Transepithelial fluxes of adenosine and 2'-deoxyadenosine across human renal proximal tubule cells: roles of nucleoside transporters hENT1, hENT2, and hCNT3

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Submitted 14 July 2008; accepted in final form 11 March 2009

ELWI AN, DAMARAJU VL, KUZMA ML, MOWLES DA, BALDWIN SA, YOUNG JD, SAWYER MB, CASS CE. Transepithelial fluxes of adenosine and 2'-deoxyadenosine across human renal proximal tubule cells: roles of nucleoside transporters hENT1, hENT2, and hCNT3. Am J Physiol Renal Physiol 296: F1439–F1451, 2009. First published March 18, 2009; doi:10.1152/ajprenal.90411.2008.—This study examined the roles of human nucleoside transporters (hNTs) in mediating transepithelial fluxes of adenosine, 2'-deoxyadenosine, and three purine nucleoside anti-cancer drugs across polarized monolayers of human renal proximal tubule cells (hRPTCs), which were shown in previous studies to have human equilibrative NT 1 (hENT1) and 2 (hENT2) and human concentrative NT 3 (hCNT3) activities (11). Early passage hRPTCs were cultured on transwell inserts under conditions that induced formation of polarized monolayers with experimentally accessible apical and basolateral domains. Polarized hRPTCs were monitored for inhibitor sensitivities and sodium-dependence of the following: 1) transepithelial fluxes of radiolabeled adenosine, 2'-deoxyadenosine, fludarabine (9-β-D-arabinosyl-2-fluoroadenine), cladribine (2-chloro-2'-deoxyadenosine), and clofarabine (2-chloro-2'-fluoro-deoxy-9-β-D-arabinofuranosyladenine); 2) mediated uptake of radiolabeled adenosine, 2'-deoxyadenosine, fludarabine, cladribine, and clofarabine from either apical or basolateral surfaces; and 3) relative apical cell surface hCNT3 protein levels. Transepithelial fluxes of adenosine were mediated from apical-to-basolateral sides by apical hCNT3 and basolateral hENT2, whereas transepithelial fluxes of 2'-deoxyadenosine were mediated from basolateral-to-apical sides by apical hENT1 and basolateral human organic anion transporters (hOATs). The transepithelial fluxes of adenosine, hCNT3-mediated cellular uptake of adenosine, and relative apical cell surface hCNT3 protein levels correlated positively in polarized hRPTCs. The purine nucleoside anti-cancer drugs fludarabine, cladribine, and clofarabine, like adenosine exhibited apical-to-basolateral fluxes. Collectively, this evidence suggested that apical hCNT3 and basolateral hENT2 are involved in proximal tubular reabsorption of adenosine and some nucleoside drugs and that apical hENT1 and basolateral hOATs are involved in proximal tubular secretion of 2'-deoxyadenosine.

Kidney; fludarabine; cladribine; clofarabine

RENAL HANDLING OF VARIOUS SOLUTES, SUCH AS NUCLEOSIDES (24, 34, 35), IN THE KIDNEY IS ACCOMPLISHED BY THE ASYMMETRICAL DISTRIBUTION OF TRANSPORTERS TO APICAL OR BASOLATERAL SURFACES OF EPITHELIAL CELLS. HUMAN NUCLEOSIDE TRANSPORTERS (hNTs) MEDIATE THE PASSAGE OF PHYSIOLOGICAL NUCLEOSIDES AND NUCLEOSIDE ANALOGS ACROSS BIOLOGICAL MEMBRANES (1, 17), WHICH COMprise TWO SOLUTE CARRIERS (SLC) FAMILIES, HUMAN EQUILIBRATIVE NTs (SLC29/hENTs: hENT1/2/3; Refs. 1–3, 18, 19), AND HUMAN CONCENTRATIVE NTs (SLC28/hCNTs: hCNT1/2/3; Refs. 17, 37–39, 44). COUPLING OF APICAL hCNT1/2/3, WHICH ARE Na+-nucleoside cotransporters with differing permeant selectivities (37–39, 44), TO BASOLATERAL hENT1/2, WHICH ARE FACILITATIVE TRANSPORTERS WITH BROAD SELECTIVITIES (18, 19), IS THOUGHT TO MEDIATE PROXIMAL TUBULAR NUCLEOSIDE REABSORPTION (25–27, 29). ENDogenous CNT3 IN MURINE PROXIMAL TUBULE CELLS AND hCNT3 IN TRANSFECTED MADIN-DARBY CANINE KIDNEY CELLS WERE SHOWN TO MEDIATE SODIUM-DEPENDENT APICAL-TO-BASOLATERAL (i.e., “REABSORPTIVE”) TRANSEPILETAL FLUXES OF VARIOUS NUCLEOSIDES, INCLUDING ADENOSINE AND FLUDARABINE (13). hENT3, WHICH IS AN INTRACELLULAR TRANSPORTER FOUND IN LYSOSOMES AND MITOCHONDRIA (2, 16), HAS MINIMAL EXPRESSION IN THE KIDNEY (2), AND hENT4, WHICH IS AN ADENOSINE TRANSPORTER LOCALIZED TO APICAL MEMBRANES OF TRANSFECTED MADIN-DARBY CANINE KIDNEY CELLS (3, 48), IS PRESENT IN KIDNEY TISSUE LYSATES (48).

The role of hNTs in renal secretion of other nucleosides is less clear. While adenosine reabsorption in vivo in mice is unaffected by the ENT1 inhibitor nitrobenzylmercapto purine ribonucleoside (NBMPR), 2'-deoxyadenosine is secreted in mice by ENT1-dependent processes in the kidney (24, 34, 35). Previous work (25) with hCNT1 and hENT1 cotransfected renal epithelial cell lines suggested that basolateral-to-apical transepithelial (i.e., “secretive”) fluxes of 2'-deoxyadenosine were due to the lower apparent affinity of hCNT1 for 2'-deoxyadenosine than for adenosine at physiological concentrations. An activity similar to that of pyrimidine nucleoside-selective hCNT1 but that also transports guanosine has been previously observed in human kidney brush border membrane vesicles (20), and hCNT1 has been detected in apical membranes of human kidney proximal tubules by immunostaining (15). While hCNT1 can also mediate transport of adenosine and 2'-deoxyadenosine, hCNT2 and hCNT3 both transport nucleosides at much higher capacities (37–39, 44) and both have been detected in apical membranes of human kidney proximal tubules by immunostaining (8, 15). The contribution of hCNT1 to purine nucleoside proximal tubular handling in the presence of hCNT2 and hCNT3 is uncertain.

The location of hENT1 in proximal tubules is controversial. In transfected renal proximal tubule epithelial cells, hENT1 is found primarily on basolateral membranes, with minor amounts present on apical membranes (25, 27, 29). One im-

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munostaining study (8) reported hENT1 in apical, but not basolateral, membranes of human kidney proximal tubules, while (15) another study reported hENT1 in both apical and basolateral membranes. Apical hENT1 may have a role in proximal tubular secretion of nucleosides by equilibration of nucleosides at luminal surfaces, as 2'-deoxyadenosine secretion in vivo is known to be ENT1 dependent (24, 35). The roles of hENT1 in proximal tubular secretion of nucleosides are currently unclear. Members of the human organic cation and anion transporter families (hOCTs and hOATs, respectively) may be involved along with hENT1 in selective secretion of some nucleosides (5–7, 35). hOCT1, which can mediate 7-deaza-2'-deoxyadenosine (2'-deoxytubercidin) transport (5, 6), and hOAT2, which can mediate 2'-deoxyadenosine and 2'-deoxyguanosine transport (7), are both present on basolateral membranes of human kidney proximal tubules (36).

