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Evaluation of 4′,6-Diamidino-2-phenylindole as a Fluorescent Probe Substrate for Rapid Assays of the Functionality of Human Multidrug and Toxin Extrusion Proteins

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ABSTRACT:

Multidrug and toxin extrusion protein 1 (MATE1) and MATE2-K are organic cation/H^+ antiporters that have recently been identified and suggested to be responsible for the brush border secretory transport of many cationic drugs in renal tubules. We here report our finding that 4′,6-diamidino-2-phenylindole (DAPI) can be used as a probe substrate for rapid assays of the functionality of the human MATEs, hMATE1, and hMATE2-K, by taking advantage of its fluorescent nature. The specific cellular uptakes of DAPI by cloned hMATE1 and hMATE2-K, which were assessed by fluorescence intensity, were found to be rapid and saturable with the Michaelis constants of 1.13 and 3.16 μM, respectively, indicating that DAPI is a good substrate of both hMATEs. It was found that many organic cations inhibit the specific uptake of DAPI by hMATE1 and hMATE2-K, and the extents of inhibition are in good correlation with those of inhibition of the specific uptake of [^{3}H]cimetidine as a typical substrate, indicating comparable performances of both substrates as probes in identifying inhibitors. Thus, DAPI can be an alternative probe substrate that enables fluorometric rapid assays of the functionality of both hMATEs. It was also found that the other major renal organic cation transporters, human organic cation transporter 2 (hOCT2), hOCT3, human novel organic cation transporter 1 (hOCTN1), and hOCTN2, cannot transport DAPI, although hOCT1, which is mainly expressed in the liver, can. Therefore, the DAPI uptake assay can be a method specific to the hMATEs among organic cation transporters in the human kidney.

Multidrug and toxin extrusion protein 1 (MATE1) and MATE2-K are organic cation/H^+ antiporters that have recently been identified and suggested to be responsible for the brush border secretory transport of many cationic drugs, such as cimetidine, tetraethylammonium (TEA), *N*-methylnicotinamide, and metformin in renal tubules (Terada and Inui, 2007; Tsuda et al., 2007). Two human MATEs, hMATE1 and hMATE2, were originally identified in 2005 by Otsuka et al. (2005). Shortly afterward, two splice variants of hMATE2, hMATE2-K and hMATE2-B, were identified, and it has been suggested that, among the hMATE2 variants, hMATE2-K is expressed most abundantly and specifically in the kidney, whereas the other hMATE2 variants, hMATE2 and hMATE2-B, are expressed only minimally or negligibly throughout the body (Masuda et al., 2006). It is notable that a recent study using *Mate1*(−/−) mice has successfully proved the role of MATE1 in renal drug secretion by demonstrating that knocking out Mate1 results in a decrease in the renal excretion of metformin, which is accompanied by an increase in the plasma concentration of the drug (Tsuda et al., 2009).

MATEs have also been shown to recognize some anionic, amphoteric, and neutral compounds as inhibitors and/or substrates (Ohta et al., 2006, 2009; Tanihara et al., 2007). They include estrone sulfate, fluoroquinolones, such as norfloxacin, and corticosterone. Characteristics of the recognition of inhibitors and substrates by MATE1 and MATE2-K are of interest but are complicated and difficult to delineate. It is important to perform extensive transport assays to identify inhibitors and substrates and thereby clarify recognition characteristics.

4′,6-Diamidino-2-phenylindole (DAPI) is a fluorescent dye widely used for nuclear staining (Fig. 1). It emits blue fluorescence by excitation with ultraviolet light only after intercalation into DNA double strands. Because this cationic compound is practically impermeable through the plasma membrane of most live cells, it is typically used for staining nuclei of fixed and dead cells (Kapuscinski, 1995).

We here report our finding that DAPI is a good substrate for both hMATE1 and hMATE2-K. Taking advantage of its fluorescent nature, we have used it as a probe substrate for rapid assays of the functionality of the hMATEs.

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ABBREVIATIONS: MATE, multidrug and toxin extrusion protein; h, human; TEA, tetraethylammonium; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT, reverse transcription; PCR, polymerase chain reaction; OCT, organic cation transporter; OCTN, novel organic cation transporter; MDCK, Madin-Darby canine kidney; HEK, human embryonic kidney; GFP, green fluorescence protein; MES, 4-morpholineethanesulfonic acid; FI, fluorescence intensity in arbitrary units; r, rat; pCMBS, *p*-chloromercuribenzene sulfonate; DTT, dithiothreitol.

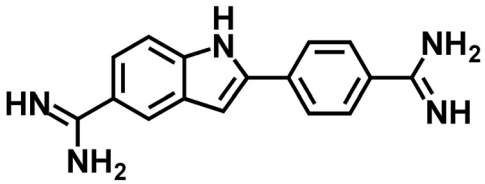

FIG. 1. Chemical structure of DAPI.

Materials and Methods

Materials. [^{3}H]Cimetidine (25.0 Ci/mmol) and [^{14}C]tetraethylammonium bromide (55.0 Ci/mmol) were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK), [^{3}H]L-carnitine (81.0 Ci/mmol) was from Moravek Biochemicals (Brea, CA), and cimetidine and DAPI were from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and obtained commercially.

