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Organic Cation Transporter 1 (OCT1/mOct1) Is Localized in the Apical Membrane of Caco-2 Cell Monolayers and Enterocytes[S]

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ABSTRACT

Organic cation transporters (OCTs) are members of the solute carrier 22 family of transporter proteins that are involved in absorption, distribution, and excretion of organic cations. OCT3 is localized in the apical (AP) membrane of enterocytes, but the literature is ambiguous about OCT1 (mOct1) localization, with some evidence suggesting a basolateral (BL) localization in human and mouse enterocytes. This is contrary to our preliminary findings showing AP localization of OCT1 in Caco-2 cell monolayers, an established model of human intestinal epithelium. Therefore, this study aims at determining the localization of OCT1 (mOct1) in Caco-2 cells, and human and mouse enterocytes. Functional studies using OCT1-specific substrate pentamidine showed transporter-mediated AP but not BL uptake in Caco-2 cells and human and mouse intestinal tissues. OCT1 inhibition decreased AP uptake of pentamidine by ~50% in all three systems with no effect on BL uptake. A short hairpin RNA-mediated OCT1 knockdown in Caco-2 cells decreased AP uptake of pentamidine by ~50% but did not alter BL uptake. Immunostaining and confocal microscopy in all three systems confirmed AP localization of OCT1 (mOct1). Our studies unequivocally show AP membrane localization of OCT1 (mOct1) in Caco-2 cells and human and mouse intestine. These results are highly significant as they will require reinterpretation of previous drug disposition and drug-drug interaction studies where conclusions were drawn assuming BL localization of OCT1 in enterocytes. Most importantly, these results will require revision of the regulatory guidance for industry in the United States and elsewhere because it has stated that OCT1 is basolaterally localized in enterocytes.

Introduction

The intestine plays a critical role in the absorption of nutrients and drugs. The intestinal epithelial cells (enterocytes) are polarized by the presence of tight junctions, and form a monolayer with the apical (AP) membrane facing the luminal side and the basolateral (BL) membrane facing the serosal side. Intestinal epithelium is the major barrier in the intestine and controls the absorption of nutrients and drugs. Lipophilic compounds can cross this barrier by passive diffusion across AP and BL membranes of epithelial cells. However, hydrophilic and charged compounds can cross the epithelial barrier either via a paracellular route, which is highly inefficient, or transcellularly with the assistance of one or more transporters that are present in epithelial cell membranes.

Epithelial cells in the intestine, liver, and kidney have specific transporters localized in either AP or BL membrane to facilitate directional transport of compounds and ensure their absorption by the intestine and excretion by the kidney and/or liver. Organic cation transporters (OCTs) belong to the solute carrier family 22 of polyspecific transporter proteins that are expressed in the liver, kidney, intestine, and other organs, transporting organic cationic compounds such as nutrients, endogenous amines, and many cationic drugs. OCTs are expressed as three different isoforms, namely, OCT1, OCT2, and OCT3, and exhibit organ-specific distribution.

Like other transporters, OCTs are expressed in specific cell membranes in epithelial tissues. For example, it is well established that OCTs such as OCT1 and OCT3 are localized in the sinusoidal (BL) membrane of human hepatocytes and are responsible for the uptake of cations from blood into the hepatocytes (Faber et al., 2003; Nies et al., 2009; Chen et al., 2010); similarly, in the kidney OCT2 is localized in the BL membrane of human renal proximal tubules and is responsible

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ABBREVIATIONS: AP, apical; BL, basolateral; CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylindole; KBR, Krebs' bicarbonate–Ringer's buffer; K_m, Michaelis-Menten constant; OCT, organic cation transporter; NGS, normal goat serum; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA.

for the uptake of cations from the blood (Okuda et al., 1996; Karbach et al., 2000; Motohashi et al., 2002). In the intestine, OCT3 is expressed in the AP membrane of enterocytes and mediates the uptake of cations from the lumen into the enterocytes (Muller et al., 2005; Koepsell et al., 2007). However, OCT1, which belongs to the same family of transporters as OCT3, has been reported to be localized in the BL membrane (Jonker et al., 2001; Wang et al., 2002; Watanabe et al., 2002a,b; Muller et al., 2005; Koepsell et al., 2007; Giacomini et al., 2010). This is surprising considering that other members of the OCT family, when expressed in the intestinal epithelium, are expressed in the AP membrane. It can be argued that OCT1 is expressed in the BL membrane to take up circulating organic cations into enterocytes; however, the affinities of OCTs for most substrates are low [e.g., apparent Michaelis-Menten constant (K_m) values $> 500\ \mu M$] (Koepsell et al., 2007) and hence a very high expression of the transporter would be needed to play a role in the serosal uptake and intestinal excretion of circulating organic cations.

Previous functional studies in our laboratory have suggested that OCT1 is not localized in the BL membrane of Caco-2 cell monolayers. The organic cation drug ranitidine, which is a substrate for OCTs, showed saturable AP uptake into Caco-2 cell monolayers with egress across the BL membrane via passive diffusion processes (Bourdet et al., 2005; Bourdet et al., 2006). BL uptake of ranitidine was mediated by a transporter with a very high apparent K_m value of 67 mM (Lee et al., 2002); this kinetic behavior is not consistent with the presence of OCT1 in the BL membrane of Caco-2 cell monolayers. Another organic cation drug metformin, also a substrate for OCTs (Kimura et al., 2005), showed saturable AP uptake and nonsaturable inefficient BL uptake into Caco-2 cell monolayers (Proctor et al., 2008).