Little is known about renal handling of fludarabine, cladribine, and clofarabine, analogs of adenosine and 2'-deoxyadenosine that are used to treat a variety of hematological malignancies. Fludarabine, cladribine, and clofarabine are renally eliminated with terminal half-lives of 30 h and plasma concentrations of free drug (<0.1 µM) that persist for 24–72 h after administration (14, 23).

To investigate the roles of hNTs in renal handling of adenosine, 2'-deoxyadenosine, fludarabine, cladribine, and clofarabine, the present study used polarized monolayers of hRPTCs because they were previously shown to have endogenous hENT1, hENT2, and hCNT3 activities (11). Transepithelial fluxes and cellular uptake of nucleosides were monitored to assess the potential involvement of hNTs, hOCTs, and hOATs in proximal tubular reabsorption and secretion processes. Adenosine “reabsorptive” fluxes (i.e., apical-to-basolateral) were mediated by apical hCNT3 and basolateral hENT2, while 2'-deoxyadenosine “secretory” fluxes (i.e., basolateral-to-apical) were mediated by apical hENT1 and basolateral hOATs. Fludarabine, cladribine, and clofarabine exhibited reabsorptive fluxes across polarized monolayers of hRPTCs. The extent of reabsorptive fluxes of adenosine, fludarabine, and cladribine across polarized monolayers of various hRPTCs correlated positively with apical hCNT3 activities.

MATERIALS AND METHODS

MATERIALS. Reagents and their sources were as follows: trypsin-EDTA, DMEM, Ham’s F-12 medium, glutamine, penicillin-streptomycin-ampicillin B, and FBS from Invitrogen (Burlington, ON, Canada); collagen type I from Inamed Biomaterials (Fremont, CA); selenium-insulin-transferrin, hydrocortisone, and epidermal growth factor from BD Biosciences (Mississauga, ON, Canada); polyester membrane 12-well, polycarbonate membrane 6-well, or HTS 24-well Transwell permeable supports from Corning Life Sciences (3 mM K₂HPO₄, 1.2 mM CaCl₂, 1 mM MgCl₂, 20 mM Tris-carbonate, pH 7.4), cells were incubated with anti-ZO-1 or anti-β-CAD monoclonal antibodies (1 µg/ml) for 30 min followed by AlexaFluor 488 goat anti-mouse secondary antibody (2 µg/ml) for 30 min. Slides were mounted with #1 coverslips using Mowiol mounting medium with p-phenylenediamine as anti-fade and with 1 µg/ml DAPI. Labelled cells were viewed on a Zeiss laser scanning confocal microscope (LSM 510 version 3.2, Jena, Germany) mounted on an Axiovert 100M inverted microscope with a plan Neofluar 40/1.3 oil immersion lens. An argon laser was used to scan at a wavelength of 488 nm, and an ultraviolet (UV) laser (364 nm) was used to excite DAPI-stained cells. Images were collected according to Nyquist sampling with a 505–550 nm band-pass filter for the AlexaFluor 488 signal and a band-pass filter of 385–470 nm for the DAPI signal.

Transepithelial flux assays. Transepithelial fluxes of radiolabeled nucleosides across polarized monolayers of hRPTCs cultured on HTS 24-well Transwell permeable supports were measured as described previously at room temperature (31, 32). Briefly, apical and basolateral chambers were 1) washed twice at room temperature with buffer (3 mM K₂HPO₄, 1.2 mM CaCl₂, 1 mM MgCl₂, 20 mM Tris·HCl, and 5 mM D-glucose pH 7.4) containing sodium (144 mM NaCl), referred to as sodium-containing buffer, or 2) incubated with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) where indicated, and nontradelabeled nucleoside or mannitol on both apical and basolateral sides in either sodium-containing or sodium-free buffer with or without various additives (i.e., potential permeants, inhibitors) for 60 min. [³H]nucleoside or [³H]mannitol (10 µCi/ml) was then added to apical or basolateral chambers, and 10-µl samples were collected from the opposite side at timed intervals, with subsequent replacement of removed samples with equal volumes of sodium-containing or sodium-free buffer, and transferred to vials that contained 10 ml of

Alberta/Canada Health Research Ethics Board. Informed consent was obtained in accordance with ethics approval guidelines. Apparently normal regions of human kidney tissue after complete or partial nephrectomy were used for isolation of cultures of hRPTCs from five individuals using an enzyme-dissociation method with type II collagenase (1 mg/ml) as described previously (4). hRPTC cultures were maintained on collagen-coated cell culture dishes in serum-free DMEM-Ham’s F-12 medium (50:50 by vol) supplemented with sodium (5 µg/l), insulin (5 µg/l), transferrin (5 µg/l), hydrocortisone (36 µg/l), epidermal growth factor (10 µg/l), triiodothyronine (4 ng/l), glutamine (2 mM), and penicillin-streptomycin-ampicillin B (0.1 U/l, 100 ng/l, and 250 µg/l, respectively) at 37°C in a humidified atmosphere containing 5% CO₂. Subculturing of hRPTCs was performed by detachment with trypsin-EDTA (0.5 and 0.2 g/l, respectively) for five passages. Addition of FBS was used to stop trypsinization. All experiments were performed with hRPTCs after five passages using cultures that were initiated at confluent densities 10 days earlier on Transwell permeable supports to induce formation of polarized monolayers as described previously (31).
Ecolite scintillation fluid for analysis of radioactivity. Flux values were normalized to total protein content per well determined by Bio-Rad protein assay of triplicate cultures. Mannitol fluxes were normalized to total protein content per well, which was determined by Bio-Rad protein assay of triplicate cultures. Mannitol fluxes were used to estimate paracellular fluxes and were subtracted from total nucleoside fluxes to obtain mediated nucleoside fluxes. Three independent flux experiments, each with triplicate measurements, were performed.

Uptake assays. Uptake of radiolabeled nucleosides into polarized monolayers of hRPTCs cultured on HTS 24-well Transwell permeable supports was measured as described previously at room temperature (31, 32). Apical and basolateral chambers of Transwell inserts containing cells were 1) washed twice with sodium-containing or sodium-free buffer; and then 2) incubated for 60 min with 500 μM EHNA where indicated and 1 μM nonradiolabeled adenosine or 2'-deoxyadenosine in either sodium-containing or sodium-free buffer at pH 7.4 with or without various additives (i.e., potential permeants, inhibitors). Uptake assays were initiated by adding [3H]nucleoside (10 μCi/ml) to apical or basolateral chambers. Uptake assays were stopped by washing cells three times with ice-cold sodium-containing buffer after which inserts were air dried, solubilized in 5% (wt/vol) Trition X-100, and then transferred to vials with 10 ml of Ecolite scintillation fluid for analysis of radioactivity. Uptake values were normalized to total protein content per well determined by Bio-Rad protein assay of triplicate cultures. Three independent uptake experiments, each with triplicate measurements, were performed.

