Cancer Chemother Pharmacol (2009) 63:289–301
DOI 10.1007/s00280-008-0739-1

ORIGINAL ARTICLE

Human concentrative nucleoside transporter 3 is a determinant of fludarabine transportability and cytotoxicity in human renal proximal tubule cell cultures

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Received: 23 November 2007 / Accepted: 11 March 2008 / Published online: 24 May 2008

Abstract
Purpose Interpatient variability in renal elimination of fludarabine (9-β-D-arabinosyl-2-fluoroadenine) by renal human nucleoside transporters (hNTs) may contribute to unpredictable toxicities including rare nephrotoxicities. This study assessed relationships between hNT levels and fludarabine uptake and cytotoxicity in cultures of human renal proximal tubule cells (hRPTCs) that produce multiple transporter types.
Methods hRPTC cultures were established from ten different individuals and their hNT characteristics were assessed by measuring RNA expression by TaqMan™ reverse transcriptase polymerase chain reaction, protein abundance by quantitative immunoblotting of cell surface protein preparations, and uptake by radiolabeled nucleoside uptake assays. Fludarabine cytotoxicity against hRPTC cultures was quantified using methoxyphenyl tetrazolium inner salt (MTS) assays.
Results RNA, protein and activities for human equilibrative NT 1 (hENT1) and 2 (hENT2) and human concentrative NT 3 (hCNT3) were identified in cultures of hRPTCs from ten different individuals. Significant differences in hCNT3 activities were exhibited among hRPTC cultures and correlated positively with cell surface levels of hCNT3 protein, but did not correlate with hCNT3 mRNA levels.

This research was funded by the Alberta Cancer Board Research Initiative Program and National Cancer Institute of Canada. C.E.C. is Canada Research Chair in Oncology. J.D.Y. is Heritage Scientist of the Alberta Heritage Foundation for Medical Research. M.B S. received an American Society of Clinical Oncology Career Development Award. A.N.E. 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, National Cancer Institute of Canada and Alberta Cancer Foundation.

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Conclusions Observed differences in hCNT3-mediated uptake activities, hNT-mediated fludarabine uptake activities, and fludarabine cytotoxicities correlated positively with each other, suggesting that hCNT3 is a primary determinant of fludarabine uptake and cytotoxicity in hRPTC cultures. Variations in hCNT3 abundance in renal proximal tubules, and hence nucleoside reabsorption, may explain interpatient variability in fludarabine's pharmacokinetics and toxicities.

Keywords Nucleoside transporter · Fludarabine · Renal proximal tubules

Abbreviations

IC_{50}	50% Inhibitory concentration
EC_{50}	50% Cell killing concentration
CNT	Concentrative nucleoside transporter
ENT	Equilibrative nucleoside transporter
hCNT1	Human concentrative nucleoside transporter 1
hCNT2	Human concentrative nucleoside transporter 2
hCNT3	Human concentrative nucleoside transporter 3
hENT1	Human equilibrative nucleoside transporter 1
hENT2	Human equilibrative nucleoside transporter 2
hENT3	Human equilibrative nucleoside transporter 3
hENT4	Human equilibrative nucleoside transporter 4
hNT	Human nucleoside transporter
hRPTC	Human renal proximal tubule cell
MTS	Methoxyphenyl tetrazolium inner salt
NBMPR	Nitrobenzylmercaptopurine ribonucleoside
NT	Nucleoside transporter
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulfate
SLC28	Solute Carrier 28
SLC29	Solute Carrier 29

Introduction

Nucleoside analogs play a central role in chemotherapy. Fludarabine (9-β-D-arabinosyl-2-fluoroadenine) is a purine nucleoside analog used to treat hematological malignancies and has become a standard treatment for indolent lymphoproliferative disorders [23, 29]. Fludarabine is administered as a more soluble pro-drug, fludarabine 5′-monophosphate (Fludara®), orally or intravenously [1, 44]. Fludarabine 5′-monophosphate is quickly dephosphorylated in the small intestine or in plasma to fludarabine by 5′-nucleotidases [12]. Following uptake into cancer cells, fludarabine is sequentially phosphorylated to its monophosphate, diphosphate, and triphosphate forms by intracellular kinases [8]. In proliferating cells, the main mechanisms of cytotoxic action of fludarabine 5′-triphosphate are inhibition of ribonucleotide reductase and inhibition of DNA synthesis [7, 45]. Cytotoxicity of fludarabine to non-dividing cells has been observed and is related to inhibition of DNA repair processes [37]. Fludarabine is eliminated from the body by renal excretion [33]. Significant interpatient variability in fludarabine pharmacokinetics, with long terminal half lives of 30 h and persistent low plasma concentrations of free drug (<0.1 μM) 24–72 h after administration, suggests variable renal handling [16]. Inter-patient variability in drug elimination can result in unpredictable toxicities from empirical dosing methodologies.

Uptake of nucleoside analogs is accomplished primarily by nucleoside transporters (NTs) [13]. Human members belong to the Solute Carrier (SLC) 29 and SLC 28 gene families, known, respectively, as equilibrative and concentrative NTs (ENTs and CNTs) [2, 19]. In the ENT family, four human ENT (hENT) protein isoforms (hENT1, hENT2, hENT3, hENT4) have been identified [3, 4, 14, 20, 21]. hENT1 and hENT2 are facilitative transporters with broad selectivities and hENT3 and hENT4 appear to be proton-nucleoside co-transporters. In the human CNT (hCNT) family, three isoforms (hCNT1, hCNT2, hCNT3) have been identified and mediate pyrimidine-nucleoside, purine-nucleoside, and broadly selective activities, respectively [38–40, 47]. hCNTs are sodium-nucleoside co-transporters; hCNT3 is also a proton-nucleoside co-transporter [41]. Fludarabine is transported by hENT1, hENT2, hCNT2, and hCNT3 [25].

Several studies have localized CNT1, CNT2, CNT3, and ENT4 to apical membranes and ENT1 and ENT2 to basolateral membranes of cultured animal kidney epithelial cells [27, 34–36, 49]. We have recently localized hENT1 and hCNT3 in human kidney tissues to apical brush border membranes of proximal tubules and identified hENT1, hENT2, and hCNT3 activities in human renal proximal tubule cell (hRPTC) cultures [11]. A recent immunolocalization study has confirmed hENT1's apical localization, but has also demonstrated hENT1 on basolateral membranes, in proximal tubules [17]. Conflicting results in hENT1 localization can be attributed to overexpression of recombinant NTs and antibody detection limits. Asymmetric distribution of hCNT3, hENT1, and hENT4 to apical membranes and hENT2 to basolateral membranes of kidney epithelial cells may result in reabsorptive fluxes of some nucleosides, driven by sodium and proton gradients, and secretory fluxes of other nucleosides, driven by, as yet, unidentified transporter processes [13].

Apical localization of hENT1 and hCNT3 in human kidney proximal tubules points to their roles in renal handling of nucleoside analogs [11]. Interpatient variability in fludarabine renal handling may be a result of variable hNT levels in proximal tubule cells between individual patients. To

address this question, an in vitro model system was used to study mechanisms underlying fludarabine renal elimination. Cultures of hRPTCs were produced and their hNT processes characterized by reverse transcriptase polymerase chain reaction (RT-PCR), immunoblotting, and nucleoside uptake assays. We observed hENT1, hENT2, and hCNT3 activities in cultures of hRPTCs from ten different individuals. In addition, relationships between hCNT3 mRNA, protein, and activity levels with hNT-mediated fludarabine uptake activities in hRPTC cultures were assessed. Although fludarabine nephrotoxicity is uncommon, it can be life threatening [24]. Since there are no reported studies of fludarabine cytotoxicity to renal proximal tubule cells, cytotoxicity of fludarabine against hRPTC cultures was assessed to determine if fludarabine is directly toxic to proximal tubule cells. We found positive correlations between hCNT3 activities, hCNT3 cell surface protein abundances, hNT-mediated fludarabine uptake activities, and fludarabine cytotoxicity against hRPTC cultures. We also found a positive correlation between hNT-mediated fludarabine uptake activities and 50% inhibitory concentration (IC_{50}) values for fludarabine inhibition of hNT-mediated uridine uptake activities, indicating that apparent composite affinities of different hRPTC cultures for fludarabine correlated negatively with hNT-mediated fludarabine uptake activities. These results add to mounting evidence for a role of hCNT3 in renal handling of nucleosides and nucleoside analogs.