These data suggest, but do not provide definitive evidence that OCT1 is apically localized in Caco-2 cell monolayers; however, the results seem to rule out BL localization of the transporter in this cell model. Preliminary immunolocalization studies in Caco-2 cell monolayers suggested that OCT1 was localized in the AP membrane (Supplemental Fig. 1; associated Supplemental Materials and Methods) (Ng, 2002). In this study, we investigated the cellular localization of OCT1 in Caco-2 cell monolayers as well as in mouse and human intestinal epithelia so as to resolve the contradiction between the ambiguous reports in the literature that suggest BL localization of this transporter and the preliminary findings in our laboratory that suggest AP localization of OCT1 in Caco-2 cell monolayers.

Materials and Methods

Materials

Minimum essential medium with Eagle's salts and L-glutamate, HEPES (1M), penicillin-streptomycin-amphotericin B solution (100×), nonessential amino acids (100×), geneticin, SuperScript III First-Strand Synthesis SuperMix, TaqMan Gene Expression Master Mix, and TaqMan Gene Expression Assays for OCT1 were obtained from Life Technologies Corporation (Grand Island, NY). Restore Western Blot Stripping Buffer and SuperSignal West Dura Extended Duration Substrate Kit were purchased from Thermo Scientific (Rockford, IL). Rabbit anti-human OCT1 antibody was purchased from Sigma-Aldrich (cat. no. AV41516; St. Louis, MO). Alexa Fluor 568 goat anti-rabbit IgG (A11036), Alexa Fluor 488 donkey anti-goat IgG (A11055), normal goat serum (NGS), nitrocellulose membrane filter paper sandwich, Nupage Western blot buffers, Bis-Tris gels, and other Western blotting reagents were purchased from Invitrogen (Carlsbad, CA). Radioimmunoprecipitation assay lysis buffer system, rabbit anti-human P-glycoprotein (P-gp) antibody (sc-8313), rabbit anti-human Na^+/K^+ ATPase antibody (sc-28800), goat anti-human villin antibody (sc-7672), goat anti-rabbit IgG-CFL488 (sc-362262), and goat anti-rabbit IgG-horseradish peroxidase (sc-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human and mouse jejunum paraffin sections were purchased from Zyagen (San Diego, CA). Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole) was purchased from Vector Laboratories (Burlingame, CA). [^{3}H]Pentamidine (4,4′-[pentane-1,5-diylbis(oxy)]dibenzenecarboximidamide) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA), and [^{14}C]TEA was purchased from American Radiolabeled Chemical Inc. (St. Louis, MO). Mitoxantrone (1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethylamino]-anthracene-9,10-dione), desipramine (3-(10,11-dihydro-5*H*-dibenzo[b, f]azepin-5-yl)-*N*-methylpropan-1-amine), and quinidine ((*S*)-(6-methoxyquinolin-4-yl)((2*R*,4*S*,8*R*)-8-vinylquinuclidin-2-yl)methanol) were purchased from Sigma-Aldrich. The Caco-2 (HTB-37) cell line was obtained from the American Type Culture Collection (Manassas, VA).

Caco-2 Cell Culture

Caco-2 cells were cultured as described previously elsewhere (Proctor et al., 2008). Briefly, the cells (passage numbers 30–40) were seeded at a density of 60,000 cells/cm^2 on polycarbonate membranes of Transwells. The culture medium was changed the day after seeding and every other day thereafter. Cell monolayers with transepithelial electrical resistance greater than 300 $\Omega \cdot cm^2$ were used for experimentation at 21–28 days after seeding.

Generation of OCT1-Knockdown Caco-2 Clones

Three OCT1-specific small interfering RNA sequences (Sigma-Aldrich) were evaluated for their ability to silence OCT1 gene expression. Sequence 1 (sense strand: 5′-GCUAUGAAGUGGACUGGAA-3′; antisense strand: 5′-UUCCAGUCCACUUCAUAGC-3′) and sequence 3 (sense strand: 5′-CCAUCUGUGUGGGCAUCGU-3′; antisense strand: 5′-ACGAUGCCCACACAGAUGG-3′) had the highest gene silencing activity (unpublished data). The antisense and sense oligonucleotides were linked with a hairpin loop and annealed with the respective complementary DNA oligonucleotides. Each resulting double-stranded DNA was inserted into BamHI and HindIII sites of the pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ) to generate a short hairpin RNA (shRNA) plasmid. Caco-2 cells at 90% confluency were transfected with the shRNA plasmid, using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol. Transfectants were selected by treatment with 0.2 mg/ml hygromycin B for 3 weeks, and were screened by quantitative real-time polymerase chain reaction (qRT-PCR) for OCT1 expression. Three clones (sequence 1 clone 43, and sequence 3 clones 21 and 27; i.e., 1-43, 3-21, 3-27) with the lowest gene expression, and normal morphology and growth rate were chosen for functional studies.

qRT-PCR for OCT1 Gene Expression in Caco-2 Cells

The mRNA expression of human OCT1 relative to 18s rRNA (18s) was determined by qRT-PCR analysis. The qRT-PCR experiments were conducted using established methods (Holmes et al., 2006) with minor deviations. Total RNA was isolated from Caco-2 cell monolayers using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA). We synthesized cDNA from the total RNA (5 μg) using Superscript III reverse transcriptase. An equal amount of RNA was included in a no-reverse transcriptase control for each separate RNA sample. We performed qRT-PCR using 1:20 dilutions of the cDNA. The PCR reactions (45 cycles) were conducted using TaqMan Gene Expression

Assays in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The expression of the 18s housekeeping gene was determined in each RT-PCR experiment and served as the normalization control ($n = 1$).