TLC analysis. Transepithelial fluxes and uptake of [3H]adenosine and 2'-[3H]deoxyadenosine in polarized hRPTC cultures, grown on HTS 24-well Transwell permeable supports, were assayed for 60 min as described above. After 50-μl samples from apical or basolateral compartments were collected and Transwell permeable supports containing cells were cut out, they were incubated in ice-cold 7% (vol/vol) perchloric acid for 1 h. Acid-soluble extracts containing nucleoside metabolites were removed and neutralized with equal volumes of ice-cold 1 M KOH, followed by centrifugation at 10,000 g for 10 min at 4°C. Supernatants were evaporated and resuspended in 10 μl of deionized H2O. Aluminum-backed Silica Gel 60 TLC plates containing fluorescent indicator F254 (EMD Chemicals, Gibbstown, NJ) were spotted with 1-μl portions of each 10 mM standard alone: ATP, dATP, ADP, dADP, AMP, dAMP, adenosine, 2'-deoxyadenosine, inosine, 2'-deoxyinosine, and hypoxanthine. Plates were developed using n-butanol/ethyl acetate/methanol/ammonium hydroxide (7:4:3:4 vol/vol/vol/vol) as described previously (41). Metabolites were identified under UV light and retention factor values were calculated and compared with individual standards for identification. The UV-identified spots were cut out and dissolved in 10 ml of Ecolite scintillation fluid overnight at room temperature for analysis of radioactivity. Results are expressed as a percentage of radioactivity loaded (determined by scintillation counting of 1-μl portions of experimental extracts) for each identified purine metabolite.

RT-PCR analysis. Total RNA was isolated from each of the five different hRPTC cultures, grown as polarized monolayers on six-well Transwell permeable supports, using the GenElute Mammalian Total RNA Kit from Sigma. RT-PCR using gene-specific primers for hOAT2 was performed with One-Step RT-PCR with Platinum Taq from Invitrogen using oligonucleotides (5'-gagagttaacgctgcaacctg-3'; 5'-ctggcagctggagcagta-3') and the following cycling parameters for 40 cycles: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. To control for contaminating genomic DNA, reactions that lacked reverse transcriptase were included. The expected PCR product size for hOAT2 was 0.59 kb. PCR products were purified using a QIAquick Gel Extraction kit from Qiagen (Mississauga, ON, Canada) and sequenced to confirm their identities.

Immunoblotting analysis. Crude membranes were isolated from 1 g of kidney cortex tissue from each of the five kidneys from which the five different hRPTC cultures were produced, using previously described methods (8). Apical cell surface protein preparations were isolated from each of the five hRPTC cultures, grown as polarized monolayers on six-well Transwell permeable supports, using a Cell Surface Protein Isolation kit from Pierce (Rockford, IL). Crude membranes (20 μg protein) and apical cell surface protein preparations (20 μg protein) were run on SDS-polyacrylamide gels (10% wt/vol), transferred to Immobilon-P polyvinylidene fluoride membranes from Millipore (Bedford, MA), or 3) immunoblotted with anti-hCNT3 monoclonal antibodies and anti-mouse IgG Alexa Fluor 488 or anti-mouse IgG horseradish peroxidase secondary antibodies to visualize or quantify relative immunoreactive band intensities, respectively, by Enhanced Chemiluminescence from Amersharm Pharmacia Biotech (Uppsala, Sweden) or by fluorescence imaging using a Typhoon 8600 Variable Mode Imager from Amersharm Biosciences (Baie d’Urfé, QC, Canada), respectively. To control for protein loading, immunoblots were incubated with stripping buffer (50 mM Tris pH 6.8, 2% SDS, and 1% vol/vol β-mercaptoethanol) at 50°C for 30 min and reprobed with anti-β-actin antibodies. Immunoblots were produced in triplicate.

Production of recombinant transporters and measurement of uptake in S. cerevisiae. hCNT3 cDNAs were inserted into the yeast expression vector pYPGE15 and the resulting plasmids were transformed into the strain fuil1::TRP1 (MATa, gal, ura3–52, trpl, lys2, ade2, hisd2000, and Δflu1::TRP1: Ref. 50). Uptake of varying concentrations of [3H]adenosine and 2'-[3H]deoxyadenosine into yeast was measured at timed intervals using a semi-automated cell harvester (Micro96 HARVEST; Skatron Instruments, Lier, Norway) as described previously (50). Experiments were performed in triplicate and kinetic parameters were calculated using GraphPad Prism 4.0 software (GraphPad Software; http://www.graphpad.com).

Statistical analysis. Statistical comparisons of experimentally determined values for measurements obtained in the presence or absence of various inhibitors for each hRPTC culture were done by unpaired t-tests. Statistical comparisons of experimentally determined values between different hRPTC cultures were done by one-way ANOVA multiple comparison tests. When overall P values <0.05, Tukey’s posttest was performed to determine which values were significantly different from each other. Correlations of experimentally determined values between different measurements obtained in hRPTCs analyses were performed by Pearson correlation analysis. All calculations and graphs were generated using GraphPad Prism 4.0 software.

RESULTS

Formation of polarized monolayers. The experiments shown in Fig. 1 were undertaken to demonstrate the formation of polarized monolayers of hRPTC11 cultures prepared on collagen-coated transwell inserts. TEER values rose to a plateau of ~100 Ω·cm² by day 10 for hRPTC11 cultures (Fig. 1A), indicating formation of polarized monolayers with leaky tight junctions (31, 45).

To demonstrate distinct apical and basolateral membrane domains of the polarized monolayers, the locations of tight junction protein ZO-1 and cell adhesion protein E-CAD were determined in hRPTC11 cultures prepared on collagen-coated transwell inserts by immunofluorescent staining and confocal imaging. Immunofluorescent staining with anti-ZO-1 antibodies showed ZO-1 in apical tight junctions between cells in xy image sections and orthogonal projections of z-stack images (Fig. 1, B-E), as expected (42). Some minimal intracellular background staining of ZO-1 was seen in orthogonal projections of z-stack images (Fig. 1E). On the other hand, immunofluorescent staining with anti-E-CAD antibodies showed E-CAD in basolateral membrane domains between cells in xy image sections and orthogonal projections of z-stack images (Fig. 1, F-I), as expected (30, 47). No E-CAD
staining was observed between cells and inserts, perhaps due to limited resolution at insert boundaries (Fig. 1). No staining was observed with isotype control antibodies (data not shown). DAPI counterstaining was used to delineate cell positions (Fig. 1, B-I). Formation of polarized monolayers for four other cultures of hRPTCs (hRPTC12, hRPTC13, hRPTC14, and hRPTC15) on transwell inserts was confirmed in parallel experiments that measured rises in TEER values and the locations of ZO-1 and E-CAD (data not shown). Collectively, these results demonstrated that hRPTC cultures isolated from different individuals consistently formed polarized monolayers with distinct apical and basolateral domains.

Transepithelial fluxes of adenosine and 2′-deoxyadenosine.

To investigate the role of hNTs in proximal tubular handling of adenosine and 2′-deoxyadenosine, their transepithelial fluxes were assessed across polarized hRPTC11 monolayer cultures. Fluxes of [3H]mannitol were used to estimate paracellular permeability, and these values were subtracted from total [3H]nucleoside fluxes to determine mediated fluxes. Concentrations of [3H]adenosine and 2′-[3H]deoxyadenosine were 1 μM, since circulating physiological and renal concentrations are ≤1 μM (39, 50). In the experiments shown in Fig. 2, substantial apical-to-basolateral fluxes of adenosine were observed across polarized hRPTC11 cultures with significantly higher levels in the presence of 500 μM EHNA, an adenosine deaminase inhibitor (P < 0.01; Fig. 2A; Ref. 45), whereas only very small basolateral-to-apical fluxes were observed and these were not enhanced by EHNA (Fig. 2B). In contrast, substantial basolateral-to-apical fluxes of 2′-deoxyadenosine were observed across polarized hRPTC11 cultures with a small but significant increases in the presence of EHNA (P < 0.01; Fig. 2B), whereas only very small apical-to-basolateral fluxes were observed and these were not enhanced by EHNA (Fig. 2C). In contrast, substantial basolateral-to-apical fluxes of 2′-deoxyadenosine were observed across polarized hRPTC11 cultures with a small but significant increases in the presence of EHNA (P < 0.01; Fig. 2B), whereas only very small apical-to-basolateral fluxes were observed and these were not enhanced by EHNA (Fig. 2C). Apical-to-basolateral fluxes of adenosine were higher than basolateral-to-apical fluxes of 2′-deoxyadenosine in both the presence and absence of EHNA (P < 0.01; Fig. 2, A and B).

