Characterization of Human OCT1-Mediated Transport of DAPI as a Fluorescent Probe Substrate

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ABSTRACT: The present study was conducted to assess the functional characteristics of human organic cation transporter 1 (hOCT1) for the transport of 4′,6-diamidino-2-phenylindol (DAPI), a fluorescent compound that may be used as a probe substrate for rapid assays of its functionality. The specific uptake of DAPI by hOCT1 heterologously introduced into Madin–Darby canine kidney II cells by stable transfection was found to be, when assessed by DAPI-derived fluorescence intensity, rapid and saturable with a Michaelis constant of 8.94 μM, indicating that DAPI is a good substrate of hOCT1. The specific uptake of DAPI was insensitive to the membrane potential and extracellular pH, indicating a mode of operation different from that for typical cationic substrates such as tetraethylammonium (TEA), for which hOCT1 has been suggested to be driven by an inside-negative membrane potential and favor higher pH for optimal operation. However, many organic cations were found to inhibit the specific DAPI uptake with extents well correlated with those of inhibition of the specific uptake of [^{14}C]TEA, 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 hOCT1. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association
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INTRODUCTION

Organic cation transporter (OCT)1/SLC22A1 is a liver-specific member of OCTs and is suggested to be involved in the hepatic uptake of various cationic compounds.[1,2] Owing to its polyspecific nature, evaluations of its functionality, including those for identifying its substrates and inhibitors, have been of great interest in various aspects of drug development and drug therapy.[3–6] For such evaluations, simple and efficient methods are preferred generally and especially when many compounds are enrolled to be tested, for example, in drug development. It is expected that the use of a fluorescent probe substrate, if available, can help that because the fluorometric method using a plate reader is easier and faster than ordinary methods such as high-performance liquid chromatography (HPLC) analysis and liquid scintillation counting analysis of a radiolabeled probe compound.

We had recently reported that 4′,6-diamidino-2-phenylindol (DAPI) can be used as a fluorescent probe substrate for rapid assays of the functionality of human multidrug and toxin extrusion protein 1 (hMATE1/SLC47A1) and hMATE2-K/SLC47A2.[7] It was also suggested that human organic cation transporter 1 (hOCT1) can be another candidate for which DAPI is effective as a probe substrate. DAPI, which is widely used for nuclear staining, is unique as a fluorescent probe in that it can emit fluorescence only when intercalated, with a high capacity of irreversible binding characteristic, into DNA double strands.[8] This characteristic enables it to be retained at high levels in the cells after transport and, hence, it is advantageous in fluorometric detection over 4-(4-(dimethylamino)-styryl)-*N*-methylpyridinium,[9] [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium,[10] and berberine,[11] which are ordinary fluorescent agents transported by hOCT1. It is also notable that DAPI enables real-time

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visualization of its uptake process because it does not emit fluorescence in the extracellular medium.

The present study was therefore conducted to demonstrate that DAPI can be used to assess the functionality of hOCT1, characterizing the hOCT1-mediated transport of DAPI in detail and comparing it with that of tetraethylammonium (TEA), which is available in ^{14}C-labeled form as a typical organic cation.

MATERIALS AND METHODS

Materials

[^{14}C]TEA bromide (55.0 Ci/mmol) was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK) and DAPI was purchased from Sigma–Aldrich (St. Louis, Missouri). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, California). All other reagents were of analytical grade and commercially obtained.

Preparation of Madin–Darby Canine Kidney II Cells Stably Expressing hOCT1

Madin–Darby canine kidney (MDCK) II cells were transfected with the plasmid carrying the complementary DNA of hOCT1, which was prepared in our previous study,[7] by using Lipofectamine 2000 (Invitrogen) as a transfection reagent, according to the manufacturer's instructions, and cultured in DMEM supplemented with 10% FBS and 400 μg/mL G418 for 2 to 3 weeks. Antibiotic-resistant clones were selected and tested for the transport of DAPI as a probe substrate.

Uptake Study in MDCKII Cells Stably Expressing hOCT1

For the assays of DAPI uptake, MDCKII cells stably expressing hOCT1 (0.5×10^5 cells/well initially) were grown on 96-well plates for 48 h at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were used without polarization. In regular uptake assays, the cells in each well were preincubated in 0.25 mL of substrate-free uptake buffer (5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4 \cdot 7H_2O$, 136.7 mM NaCl, 0.385 mM $Na_2HPO_4 \cdot 12H_2O$, 0.952 mM $CaCl_2$, 25 mM D-glucose, and 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 8.5) for 20 min. In uptake assays at a low extracellular pH of 6.0, 10 mM HEPES was replaced with 10 mM 2-(*N*-morpholino)ethanesulfonic acid. Uptake assays were started by replacing the substrate-free uptake buffer for preincubation with uptake buffer containing DAPI (0.1 mL). All the procedures were conducted at 37°C. Assays were stopped by addition of ice-cold substrate-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 substrate-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. Fluorescence intensity was measured in arbitrary units and it is indicated to be FI in data presentation.

For the assays of [^{14}C]TEA uptake, MDCKII cells stably expressing hOCT1 (1.5×10^5 cells/well initially) were grown on 24-well plates for 72 h. The cells in each well were preincubated in 1 mL of substrate-free uptake buffer for 20 min and uptake assays were started by replacing the substrate-free uptake buffer for preincubation with uptake buffer containing [^{14}C]TEA (0.25 mL). All the procedures were conducted at 37°C. Assays were stopped by addition of ice-cold substrate-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% sodium dodecyl sulphate 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.,[12] 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.

Data Analysis

The specific uptake by hOCT1 was estimated by subtracting the uptake in mock cells from that in the hOCT1-transfected cells. The saturable transport was analyzed by assuming Michaelis–Menten type carrier-mediated transport represented by the following equation: $v = V_{\max} \times s/(K_{\mathrm{m}} + s)$. 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 concentration (s), using a nonlinear least-squares regression analysis program, WinNonlin (Pharsight, Mountain View, California), and the reciprocal of variance as the weight. The parameters are presented as the computer-fitted ones with standard error (SE).

Experimental data are presented as the means ± SE. Statistical analysis was performed using analysis of variance followed by Dunnett's test, with a p value of less than 0.05 was considered significant.

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