Isolation of hMATEs. The cDNA of hMATE1 was cloned from the human kidney total RNA (Clontech, Mountain View, CA) by reverse transcription (RT) and subsequent polymerase chain reaction (PCR). In brief, an RT reaction was performed using 3 μg of the total RNA, an oligo(dT) primer and ReverTra Ace (Toyobo, Osaka, Japan) as a reverse transcriptase. The cDNA of hMATE1 was isolated from the thus obtained cDNA mixture by PCR using KOD FX DNA polymerase (Toyobo) and the following primers: forward primer, 5′-CAT GGA AGC TCC TGA GGA GCC C-3′ and reverse primer, 5′-TTT CTT TCC TAC CAC GTC ACT GAA TTC-3′. These primers were designed on the basis of the sequence in GenBank (accession number NM_018242.2). PCR was performed using the following conditions: predenature at 94°C for 2 min and 35 cycles of 1) denature at 98°C for 10 s, 2) annealing at 56°C for 20 s, and 3) extension at 68°C for 90 s. The second PCR was performed using the PCR product as a template and a forward primer containing a SalI restriction site (underlined), 5′-GTC GAC GCC ACC ATG GAA GCT CCT GAG GAG CCC-3′ and a reverse primer containing a SmaI restriction site (underlined), 5′-ACC CGG GTT TCT TTC CTA CCA CGT CAC-3′. The amplified cDNA product was subcloned into pME18 vector, digested with SalI and SmaI, and then transferred into a mammalian expression vector, pCI-neo (Promega, Madison, WI). The sequence of the final product was determined with an automated sequencer. The cDNA of hMATE2-K (GenBank accession number NM_001093116.1) was isolated similarly by RT-PCR cloning. The primers for PCR were as follows: forward primer containing an EcoRI restriction site (underlined), 5′-GAA TTC CGC CAC CAT GGA CAG CCT CCA GGA CAC AG-3′ and reverse primer, 5′-GCTAGTGCCTGGTGGCTAGGATC-3′. The amplified cDNA product was, without the second PCR, subcloned into pME18 vector, digested with EcoRI and SalI, which is for a restriction site in pME18 vector, and then transferred into pCI-neo vector.

Isolation of hOCTs and hOCTNs. The cDNAs of OCTs and OCTNs of humans, hOCT1, hOCT2, hOCT3, hOCTN1, and hOCTN2, were cloned by an RT-PCR method similar to that for the cloning of hMATE1, using PCR primers designed on the basis of the sequences in GenBank under the accession numbers NM_003057, NM_003058, NM_021977, NM_003059, and NM_003060, respectively. hOCT1 was cloned from the human liver total RNA (Clontech), hOCT2 and hOCTN1 were from the human kidney total RNA (Clontech), hOCT3 was from total RNA prepared from the Caki-1 human kidney cancer cell line by a guanidine isothiocyanate extraction method (Chomczynski and Sacchi, 1987), and hOCTN2 was from the human small intestine total RNA (Clontech). The primers for the initial PCR were as follows: forward primer for hOCT1, 5′-AGC CAT CAT GCC CAC CGT GG-3′; reverse primer for hOCT1, 5′-ATC TTC ATC CCT CCA ACA CG-3′; forward primer for hOCT2, 5′-CCG CTC TCA GCC TCG CTC C-3′; reverse primer for hOCT2, 5′-GGT CAT GAC AGC AGC AAC GGT C-3′; forward primer for hOCT3, 5′-CGG GCG GCG GGC GCA CCA TGC CCT CCT TCG-3′; reverse primer for hOCT3, 5′-GTC GGG GGC CTC AAA GGT GAG AG-3′; forward primer for hOCTN1, 5′-GCA AGT TTC GGA GCG GCA GT-3′; reverse primer for hOCTN1, 5′-ACG AAT TTC TCC ACA GGG TCT TA-3′; forward primer for hOCTN2, 5′-GGA CGG TCT TGG GTC GCC TGC-3′; and reverse primer for hOCTN2, 5′-GGG GAA AGG GGT GGG GGA CTT A-3′. The primers for the second PCR were as follows: forward primer containing an EcoRI restriction site (underlined) for hOCT1, 5′-AGA ATT CAG CCA TCA TGC CCA CCG TG-3′; reverse primer containing an XbaI restriction site (underlined) for hOCT1, 5′-TTT CTA GAC TCT CAG GTG CCC GAG GGT-3′; forward primer containing a XhoI restriction site (underlined) for hOCT2, 5′-GCC TCG AGG TCG ACA GAT CTG ATT CCG C-3′; reverse primer containing an XbaI restriction site (underlined) for hOCT2, 5′-TGT CTA GAG TCA TGA CAG CAG CAA CGG TCT-3′; forward primer containing an EcoRI restriction site (underlined) for hOCT3, 5′-GAG AAT TCG ATG GGC GGC GGG CGC ACC A-3′; reverse primer containing a SalI restriction site (underlined) for hOCT3, 5′-TAG TCG ACG TCG GGG GCC TCA AAG GTG AGA G-3′; forward primer containing an EcoRI restriction site (underlined) for hOCTN1, 5′-AGA ATT CCA TGC GGG ACT ACG ACG AGG T-3′; reverse primer containing an XbaI restriction site (underlined) for hOCTN1, 5′-GTT CTA GAA TTT CTC CAC AGG GTC TT-3′; forward primer containing an EcoRI restriction site (underlined) for hOCTN2, 5′-AGG AAT TCT TGG GTC GCC TGC TGC-3′; and reverse primer containing an XbaI restriction site (underlined) for hOCTN2, 5′-TCC TCT AGA CAG TCT TTC CTC TTC-3′. The amplified cDNA products were subcloned into pME18 vector and then transferred into pCI-neo vector.

Cell Culture. Madin-Darby canine kidney (MDCK) II cells and human embryonic kidney (HEK) 293 cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of MDCKII Cells Stably Expressing hMATEs. MDCKII cells were transfected with the plasmid carrying the cDNA of hMATE1 or hMATE2-K by using Lipofectamine 2000 (Invitrogen) as a transfection reagent, according to the manufacturer's instructions, and cultured in DMEM supplemented with 10% FBS and 800 μg/ml Geneticin for 2 to 3 weeks. Antibiotic-resistant clones were selected and tested for the transport of [^{3}H]cimetidine as a probe substrate.