Results of studies of metabolism of [3H]adenosine and 2′-[3H]deoxyadenosine after transepithelial flux assays are...
shown in Table 1. In the presence of EHNA, the majority of radioactivity recovered when adenosine moved from apical-to-basolateral compartments and when 2'-deoxyadenosine moved from basolateral-to-apical compartments was unaltered nucleoside. In contrast, in the absence of EHNA, there was significant degradation of the fluxed adenosine to inosine and hypoxanthine and of the fluxed 2'-deoxyadenosine to 2'-deoxyxanthine and hypoxanthine (P < 0.01; Table 1). The majority of intracellular recovered radioactivity after apical-to-basolateral transepithelial fluxes of adenosine in the presence of EHNA was adenosine and phosphorylated forms of adenosine and in the absence of EHNA was hypoxanthine and phosphorylated forms of adenosine (Table 1). Similarly, intracellular recovered radioactivity after basolateral-to-apical transepithelial fluxes of 2'-deoxyadenosine in the presence of EHNA was unaltered 2'-deoxyadenosine and in the absence of EHNA was hypoxanthine (Table 1). In this study, subsequent experiments with adenosine and 2'-deoxyadenosine were performed in the presence of 500 μM EHNA to inhibit deamination. Preferential apical-to-basolateral transepithelial fluxes of adenosine and basolateral-to-apical transepithelial fluxes of 2'-deoxyadenosine were confirmed for the five different hRPTC cultures (i.e., isolated from kidneys of 5 different individuals), and the results are summarized in Tables 2 and 3.

Table 1. Analysis of adenosine and 2'-deoxyadenosine metabolites after apical-to-basolateral transepithelial fluxes of 1 μM [3H]adenosine and after basolateral-to-apical transepithelial fluxes of 1 μM 2'-[3H]deoxyadenosine across polarized hRPTC11 monolayers after 60 min

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Adenosine Metabolites Recovered in Basolateral Compartment, %radioactivity loaded</th>
<th>Adenosine Metabolites Recovered Intracellularly, %radioactivity loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺</td>
<td>Na⁺ + 500 μM EHNA</td>
</tr>
<tr>
<td>Phosphorylated</td>
<td>10.7±1.8</td>
<td>15.6±2.1</td>
</tr>
<tr>
<td>Adenosine</td>
<td>31.5±3.3</td>
<td>72.3±5.8</td>
</tr>
<tr>
<td>Inosine</td>
<td>21.2±2.6</td>
<td>3.2±2.6</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>20.9±4.1</td>
<td>4.3±1.9</td>
</tr>
<tr>
<td>Other</td>
<td>15.7±2.5</td>
<td>4.6±2.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2'-Deoxyadenosine Metabolites Recovered in Apical Compartment, %radioactivity loaded</th>
<th>2'-Deoxyadenosine Metabolites Recovered Intracellularly, %radioactivity loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>Na⁺ + 500 μM EHNA</td>
</tr>
<tr>
<td>Phosphorylated</td>
<td>15.2±1.7</td>
</tr>
<tr>
<td>2'-Deoxyadenosine</td>
<td>35.6±4.5</td>
</tr>
<tr>
<td>2'-Deoxynosine</td>
<td>10.1±3.2</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>30.4±2.7</td>
</tr>
<tr>
<td>Other</td>
<td>9.2±2.5</td>
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hRPTC, human renal proximal tubule cells. TLC analyses of metabolites were performed as described in MATERIALS AND METHODS. “Other” constitutes unidentified spots on TLC plates that were pooled.

Fig. 2. Demonstration of apical-to-basolateral fluxes of adenosine and basolateral-to-apical fluxes of 2'-deoxyadenosine across polarized hRPTC11 cultures. Apical-to-basolateral fluxes of 1 μM [3H]adenosine (A) or 2'-[3H]deoxyadenosine (D) and basolateral-to-apical fluxes of 1 μM [3H]adenosine (C) or 2'-[3H]deoxyadenosine (B) were measured over time across polarized monolayers of hRPTC11 cultures in sodium-containing buffer with (●) or without (◆) 500 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) as described in MATERIALS AND METHODS. *Significantly different values in the presence vs. the absence of EHNA (P < 0.01). Values are means ± SD for 3 experiments each with triplicate measurements. Error bars are absent where data symbols were larger than SD values.
The observed lag times in which no transepithelial fluxes were observed (< 30 min; see Fig. 2 for results with hRPTC11 cultures) may have been due to the accumulation of phosphorylated forms of adenosine and 2′-deoxyadenosine within cells before commencement of subsequent effluxes into the opposite extracellular compartments.

Apical-to-basolateral fluxes of adenosine and basolateral-to-apical fluxes of 2′-deoxyadenosine across polarized hRPTC11 cultures were almost completely ablated in the absence of sodium and completely inhibited by dilazep at 200 μM (P < 0.001), a concentration that inhibits both hENT1 and hENT2, the hENTs known to be present in hRPTC cultures (Fig. 3, A and B). On the other hand, apical-to-basolateral fluxes of adenosine were not affected by NBMPR at 0.1 μM, a concentration that inhibits hENT1 but not hENT2, whereas basolateral-to-apical fluxes of 2′-deoxyadenosine were inhibited by 0.1 μM NBMPR (P < 0.001; Fig. 3, A and B). These results indicated that adenosine “reabsorptive” fluxes were dependent on hCNTs and hENT2 but not hENT1 and 2′-deoxyadenosine “secretive” fluxes were dependent on hENT1. Also, apical-to-basolateral fluxes of adenosine were slightly increased in the presence of NBMPR (P < 0.05; Fig. 3A), possibly due to inhibition of efflux through hENT1 at apical membranes.

In five different polarized hRPTC cultures, the apical-to-basolateral fluxes of adenosine were higher in the presence of sodium than in its absence (P < 0.01) and the basolateral-to-apical fluxes of 2′-deoxyadenosine were lower in the presence of NBMPR than in its absence (P < 0.01; Table 2). These results suggested that apical-to-basolateral fluxes of adenosine were sodium dependent and mediated by apical hCNTs and that basolateral-to-apical fluxes of 2′-deoxyadenosine were dependent on hENT1. Attempts to localize hENT1, hENT2, and hCNT3 by immunofluorescent staining with anti-hNT-specific monoclonal antibodies in polarized monolayer cultures of hRPTCs were unsuccessful, possibly due to low protein abundance.

To investigate whether or not hOCTs and hOATs are involved in proximal tubular secretion of 2′-deoxyadenosine, the effects of 500 μM cimetidine or probenecid, inhibitors of hOCTs and hOATs, respectively, on basolateral-to-apical fluxes of 2′-deoxyadenosine across polarized hRPTC11 cultures were examined in the experiments of Fig. 3B. Although cimetidine had no effect on apical-to-basolateral fluxes of 2′-deoxyadenosine, probenecid reduced basolateral-to-apical fluxes (P < 0.05; Fig. 3B), implicating hOATs in the observed 2′-deoxyadenosine secretive fluxes. The reduction of basolateral-to-apical fluxes of 2′-deoxyadenosine by probenecid was confirmed for the five different hRPTC cultures, and the results are summarized in Table 2 (P < 0.05).