Materials and methods

Materials

Cell culture reagents and oligonucleotides were purchased from Invitrogen (Burlington, ON, Canada). Collagen type I was purchased from Inamed Biomaterials (Fremont, CA, USA). Selenium-insulin-transferrin, hydrocortisone, and epidermal growth factor were purchased from BD Biosciences (Mississauga, ON, Canada). Anti-hENT1, -hENT2, -hCNT2, and -hCNT3 monoclonal antibodies were developed previously [30, 46, 50, 51] using synthetic peptide conjugates (residues 254–273 from hENT1, 261–280 from hENT2, 30–51 from hCNT2, and 45–69 from hCNT3). [5-^{3}H]-Uridine (40 Ci/mmol), [methyl-^{3}H]-thymidine (20 Ci/mmol), [2,8-^{3}H]-inosine (50 Ci/mmol), and [8-^{3}H]-fludarabine (15 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). Other supplies were purchased from Sigma (Oakville, ON, Canada).

Cell culture

Normal regions of human kidney tissue after nephrectomy for renal cell carcinoma were used for isolation of ten hRPTC cultures, each from a different individual, using an enzyme-dissociation method with collagenase type II (1 mg/mL) and deoxyribonucleotidase I (0.2 mg/mL) [5]. hRPTCs were maintained on collagen-coated cell culture ware in serum-free Dulbecco's modified Eagle's–Ham's F-12 (50:50 by volume) medium supplemented with selenium-insulin-transferrin (5 μg/L, 5, 5 mg/L, respectively), hydrocortisone (36 μg/L), epidermal growth factor (10 μg/L), triiodothyronine (4 ng/L), glutamine (2 mmol/L), and penicillin-streptomycin-amphotericin B (0.1 U/L, 100 ng/L, and 250 pg/L, respectively) at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were performed on hRPTC cultures after five passages that were 5–7 days post-confluency and that contained phenotypically differentiated proximal tubule cells as shown in representative cultures by: (1) parathyroid hormone sensitive cAMP synthesis; (2) anti-diuretic hormone insensitivity; (3) phloridzin sensitive α-methyl glucoside transport; (4) apical localized zonula occludens 1 at tight junctions; (5) basolateral localized E-cadherin; and (6) the presence of the brush border enzymes alkaline phosphatase, dipeptidyl peptidase IV, and γ-glutamyl transferase, using methods described elsewhere [5]. Preliminary experiments indicated that proximal tubular phenotypes were similar in second and fifth passage cultures (data not shown). Ethics approval was obtained from the Research Ethics Board of the Alberta Cancer Board and the University of Alberta/Capital Health Research Ethics Board, ensuring that informed consent was obtained from all patients in accordance with all Ethics Board guidelines.

RT-PCR

Total RNA was isolated from 5×10^6 cells of the ten different hRPTC cultures using GenElute Mammalian Total RNA kit from Sigma. RT-PCR using gene-specific primers for hENT1, hENT2, hCNT1, hCNT2, and hCNT3 was performed with One-Step RT-PCR with Platinum® Taq from Invitrogen using oligonucleotides and cycling parameters as previously described [11]. Polymerase chain reaction (PCR) amplification of (1) positive or (2) negative controls was performed on plasmid pYpGE15 constructs previously described [46, 50, 51] that contained either (1) hENT1, hENT2, hCNT1, hCNT2 or hCNT3 full length inserts, or (2) no insert. Expected PCR product sizes were 0.50 kb for hENT1, 0.43 kb for hENT2, 0.80 kb for hCNT1, 0.61 kb for hCNT2, and 0.48 kb for hCNT3. PCR products were purified using a QIAquick Gel Extraction kit from QIAGEN (Mississauga, ON, Canada) and sequenced to confirm their identities. Quantitative real time TaqMan™ RT-PCR using gene-specific primers and fluorescent dye-labeled probes for hCNT3 (Applied Biosystems; Streetsville, ON, Canada) was performed in triplicate on total RNA samples from hRPTC cultures as previously described [40]. Oligonucleotide probes and primers for

glyceraldehyde-3-phosphate dehydrogenase were used to control for RNA pipetting. Reactions that lacked template were used as negative controls. Relative expression values were calculated as described previously [40].

Immunoblotting

Crude membranes were isolated from 5×10^7 cells of each of the ten different hRPTC cultures using previously described methods [11]. Cell surface protein preparations were isolated from 5×10^6 cells of each of the ten different hRPTC cultures using a Cell Surface Protein Isolation kit from Pierce (Rockford, IL, USA) according to the manufacturer's instructions [43]. Crude membranes of yeast transfected with plasmid pYpGE15 containing either (1) no insert, or (2) hENT1, hENT2, hCNT2, or hCNT3 gene inserts, were prepared as previously described and served, respectively, as (1) negative and (2) positive controls [46, 50, 51]. Crude membranes (20 μg), cell surface protein preparations (20 μL, purified from equal numbers of cells), and yeast crude membranes (0.1 μg) were: (1) run on sodium dodecyl sulfate (SDS) polyacrylamide gels (10% *w/v*), (2) transferred to Immobilon-P polyvinylidene fluoride membranes, and (3) immunoblotted with anti-hENT1, -hENT2, -hCNT2, or -hCNT3 monoclonal antibodies as previously described [11]. Alexa Fluor 488 conjugated secondary antibodies were used in place of horseradish peroxidase conjugated secondary antibodies for immunoblots with cell surface protein preparations. Immunoblots with cell surface protein preparations had immunoreactive band intensities quantified using a Typhoon 8600 Variable Mode Imager from Amersham Biosciences (Baie d'Urfé, QC, Canada). To control for protein loading, immunoblots were incubated with stripping buffer (50 mM Tris pH 6.8, 2% SDS, 1% *v/v* β-mercaptoethanol) at 50°C for 30 min and re-probed with anti-β-actin antibodies. Immunoblots were produced in triplicate.

Uptake assays

Assays were performed by assessing accumulation of radiolabeled nucleosides over time into hRPTC cultures [40]. Preliminary experiments indicated that hRPTC cultures at passage two and five had similar hNT activities (data not shown). hRPTC cultures were maintained on 12-well plates, then: (1) washed twice at room temperature with buffer (3 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Tris/HCl, and 5 mM D-glucose pH 7.4) containing sodium (144 mM NaCl), referred to as sodium-containing buffer, or containing *N*-methyl-D-glucamine (144 mM), referred to as sodium-free buffer; (2) incubated with 1 μM [^{3}H]-uridine, -thymidine, -inosine, or -fludarabine (1 μCi/mL) in sodium-containing or sodium-free buffer with or without inhibitors for time intervals up to 12 min at room temperature. Cultures were pre-incubated with buffer containing dilazep or nitrobenzylmercaptopurine ribonucleoside (NBMPR) for 30 min when uptake assays involved these inhibitors to allow steady-state inhibitor binding to NTs [6]. To end uptake assays, cells were washed three times with ice-cold sodium-containing buffer, air dried, solubilized in 5% Triton X-100, and transferred to vials with 10 mL of Ecolite scintillation fluid for scintillation counting. Uptake values were normalized to cell number per well. Uptake mediated by hENT2 and hCNT3 was calculated by subtracting uptake values of 1 μM [^{3}H]-uridine obtained in the presence of excess non-radiolabeled uridine (10 mM) from that obtained, respectively, in sodium-free buffer with 0.1 μM NBMPR or in sodium-containing buffer with 200 μM dilazep. Uptake mediated by hENT1 was calculated by subtracting hENT2 activities from uptake values of 1 μM [^{3}H]-uridine obtained in sodium-free buffer. Three independent experiments, each with triplicate measurements, were performed.

Cytotoxicity assays

Assays were performed using a CellTiter Proliferation Assay from Promega (Madison, WI, USA) that monitors conversion of methoxyphenyl tetrazolium inner salt (MTS) to formazan by mitochondrial dehydrogenase in viable cells [32]. Briefly, the ten different hRPTC cultures were maintained on 96-well plates, incubated for 72 h with or without various concentrations of fludarabine, and incubated with MTS reagent for 1 h. Absorbance at 490 nm was measured using a 96-well plate reader from Molecular Devices (Sunnyvale, CA, USA). Three independent experiments, each with triplicate measurements, were performed.