Preparation of HEK293 Cells Transiently Expressing hMATEs, hOCTs, and hOCTNs. HEK293 cells (1.5×10^5 cells/well initially) were grown on 24-well plates coated with poly-L-lysine for 12 h, transfected with 1 μg/well of the plasmid carrying the cDNA of hMATE1, hMATE2-K, hOCT1, hOCT2, hOCT3, hOCTN1, or hOCTN2 by using 1 μg/well of Lipofectamine 2000, and cultured for 24 h for transient expression. For fluorescent microscopic observation, pEGFP plasmid (0.1 μg/well) was cotransfected.

Uptake Study in MDCKII Cells Stably Expressing hMATEs. For the assays of DAPI uptake, MDCKII cells stably expressing hMATE1 or hMATE2-K (0.5×10^5 cells/well initially) were grown on 96-well plates for 72 h. In regular uptake assays, the cells in each well were preincubated in 0.25 ml of DAPI-free uptake buffer (130 mM KCl, 2 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5 mM glucose, and 20 mM HEPES, pH 7.4) for 10 min. In uptake assays to examine the effect of intracellular acidification, we used a NH_4Cl prepulse method (Boron and De Weer, 1976), in which the cells were preincubated in the uptake buffer free of DAPI and supplemented with 20 mM NH_4Cl (0.25 ml) for 10 min and thereafter in the uptake buffer free of DAPI and NH_4Cl (0.25 ml) for 5 min. In uptake assays to examine the effect of extracellular pH, 20 mM HEPES was replaced with 20 mM MES for pH 6.0 and below. Uptake assays were started by replacing the DAPI-free uptake buffer for preincubation with buffer containing DAPI (0.1 ml). All of the procedures were conducted at 37°C. Assays were stopped by addition of ice-cold DAPI-free uptake buffer (0.25 ml), and the cells were washed two times with 0.25 ml of the same buffer. Then, each well was filled with 0.25 ml of ice-cold DAPI-free uptake buffer, and the intensity of fluorescence from DAPI was measured with a fluorescence plate reader, using the wavelengths of 360 nm for excitation and 460 nm for emission, for the evaluation of uptake.

For the assays of [^{3}H]cimetidine uptake, MDCKII cells stably expressing hMATE1 or hMATE2-K (1.5×10^5 cells/well initially) were grown on 24-well plates for 72 h. Because cimetidine is transported by hMATEs by an H^+-coupled antiport mechanism, we used the NH_4Cl prepulse method to generate an outward H^+ gradient by intracellular acidification for the evaluation of transport by uptake measurements. The cells in each well were pretreated as described above, using 1 ml each of preincubation buffers, and uptake assays were started by replacing the cimetidine-free uptake buffer for preincubation with one containing [^{3}H]cimetidine (0.25 ml). All of the procedures were conducted at 37°C. Assays were stopped by addition of ice-cold cimetidine-free uptake buffer (2 ml), and the cells were washed two times with 2 ml of the same buffer. The cells were solubilized in 0.5 ml of 0.2 M NaOH

solution containing 0.5% SDS at room temperature for 1 h, and the associated radioactivity was measured by liquid scintillation counting, using 3 ml of Clear-sol I (Nakarai Tesque, Kyoto, Japan) as a scintillation fluid, for the evaluation of uptake.

Cellular protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Uptake assays were also conducted in mock cells, which were transfected with empty pCI-neo vector, to estimate nonspecific uptake.

Fluorescent Microscopy. HEK293 cells transiently expressing one of the cloned transporters and cultured on 24-well plates coated with poly-L-lysine were detached using 0.05% EDTA in phosphate-buffered saline (pH 7.4), seeded at a density of 0.5×10^5 cells on a 35-mm glass-bottom dish coated with poly-L-lysine, and cultured overnight. After incubation of the cells in uptake buffer containing DAPI (0.5 μM) at 37°C for 20 min, the fluorescent images of DAPI and GFP were directly visualized by using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

Uptake Study in HEK293 Cells Transiently Expressing hOCTs and hOCTNs. HEK293 cells transiently expressing each of the hOCTs and hOCTNs and cultured on the 24-well plates were used to assess the uptake of L-[^{3}H]carnitine, for hOCTN2 or [^{14}C]TEA, for the others. The uptake buffer was prepared by using Hanks' solution (136.7 mM NaCl, 5.36 mM KCl, 0.952 mM $CaCl_2$, 0.812 mM $MgSO_4$, 0.441 mM KH_2PO_4, 0.385 mM Na_2HPO_4, and 25 mM D-glucose) and supplemented with 20 mM HEPES (pH 8.5). The cells in each well were preincubated in substrate-free uptake buffer (1 ml) at 37°C for 10 min. Uptake assays were started by replacing the substrate-free uptake buffer for preincubation with one containing one of the radiolabeled substrates (0.25 ml) and, thereafter, conducted in the same manner as the assays of [^{3}H]cimetidine uptake.

Data Analysis. Specific uptake by the introduced transporter, hMATE1 or hMATE2-K, was estimated by subtracting the uptake in mock cells from that in the transporter-transfected cells.

Saturable transport of DAPI was analyzed by assuming Michaelis-Menten type carrier-mediated transport represented by the following equation: $v = V_{max} \times s/(K_m + s)$. The maximum transport rate (V_{max}) and the Michaelis constant (K_m) were estimated by fitting this equation to the experimental profile of the uptake rate (v) versus the substrate (DAPI) concentration (s), using a nonlinear least-squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA), and the reciprocal of variance as the weight. The parameters are presented as the computer-fitted values with S.E.

Experimental data are presented as the means ± S.E., and statistical analysis was performed using two-tailed, unpaired Student's t test or, when multiple comparisons were needed, analysis of variance followed by Dunnett's test, with $p < 0.05$ considered significant.