**Table 2. Summary of 1 μM [3H]adenosine and 2′-[3H]deoxyadenosine transepithelial fluxes and uptake in polarized hRPTC monolayers**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Apical-to-basolateral fluxes</th>
<th>Basolateral-to-apical fluxes</th>
<th>Basolateral-mediated uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sodium-containing buffer (Control), pmol/mg protein -1·60 min -1</td>
<td>Sodium-free buffer, %reduction of control values</td>
<td>Apical hCNT3-mediated uptake, pmol/mg protein -1·10 min -1</td>
</tr>
<tr>
<td></td>
<td>2′-Deoxyadenosine</td>
<td>Control, pmol/mg protein -1·60 min -1</td>
<td>0.1 μM NBMPR, %reduction of control values</td>
</tr>
<tr>
<td>hRPTC11</td>
<td>133±17</td>
<td>85.3±9.8</td>
<td>352±14</td>
</tr>
<tr>
<td>hRPTC12</td>
<td>42±8</td>
<td>88.8±7.8</td>
<td>97±7</td>
</tr>
<tr>
<td>hRPTC13</td>
<td>79±8</td>
<td>93.7±10.7</td>
<td>221±13</td>
</tr>
<tr>
<td>hRPTC14</td>
<td>118±13</td>
<td>90.6±11.1</td>
<td>307±15</td>
</tr>
<tr>
<td>hRPTC15</td>
<td>71±6</td>
<td>80.8±7.5</td>
<td>192±11</td>
</tr>
<tr>
<td>hRPTC11</td>
<td>68±7</td>
<td>99.9±9.1</td>
<td>17.6±4.2</td>
</tr>
<tr>
<td>hRPTC12</td>
<td>88±9</td>
<td>100.9±7.9</td>
<td>23.2±4.1</td>
</tr>
<tr>
<td>hRPTC13</td>
<td>95±10</td>
<td>97.1±9.8</td>
<td>15.2±4.2</td>
</tr>
<tr>
<td>hRPTC14</td>
<td>92±7</td>
<td>99.1±8.9</td>
<td>20.2±5.9</td>
</tr>
<tr>
<td>hRPTC15</td>
<td>49±6</td>
<td>98.9±9.7</td>
<td>12.6±4.3</td>
</tr>
</tbody>
</table>

Measurements of transepithelial fluxes of radiolabeled adenosine or 2′-deoxyadenosine were performed as described in MATERIALS AND METHODS. Measurements of uptake of radiolabeled adenosine or 2′-deoxyadenosine were performed as described in MATERIALS AND METHODS. Apical-to-basolateral fluxes of 1 μM [3H]adenosine across polarized hRPTC monolayers in sodium-containing buffer (Control) or in sodium-free buffer were measured as described in MATERIALS AND METHODS. Apical hCNT3-mediated uptake and basolateral-mediated uptake were calculated as described in RESULTS. Basolateral-to-apical fluxes of 1 μM 2′-[3H]deoxyadenosine across polarized hRPTC monolayers in sodium-containing buffer in the presence or absence (Control) of 0.1 μM nitrobenzylmercapturine ribonucleoside (NBMPR) or 500 μM probenecid were measured as described in MATERIALS AND METHODS. Basolateral-mediated uptake of 1 μM 2′-[3H]deoxyadenosine across polarized hRPTC monolayers in sodium-containing buffer in the presence or absence (Control) of 200 μM dilazep or 200 μM dilazep and 500 μM probenecid were measured as described in MATERIALS AND METHODS.
estimate of initial rates of cellular uptake (i.e., transport; Ref. 8); and 2) transepithelial fluxes were not evident at 10 min (see Fig. 2). Apical uptake of adenosine (Fig. 4A) and 2'-deoxyadenosine (Fig. 4B) in sodium-free buffer was I substantially lower than uptake in sodium-containing buffer (P < 0.001), indicating the presence of apical sodium-dependent transport activity; 2) further reduced by the inclusion of 0.1 μM NBMPR (P < 0.001), indicating the presence of apical hENT1 activity; and 3) still further inhibited by 200 μM dilazep (P < 0.001; i.e., to levels similar to those observed in sodium-free buffer with dilazep and excess nonradioabeled adenosine or 2'-deoxyadenosine), indicating the presence of apical hENT2 activity. Apical uptake of adenosine was higher than that of 2'-deoxyadenosine in both sodium-containing and sodium-free buffers (P < 0.001; Fig. 4, A and B). The reduction of apical uptake of adenosine and 2'-deoxyadenosine by high concentrations of either thymidine or inosine in sodium- and dilazep-containing buffer (P < 0.001; Fig. 4, A and B) demonstrated that apical sodium-dependent transport of both adenosine and 2'-deoxyadenosine was mediated by apical hCNT3. The slightly increased uptake of adenosine and 2'-deoxyadenosine observed in sodium-containing buffer in the presence of dilazep compared with that in its absence (Fig. 4, A and B; P < 0.01) was consistent with intracellular trapping due to inhibition of efflux through hENTs.

Basolateral uptake of 1 μM [3H]adenosine (Fig. 4C) and 2'-[3H]deoxyadenosine (Fig. 4D) was also assessed at 10 min in sodium-containing or sodium-free buffer. For both nucleosides, uptake rates were unaffected by I) removal of sodium, indicating the absence of basolateral sodium-dependent transport activity; or 2) the presence of 0.1 μM NBMPR, indicating the absence of basolateral hENT1 activity. Adenosine and 2'-deoxyadenosine basolateral uptake differed in their sensitivities to inhibition by 200 μM dilazep in that basolateral uptake of adenosine was reduced to nonmediated levels (P < 0.001; i.e., those observed in the presence of excess nonradioabeled adenosine; Fig. 4C), whereas that of 2'-deoxyadenosine was only partially reduced (P < 0.001; Fig. 4D). These results, which demonstrated basolateral hENT2 activity for both nucleosides, suggested the presence of a basolateral dilazep-insensitive uptake process for 2'-deoxyadenosine. Basolateral 2'-deoxyadenosine uptake was significantly higher than apical 2'-deoxyadenosine uptake or basolateral adenosine uptake in sodium-containing buffer (P < 0.01; Fig. 4, B, C, and D). These results were consistent with the observed vectorial fluxes of 2'-deoxyadenosine from basolateral-to-apical compartments.

To determine if apical hCNT3 activities were a determinant of apical-to-basolateral fluxes of adenosine, uptake and transepithelial fluxes of adenosine were assessed in hRPTC cultures.