Functional Uptake Studies

In Caco-2 cell monolayers, the initial AP and BL uptake of [^{14}C]TEA and [^{3}H]pentamidine was quantified using methods outlined previously elsewhere (Proctor et al., 2008) with minor deviations. Uptake of [^{14}C]TEA (5 μM, 0.05 μCi/ml, 10 minutes) across the AP and BL membranes was measured in the absence or presence of 500 μM unlabeled TEA or 500 μM quinidine. [^{3}H]Pentamidine (0.1 μM, 0.05 μCi/ml, 5 minutes) uptake was performed in control and OCT1-knockdown cells. Mitoxantrone (25 μM) was used as the OCT1-specific inhibitor (Koepsell et al., 2007; Han et al., 2012), and 500 μM quinidine was used as the OCT pan inhibitor.

Human intestinal tissues collected from gastric bypass surgical patients were procured from the University of North Carolina Hospitals (Chapel Hill, NC), and all investigational experiments were performed in accordance with approval from the institutional review board at the Office of Human Research Ethics at the University of North Carolina at Chapel Hill and in compliance with federal regulations. The tissues were stripped of the exterior seromusculature and serosa layers by sharp resection, and the intestinal epithelium was mounted between two halves of a diffusion chamber insert. The entire procedure was completed within 1 hour from collection so as to preserve viability of the tissues.

The inserts were placed between two side-by-side diffusion chambers. Krebs' bicarbonate–Ringer's buffer (KBR; 3 ml) at 37°C was added to the AP and BL chambers and bubbled with oxygen/carbon dioxide (95:5) gas to maintain tissue viability (Johnson et al., 2002). For AP uptake studies, three intestinal epithelial samples were preincubated for 30 minutes at 37°C with 3 ml of KBR buffer in the AP and BL compartments.

After equilibration, the transepithelial electrical resistance was measured (greater than 250 $\Omega \cdot cm^2$) to ensure the epithelial integrity of the intestinal tissue. KBR buffer from the BL chamber was removed and replaced with fresh buffer; buffer in the AP chamber was replaced with [^{3}H]pentamidine [0.1 μM, 0.05 μCi/ml] in KBR, or [^{3}H]pentamidine (0.1 μM, 0.05 μCi/ml) with 25 μM mitoxantrone or 500 μM quinidine for inhibition studies. The intestinal epithelial segments were incubated for 5 minutes at 37°C, the buffer from both chambers was aspirated, and the tissues were washed 10 times in ice-cold KBR buffer, digested for 10 minutes with 0.2 ml of 2N NaOH at 50°C, and neutralized with 0.2 ml of 2N HCl and 0.2 ml of KBR. The fully digested tissue solution was analyzed by liquid scintillation spectrometry, and the rate of initial uptake of [^{3}H]pentamidine was determined.

For the mouse studies, male C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME) were housed according to approved Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC) requirements and protocols and in compliance with the Animal Welfare Act public laws. Mice were fasted overnight and then anesthetized with an i.p. injection of urethane (1.2–1.5 g/kg). A 10-cm segment of jejunum was dissected and flushed with 10 ml ice-cold KBR with 50% complete EDTA-free protease inhibitors. The intestinal segment was separated into 2-cm segments and mounted in a diffusion chamber, and the [^{3}H]pentamidine uptake in mouse intestinal tissue was determined as described with the exception of a mOct1 specific inhibitor, desipramine (10 μM), being used instead of mitoxantrone (Costales et al., 2011). Quinidine (500 μM) was used as a pan inhibitor.

Western Blot Analysis

The specificity of the primary rabbit anti-human OCT1 antibody was confirmed by Western blot analysis. Chinese hamster ovary (CHO) cells were cultured as described previously elsewhere (Ming et al., 2009). CHO cells singly transfected with human OCT1-3 or mouse mOct1-3, parental CHO K1 cells, Caco-2 cells (including wild-type and OCT1 knockdown Caco-2 cells), human liver, mouse liver, and mouse kidney specimens were lysed and homogenized in a radioimmunoprecipitation assay buffer system according to the manufacturer's protocol (Santa Cruz Biotechnology). The homogenized lysates were centrifuged at 14,000g for 15 minutes at 4°C. The pellets were discarded, and protein content in the supernatants was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Proteins were denatured with Nupage loading buffer and reducing agent, and heated at 70°C for 10 minutes. After being cooled to room temperature, the protein samples (20 μg each lane) were loaded to 4–12% (gradient) Bis-Tris gel, and separated by electrophoresis (200 V, 160 mA for 1 hour) and transferred to nitrocellulose membranes (30 V, 160 mA for 1 hour). The membranes were probed with rabbit anti-human OCT1 antibody followed by goat anti-rabbit IgG-horseradish peroxidase secondary antibody. Positive protein bands were detected with SuperSignal West Dura Extended Duration Substrate Kit (Thermo Scientific) and imaged by the Versa Doc Imaging System Model 1000 (Bio-Rad Laboratories, Hercules, CA). After imaging, the membrane was stripped, washed, and analyzed for glyceraldehyde-3-phosphate dehydrogenase.