Data analysis

Fludarabine concentrations that reduced hNT-mediated uridine uptake and cell viability by 50% (IC_{50} and EC_{50} values, respectively) were determined by regression analyses of concentration-effect curves. Statistical analyses were done with unpaired *t* tests and correlations. Graphs were generated using GraphPad Prism® 4.0 (GraphPad Software Inc.; San Diego, CA, USA).

Results

NT mRNA expression

In a previous study [11], we demonstrated the presence of hENT1, hENT2, hCNT1, hCNT2, and hCNT3 transcripts in total RNA from human kidney cortex, the bulk of which was

proximal tubules. To establish that hRPTC cultures were a suitable model for studying kidney NTs, we first examined their NT mRNA expression. Transcripts for hENT1, hENT2, hCNT1, hCNT2, and hCNT3 were observed in the ten different hRPTC cultures as determined by RT-PCR with gene-specific primers (Fig. 1, data shown for four different cultures). Identities of amplified bands were confirmed from their predicted PCR product sizes and by sequence analyses of representative bands that were excised from the gels (data not shown). Genomic DNA contamination was not detected in RT–PCR reactions without reverse transcriptase (data not shown). The presence of transcripts for hCNT1 and hCNT2 in hRPTC cultures was expected since cDNAs of both were isolated from kidney cDNA libraries [38, 39, 47].

NT protein abundance

The presence of hENT1 and hCNT3 proteins in crude membrane preparations from human kidney cortex tissues was previously shown by immunoblotting [11]. Since expression studies demonstrated transcripts for hENT1, hENT2, hCNT1, hCNT2, and hCNT3 in hRPTC cultures, immunoblotting experiments were undertaken to determine which NT proteins were present in their crude membranes. hENT1, hENT2, and hCNT3, but not hCNT2, were detected by immunoblotting in crude membranes from all ten cultures with anti-hNT specific monoclonal antibodies (Fig. 2). Efforts to undertake immunoblotting studies to detect hCNT1 in crude membranes of hRPTC cultures were unsuccessful since antibodies raised against a hCNT1-derived synthetic peptide also failed to recognize hCNT1 in the positive controls. Visualized bands (Fig. 2) exhibited the expected gel mobilities of NT proteins—i.e., 45–55 and 90 kDa, respectively, for hENT1/hENT2 and hCNT3 and 35–45 and 90 kDA, respectively, for recombinant hENT1/hENT2 and hCNT3 produced in yeast. hENT1 and hENT2 are known to be heterogeneously glycosylated [2] and the presence of diffuse bands with both anti-hENT1 and hENT2 antibodies was most likely a result of different glycosylation states. The presence of multiple bands in hENT2 immunoblots has been reported previously [46] and may be a result of proteolysis during preparation. Despite the presence of mRNA transcripts for hCNT2 in the hRPTC cultures that were analyzed, no hCNT2 protein was detected in any of the corresponding crude membrane preparations, indicating that hCNT2 was either not present or below the limits of detection of the assay. Single bands for hCNT3 migrating at 90 kDa were detected in immunoblots of hRPTC culture crude membranes, indicating the presence of hCNT3. Because NT mRNA and proteins that were detected in hRPTCs corresponded well with those found in human kidney cortex tissues in previous work [11],

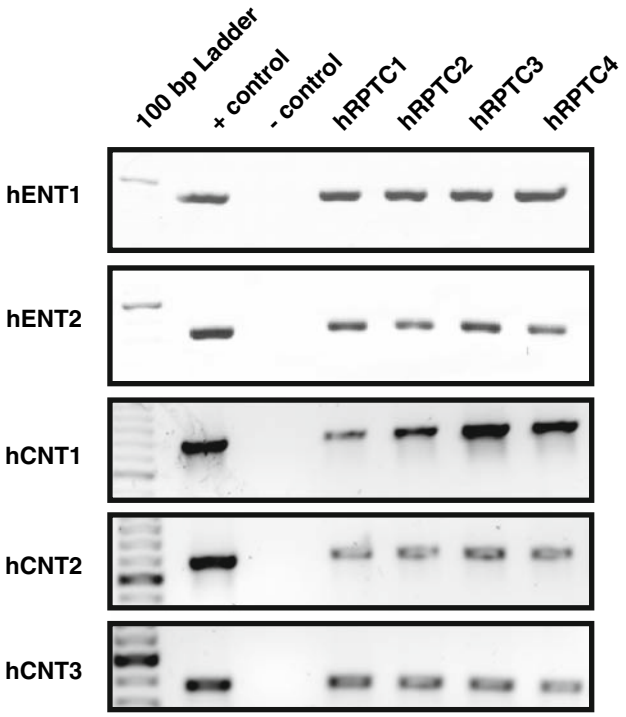

Fig. 1 Demonstration of hENT1, hENT2, hCNT1, hCNT2, and hCNT3 mRNAs in hRPTC cultures. Transcripts were assessed by RT-PCR analysis using gene-specific primers in total RNA preparations isolated from each of ten different hRPTC cultures of which four are shown. Positive controls (+) consisted of plasmid DNA with the indicated hNT cDNA insert and negative controls (−) consisted of plasmid DNA without insert. Parallel reactions that were performed in absence of reverse transcriptase to control for contaminating genomic DNA in total RNA preparations did not yield products (data not shown)

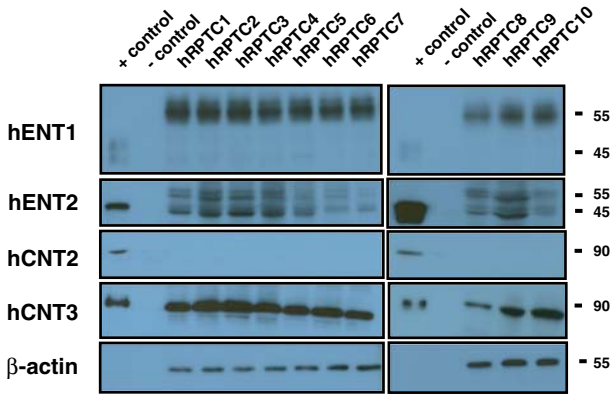

Fig. 2 Demonstration of hENT1, hENT2, and hCNT3, but not hCNT2, proteins in crude membrane preparations of hRPTC cultures. hENT1, hENT2, hCNT2, and hCNT3 proteins were assessed by immunoblotting using anti-hNT specific monoclonal antibodies in membrane preparations isolated from each of ten different hRPTC cultures. Positive controls (+) consisted of crude membrane preparations from yeast transfected with plasmids that contained the indicated hNT cDNA insert and negative controls (−) consisted of crude membrane preparations from yeast transfected with plasmids without inserts. Bands were visualized using horseradish peroxidase conjugated anti-mouse IgG antibodies and enhanced chemiluminescence

non-proliferating hRPTC cultures were used to further study the roles of kidney NTs in renal handling of nucleosides and nucleoside analogs.

Relative hCNT3 mRNA expression and protein abundance

Previous work demonstrated the presence of hENT1 and hCNT3 proteins in apical brush border membranes of proximal tubules in human kidney tissues [11], suggesting involvement of hCNT3 in nucleoside reabsorption from proximal tubule lumens and of hENT1, which was not detected in basolateral membranes, in nucleoside secretion into proximal tubule lumens. The presence of functional hENT1, hENT2, and hCNT3 was demonstrated in hRPTC cultures, shown by measuring hNT-mediated uridine uptake, and suggested that hCNT3 was a contributor to nucleoside reabsorption in proximal tubules [11]. Development of hRPTC cultures from ten different patients' kidneys provided the opportunity to assess relationships between hCNT3 mRNA levels, protein abundance, and activities.