Table 3. Recovery of adenosine in basolateral compartments and 2'-deoxyadenosine in apical compartments after apical-to-basolateral transepithelial fluxes of 1 μM [3H]adenosine and after basolateral-to-apical transepithelial fluxes of 1 μM 2'-[3H]deoxyadenosine across polarized hRPTC monolayers after 60 min

<table>
<thead>
<tr>
<th>Culture</th>
<th>Adenosine Recovered in Basolateral Compartments, %radioactivity loaded</th>
<th>2'-Deoxyadenosine Recovered in Apical Compartments, %radioactivity loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na+</td>
<td>Na+ + 500 μM EHNA</td>
</tr>
<tr>
<td>hRPTC11</td>
<td>31.5±3.3</td>
<td>72.3±5.8</td>
</tr>
<tr>
<td>hRPTC12</td>
<td>24.3±4.5</td>
<td>60.7±4.2</td>
</tr>
<tr>
<td>hRPTC13</td>
<td>36.6±3.1</td>
<td>79.8±4.7</td>
</tr>
<tr>
<td>hRPTC14</td>
<td>30.1±3.6</td>
<td>75.9±6.2</td>
</tr>
<tr>
<td>hRPTC15</td>
<td>28.6±3.8</td>
<td>72.3±5.7</td>
</tr>
</tbody>
</table>

TLC analyses of metabolites were performed as described in MATERIALS AND METHODS.
from five different individuals (Table 2). Apical uptake of 1 μM \(^{3}\text{H}\)adenosine by polarized hRPTC cultures was measured in sodium-containing buffer with 200 μM dilazep (to inhibit hENT1 and hENT2) in the absence or presence of 1 mM nonradiolabeled adenosine (to inhibit all mediated uptake), and the hCNT3-mediated component was calculated by subtracting nonmediated uptake values from the corresponding total uptake values. Table 2 shows that apical hCNT3-mediated adenosine uptake varied significantly between cultures (\(P < 0.05\)), consistent with previous work (11). When apical-to-basolateral fluxes of 1 μM \(^{3}\text{H}\)adenosine across polarized monolayers of the five hRPTC cultures were measured in sodium-containing buffer, the fluxes varied significantly between cultures (\(P < 0.05\)), and 2) correlated positively with apical hCNT3-mediated adenosine uptake levels (\(r^2 = 0.9908; P < 0.001\); Table 2). These results demonstrated that apical hCNT3-mediated uptake activities were determinants of apical-to-basolateral fluxes of adenosine in polarized monolayers of hRPTCs.

Basolateral uptake of 1 μM 2′-\(^{3}\text{H}\)deoxyadenosine by polarized hRPTC cultures was determined in sodium-containing buffer with 200 μM dilazep (to inhibit hENT1 and hENT2 and basolateral hENT2 activities) was measured at 10 min into polarized monolayers of hRPTC11 in various buffers containing 500 μM EHNA with or without the indicated inhibitors as described in MATERIALS AND METHODS. Values are means ± SD for 3 experiments each with triplicate measurements. Error bars are absent where data symbols were larger than SD values.

was calculated by subtracting nonmediated uptake determined in the presence of 1 mM nonradiolabeled 2′-deoxyadenosine. Basolateral-mediated uptake of 2′-deoxyadenosine varied between cultures (\(P < 0.05\)) as did basolateral-to-apical fluxes of 1 μM 2′-\(^{3}\text{H}\)deoxyadenosine across polarized monolayers (\(P < 0.05\); Table 2), and the values of these parameters exhibited a positive correlation (\(r^2 = 0.9566; P < 0.01\); Table 2). These results demonstrated that basolateral transporters were determinants of basolateral-to-apical fluxes of 2′-deoxyadenosine in polarized monolayers of hRPTCs.

To determine if hOCTs and hOATs were involved in apical and basolateral uptake of 2′-deoxyadenosine, uptake was assessed in the presence or absence of 500 μM cimetidine or probenecid, nonspecific inhibitors, respectively, of hOCTs and hOATs in the experiments shown in Fig. 4, B and D. Neither apical (Fig. 4B) nor basolateral (Fig. 4D) uptake of 2′-deoxyadenosine in sodium-containing buffer with 200 μM dilazep (to block apical hENT1 and hENT2 and basolateral hENT2 activities) was inhibited by cimetidine. Although apical uptake of 2′-deoxyadenosine under these conditions was not inhibited by probenecid (Fig. 4B), basolateral uptake was reduced to non-
mediated levels \((P < 0.001; \text{Fig. 4D})\), a finding consistent with the presence of basolateral hOAT-mediated 2'-deoxyadenosine uptake. Inhibition of basolateral-mediated 2'-deoxyadenosine uptake by probenecid was confirmed for the five different hRPTC cultures, and the results are summarized in Table 2 \((P < 0.01)\).

Expression of hOAT2 mRNA. Since 1) functional studies suggested that hOATs mediated basolateral uptake and basolateral-to-apical transepithelial fluxes of 2'-deoxyadenosine in polarized hRPTC monolayers, 2) 2'-deoxyadenosine is a permeant of hOAT2 \((7, 8)\), and 3) hOAT2 is present in basolateral membranes of kidney proximal tubules \((35)\), the expression of hOAT2 mRNA in five different polarized hRPTC monolayers was determined by RT-PCR \((\text{Fig. 5A})\). The identities of the amplified bands were confirmed by their predicted PCR product sizes \((0.59 \text{ kb}; \text{Fig. 5A})\) and by sequence analysis of amplified PCR products \((\text{data not shown})\). Genomic DNA contamination was not detected in RT-PCR reactions without reverse transcriptase \((\text{Fig. 5A})\). These results suggested involvement of hOAT2 in basolateral-to-apical transepithelial fluxes of 2'-deoxyadenosine.

hCNT3 protein abundance. To determine if the different apical hCNT3 activity levels were due to differences in hCNT3 protein levels in apical cell surfaces of polarized hRPTC cultures, as observed previously \((11)\), the relative apical cell surface abundance of hCNT3 was investigated in cultures derived from five different individuals. hCNT3 was identified by its immunoreactivity at 90 kDa in apical cell surface protein preparations \((\text{Fig. 5B})\), and its relative abundance varied over a fivefold range \((P < 0.05; \text{Fig. 5C})\). Apical hCNT3 mediated adenosine uptake, and the extent of apical-to-basolateral fluxes of adenosine correlated positively with the relative levels of apical cell surface hCNT3 \((r^2 = 0.8179 \text{ and } r^2 = 0.8055\), respectively; \(P < 0.05; \text{data from Table 2; Fig. 5C})\).

To determine if the polarized hRPTC cultures retained apical cell surface hCNT3 at relative levels similar to those of human kidney cortical proximal tubules, the relative abundance of hCNT3 was examined in crude membranes from the human kidney cortex tissues from which the hRPTC cultures had been isolated. Immunoreactive bands for hCNT3 at 90 kDa were identified in human kidney cortex crude membrane preparations \((\text{Fig. 5B})\), as previously observed \((8)\), and their relative abundance correlated positively with apical cell surface hCNT3 protein abundance in the corresponding polarized hRPTC cultures \((r^2 = 0.8107; P < 0.05; \text{Fig. 5C})\). Collectively, these results showed that polarized hRPTC monolayer cultures retained the same relative hCNT3 protein levels as their tissue cells of origin and suggested that apical hCNT3 protein levels \((\text{and activities})\) are determinants of the extent of adenosine reabsorptive fluxes.