Laser Scanning Confocal Microscopy

Caco-2 Cell Monolayers. Caco-2 cells grown on Transwells for 21–28 days were washed 3 times in phosphate-buffered saline (PBS), frozen in Tissue-Tek Cryo-OCT Compound (Sakura Fineteck, Torrance, CA), cut longitudinally into 5 μM thick sections, and fixed on glass slides with the AP side of the Caco-2 cells facing up so that the polarity of the cells could be readily identified. The sections were rinsed with PBS and permeabilized for 10 minutes in 0.5% Triton X-100 in PBS. The sections were blocked for 30 minutes with PBS containing 5% NGS followed by incubation with rabbit anti-human OCT1 primary antibodies (1:75 dilution) or rabbit anti-human P-gp primary antibodies (1:100 dilution) for 1 hour at room temperature. The slides were rinsed 3 times in PBS and then incubated with a secondary antibody (Alexa Fluor 568-conjugated goat anti-rabbit IgG for OCT1 or Alexa Fluor 488-conjugated goat anti-rabbit IgG for P-gp) for 1 hour at room temperature in the dark. After washing the slides 3 times in PBS, mounting medium with DAPI was applied for nuclear staining, and confocal images were obtained with a Leica SP2 AOBS laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

To visualize the localization of a BL biomarker against which AP localization of OCT1 can be compared, rabbit anti-Na^+/K^+ ATPase primary antibody (1:100 dilution) was used to detect Na^+/K^+ ATPase which is known to be confined to the BL membrane of Caco-2 cells and enterocytes. Alexa Fluor 568-conjugated goat anti-rabbit IgG was used as a secondary antibody.

Human and Mouse Intestinal Tissue. Slides containing paraffin-fixed human or mouse jejunum tissue sections were deparaffinized in xylene 3 times for 5 minutes, rehydrated in serial ethanol dilutions (2 times for 3 minutes in 100% ethanol, 1 minute in 95%, 1 minute in 80%, then rinsed in distilled water), and transferred to PBS. Slides were incubated in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) at 95–100°C for 30 minutes for antigen retrieval, and cooled down to room temperature.

The tissues were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, transferred back to PBS and blocked for 30 minutes with PBS containing 5% NGS, then incubated with rabbit anti-human OCT1 primary antibodies (1:75 dilution), rabbit anti-human P-gp primary antibodies (1:100 dilution), or rabbit anti-Na^+/K^+ ATPase primary antibody (1:100 dilution) for 1 hour at room temperature. The tissues were rinsed 3 times in PBS and then incubated with the secondary antibody (Alexa Fluor 568-conjugated goat anti-rabbit IgG for OCT1 and Na^+/K^+ ATPase; or Alexa Fluor 488-conjugated goat

anti-rabbit IgG for P-gp) for 1 hour at room temperature in the dark. After the tissues were washed three times in PBS, the mounting medium containing DAPI was applied, and confocal images were obtained with a Leica SP2 AOBS laser scanning confocal microscope.

To demonstrate colocalization of OCT1 (mOct1) with villin in Caco-2 cells and intestinal tissues, rabbit anti-human OCT1 and goat anti-human villin primary antibodies were applied together for 1 hour at room temperature, washed, and Alexa Fluor 568-goat anti-rabbit IgG and Alexa Fluor-488 donkey anti-goat IgG secondary antibodies were applied together to the cells or tissue slides. After washing, mounting medium with DAPI was applied, and confocal images were obtained with a Leica SP2 AOBS laser scanning confocal microscope.

Results

Functional Evidence for the Absence of Cation-Selective BL Transporters in Caco-2 Cell Monolayers and Enterocytes. TEA, a substrate for cation-selective transporters, was not taken up across the BL membrane of Caco-2 cell monolayers via a transporter-mediated process. This was evidenced by the result: the BL uptake of [^{14}C]TEA was not inhibited by unlabeled TEA at a 100-fold greater concentration or by the potent inhibitor of organic cation transporters quinidine (Fig. 1A). In contrast, unlabeled TEA and quinidine inhibited the uptake of [^{14}C]TEA across the AP membrane of Caco-2 cell monolayers by approximately 50 and 90%, respectively (Fig. 1A).

These results suggest that the BL membrane of Caco-2 cell monolayers does not express cation-selective transporters that can facilitate the transport of TEA, whereas one or more cation-selective transporters in the AP membrane mediate TEA uptake. Similarly, our results demonstrate that uptake of pentamidine, a selective substrate for OCT1 over OCT2 or OCT3 (Ming et al., 2009), was not mediated by OCT1 at the BL membrane of Caco-2 cell monolayers because the uptake was not inhibited by the selective OCT1 inhibitor mitoxantrone (Koepsell et al., 2007; Han et al., 2012) or the cationic transporter pan inhibitor quinidine. In contrast, ~50% of pentamidine uptake across the AP membrane was inhibited by mitoxantrone or quinidine (Fig. 1B). Collectively, these results provide strong evidence that the BL membrane of Caco-2 cell monolayers is devoid of OCT transporters, and that these transporters, including OCT1, are functional in the AP membrane.

Similar results were obtained with human intestinal epithelium and mouse intestinal tissue, where uptake of pentamidine across the BL membrane was not inhibited by the selective inhibitor of OCT1 (mitoxantrone) and mOct1 [desipramine 10 μM; IC_{50} values for mOct1 = 1.2 ± 1.1 μM, mOct2 = 11 ± 1.3 μM, mOct3 = 84 ± 1.1 μM (unpublished data)] (Costales et al., 2011), or the pan inhibitor quinidine; but these inhibitors inhibited pentamidine uptake across the AP membrane by ~50% (Fig. 1, C and D). These data imply that functionally active OCT1 localizes in the AP membrane of Caco-2 cell monolayers and human and mouse intestinal tissues, and is absent in the BL membrane.

