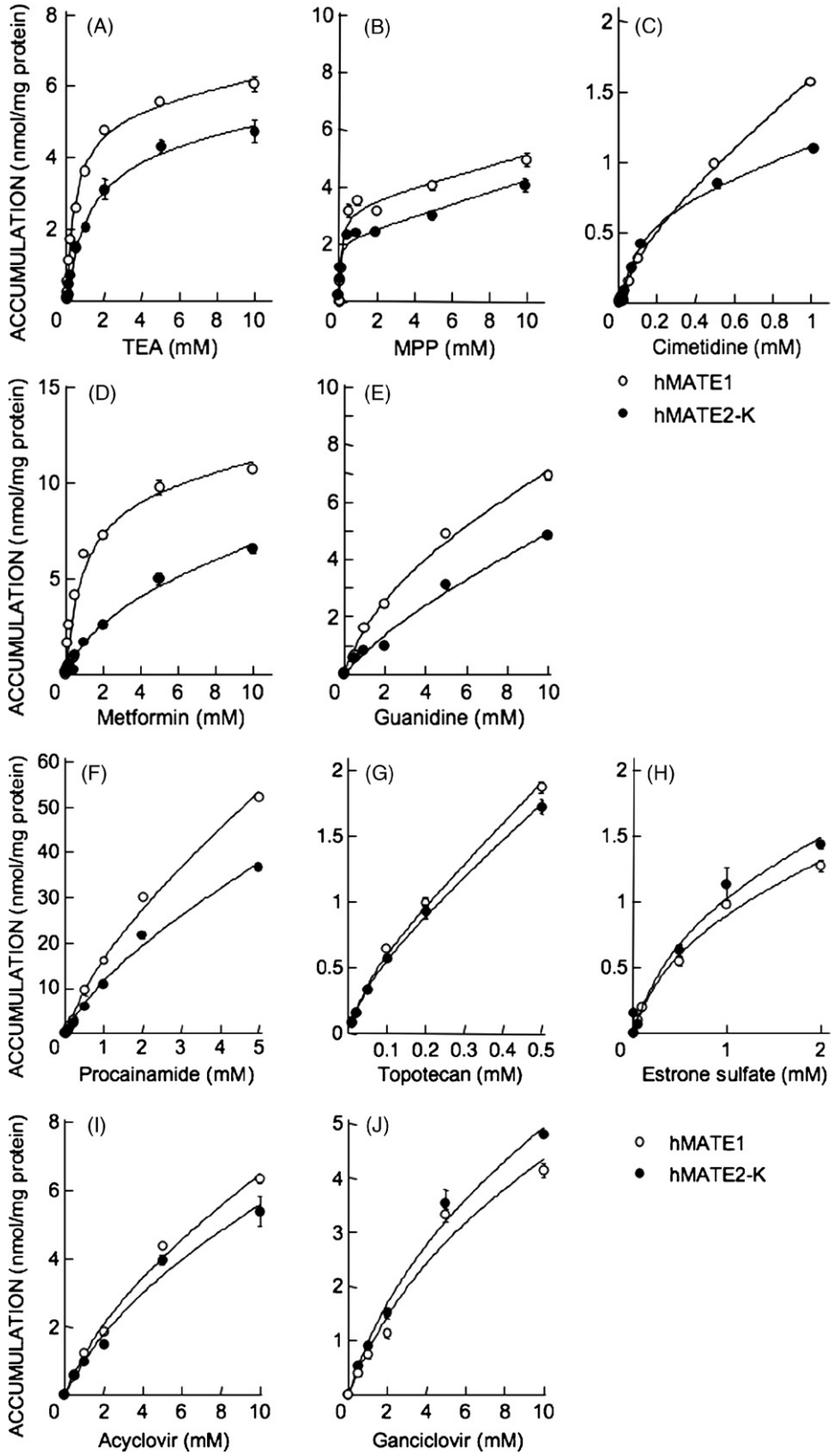

Fig. 6 – Concentration-dependence of substrates for hMATE1 and hMATE2-K using stable transfectants. HEK293 cells stably expressing hMATE1 (open circle) or hMATE2-K (closed circle) were incubated with various concentrations of [^{14}C]TEA (A), [^{3}H]MPP (B), [^{3}H]cimetidine (C), [^{14}C]metformin (D), [^{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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