Transcript levels for hCNT3, determined by TaqMan® quantitative RT-PCR, varied over a 16-fold range (P values < 0.01) between the different hRPTCs (Fig. 3a). The relative abundance of hCNT3 protein was determined in cell surface protein preparations by immunoblotting analysis of biotinylated cell surface proteins purified by avidin chromatography. Abundance of cell surface hCNT3 varied over a 35-fold range (P values < 0.01) between different hRPTC cultures (Fig. 3b, Table 1). There was no correlation between relative hCNT3 mRNA levels and hCNT3 cell surface protein abundance for the ten different hRPTC cultures (r^2 0.0529, $P > 0.5$).

hNT-mediated uptake activities

In previous work, the presence of hENT1, hENT2, and hCNT3-mediated uptake was demonstrated in hRPTC cultures [11]. Since cell surface abundance of hCNT3 varied significantly between different hRPTC cultures analyzed in the current work, experiments were undertaken to determine if hNT-mediated uptake also varied. Uptake of radiolabeled nucleosides into hRPTC cultures was monitored over time in sodium-containing or sodium-free buffers in the presence or absence of potential inhibitors to functionally dissect the hNT processes that were responsible. Uptake time courses for uridine, thymidine, and inosine were linear for up to 8 min (Fig. 4), indicating that the time courses provided a good approximation of initial rates of uptake—i.e., of transport activities. Confirmation that these time courses represented initial rates of uptake was demonstrated in experiments in which uptake was measured for 1 min at 5-s intervals (data not shown). Since hRPTC cultures were differentiated into polarized monolayers,

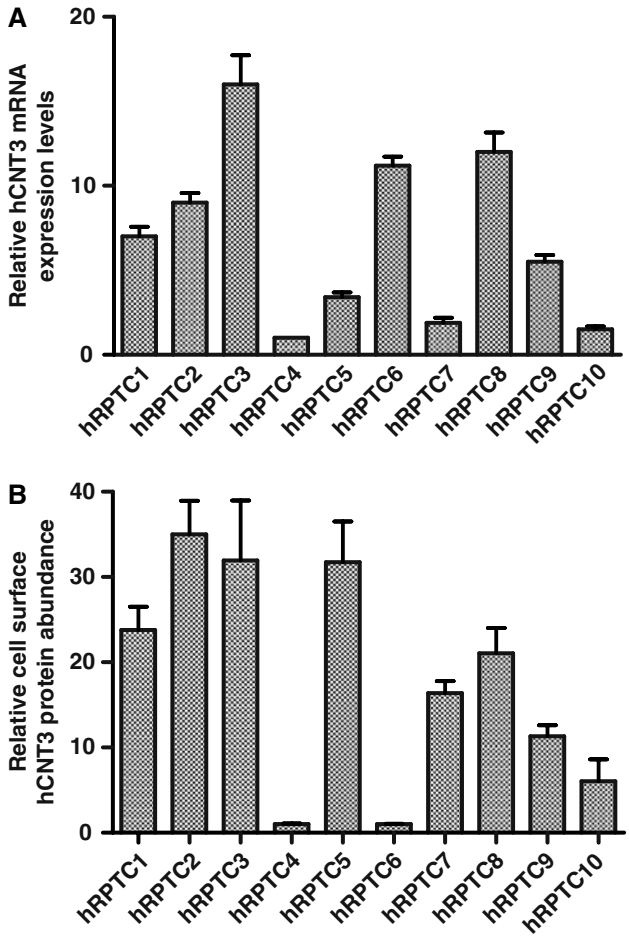

Fig. 3 Relative abundance of hCNT3 mRNA and cell surface protein in hRPTC cultures. **a** Relative hCNT3 mRNA levels in total RNA preparations from each of ten different hRPTC cultures were assessed by TaqMan® quantitative RT-PCR using hCNT3-specific oligonucleotide primers and fluorescently labeled probes as described in "Materials and methods". Values were normalized to the levels of human glyceraldehyde-3-phosphate dehydrogenase, which was used to control for the amounts of RNA added to reaction mixtures. **b** Cell surface hCNT3 protein abundance in cell surface protein preparations from each of ten different hRPTC cultures was assessed by quantitative immunoblotting analysis using anti-hCNT3 monoclonal antibodies. Cell surface biotinylation was performed on cultures that were produced under conditions that yielded similar population densities and equal volumes of samples were loaded onto SDS polyacrylamide gels as described in "Materials and methods". Bands corresponding to hCNT3 were quantified using AlexaFluor 488 conjugated anti-IgG antibodies and a Typhoon multimode scanner. For both **a** and **b** values are means (±standard errors of measurement) from triplicate experiments. Samples for which error bars are absent had errors equal or smaller than the border size of the bars

uptake experiments assessed cellular accumulation that was mediated primarily by apical NTs.

Uptake of 1 μM [^{3}H]-uridine in sodium-containing buffer was inhibited almost completely in the presence of excess (10 mM) non-radiolabeled uridine (Figs. 4a, 5a), indicating that uridine uptake was primarily mediated. The hENT inhibitor dilazep, when present in sodium-containing buffer

Table 1 Summary of relative hCNT3 mRNA expression levels, relative hCNT3 cell surface protein abundances, hCNT3-mediated uridine uptake, hNT-mediated fludarabine uptake, fludarabine inhibition of uridine uptake, and fludarabine cytotoxicity in hRPTC cultures

Culture	Relative hCNT3 mRNA expression levels[a]	Relative cell surface hCNT3 protein abundances[b]	hCNT3-mediated uridine uptake (pmol/10 min/10^6 cells)[c]	hNT-mediated fludarabine uptake (pmol/10 min/10^6 cells)[d]	Fludarabine inhibition of uridine uptake IC_{50} (μM)[e]	Fludarabine cytotoxicity EC_{50} (μM)[f]
hRPTC1	7 ± 2	24 ± 5	36 ± 3	3.8 ± 0.4	90 ± 9	50 ± 17
hRPTC2	1.01 ± 0.01	35 ± 7	49 ± 7	4.5 ± 0.1	122 ± 12	43 ± 11
hRPTC3	16 ± 3	32 ± 12	35 ± 3	4.0 ± 0.2	95 ± 12	45 ± 10
hRPTC4	3.4 ± 0.5	1.0 ± 0.1	1.9 ± 0.1	1.5 ± 0.3	12 ± 5	99 ± 13
hRPTC5	9 ± 1	32 ± 8	50 ± 3	5.4 ± 0.1	142 ± 30	40 ± 8
hRPTC6	11.4 ± 0.8	1.0 ± 0.1	1.8 ± 0.1	1.4 ± 0.1	13 ± 3	132 ± 45
hRPTC7	1.9 ± 0.4	16 ± 2	23.1 ± 0.7	3.0 ± 0.1	71 ± 17	52 ± 10
hRPTC8	12 ± 2	21 ± 5	31 ± 2	3.5 ± 0.1	95 ± 10	47 ± 15
hRPTC9	5.6 ± 0.7	11 ± 2	17.5 ± 0.9	1.9 ± 0.3	22 ± 5	>200
hRPTC10	1.5 ± 0.3	6 ± 4	1.7 ± 0.5	1.3 ± 0.1	15 ± 2	84 ± 4

[a] Determined from quantitative TAQMAN® RT–PCR analysis; see Fig. 3. Mean ± standard errors of measurement

[b] Determined from quantitative immunoblotting analysis; see Fig. 3. Mean ± standard errors of measurement

[c] Determined from calculated hCNT3 functional activities; see Fig. 4. Mean ± standard deviations

[d] Determined from radiolabeled fludarabine uptake assays; see Fig. 5. Mean ± standard deviations

[e] Determined from fludarabine inhibition of uridine uptake curves; see Fig. 6. Mean ± standard deviations

[f] Determined from fludarabine cytotoxicity curves; see Fig. 6. Mean ± standard deviations