Results

Fluorescent Microscopic Observation of DAPI Uptake by hMATEs. The uptake of DAPI (0.5 μM) was first examined by fluorescent microscopic observation in HEK293 cells transiently expressing hMATE1 and those expressing hMATE2-K. As shown in Fig. 2, blue fluorescence from DAPI was observed in some cells in both preparations treated for transfection with hMATE1 and with hMATE2-K but not in mock cells. Furthermore, the fluorescence from DAPI was almost completely overlapped with green fluorescence from cotransfected GFP, which serves as a marker of successful transfection. These results indicate that DAPI was taken up into live cells expressing hMATE1 and those expressing hMATE2-K by these transporters.

Time Courses of DAPI Uptake by hMATEs. The uptake of DAPI (0.5 μM) was greater in MDCKII cells stably expressing hMATE1 and those expressing hMATE2-K than in mock cells (Fig. 3), consistent with the fluorescent microscopic observation (Fig. 2) and indicating its efficient uptake by both hMATEs. Because the uptakes increased in proportion to time up to 10 min in both cells expressing hMATE1 and expressing hMATE2-K, we set the uptake period to be 10 min in subsequent experiments to evaluate transport across the plasma membrane within the initial uptake phase. Because DAPI can

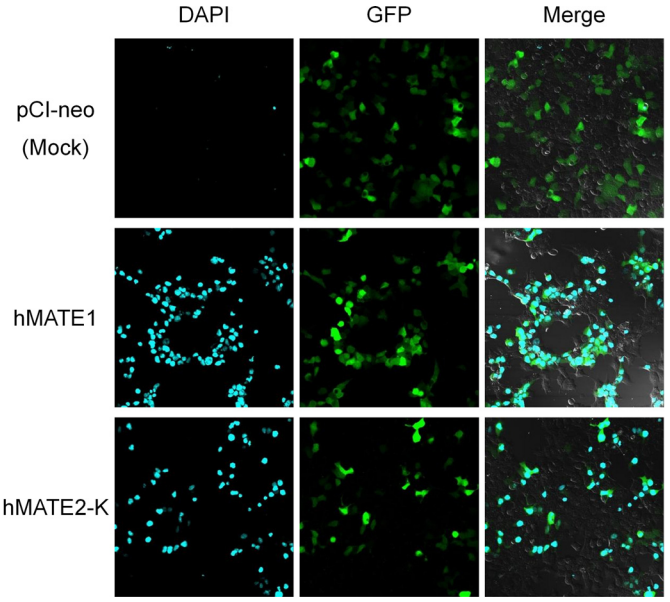

FIG. 2. Uptake of DAPI in HEK293 cells transiently expressing hMATEs. The fluorescent microscopic images of DAPI were obtained after incubation of HEK293 cells transiently expressing hMATE1, those expressing hMATE2-K, and mock cells with DAPI (0.5 μM) at 37°C and pH 7.4 for 20 min. Also shown are the images of cotransfected GFP.

emit fluorescence only after intercalation into DNA double strands, DAPI must be transported across the plasma membrane, distributed into the nucleus, and bound to DNA to be detected fluorometrically. However, because the plasma membrane is presumably the primary barrier in the whole process, the assumption here is that the fluorometrically evaluated uptake process would mainly represent transport across the plasma membrane.

Functional Characteristics of hMATEs for DAPI Transport. As shown in Fig. 4, the specific uptakes of DAPI by hMATE1 and hMATE2-K stably expressed in MDCKII cells were both saturable, conforming to Michaelis-Menten kinetics. The K_m of hMATE1 for DAPI (1.13 μM) was smaller than that of hMATE2-K (3.16 μM) by a factor of approximately 3, indicating that the former has a higher affinity for DAPI. Consistent K_m values, 1.53 μM for hMATE1 and 2.93 μM for hMATE2-K, were observed for DAPI uptake for a shorter period of 1 min. This moderate difference in the affinity between the two hMATEs is within a range of differences reported by Tanihara et al. (2007) for several cationic compounds, for which the K_m values of hMATE1 were comparable with or smaller by a factor

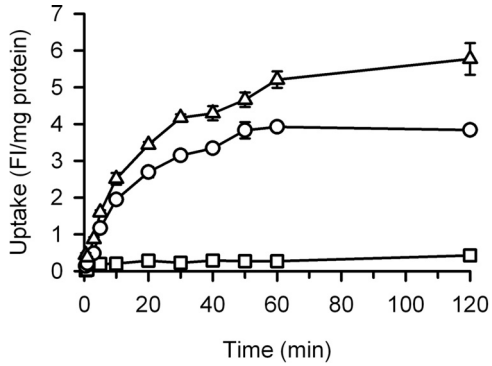

FIG. 3. Time courses of DAPI uptake in MDCKII cells stably expressing hMATEs. The uptake of DAPI (0.5 μM) was evaluated at 37°C and pH 7.4 in MDCKII cells stably expressing hMATE1 (○), those expressing hMATE2-K (▲), and mock cells (□). Data are presented as means ± S.E. ($n = 8$).