Transportability of adenosine and 2'-deoxyadenosine by recombinant hCNT3. Despite the observed apical sodium-dependent uptake of 2'-deoxyadenosine in polarized hRPTC monolayers, 2'-deoxyadenosine exhibited preferential basolateral-to-apical transepithelial fluxes. In the experiments shown in Fig. 4, A and B, apical hCNT3-mediated uptake of 2'-deoxyadenosine was lower than that of adenosine in the same polarized hRPTC monolayers \((P < 0.01)\), suggesting that adenosine is a better permeant of hCNT3 than 2'-deoxyadenosine. To compare the transportabilities of adenosine and 2'-deoxyadenosine by hCNT3, kinetic studies were undertaken in yeast-producing recombinant hCNT3 \((\text{Fig. 6, A and B})\). The initial rates of uptake of adenosine and 2'-deoxyadenosine were saturable and conformed to Michaelis-Menten kinetics with apparent \(K_m\) values of 1.3 ± 0.2 and 3.6 ± 0.5 μM, respectively, and \(V_{\text{max}}\) values of 110.3 ± 5.4 and 80.4 ± 4.8 pmol·mg protein \(^{-1}\)·min \(^{-1}\), respectively \((\text{Fig. 6, A and B})\). The efficiencies of transport of adenosine and 2'-deoxyadenosine by hCNT3 \((V_{\text{max}}/K_m\) ratio) were 83.1 and 22.4 pmol·mg protein \(^{-1}\)·min \(^{-1}\)·μM \(^{-1}\), respectively, indicating greater hCNT3 transportability of adenosine than 2'-deoxyadenosine. These results were consistent with the higher observed apical hCNT3-mediated uptake of adenosine than 2'-deoxyadenosine in polarized hRPTC monolayers.
Fludarabine, cladribine, and clofarabine transepithelial fluxes and cellular uptake. Because little is known about renal handling of the purine nucleoside analogs fludarabine, cladribine, and clofarabine, all of which are transported by hENT1, hENT2, hCNT2, and hCNT3 but not by hCNT1 (22, 23), their transepithelial fluxes and polarized uptake were investigated in polarized hRPTC cultures in the experiments of Fig. 7. Transepithelial fluxes of $^{3}$Hfludarabine, $^{3}$Hcladribine, or $^{3}$Hclofarabine across polarized hRPTC cultures were measured in sodium-containing buffer. Apical-to-basolateral fluxes of all three analogs were observed (Fig. 7A), whereas basolateral-to-apical fluxes were not (Fig. 7B). Thus the directionality of transepithelial fluxes of fludarabine, cladribine, and clofarabine resembled that of adenosine, which is reabsorbed in human kidney proximal tubules. Apical and basolateral cellular uptake of $^{3}$Hfludarabine, $^{3}$Hcladribine, and $^{3}$Hclofarabine was measured in sodium-containing buffer in the absence (total uptake) or presence (nonmediated uptake) of 1 mM nonradiolabeled adenosine (Fig. 7, C and D). For all three analogs, apical uptake was higher than basolateral uptake ($P$ values < 0.01; Fig. 7, C and D), similar to adenosine (Fig. 4, A and C).

Because of the observed positive correlations for adenosine between relative apical-to-basolateral fluxes and apical hCNT3-mediated uptake in polarized hRPTC monolayers from different individuals, similar studies were undertaken for fludarabine, cladribine, and clofarabine. Apical-to-basolateral fluxes and apical-mediated cellular uptake of all three analogs were assayed in the five different polarized hRPTC monolayer cultures isolated from different individuals (Table 4). Positive correlations were found between apical-to-basolateral fluxes and mediated apical uptake for fludarabine and clofarabine ($r^2 = 0.9218$ and 0.9372, respectively; $P < 0.01$) but not for cladribine ($r^2 = 0.7448$; Table 4) and between apical hCNT3 activities (Table 2) and apical-to-basolateral fluxes for fludarabine and clofarabine ($r^2 = 0.7944$ and 0.8183, respectively; $P < 0.05$) but not for cladribine ($r^2 = 0.4204$; Table 4).

**DISCUSSION**

Evidence presented in this study supports a model for proximal tubular reabsorption of adenosine, fludarabine, cladribine, and clofarabine mediated by asymmetrically di-
TRANSEPIHELIAL FLUXES OF ADENOSINE AND 2′-DEOXYADENOSINE

Table 4. Summary of apical-to-basolateral transepithelial fluxes and apical-mediated uptake of 1 μM [3H]fludarabine, [3H]cladribine, and [3H]clofarabine in polarized hRPTC monolayers

<table>
<thead>
<tr>
<th>Culture</th>
<th>Apical-Mediated Uptake, pmol·mg protein⁻¹·10 min⁻¹</th>
<th>Apical-to-Basolateral fluxes, pmol·mg protein⁻¹·60 min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fludarabine</td>
<td>Cladribine</td>
</tr>
<tr>
<td>hRPTC11</td>
<td>22±5</td>
<td>19±5</td>
</tr>
<tr>
<td>hRPTC12</td>
<td>8±2</td>
<td>7±1</td>
</tr>
<tr>
<td>hRPTC13</td>
<td>19±4</td>
<td>28±5</td>
</tr>
<tr>
<td>hRPTC14</td>
<td>24±3</td>
<td>18±5</td>
</tr>
<tr>
<td>hRPTC15</td>
<td>15±3</td>
<td>19±4</td>
</tr>
</tbody>
</table>

Uptake and transepithelial flux assays performed as described in MATERIALS AND METHODS. Apical-mediated uptake was calculated as described in RESULTS.


distributed hCNT3 and hENT2 to apical and basolateral membranes, respectively. Furthermore, a model for proximal tubular secretion of 2′-deoxyadenosine is proposed from the evidence presented in this study that involves asymmetrically distributed hOATs and hENT1 to basolateral and apical membranes, respectively. Both of these models fit well with the current evidence in the literature. This study also extended previous findings that suggested that hCNT3 is a determinant of proximal tubular reabsorption of nucleosides, including adenosine and fludarabine (13), to clofarabine.

Apical-to-basolateral (i.e., reabsorptive) fluxes of adenosine were mediated by the coupling of sodium-driven apical hCNT3 to basolateral hENT2 (Figs. 2, 3, and 4; Table 2). These findings agree with previous results (13) that attributed transepithelial fluxes of cytidine and adenosine across murine proximal convoluted tubule cells to endogenous CNT3 activities and hCNT3-transfected renal epithelial cell lines to hCNT3 activities. Theoretically, inhibition of transport at either apical or basolateral sides could inhibit transepithelial fluxes; however, hENT1, found on apical membranes, did not contribute to apical-to-basolateral fluxes of adenosine across polarized hRPTC monolayers in the current study (Fig. 3). The lack of involvement of hENT1 in adenosine apical-to-basolateral fluxes may reflect its lower apparent affinity for adenosine than hCNT3 and the presence of abundant hCNT3 at the apical side (18, 39).

This study extended the role of hCNT3 in mediating transepithelial fluxes of nucleosides to include cladribine and clofarabine in human kidney proximal tubule cells with endogenous hCNT3 activities (Fig. 7; Table 4). Interestingly, cladribine, which, like 2′-deoxyadenosine, contains a 2′-deoxyriboyl moiety, also exhibited apical-to-basolateral (i.e., reabsorptive) transepithelial fluxes across polarized hRPTC cultures (Fig. 7A; Table 4). These results may explain, in part, the observed delayed elimination of some nucleoside analog drugs from the body (14) and highlight the high selectivity of transport machinery involved in renal handling of physiological nucleosides and therapeutic nucleoside analogs. hCNT3 was previously shown to be involved in transepithelial fluxes of various nucleoside analogs in transfected renal epithelial cell lines (13), and endogenous hCNT3 was previously shown to be a determinant of fludarabine-mediated uptake and cytotoxicity in hRPTC cultures (11). In this study, relative levels of apical-mediated uptake of fludarabine and clofarabine were determinants of their apical-to-basolateral transepithelial fluxes (Fig. 7; Table 4). These results suggested that differences in kidney proximal tubule hCNT3 levels may result in differences in renal handling of physiological nucleosides and nucleoside analogs that affect their pharmacokinetics and, for nucleoside analogs, their normal tissue toxicities.