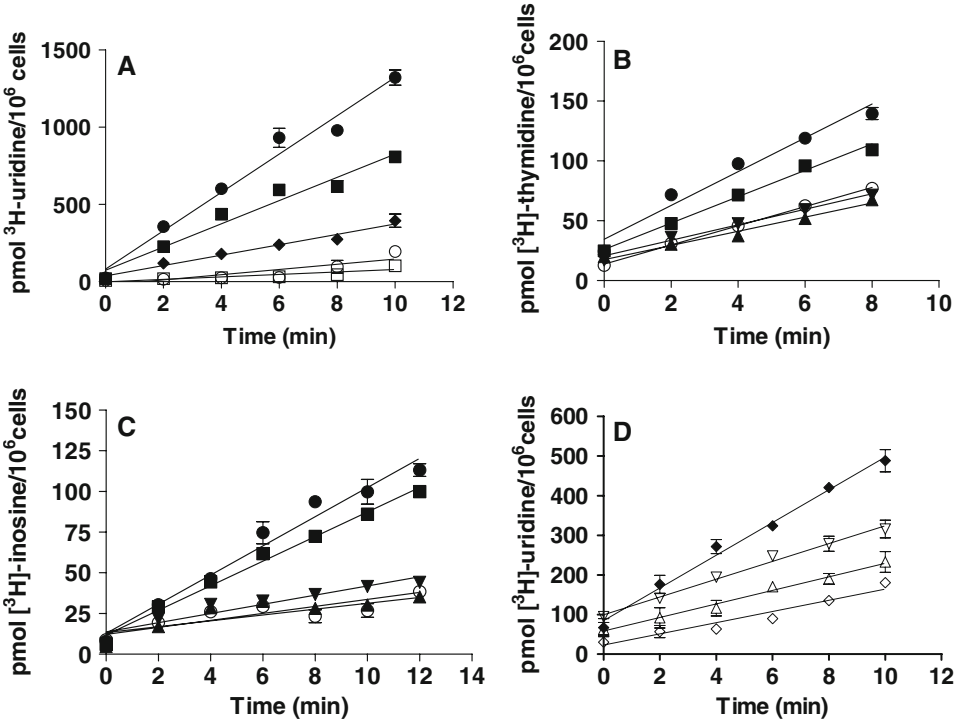

Fig. 4 Time courses of uptake of uridine, thymidine, and inosine by hRPTC cultures. Uptake of 1 μM **a** [^{3}H]-uridine, **b** [^{3}H]-thymidine, and **c** [^{3}H]-inosine in sodium-containing buffer and **d** 1 μM [^{3}H]-uridine in sodium-free buffer with or without various inhibitors was monitored over time into one (hRPTC1) of ten different hRPTC cultures. The symbols denote the following buffers and inhibitors: sodium-containing buffer alone (*filled square*) or with 10 mM uridine (*open square*) or 200 μM dilazep alone (*filled circle*) or with 10 mM uridine (*open circle*), 1 mM thymidine (*filled inverted triangle*), or 1 mM inosine (*filled triangle*); sodium-free buffer (*filled diamond*) with 0.1 μM NBMPR (*open inverted triangle*), 200 μM dilazep (*open triangle*), or 10 mM uridine (*open diamond*). Values plotted are means (±standard deviations) from three independent experiments. Points for which error bars are absent had errors equal or smaller than the border size of the points

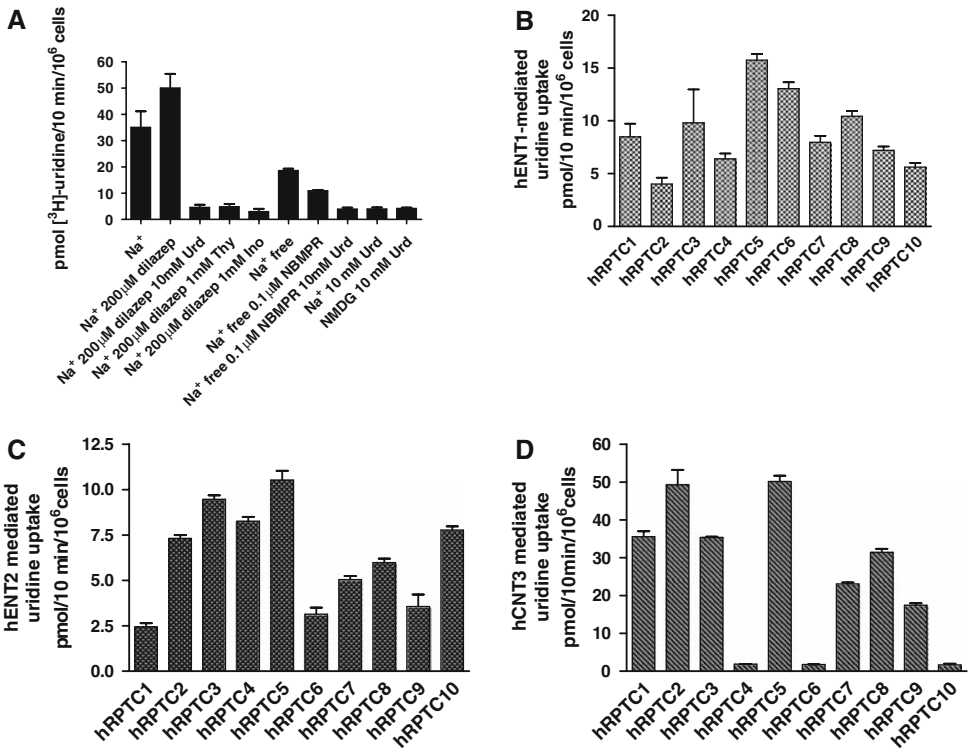

Fig. 5 hRPTC cultures exhibit hENT1-, hENT2-, and hCNT3-mediated uptake activities. **a** Values obtained for uptake of 1 μM [^{3}H]-uridine into hRPTC1 cultures in sodium-containing and sodium-free buffers with and without various NT inhibitors (see Fig. 4a, d) were used to calculate NT activities as described in "Materials and methods". A similar approach was used for the other nine hRPTC cultures (not shown) and resulting uptake values for all ten cultures are shown for **b** hENT1, **c** hENT2, and **d** hCNT3. Values are means (±standard deviations) of three independent experiments each with triplicate measurements. Samples for which error bars are absent had errors equal or smaller than the border size of the bars

at a concentration (200 μM) that inhibits both hENT1 and hENT2 activities [6, 48], increased uridine uptake ($P < 0.01$) (Figs. 4a, 5a), a result that can be explained by inhibition of uridine efflux through bi-directional hENTs while still allowing uptake via uni-directional hCNTs. Uptake of [^{3}H]-uridine in sodium-free buffer was lower than in sodium-containing buffer ($P < 0.01$) and could be further reduced by 200 μM dilazep to levels observed in the presence of 10 mM non-radiolabeled uridine (Fig. 4d), indicating the presence of both hCNT- and hENT-mediated uptake processes in hRPTC cultures. Uptake of [^{3}H]-uridine in sodium-free buffer was only partially inhibited by NBMPR at 0.1 μM, a concentration that inhibits hENT1 but not hENT2 [6, 48], indicating the presence of both hENT1- and hENT2-mediated uptake processes in hRPTCs (Figs.4d, 5a). Uptake of 1 μM [^{3}H]-thymidine, which is transported by hCNT1 and hCNT3 but not by hCNT2, in sodium-containing buffer with 200 μM dilazep was completely inhibited by either 1 mM non-radiolabeled thymidine or inosine, both of which are transported by hCNT3 but not by hCNT1 [38–40, 46]; this result (Fig. 4b) demonstrated the presence of hCNT3-mediated uptake processes in hRPTC cultures. Similarly, uptake of 1 μM [^{3}H]-inosine in sodium-containing buffer with 200 μM dilazep was completely inhibited by either 1 mM non-radiolabeled thymidine or inosine (Fig. 4c), also demonstrating the presence of hCNT3-mediated, but not of hCNT1- or hCNT2-mediated, uptake processes in hRPTC cultures.

To compare hENT1-, hENT2-, and hCNT3-mediated uptake activities in hRPTC cultures, uptake of 1 μM [^{3}H]-uridine was monitored over ten min under conditions that allowed estimation of NT activities (see Fig. 5a) as follows: (1) hCNT3-mediated activities were obtained by subtracting non-mediated uptake values from those in sodium-containing buffer with 200 μM dilazep; (2) hENT2-mediated activities were obtained by subtracting non-mediated uptake values from those in sodium-free buffer with 0.1 μM NBMPR; and (3) hENT1-mediated uridine uptake activities were obtained by subtracting hENT2-mediated and non-mediated uptake values from total uptake values in sodium-free buffer. hCNT3 activities (Fig. 5d) varied over 30-fold (P values < 0.01), hENT2 activities (Fig. 5c) varied over fourfold (P values < 0.01), and hENT1 activities (Fig. 5b) varied over 16-fold (P values < 0.01). Results are summarized in Table 1.

While a direct and significant correlation between cell surface hCNT3 protein levels and hCNT3 activities in the different hRPTC cultures (Table 1) was found as expected (r^2 0.9439, $P < 0.0001$), no correlation was found between hCNT3 activities and relative hCNT3 transcript levels. hENT1 and hENT2 activities in hRPTC cultures tended to be relatively low compared to hCNT3 activities (Fig. 5b–d, $P < 0.01$), suggesting that hCNT3 was the major contributor to nucleoside uptake.