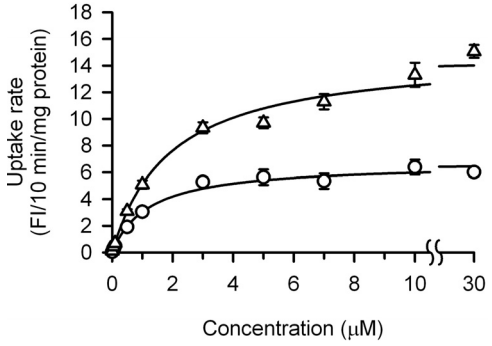

FIG. 4. Concentration dependence of DAPI uptake mediated by hMATEs stably expressed in MDCKII cells. The uptake of DAPI was evaluated at 37°C and pH 7.4 for 10 min in MDCKII cells stably expressing hMATE1, those expressing hMATE2-K, and mock cells. Data show the specific uptake rates by hMATE1 (○) and hMATE2-K (△). Solid lines represent the computer-fitted profiles. The K_m and V_{max} are 1.13 ± 0.21 μM and 6.78 ± 0.17 FI/10 min/mg protein, respectively, for hMATE1, and 3.16 ± 0.36 μM and 17.23 ± 0.36 FI/10 min/mg protein, respectively, for hMATE2-K, as the computer-fitted parameters with S.E. Data are presented as means ± S.E. ($n = 8$).

of approximately 3 or less than the respective K_m values of hMATE2-K. It is notable that the K_m values for DAPI are much smaller than the smallest ones, which are 70 μM (hMATE1) and 60 μM (hMATE2-K) for topotecan, in their study, indicating much higher affinities of the hMATEs for DAPI.

When MDCKII cells expressing hMATE2-K were permeabilized by pretreatment with 40 μg/ml digitonin for 10 min and incubated with DAPI (30 μM), DAPI uptake was as much as 80.1 ± 5.7 FI/mg protein ($n = 4$), being approximately 5-fold larger than the maximum uptake observed in uptake assays (Fig. 4) and, hence, indicating that the nuclear DNA of the cells has sufficient binding capacity for DAPI not to bias the evaluation of its transport. DAPI uptake (DNA binding) was found to be large enough also in permeabilized MDCKII cells expressing hMATE1 (79.9 ± 6.0 FI/mg protein, $n = 4$).

Both hMATEs were originally been identified as organic cation/H^+ antiporters (Otsuka et al., 2005; Masuda et al., 2006). We therefore examined the role of H^+ in DAPI transport by them. However, as shown in Fig. 5, the enhancements of the uptake of DAPI (0.5 μM) by intracellular acidification by the NH_4Cl prepulse method were only minimal or insignificant in both cells expressing hMATE1 and cells expressing hMATE2-K as well as in mock cells. Therefore, it is likely that both hMATEs mediate DAPI transport by a facilitative manner, without requiring a transmembrane proton gradient. Differences in DAPI uptake were also minimal or insignificant in any type of cells between the depolarized condition (K^+ buffer) and the polarized condition (Na^+ buffer), indicating that the membrane potential is not involved, either. However, when the extracellular pH was varied, the uptake of DAPI changed dramatically in both cells expressing hMATE1 and cells expressing hMATE2-K, whereas it remained minimal in mock cells (Fig. 6). DAPI uptake was greatest at approximately pH 7.5 in both cells expressing hMATE1 and cells expressing hMATE2-K and decreased when pH became more acidic. Because DAPI, for which the pK_a is 11.65 as estimated by the ChemAxon pK_a calculator at http://www.chemaxon.com/marvin/sketch/index.jsp (Pirok et al., 2006), is presumed to be almost completely dissociated in the entire pH range of 5.5 to 8.0, the pH-dependent change in its uptake cannot be attributed to the change in its fraction un-ionized, with an assumption that the un-ionized form is transported. Both hMATEs seem to be functionally sensitive to extracellular pH for unknown mechanism.

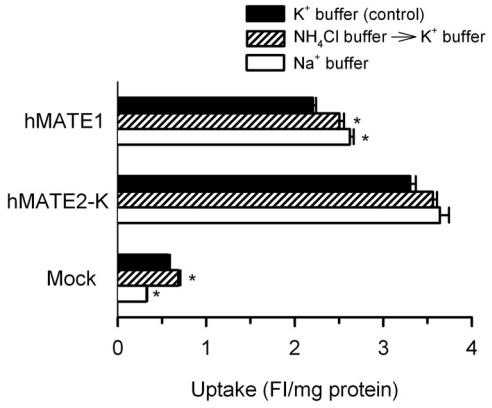

FIG. 5. Effects of intracellular acidification and membrane potential on DAPI uptake in MDCKII cells stably expressing hMATEs. MDCKII cells stably expressing hMATE1, those expressing hMATE2-K, and mock cells were incubated at 37°C in K^+-based regular uptake buffer (pH 7.4) for 15 min, in the K^+-based buffer supplemented with 20 mM NH_4Cl for 10 min, and subsequently in the K^+-based buffer (NH_4Cl-free) for 5 min, or in the Na^+-based buffer, in which K^+ in the K^+-based buffer was replaced with Na^+, for 15 min, before DAPI uptake was evaluated using the K^+-based buffer containing DAPI (0.5 μM) for 10 min. Data are presented as means ± S.E. ($n = 8$). *, significantly different from the value for control at $p < 0.05$.

Effect of Various Compounds on DAPI Uptake by hMATEs. It is likely that, as indicated above, the mechanism of DAPI transport by both hMATEs is different from the H^+-coupled antiport mechanism for organic cations, such as cimetidine and TEA. In this section, we therefore examined whether various compounds, which inhibit organic cation/H^+ antiport by the hMATEs, still inhibit the transport of DAPI (0.5 μM) by them. As shown in Table 1 and Fig. 7, it was found that many organic cations are effective as inhibitors of the specific uptake of DAPI by hMATE1 stably expressed in MDCKII cells and the extents of inhibition were in good correlation with those of inhibition of the specific uptake of [^{3}H]cimetidine as a typical substrate at its trace concentration of 50 nM. Specific DAPI uptake by hMATE2-K was also found to be inhibited by many organic cations with extents well correlated to those of inhibition of specific [^{3}H]cimetidine uptake.