AP and BL Uptake of Pentamidine in OCT1 Knockdown Caco-2 Clones. To confirm results from the chemical inhibition studies that provided evidence for functional localization of OCT1 in Caco-2 cells monolayers, we evaluated pentamidine uptake in Caco-2 cell monolayers in which OCT1 expression was downregulated by OCT1-specific shRNA. Three clones in which OCT1 expression was reduced by 70% or more were selected for experimentation (Fig. 2A). Uptake of pentamidine across the AP membrane was decreased by 50%

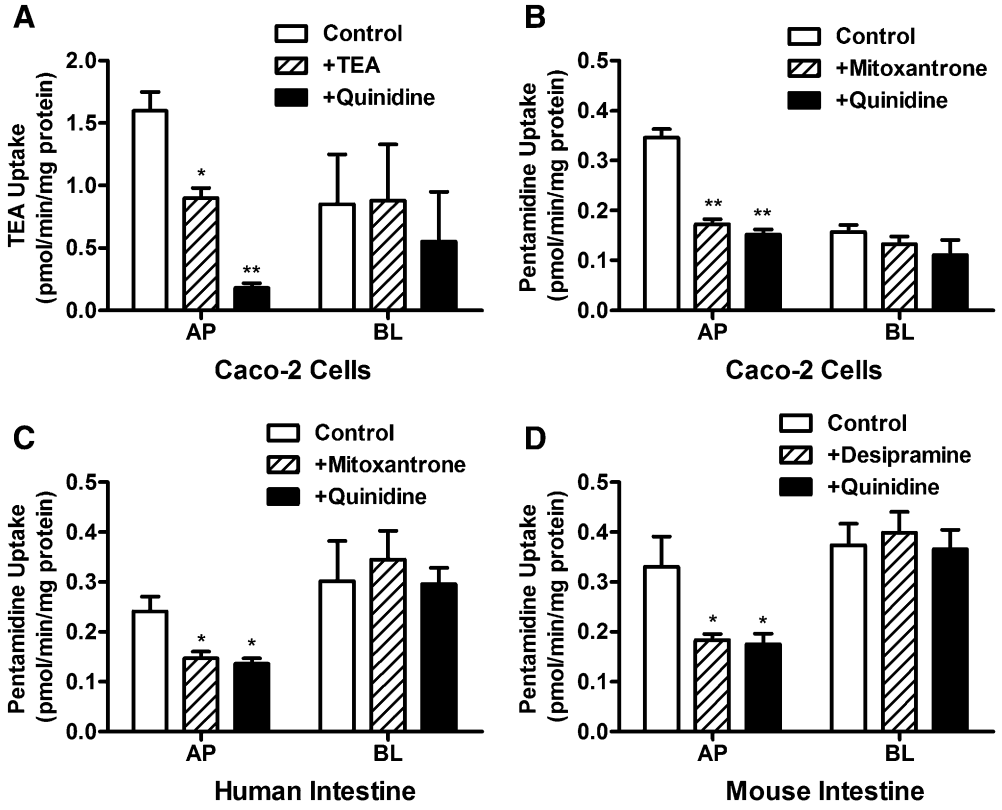

Fig. 1. AP and not BL uptake of TEA and pentamidine is reduced by OCT1 inhibitors in Caco-2 cell monolayers, human intestinal epithelium, and mouse intestinal tissue. (A) Initial uptake (10 minutes) of [^{14}C]TEA (5 μM, 0.05 μCi/ml) across the AP and BL membranes of Caco-2 cells was measured in the absence (open bars) or presence of unlabeled TEA (500 μM) (hatched bars) or quinidine (500 μM) (solid bars). Initial uptake (5 minutes) of [^{3}H]pentamidine (0.1 μM, 0.05 μCi/ml) in Caco-2 cell monolayers (B), human intestinal epithelium (C), and mouse intestinal tissue (D) across AP and BL membrane in the absence (open bars) and presence of mitoxantrone (25 μM) for OCT1 or desipramine (10 μM) for mOct1 (hatched bars), or quinidine (500 μM) (solid bars). Data represent the mean ± S.D. (n = 3). *$P < 0.05$; **$P < 0.01$, compared with control.

in OCT1-knockdown clones compared with control Caco-2 cell monolayers (Fig. 2B). Similar to the results from the chemical inhibition studies, the BL uptake of pentamidine in the OCT1-knockdown clones was not significantly different from that of control cells (Fig. 2C). Data from chemical inhibition and OCT1-knockdown studies provided definitive evidence that OCT1 in Caco-2 cell monolayers is not localized in the BL membrane but is present in the AP membrane.

Specificity of the Rabbit Anti-Human OCT1 Antibody for OCT1 and mOct1. To use the rabbit anti-human OCT1 antibody to assess the cellular localization of OCT1 and mOct1, it was important to demonstrate by Western blot analysis that the antibody detects OCT1 and does not cross-react with OCT2 or OCT3, but does cross-react with mOct1. Protein extracts from CHO cells that are singly transfected with OCT (mOct) transporters (OCT1-CHO, OCT2-CHO, OCT3-CHO, mOct1-CHO, mOct2-CHO, mOct3-CHO), parental CHO K1 cells, wild-type Caco-2 cells, OCT1 knockdown Caco-2 cells (clone 3-27), human liver, mouse liver, and mouse kidney were probed with the rabbit anti-human OCT1 antibody. The antibody specifically bound to a ~60 kDa protein, which is the expected size of OCT1 (mOct1), in the protein extracts of the OCT1-CHO cells, mOct1-CHO cells, wild-type Caco-2 cells, and human and mouse liver homogenates (positive controls). In contrast, the antibody either did not yield a band or yielded a faint band with the protein extracts of the OCT2-3 CHO cells, mOct2-3 CHO cells, CHO K1 cells, OCT1 knockdown Caco-2 cells, and mouse kidney (negative control) (Fig. 3). These results confirm that the rabbit anti-human OCT1 antibody detects OCT1 and mOct1 without producing a false-positive signal with OCT2-3 (mOct2-3).