Fludarabine uptake

Since fludarabine is transported by hCNT3 [25], observed variations in hCNT3 protein abundance and activities in hRPTCs from different individuals grown under identical

conditions suggested that there would be corresponding variations in hNT-mediated fludarabine uptake. Fludarabine, which is widely used clinically, exhibits considerable inter-patient variability in renal elimination and normal tissue toxicities [16]. Uptake of [^{3}H]-fludarabine in sodium-containing buffer over ten min was monitored in the different hRPTCs cultures at 1 μM, since this concentration is observed during the terminal elimination phase of fludarabine in patients [16]. Uptake was almost completely inhibited in the presence of 10 mM non-radiolabeled uridine (Fig. 6a), indicating that fludarabine uptake was mediated. Average uptake values of fludarabine among the different hRPTC cultures were significantly different (P values < 0.01) (Fig. 6a, Table 1) and correlated positively with estimated hCNT3 activities also shown in Table 1 (r^2 0.9534, $P < 0.0001$), suggesting that hCNT3 was a primary determinant of fludarabine uptake by hRPTC cultures.

To further study relationships between hCNT3 activities and fludarabine uptake, concentration-dependent fludarabine inhibition of uptake of [^{3}H]-uridine, which is transported by hENT1, hENT2 and hCNT3, was assayed in the different hRPTC cultures under conditions for which these hNTs were all functional (Fig. 6b shows results for hRPTC2 cultures). Fludarabine inhibited uptake of 1 μM [^{3}H]-uridine in a concentration-dependent fashion and concentrations resulting in 50% inhibition of hNT-mediated uridine uptake (IC_{50} values), which were determined by non-linear regression, ranged from 12 to 142 μM (Table 1). Because the three hNTs exhibited different activities in the different hRPTC cultures (probably reflecting different relative NT quantities), IC_{50} values provided a rough measure

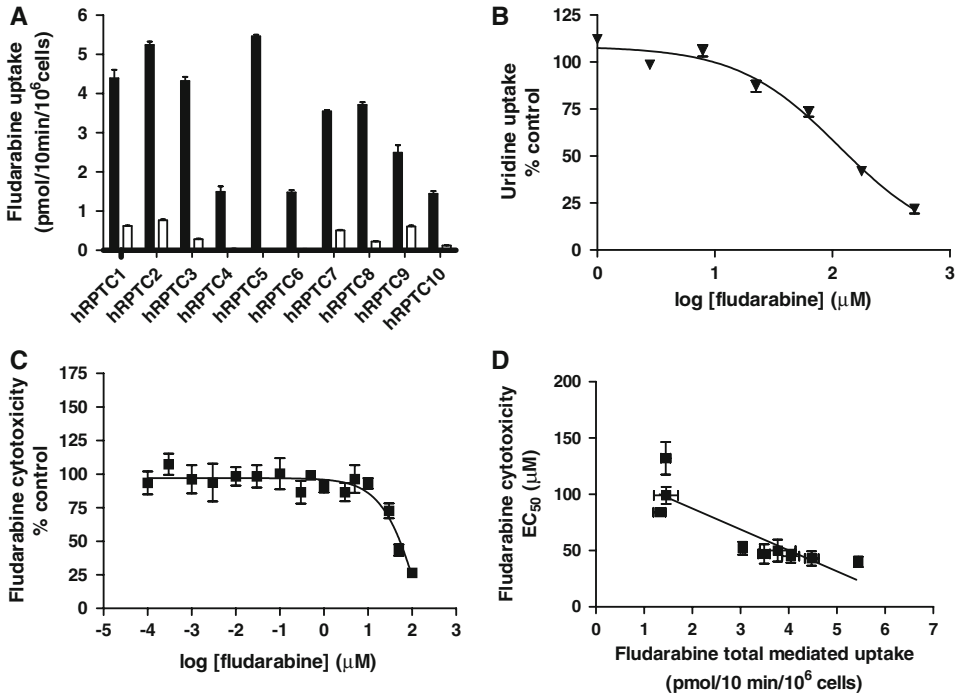

Fig. 6 hRPTC cultures exhibit different hNT-mediated fludarabine uptake activities, sensititivities of hNT-mediated to inhibition by fludarabine, and sensitivities to fludarabine cytotoxicity. **a** Uptake of 1 μM [^{3}H]-fludarabine into hRPTC cultures after 10-min exposures in sodium-containing buffer (*filled bar*) or sodium-containing buffer with 10 mM non-radiolabeled uridine (*open bar*). **b** Inhibition of 1 μM [^{3}H]-uridine uptake into cultures of hRPTC2 by fludarabine. **c** Cytotoxicities of fludarabine to hRPTC2. **d** Correlation of hNT-mediated fludarabine uptake and fludarabine cytotoxicity sensitivities in the ten different hRPTC cultures (r^2 0.7356, $P < 0.01$). In panel **b**, uptake of 1 μM [^{3}H]-uridine was measured after 10-min exposures in the presence or absence of non-radiolabeled fludarabine (1.0, 2.9, 7.9, 22.4, 63.0, 177.5, 500.0 μM) in sodium-containing buffer with or without 10 mM non-radiolabeled uridine. Uridine uptake values in the presence of fludarabine for hRPTC2 cultures are expressed as percentage of values in its absence, correcting all values for non-mediated uptake in the presence of 10 mM non-radiolabeled uridine. In panel **c**, cultures of hRPTC2 were incubated for 72-h in the presence or absence of fludarabine (0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 0.5, 1, 3, 5, 10, 30, 50, 100 μM), and cytotoxicities were determined using the MTS assay as described in "Materials and methods". Cytotoxicity values are absorbance at 490 nM of cultures treated with various fludarabine concentrations expressed as a percentage of absorbance of untreated cells. The IC_{50} value in **b** was 122 ± 12 μM for fludarabine inhibition of hNT-mediated [^{3}H]-uridine uptake and the EC_{50} value in **c** was 43 ± 10 μM for fludarabine cytotoxicity sensitivity, both calculated from non-linear regression analysis of sigmoidal dose-response curves. Similar experiments were conducted for the other nine hRPTC cultures and IC_{50} (50% inhibition by fludarabine of hNT-mediated uridine uptake) and EC_{50} (50% inhibition by fludarabine cytotoxicity of cell viability) values are given in Table 1. Values shown in **a**, **b**, **c**, and **d** are means (±standard deviations) of three independent experiments each with triplicate measurements. Samples for which error bars are absent had errors equal to or smaller than the border sizes of the bars or points

of the composite apparent affinities of hNTs present in hRPTC cultures for fludarabine, with the lowest IC_{50} values representing the highest composite apparent affinities. A positive correlation was found between hNT-mediated fludarabine uptake values and IC_{50} values for fludarabine inhibition of hNT-mediated uridine uptake (Table 1) (r^2 0.9831, $P < 0.0001$), suggesting that composite apparent affinities of the different hRPTC cultures for fludarabine correlated negatively with hNT-mediated uptake of fludarabine. The composite apparent affinity of a particular hRPTC culture would be expected to reflect the individual apparent affinity of the hNT present in greatest abundance (i.e., with the greatest activity) in plasma membranes.

Fludarabine cytotoxicity

Because of positive correlations between hCNT3 activities and hNT-mediated fludarabine uptake values observed in the different hRPTC cultures, the relationship between hNT-mediated fludarabine uptake and cytotoxicity was also assessed. Fludarabine cytotoxicity, which was investigated by the MTS assay in hRPTC cultures exposed to graded concentrations of fludarabine over 72-h periods, showed dose-dependent cell killing (Fig. 6c shows results for hRPTC2 cultures). The 50% cell killing concentration (EC_{50}) values of fludarabine exposures ranged from 40 to >200 μM (Table 1). Cultures used in these experiments were non-dividing and had been confluent for five to seven days. A modest positive correlation between hNT-mediated fludarabine uptake values and fludarabine cytotoxicities (EC_{50} values) was found (Fig. 6d, Table 1) (r^2 0.7356, $P < 0.01$). Closer analysis of the data revealed that the different hRPTC cultures fell into two clusters: (1) those with lower sensitivities to fludarabine cytotoxicity (EC_{50} values, 80 to >200 μM) and lower hNT-mediated fludarabine uptake values (1.3 to 1.9 pmol/10 min/10^6 cells); and (2) those with higher sensitivities to fludarabine cytotoxicity (EC_{50} values, 40–52 μM) but also higher hNT-mediated fludarabine uptake values (3.0 to 5.4 pmol/10 min/10^6 cells).