In addition, DAPI uptake was extensively reduced in both cells expressing hMATE1 and cells expressing hMATE2-K by pretreatment with *p*-chloromercuribenzene sulfonate (pCMBS), a thiol-modifying reagent, and it was reversed by subsequent treatment with dithiothreitol (DTT), whereas no effect of such treatments was observed in mock cells (Fig. 8). These results suggest the inhibition of both hMATEs by thiol modification, which is consistent with the characteristic of the organic cation/H^+ antiport system in the kidney

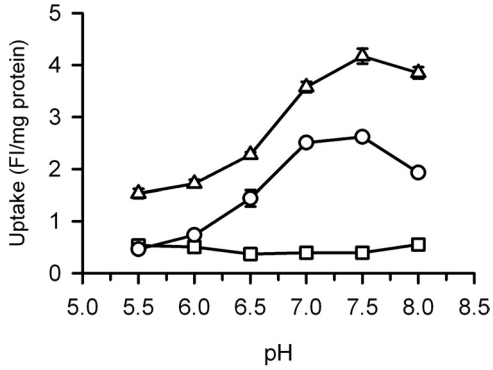

FIG. 6. Effect of extracellular pH on DAPI uptake in MDCKII cells stably expressing hMATEs. The uptake of DAPI (0.5 μM) was evaluated at 37°C for 10 min in MDCKII cells stably expressing hMATE1 (○), those expressing hMATE2-K (△), and mock cells (□). Data are presented as means ± S.E. ($n = 8$).

TABLE 1

Effect of various compounds on the uptakes of DAPI and [^{3}H]cimetidine mediated by hMATEs stably expressed in MDCKII cells

The uptakes of DAPI (0.5 μM) and [^{3}H]cimetidine (50 nM) were evaluated at 37°C and pH 7.4 for 10 min (DAPI) or 20 s ([^{3}H]cimetidine) in MDCKII cells stably expressing hMATE1, those expressing hMATE2-K, and mock cells in the presence or absence of a test compound. The specific uptakes by hMATEs are presented as means ± S.E. (n = 8). The control values for DAPI uptake were 2.34 and 3.70 FI/mg protein, respectively, for hMATE1 and hMATE2-K, and for cimetidine uptake were 0.75 and 0.39 pmol/mg protein, respectively.

Compound	Conc.	Uptake			
		hMATE1		hMATE2-K	
		DAPI	[^{3}H]Cimetidine	DAPI	[^{3}H]Cimetidine
	mM	*%control*			
None (control)		100.0 ± 1.4	100.0 ± 3.0	100.0 ± 1.5	100.0 ± 1.5
Cimetidine	0.2	N.D.	N.D.	24.6 ± 1.6*	3.1 ± 0.7*
Clonidine	0.2	45.4 ± 1.3*	18.4 ± 0.5*	65.2 ± 3.3*	24.2 ± 1.2*
Corticosterone	0.2	34.0 ± 2.0*	27.6 ± 0.4*	49.8 ± 2.6*	13.5 ± 0.5*
Diphenhydramine	0.2	34.1 ± 1.5*	23.6 ± 0.9*	57.2 ± 2.1*	41.4 ± 1.7*
Diltiazem	0.2	N.D.	5.8 ± 0.2*	47.4 ± 2.1*	33.6 ± 2.2*
Estrone-3-sulfate	0.2	80.5 ± 2.4*	65.9 ± 0.6*	100.8 ± 4.6	70.0 ± 2.8*
Guanidine	2	92.3 ± 2.6	92.9 ± 2.8	103.8 ± 1.8	87.8 ± 0.8*
Imipramine	0.2	1.0 ± 0.6*	11.5 ± 0.3*	19.2 ± 1.4*	25.9 ± 0.6*
MPP	0.2	60.6 ± 1.7*	27.7 ± 0.4*	82.8 ± 3.6*	27.3 ± 1.8*
Nicotinamide	0.2	105.1 ± 2.8	106.6 ± 5.1	112.0 ± 2.8*	97.0 ± 1.7
NMN	2	111.1 ± 1.8*	123.4 ± 5.0*	117.7 ± 4.4*	78.9 ± 1.5*
Norepinephrine	0.2	74.9 ± 1.8*	89.0 ± 1.2*	67.7 ± 2.9*	65.3 ± 3.8*
PAH	0.2	96.6 ± 6.0	132.0 ± 3.6*	105.3 ± 2.0	102.6 ± 4.1
Procainamide	0.2	96.4 ± 3.0	58.2 ± 1.0*	93.3 ± 3.3	30.5 ± 1.3*
Serotonin	0.2	93.3 ± 2.9	44.9 ± 2.0*	107.2 ± 4.4	69.2 ± 4.0*
TEA	2	54.6 ± 2.7*	23.6 ± 0.2*	88.4 ± 1.9*	27.0 ± 0.2*
TPP	0.2	12.9 ± 1.6*	13.0 ± 0.5*	67.9 ± 3.3*	26.4 ± 0.3*
Verapamil	0.2	N.D.	10.8 ± 0.6*	10.9 ± 1.4*	11.8 ± 0.7*

MPP, 1-methyl-4-phenylpyridinium; NMN, *N*-methylnicotinamide; PAH, *p*-aminohippurate; TPP, thiamine pyrophosphate; N.D., not detected.
* Significantly different from the value for control at $p < 0.05$.