Immunolocalization of OCT1 by Confocal Imaging in Caco-2 Cells and Human and Mouse Intestinal Tissues. Immunostaining and confocal microscopy studies in Caco-2 cell monolayers revealed that OCT1 is localized in the AP membrane (Fig. 4A). The efflux transporter P-gp, which is known to be exclusively localized in the AP membrane of Caco-2 cells, was found to be localized in the same membrane as OCT1 (Fig. 4B), thus confirming the AP expression of OCT1. Further, these studies confirmed that OCT1 is not expressed in the membrane where the BL membrane marker Na^+/K^+ ATPase was immunostained (Fig. 4C). Thus, the distinct difference in the polarized localization of OCT1 and Na^+/K^+ ATPase and the unmistakable overlap in the membrane localization of OCT1 and P-gp unequivocally confirm that OCT1 is localized in the AP membrane of Caco-2 cells. Similarly, OCT1 (mOct1) was found to be localized in the same membrane as P-gp in human (Fig. 4, D–F) and mouse enterocytes (Fig. 4, G–I), confirming AP localization of OCT1 (mOct1) in these tissues.

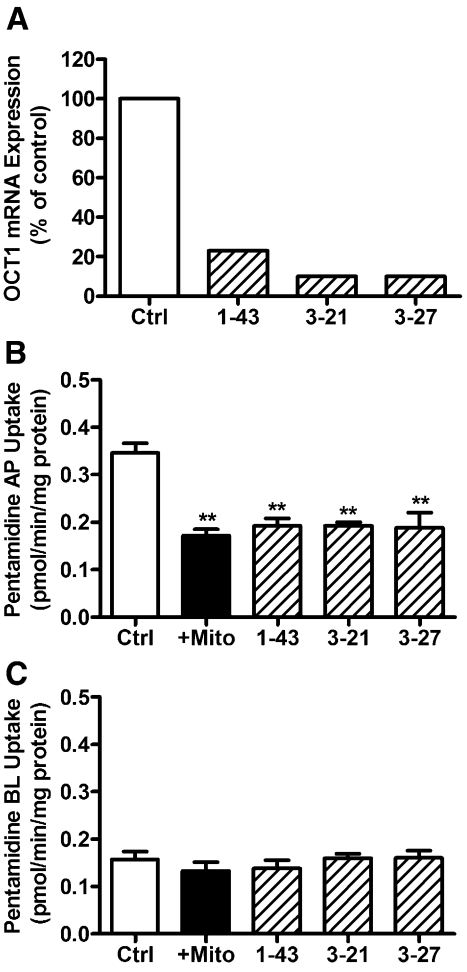

Fig. 2. AP and not BL uptake of pentamidine is reduced in OCT1 knockdown Caco-2 clones compared with wild-type Caco-2 cells. (A) OCT1 mRNA expression in control Caco-2 cells (open bar) and OCT1 knockdown Caco-2 clones 1-43, 3-21, and 3-27 (hatched bars) ($n = 1$). (B) AP and (C) BL uptake of [^{3}H]pentamidine (0.1 μM, 0.05 μCi/ml) (5 minutes) in the absence (open bars) and presence (solid bars) of mitoxantrone (25 μM) in control Caco-2 cells, and in OCT1 knockdown Caco-2 clones 1-43, 3-21, and 3-27 (hatched bars). In B and C, data represent the mean ± S.D. ($n = 3$). $^{**}P < 0.01$ compared with control.

Finally, because colocalization of OCT1 (mOct1) with P-gp in the same cell/tissue preparation did not produce a good signal due to lack of appropriate secondary antibodies, colocalization of OCT (mOct1) with villin, another apically localized protein, was investigated by immunostaining and confocal microscopy (Fig. 5). When images of antibody-labeled OCT1 (mOct1) (red fluorescence) and villin (green fluorescence)

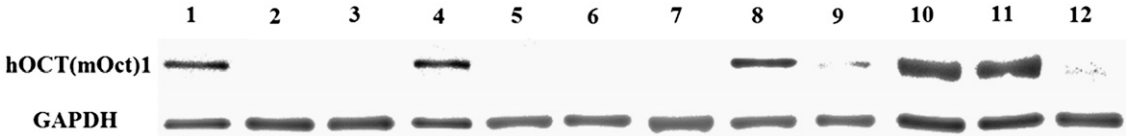

Fig. 3. Western blot analysis shows the specificity of rabbit anti-human OCT1 antibody for OCT1 and cross-reactivity with mOct1. Lanes 1–12: lane 1, OCT1-CHO; lane 2, OCT2-CHO; lane 3, OCT3-CHO; lane 4, mOct1-CHO; lane 5, mOct2-CHO; lane 6, mOct3-CHO; lane 7, parental CHO K1 cells; lane 8, wild-type Caco-2 cells; lane 9, OCT1-knockdown Caco-2 cells; lane 10, human liver homogenate; lane 11, mouse liver homogenate; lane 12, mouse kidney homogenate. Anti-human OCT1 antibody is specifically bound to a protein with an apparent molecular mass of ~60 kDa as shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Gene expression studies showed high expression of mOct1 in the mouse liver tissue and low expression in the mouse kidney tissue (data in Supplemental Fig. 2; associated Supplemental Materials and Methods).