Discussion

Because empirical dosing methodologies currently in use for chemotherapeutic nucleoside analogs do not take into account interpatient variabilities in tumor response, drug metabolism, and drug elimination, individually tailored dosing regimens will greatly benefit patient treatment. This study focused on understanding variabilities in renal elimination of the nucleoside analog fludarabine at the level of hNTs in normal human renal epithelial cells grown in vitro. Cultures of human renal proximal tubule epithelial cells, termed hRPTC cultures, were isolated from ten individuals and their in vitro hNTs were characterized at mRNA, protein, and activity levels, respectively, by RT-PCR from total RNA, immunoblotting of crude membranes and cell surface protein preparations, and measurements of radiolabeled nucleoside uptake. Although the hRPTC cultures all possessed hENT1, hENT2, and hCNT3 mRNA and protein and exhibited hENT1-, hENT2-, and hCNT3-mediated uptake (i.e., transport) activities, these parameters differed significantly between cultures derived from different individuals. hCNT3 was an important determinant of hNT-mediated fludarabine uptake in hRPTC cultures because hCNT3 cell surface protein abundances and hCNT3-mediated uptake (i.e., transport) activities correlated positively with hNT-mediated fludarabine uptake (i.e., transport) activities. A similar case for hCNT3 as a determinant of nucleoside analog transepithelial fluxes has been made in renal epithelial cell lines transfected with hCNT3 [15].

The presence of hENT1, hENT2, and hCNT3 mRNA, protein, and hENT1-, hENT2-, and hCNT3-mediated uptake (i.e., transport) activities in hRPTC cultures fits well with current models of renal nucleoside handling [13]. Because proximal tubules are the main site of reabsorption of solutes in nephrons, both hENTs and hCNTs in hRPTC cultures should be necessary to effect transepithelial vectorial fluxes in a reabsorptive direction. The lower levels of hENT2 activities and higher levels of hENT1 and hCNT3 activities observed in hRPTC cultures suggested that hENT1 and hCNT3 are the primary luminal hNT activities in proximal tubule cells. This corroborates our previous findings in localization studies in fixed human kidney tissues that hENT1 and hCNT3 are localized to brush border membranes of proximal tubules [11]. While earlier studies in human brush border membrane vesicles identified an activity now known to be mediated by hCNT1 [22], no hCNT1 activities were identified in hRPTC cultures in the current work, which was consistent with our previous findings [11]. Our results suggested that hCNT3 is the predominant hCNT in human renal proximal tubules. The ability of hCNT3 to utilize (1) the sodium gradient to a greater extent than hCNT1 or hCNT2 (2:1 versus 1:1 sodium-to-nucleoside coupling ratio) [38–40, 47] and (2) the proton gradient [41] to drive uphill transport of nucleosides fits with the conclusion that hCNT3 is the major renal hCNT, and thus drives reabsorption of nucleosides from the acidic proximal tubular lumen.

Although there was no correlation between relative hCNT3 mRNA and hCNT3 cell surface abundance in the different hRPTC cultures, a positive correlation between hCNT3 cell surface protein levels and hCNT3 activities was observed in the current work, suggesting that regulation of cell surface levels of hCNT3 may be an important method of regulating activity. In contrast, in proliferating versus differentiated HL-60 cells, a 17-fold relative difference

of hCNT3 mRNA expression levels was demonstrated and was accompanied by corresponding absence and presence of hCNT3 activities [40].

A central question that this report raises is why hNT cell surface levels varied between different hRPTC cultures. Single nucleotide polymorphisms (SNPs) in hNTs have been investigated previously as a source of interindividual differences in NT activities. Two SNPs identified for hCNT1 and hCNT3 have direct effects on their activities in recombinant expression systems [18]; however, their low prevalence discounts them as plausible explanations for the observed differences in hNT activities in the hRPTC cultures assayed. Rather, interindividual differences in regulatory proteins that affect transcriptional, translational, and/or post-translational regulation of hNTs may be responsible for the observed variations of NT activities in the different hRPTC cultures. Although it is possible that the observed differences may have been a result of different growth states of the cultures, this is unlikely as care was taken to ensure that all experiments were performed on non-proliferating cells (i.e., cells had been confluent for 5–7 days). Previous studies have identified significant variations in abundance of other transporters between different hRPTC cultures, namely the organic cation transporters [28].

Since fludarabine, and other nucleoside analogs, undergo primarily renal elimination, hNT levels in human kidney epithelial cells may determine total systemic exposures to these drugs and hence efficacy and normal tissue toxicities. Interpatient heterogeneity in renal hCNT3 abundance and therefore activity may have profound effects on the extent of fludarabine luminal uptake and hence fludarabine reabsorption. Patients with relatively higher renal hCNT3 activities may experience higher fludarabine systemic exposures and therefore different tumor responses and normal tissue toxicities. Renal epithelial cells, being effectors of transporter-mediated reabsorption and secretion in kidneys, are exposed continuously to cytotoxic xenobiotics. As such, hRPTC cultures are expected to be relatively more resistant to cytotoxic drugs such as fludarabine than other epithelial tissues although fludarabine cytotoxicity to hRPTCs was, nonetheless, observed (EC_{50} values of 40–100 μM; 72 h exposures). While the peak plasma fludarabine concentrations (3 μM) in normal patients undergoing treatment is considerably lower than the observed EC_{50} values of fludarabine cytotoxicity to hRPTCs, persistent low plasma concentrations of free drug (<0.1 μM; 24–72 h) have been observed [16]. Because kidneys filter blood plasma volume several times in a 24-h period, continuous exposure of kidney epithelial cells to even low plasma concentrations of fludarabine may lead to significant accumulation of fludarabine 5′-triphosphate levels in kidney proximal tubule cells. Additionally, the range of peak plasma concentrations of fludarabine in renally compromised patients may be much higher than in normal patients [16]. Sensitivity to fludarabine cytotoxicity of hRPTC cultures, as assessed by EC_{50} values of concentration-effect curves, was positively correlated, albeit weakly, to hNT-mediated fludarabine uptake values, hCNT3 activities and hCNT3 cell surface protein levels. The different hRPTC cultures comprised two groups with differing fludarabine transportabilities and sensitivities to fludarabine cytotoxicity. The first group exhibited lower hNT-mediated fludarabine uptake and sensitivity to fludarabine cytotoxicity, while the second group exhibited higher and varied hNT-mediated fludarabine uptake and higher sensitivity to fludarabine cytotoxicity. The first group appeared to be relatively more resistant to fludarabine because of lower hCNT3 activities, resulting in lower fludarabine cellular accumulation, than the second group. Within the second group, hRPTC cultures exhibited similar sensitivities to fludarabine despite different hCNT3 activities, which could be explained by cytotoxicity being a multi-step process, with permeation through NTs being the first step and intracellular activation by kinases being subsequent steps. Notably, fludarabine has been shown to be cytotoxic to non-proliferating plasma cells by virtue of DNA repair inhibition [37], which is consistent with the observed cytotoxicity of fludarabine to non-proliferating hRPTC cultures. Precedent for interpatient variations in hNT abundance comes from hENT1 immunohistochemistry and mRNA expression studies in primary breast cancers, non-Hodgkins lymphoma, pancreatic cancers, and chronic lymphocytic leukemia in which hENT1 levels varied significantly and independently of pathological and clinical features [9, 10, 30, 31, 42].

Although this study assessed the contribution of NT-mediated fludarabine uptake to fludarabine cytotoxicity in hRPTC cultures, it is well established that cellular uptake is only the first step in pathways leading to nucleoside analog cytotoxicity. Conversion of fludarabine to its monophosphate, diphosphate, and triphosphate forms by intracellular kinases, most notably its monophosphate form by deoxycytidine kinase, is the second step in fludarabine cytotoxicity to normal and cancerous cells [8]. Indeed, low deoxycytidine kinase levels have been shown to be determinants of fludarabine resistance [26]. It is likely that the intracellular levels of fludarabine 5′-triphosphate, the cytotoxic form of fludarabine which inhibits ribonucleotide reductase and DNA synthesis [7, 45], that are achieved in cultures of hRPTCs may also be primary determinants in fludarabine cytotoxicity to hRPTC cultures.