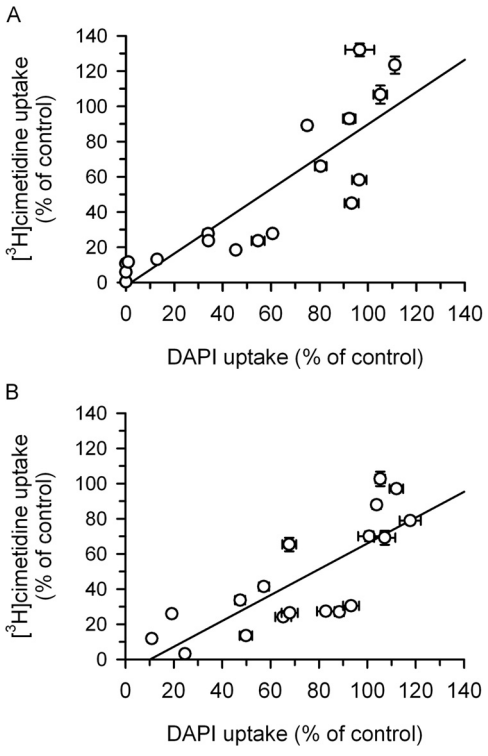

FIG. 7. Correlations between specific DAPI uptake and specific [^{3}H]cimetidine uptake for hMATE1 (A) and hMATE2-K (B) stably expressed in MDCKII cells. Data shown in Table 1 were examined for correlations. Regression equations are as follows: $y = 0.917x - 1.95$ with $r^2 = 0.749$ for hMATE1 and $y = 0.734x - 7.450$ with $r^2 = 0.612$ for hMATE2-K, where x and y represent DAPI uptake and [^{3}H]cimetidine uptake, respectively.

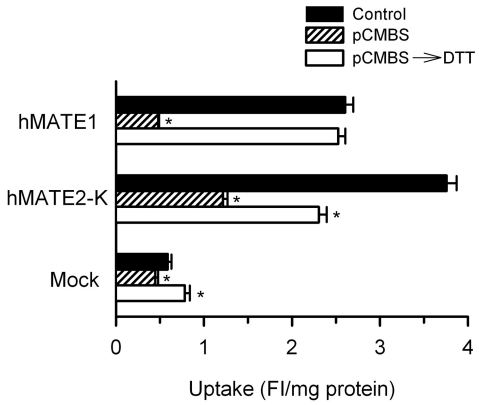

FIG. 8. Effect of pCMBS on DAPI uptake in MDCKII cells stably expressing hMATEs. MDCKII cells stably expressing hMATE1, those expressing hMATE2-K, and mock cells were incubated at 37°C and pH 7.4 in the absence of pCMBS and DTT for 10 min (control), or in the presence of pCMBS (0.5 mM) for 5 min and subsequently in the absence or presence of DTT (0.5 mM) for 5 min. Then, the uptake of DAPI (0.5 μM) was evaluated in the absence of pCMBS and DTT at 37°C and pH 7.4 for 10 min. Data are presented as means ± S.E. (n = 8). *, significantly different from the value for control at $p < 0.05$.

(Hori et al., 1987) and indicates that cysteine residues, which have a thiol group, may play an important role in the transporting function (Ohta et al., 2006; Asaka et al., 2007).

All of these results suggest that in both hMATEs DAPI may share the substrate recognition site with organic cations, despite the difference in the mode of transporting operation. Good correlations in the extents of inhibition by various compounds between uptake assays using DAPI and [^{3}H]cimetidine as substrates indicate that DAPI can be an alternative probe substrate that enables fluorometric rapid assays of the functionality of both hMATEs.

DAPI Uptake by hOCTs and hOCTNs. We further examined whether other major organic cation transporters, hOCT1 (Zhang et al., 1998), hOCT2 (Barendt and Wright, 2002), hOCT3 (Kekuda et al., 1998), hOCTN1 (Tamai et al., 1997), and hOCTN2 (Ohashi et al., 1999) could transport DAPI, using HEK293 cells transiently expressing each of them. By fluorescent microscopic observation (Fig. 9), DAPI was

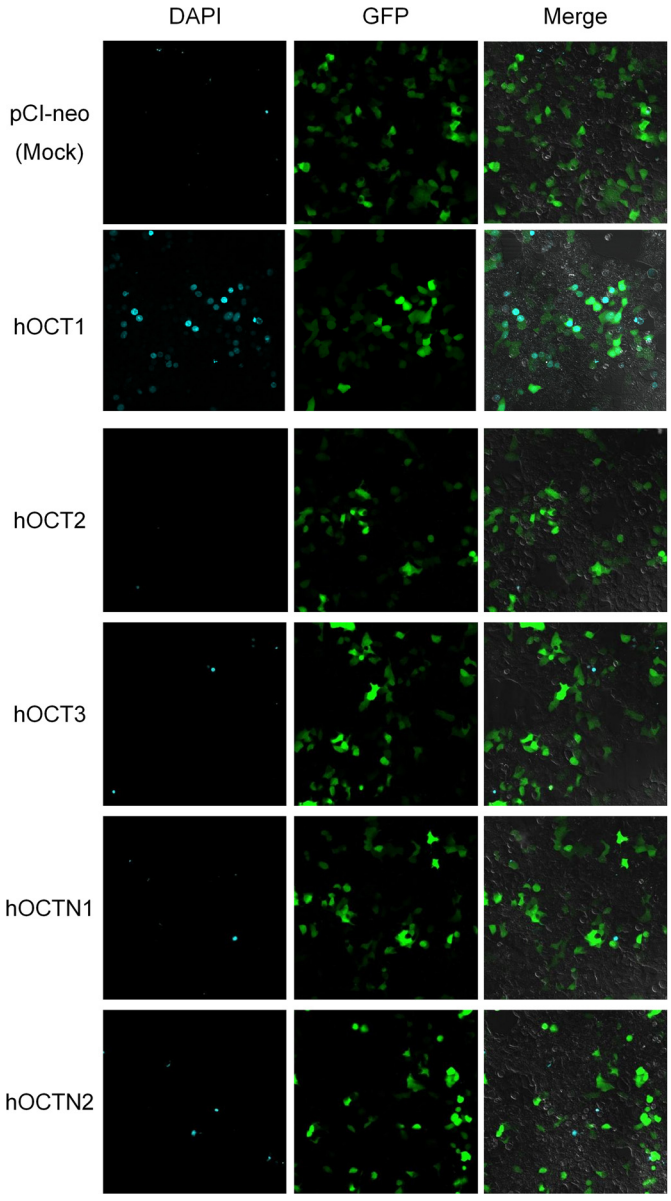

FIG. 9. Uptake of DAPI in HEK293 cells transiently expressing various human organic cation transporters. The fluorescent microscopic images of DAPI were obtained after incubating HEK293 cells transiently expressing each of hOCT1, hOCT2, hOCT3, hOCTN1, and hOCTN2, and mock cells at 37°C and pH 8.5 for 20 min. Also shown are the images of cotransfected GFP.