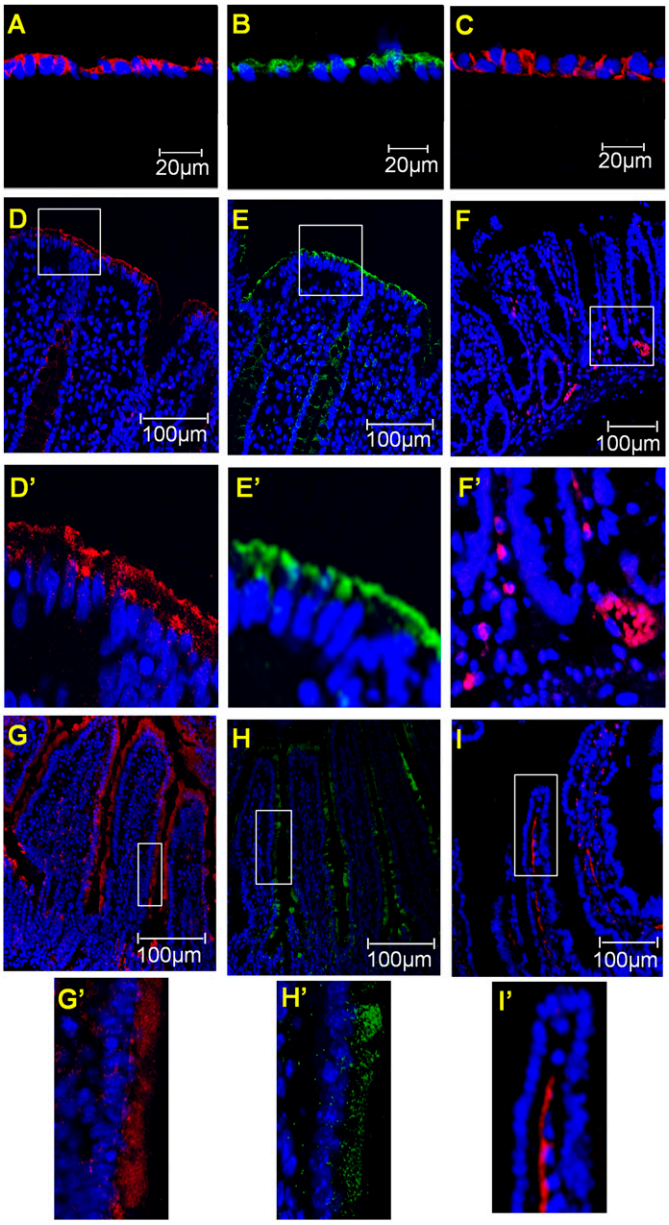

Fig. 4. Immunocytochemical localization of OCT1 (mOct1) in Caco-2 cell monolayers and in human and mouse intestinal tissues. (A) OCT1, (B) P-gp, and (C) Na^+/K^+ ATPase localization in Caco-2 cell monolayers. The orientation of the monolayers shows the AP side facing up and the basal side facing down. (D) OCT1, (E) P-gp, and (F) Na^+/K^+ ATPase localization in human intestinal tissues. (G) mOct1, (H) P-gp, and (I) Na^+/K^+ ATPase localization in mouse intestinal tissues. Blue fluorescence represents nuclear staining with DAPI. Larger amplifications of the boxed area in D–I were shown as D′–I′, respectively.

were merged, colocalization of these two proteins in Caco-2 cell as well as in human and mouse intestinal tissues was evidenced by a yellow color, thus unequivocally confirming the AP localization of OCT1 (mOct1).

Discussion

The results presented here contradict several reports that concluded that OCT1 (mOct1) is localized in the BL membrane of Caco-2 cell monolayers and enterocytes. The first suggestion that OCT1 (mOct1) is localized basolaterally in the intestinal epithelium came from studies by Jonker et al. (2001) based on their observation that approximately twice the amount of [^{14}C]TEA was found in the intestinal content of gall bladder–cannulated wild-type mice compared with mOct1$^{-/-}$ mice at 60 minutes after intravenous injection. However, it should be noted that the amount of TEA found in the intestinal content represented only 0.7 and 1.3% of the total excretion in mOct1$^{-/-}$ and wild-type mice, respectively, and that distribution of TEA in the liver was reduced by 4-fold in mOct1$^{-/-}$ mice compared with the wild-type mice. Further, the intestinal excretion of 1-methyl-4-phenylpyridinium, another mOct1 substrate, was not significantly different in mOct1$^{-/-}$ and wild-type mice. This was consistent with the findings of Bleasby et al. (2000) that showed that in Caco-2 cells OCT1 does not play a role in the BL uptake of 1-methyl-4-phenylpyridinium. Thus, the conclusion by Jonker et al. (2001) that mOct1 is basolaterally localized in the intestinal epithelium based on their results was tenuous.

Later, Wang et al. (2002) showed that the amount of metformin associated with the duodenum, jejunum, and ileum was 3- to 7-fold higher in wild-type mice than in mOct1$^{-/-}$ mice. The authors interpreted these results to be suggestive of the presence of mOct1 in the BL membrane of the intestinal epithelium because this membrane faces the serosal blood capillaries. However, they appropriately indicated that this hypothesis should be tested with further experiments.

Using functional studies of sulpiride uptake, Watanabe et al. (2002a,b) suggested that OCT1 is localized at the BL membrane of Caco-2 cells. Subsequently, in Caco-2 cell monolayers that are widely used to study intestinal drug absorption and that exhibit low but detectable levels of OCT1 mRNA (Muller et al., 2005; Englund et al., 2006; Seithel et al., 2006), BL localization of OCT1 was reported by Muller et al. (2005) based on immunostaining and confocal microscopy studies. They also reported BL localization of OCT1 in human intestinal tissue. However, the immunohistochemical images in this report show weak signals and widespread cellular distribution of OCT1, including the cytoplasm and entire cell membrane. This is likely due to the use in this study of a rat Oct1 antibody that was not sufficiently selective for the human OCT1 transporter. Taken together, this body of work has not provided conclusive evidence for BL localization of OCT1 (mOct1) in the intestinal epithelium, and it needs to be reinterpreted in light of our results.