Axial heterogeneity of hNTs in different proximal tubular cell types in hRPTC cultures from kidneys of different individuals may have contributed to the observed variability in transepithelial fluxes and cellular uptake of nucleosides. However, in a previous study (8), hCNT3 and hENT1 staining was present in apical membranes uniformly throughout cortical and corticomedullary proximal tubules of human kidney tissues. Other studies (15) have also identified hENT1 staining in apical and basolateral membranes of proximal tubules adjacent to corticomedullary junctions. In the current study, polarized hRPTC cultures exhibited similar relative hCNT3 protein levels on apical cell surfaces as those in the corresponding human kidney cortex crude membranes (Fig. 5, B and C), of which the majority were from cortical convoluted proximal tubule cells (4). This correspondence suggests that hRPTCs retained the in vivo characteristics of nucleoside transport processes present in their cortical proximal tubular cells of origin.

A model of renal secretion of 2′-deoxyadenosine through apical hCNT1 and basolateral hENT1 was previously proposed based on results of studies with hCNT1- and hENT1-overexpressing animal kidney cell lines in which it was suggested that the selectivity of adenosine reabsorption and 2′-deoxyadenosine secretion was the result of the higher affinity of hCNT1 for adenosine than for 2′-deoxyadenosine (25). However, since hCNT2 and hCNT3 are also present in apical membranes of kidney tubule epithelial cells and have higher transport capacities for adenosine and 2′-deoxyadenosine than hCNT1 (8, 15, 37–39, 44), they are also likely to contribute to the selectivity of proximal tubular handling of adenosine and 2′-deoxyadenosine. hCNT3-mediated apical uptake of 2′-deoxyadenosine was significantly lower than that of adenosine under similar conditions (Fig. 4, A and B), consistent with the higher hCNT3 transportability of adenosine than of 2′-deoxyadenosine observed in the recombinant yeast expression system (Fig. 6, A and B). The results of the present study support a new model of renal secretion of 2′-deoxyadenosine that involves apical hENT1 and basolateral hOATs (Figs. 3B and 4, B and D; Table 2). This model is supported by results of in vivo studies in mice treated with 2′-deoxycoformycin in which adenosine reabsorption was not inhibited by the classical hENT inhibitors NBMPR or dipyridamole, whereas 2′-deoxyadenosine secretion was inhibited by both (34, 35).

Recent evidence (7) shows that hOATs share some permeants with hNTs, e.g., 2′-deoxyadenosine is a permeant of hOAT2. hOAT2 is known to be present in basolateral membranes of kidney proximal tubules (36) and may account, in part, for...
the observed inhibitions of secretive fluxes and basolateral uptake of 2'-deoxyadenosine by probenecid (Figs. 2B and 3D; Table 2). In the present study, hOAT2 mRNA transcripts were found in polarized hRPTC monolayer cultures (Fig. 5A). While hOCT1 is known to transport 2'-deoxyxynucleoside, a nucleoside analog almost identical in structure to 2'-deoxyadenosine, the nonspecific hOCT inhibitor cimetidine had no inhibitory effects on renal secretion of 2'-deoxyxynucleoside in mice (34) or on apical and basolateral uptake of 2'-deoxyadenosine in the present study (Figs. 2B and 3, B and D). Both hOAT2 and hOCT1, along with other hOCTs and hOATs, are known to be asymmetrically distributed in apical and basolateral membrane domains in human kidney proximal tubules (36). hOAT1, hOAT2, and hOAT3 appear to be basolateral transporters, while hOAT4 appears to be an apical transporter in kidney epithelia (36). While hOAT1, hOAT2, and hOAT4 are facilitative transporters like hENTs, hOAT3 is a dicarboxylate-exchanger that is driven by the sodium gradient (36). Although apical sodium-driven hCNT3-mediated 2'-deoxyadenosine uptake was observed in polarized hRPTC cultures (Fig. 4B), basolateral sodium-independent 2'-deoxyadenosine uptake was higher, resulting in preferential basolateral-to-apical fluxes of 2'-deoxyadenosine (Fig. 4D; Table 2). This may have been due to more efficient transport of 2'-deoxyadenosine by basolateral hOATs than by apical hCNT3, which was shown to have a lower transport efficiency for 2'-deoxyadenosine than for adenosine (Fig. 6). Furthermore, the basolateral-mediated uptake values for 2'-deoxyadenosine were positively correlated with the extent of basolateral-to-apical fluxes. The driving forces for 2'-deoxyadenosine secretive transepithelial fluxes across polarized hRPTC cultures are unknown. Development of more transporter-specific inhibitors will allow further delineation of the roles of specific hOATs in renal secretion of 2'-deoxyadenosine (21).

Inter-individual differences in hOAT and hOCT abundances have been demonstrated previously in cultures of hRPTCs from different individuals (26). We have also observed differences in hCNT3 abundance and activities in adult cultures of hRPTC from different individuals that correlated positively with mediated uptake rates and cytotoxocities of fludarabine (11). Therefore, inter-individual variation in renal reabsorption or secretion of physiological nucleosides and nucleoside drugs may be due to differences in kidney transporter types and/or levels, as suggested by the observed variations in apical hCNT3 activities in the present study (Table 2). Because proximal tubular luminal pH is highly variable and regulated, ranging from pH 5.5 to 7.4 (9), hCNT3, which cotransports nucleosides with either sodium and/or protons (43), is ideally suited to be present in apical membranes of human kidney proximal tubules. hCNT3 can function under varying conditions and thus enable continuous reabsorption of energetically expensive physiological nucleosides for tissues that lack de novo nucleotide biosynthesis pathways.

As previously observed for adenosine in hCNT3-mediated transepithelial fluxes across polarized monolayers of transfected renal tubular epithelial cells (13), the majority of fluxed molecules across polarized hRPTC cultures in the absence of adenosine deaminase inhibition by EHNA (Tables 1 and 3) was, respectively, for adenosine and 2'-deoxyadenosine: 1) adenosine, inosine, and hypoxanthine; and 2) 2'-deoxyadenosine and hypoxanthine. The extrusion of hypoxanthine at basolateral membranes lends credence to the basolateral location of hENT2, which transports both nucleosides and nucleobases, such as hypoxanthine (49).

In the absence of adenosine deaminase inhibition, intracellular metabolites for both adenosine and 2'-deoxyadenosine were phosphorylated nucleotides and hypoxanthine (Table 1). While the contribution of various transporters to transepithelial fluxes of a given nucleoside will ultimately depend on their relative abundances, turnover numbers, and apparent affinities, the extent and nature of transepithelial fluxes of particular nucleosides will also depend on their intracellular metabolism.

In summary, the current study provides evidence for a descriptive model of purine nucleoside renal handling in which adenosine reabsorptive fluxes are mediated by hCNT3 at apical domains and hENT2 at basolateral domains and 2'-deoxyadenosine secretive fluxes are mediated by hENT1 at apical domains and hOATs at basolateral domains. A better understanding of renal handling of nucleosides by hNTs, the main route of elimination of nucleosides from the body, could lead to strategies aimed at improving drug dosing for therapeutic nucleoside analogs that maximize their efficacies and minimize their toxicities.

GRANTS

This research was funded by the Alberta Cancer Board Research Initiative Program, the Canadian Institutes of Health Research, and Canadian Cancer Society Research Institute. C. E. Cass was Canada Research Chair in Oncology during much of this work. J. D. Young is Heritage Scientist of the Alberta Heritage Foundation for Medical Research. M. B. Sawyer received an American Society of Clinical Oncology Career Development Award. A. N. Elwi is supported by an Alberta Cancer Board Legacy Graduate Award and a Studentship from the Translational Research Training in Cancer Program jointly supported by the Canadian Institutes of Health Research, the Canadian Cancer Society Research Institute, and the Alberta Cancer Foundation.

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