taken up into HEK293 cells by transiently expressed hMATE1 and hMATE2-K, as into MDCKII cells by the stably expressed one (Fig. 2). However, among hOCTs and hOCTNs, only hOCT1, which is mainly expressed in the liver, was found to transport DAPI (Fig. 9). Successful expressions of all the transporters were confirmed by the increased uptakes of their typical substrates, L-[^{3}H]carnitine (10 nM) for hOCTN2 and [^{14}C]TEA (10 μM) for the others, in transfected cells than in mock cells (Table 2). Because major renal organic cation transporters, hOCT2, hOCT3, hOCTN1, and hOCTN2, other than the hMATEs were thus found not to transport DAPI, the DAPI uptake assay can be a method specific to the hMATEs among organic cation transporters in the human kidney.

Discussion

DAPI is unique as a fluorescent probe in that it can emit fluorescence only when intercalated into DNA double strands (Kapuscinski, 1995). Thus, fluorometrically evaluated DAPI uptake in the present study represents, strictly, accumulation of DNA-bound DAPI. However, assuming swift diffusional distribution of DAPI in the small intracellular space and instantaneous binding of DAPI to DNA with high capacity, the fluorometric DAPI uptake rate would represent the rate of DAPI transport across the plasma membrane. At least, the specific entry of DAPI into the cells transfected with the hMATEs, hMATE1 and hMATE2-K, is undoubtedly mediated by them and, hence, changes in fluorometric DAPI uptake is in principle attributable to those in DAPI transport by them. This finding is supported by good correlations observed for both hMATEs between the extents of inhibition of specific DAPI uptake by various compounds and those of inhibition of specific [^{3}H]cimetidine uptake (Fig. 8). Although some deviations in correlations may indicate that we cannot exclude the possibility of the involvement of intracellular events, the uptake assay method using DAPI as a probe substrate can practically be effective for assessing the functionality of both hMATEs. Examples of its application include identification of inhibitors of these important secretory transporters with polyspecific characteristics. The fluorometric method using a plate reader is easier and faster than ordinary methods using high-performance liquid chromatography analysis and radioanalysis and can beneficially accelerate assays for, e.g., screening a large number of drugs and candidates for their potencies to inhibit the hMATEs. In addition, the irreversible DNA binding of DAPI with high capacity is also advantageous for its fluorometric detection, as it can be retained at high levels in the cells. It is also notable that DAPI enables real time visualization of its uptake process, because it does not emit fluorescence in the extracellular medium.

Even though both hMATEs were originally identified as H^+-coupled antiporters (Otsuka et al., 2005; Masuda et al., 2006), the transport of DAPI by neither of them was found to be accelerated by H^+ on the *trans* side. Reviewing our earlier study (Ohta et al., 2006), we find that although TEA transport by rat MATE1 (rMATE1) was accelerated by H^+ on the *trans* side, a small but significant level of TEA transport was also observed in the absence of a driving H^+ gradient. Low levels of apparently *trans* H^+-independent TEA transport have also been reported for both hMATEs (Otsuka et al., 2005; Masuda et al., 2006). Therefore, a possibility is that MATEs can act as facilitative transporters without H^+ and their operations can be accelerated in the presence of countering H^+, depending on substrates. *Trans* H^+ independence has also been observed for the transport of norfloxacin, an amphoteric compound that belongs to the fluoroquinolones, by rMATE1 in our recent study (Ohta et al., 2009). It is also notable that norfloxacin transport by rMATE1 was dependent on extracellular pH, similar to DAPI transport by the hMATEs. Because DAPI can be transported fairly well without H^+ by both

TABLE 2

Uptakes of probe substrates in HEK293 cells transiently expressing various human organic cation transporters

The uptake of [^{3}H]L-carnitine, for hOCTN2, or [^{14}C]TEA, for the others, was evaluated at 37°C and pH 8.5 for 10 min in HEK293 cells transiently expressing hOCT1, hOCT2, hOCT3, hOCTN1, and hOCTN2, and mock cells. Data are presented as means ± S.E. (n = 3).

Transporter	Substrate	Conc.	Uptake
		μM	*pmol/mg protein*
Mock (control)	TEA	10	6.0 ± 0.2
hOCT1			145.9 ± 1.5*
hOCT2			93.2 ± 7.6*
hOCT3			75.9 ± 1.1*
hOCTN1			48.2 ± 0.4*
Mock (control)	L-Carnitine	0.01	0.23 ± 0.01
hOCTN2			1.16 ± 0.06*

* Significantly different from the value for mock cells at $p < 0.05$.

hMATEs, the procedure of intracellular acidification, which is necessary for uptake assays using ordinary probe substrates such as cimetidine and TEA, is not required for the DAPI uptake assay. This is another merit of the assay method, which makes it easier and faster.

In conclusion, we have demonstrated that DAPI can be efficiently transported by both hMATEs, hMATE1 and hMATE2-K. By taking advantage of its fluorescent nature, it can be used as a probe substrate for rapid assays of the functionality of the hMATEs. Because the other major renal organic cation transporters were found not to transport DAPI, the DAPI uptake assay can be a method specific to the hMATEs among organic cation transporters in the human kidney.

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