Our results are highly significant because all the experimental observations since 2001 involving the intestinal absorption or secretion of cationic compounds have been interpreted based on the previously-mentioned studies that reported BL localization of OCT1 (mOct1). Our results warrant that the literature since 2001 on intestinal absorption and disposition of cationic drugs be re-evaluated. Our findings could impact not only the results involving the intestinal absorption of a single drug but also reports on drug-drug interactions of coadministered drugs that implicate intestinal OCT1 (mOct1). It is important to note that several reviews and the US Food and Drug Administration (FDA) guidance for the industry (CDER, 2012; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf) state that OCT1 is localized in

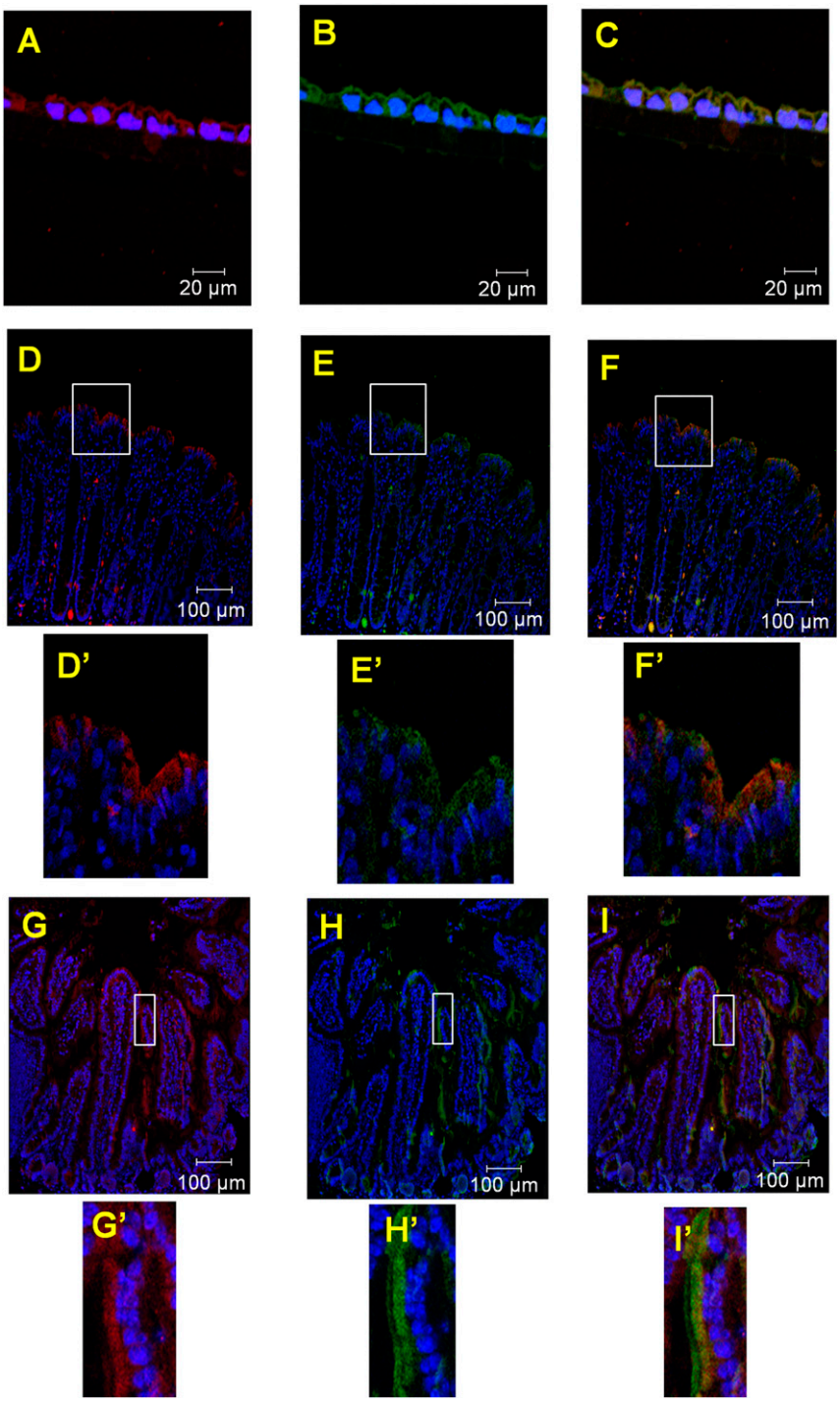

Fig. 5. Immunocytochemical colocalization of OCT1 (mOct1) with villin in Caco-2 cell monolayers and in human and mouse intestinal tissues. (A) OCT1, (B) villin, and (C) colocalization of OCT1 and villin in Caco-2 cell monolayers. The orientation of the monolayers shows the AP side facing up and the basal side facing down. (D) OCT1, (E) villin, and (F) colocalization of OCT1 and villin in human intestinal tissues. (G) mOct1, (H) villin, and (I) colocalization of mOct1 and villin in mouse intestinal tissues. Blue fluorescence represents nuclear staining with DAPI. Larger amplifications of the boxed area in D–I were shown as D′–I′, respectively.

the BL membrane of the enterocytes. The FDA guidance impacts numerous drug development decisions involving compounds that are likely or known substrates/inhibitors of OCT1. Clearly, a re-evaluation and revision of the FDA guidance is warranted.

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Authorship Contributions

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Performed data analysis: Han, Proctor, Ng, Thakker.

Wrote or contributed to the writing of the manuscript: Han, Everett, Proctor, Ng, Costales, Brouwer, Thakker.

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