A positive correlation was observed between hNT-mediated fludarabine uptake and IC_{50} values for concentration-dependent fludarabine inhibition of hNT-mediated uridine uptake: the latter provided a rough measure of the composite

apparent affinities of the hNTs of hRPTC cultures for fludarabine, with lower values representing higher affinities. The apparent affinity of hENT1 for fludarabine is higher than its apparent affinity for uridine (K_m values, 107 versus 250 μM, respectively), whereas the apparent affinity of hCNT3 for fludarabine is lower than its apparent affinity for uridine (K_m values, 353 versus 20 μM, respectively) [25]. Although hENT2 is known to transport fludarabine [26], the K_m value for fludarabine interaction with hENT2 has not been reported. The composite apparent affinities for fludarabine of individual hRPTC cultures, which exhibited different levels of hENT1, hENT2, and hCNT3 activities, was a consequence of the relative levels of these activities and their individual affinities for fludarabine. As hCNT3 was the dominant contributor to hNT-mediated fludarabine uptake in hRPTCs and the affinity of hCNT3 for fludarabine is less than that of hENT1, the composite apparent affinity for fludarabine was expected to decrease (thereby yielding increased IC_{50} values) as hCNT3 activity increased. Additionally, there is no a priori relationship between transporter affinity and transporter-mediated uptake in physiological milieus, because transporter-mediated uptake will depend upon a combination of affinity, turnover number, and total cell surface abundance of transporter.

In summary, hENT1, hENT2, and hCNT3 were present in hRPTC cultures, a differentiated model system of proximal tubules, and hCNT3 was shown to be an important determinant of fludarabine uptake and toxicity. These experiments established cultures of hRPTCs as an excellent model system for studying roles of multiple hNTs, present at endogenous levels, in renal handling of nucleosides and nucleoside analogs. A better understanding of the determinants of individual patient differences in renal elimination of nucleoside analogs could lead to approaches for individualizing chemotherapy regimens and improving patient treatment.

References

1. Avramis VI, Champagne J, Sato J et al (1990) Pharmacology of fludarabine phosphate after a phase I/II trial by a loading bolus and continuous infusion in pediatric patients. Cancer Res 50:7226–7231
2. Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD (2004) The equilibrative nucleoside transporter family, SLC29. Pflugers Arch 447:735–743
3. Baldwin SA, Yao SY, Hyde RJ et al (2005) Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. J Biol Chem 280:15880–15887
4. Barnes K, Dobrzynski H, Foppolo S et al (2006) Distribution and functional characterization of equilibrative nucleoside transporter-4, a novel cardiac adenosine transporter activated at acidic pH. Circ Res 99:510–519
5. Blackburn JG, Hazen-Martin DJ, Detrisac CJ, Sens DA (1988) Electrophysiology and ultrastructure of cultured human proximal tubule cells. Kidney Int 33:508–516
6. Boumah CE, Hogue DL, Cass CE (1992) Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells. Biochem J 288:987–996
7. Brockman RW, Schabel FM Jr, Montgomery JA (1977) Biological activity of 9-β-D-arabinofuranosyl-2-fluoroadenine, a metabolically stable analog of 9-β-D-arabinofuranosyladenine. Biochem Pharmacol 26:2193–2196
8. Carson DA, Wasson DB, Kaye J et al (1980) Deoxycytidine kinase mediated toxicity of deoxyadenosine analogs toward human lymphoblasts in vitro and toward murine L1210 leukemia in vivo. Proc Natl Acad Sci USA 77:6865–6869
9. Chow L, Lai R, Dabbagh L et al (2005) Analysis of human equilibrative nucleoside transporter 1 (hENT1) protein in non-Hodgkin's lymphoma by immunohistochemistry. Mod Pathol 18:558–564
10. Dabbagh L, Coupland RW, Cass CE, Mackey JR (2003) Immunohistochemical variation of human equilibrative nucleoside transporter 1 protein in primary breast cancers. Clin Cancer Res 9:3213–3214
11. Damaraju VL, Elwi AN, Hunter C et al (2007) Localization of broadly selective equilibrative and concentrative nucleoside transporters, hENT1 and hCNT3, in human kidney. Am J Physiol Renal Physiol 293:F200–F211
12. Danhauser L, Plunkett W, Keating M et al (1996) 9-β-D-Arabinofuranosyl-2-fluoroadenine 5′-monophosphate pharmacokinetics in plasma and tumor cells of patients with relapsed leukemia and lymphoma. Cancer Chemother Pharmacol 18:145–152
13. Elwi AN, Damaraju VL, Baldwin SA, Young JD, Sawyer MB, Cass CE (2006) Renal nucleoside transporters: physiological and clinical implications. Biochem Cell Biol 84:844–858
14. Engel K, Zhou M, Wang J (2004) Identification and characterization of a novel monoamine transporter in the human brain. J Biol Chem 279:50042–50049
15. Errasti-Murugarren E, Pastor-Anglada M, Casado FJ (2007) Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs. J Physiol 582:1249–1260
16. Ghandi V, Plunkett W (2002) Cellular and clinical pharmacology of fludarabine. Clin Pharmacokinet 41:93–103
17. Govindarajan R, Bakken AH, Hudkins KL et al (2007) In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. Am J Physiol Regul Integr Comp Physiol 293:R1809–R1822
18. Gray JH, Mangravite LM, Owen RP et al (2003) Functional and genetic diversity of concentrative nucleoside transporter 1, CNT1, in human populations. Mol Pharmacol 73:512–519
19. Gray JH, Owen RP, Giacomini KM (2004) The concentrative nucleoside transporter family, SLC28. Pflugers Arch 447:728–734
20. Griffiths M, Beaumont N, Yao SY et al (1997) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. Nat Med 3:89–93
21. Griffiths M, Yao SY, Abidi F et al (1997) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. Biochem J 328:739–743
22. Gutierrez MM, Brett CM, Ott RJ, Hui AC, Giacomini KM (1992) Nucleoside transport in brush border membrane vesicles from human kidney. Biochim Biophys Acta 1105:1–9
23. Hagenbeek A, Eghbali H, Monfardini S et al (2006) Phase III intergroup study of fludarabine phosphate compared with cyclophosphamide, vincristine, and prednisone chemotherapy in newly diagnosed patients with stage III and IV low-grade malignant Non-Hodgkin's lymphoma. J Clin Oncol 24:1590–1596

24. Hussain K, Mazza JJ, Clouse LH (2003) Tumor lysis syndrome (TLS) following fludarabine therapy for chronic lymphocytic leukemia (CLL): case report and review of the literature. Am J Hematol 72:212–215
25. King KM, Damaraju VL, Vickers MF et al (2006) A comparison of the transportability, and its role in cytotoxicity, of clofarabine, cladribine, and fludarabine by recombinant human nucleoside transporters produced in three model expression systems. Mol Pharmacol 69:346–353
26. King KM (2007) Current Cancer Therapy Rev (in press)
27. Lai Y, Bakken AH, Unadkat JD (2002) Simultaneous expression of hCNT1-CFP and hENT1-YFP in Madin-Darby canine kidney cells. Localization and vectorial transport studies. J Biol Chem 277:37711–37717
28. Lash LH, Putt DA, Cai H (2006) Membrane transport function in primary cultures of human proximal tubular cells. Toxicology 228:200–218
29. Leporrier M, Chevret S, Cazin B et al (2001) French Cooperative Group on Chronic Lymphocytic Leukemia. Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. Blood 98:2319–2325
30. Mackey JR, Jennings LL, Clarke ML et al (2002) Immunohistochemical variation of human equilibrative nucleoside transporter 1 in primary breast cancers. Clin Cancer Res 8:110–116
31. Mackey JR, Galmarini CM, Graham KA et al (2005) Quantitative analysis of nucleoside transporter and metabolism gene expression in chronic lymphocytic leukemia (CLL): identification of fludarabine-sensitive and -insensitive populations. Blood 105:767–774
32. Malich G, Markovic B, Winder C et al (1997) The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. Toxicol 124:179–192
33. Malspeis L, Grever MR, Staubus AE et al (1990) Pharmacokinetics of 2-F-ara-A (9-beta-D-arabinofuranosyl-2-fluoroadenine) in cancer patients during the phase I clinical investigation of fludarabine phosphate. Sem Oncol 17:18–32
34. Mangravite LM, Lipschutz JH, Mostov KE, Giacomini KM (2001) Localization of GFP-tagged concentrative nucleoside transporters in a renal polarized epithelial cell line. Am J Physiol Renal Physiol 280:F879–F885
35. Mangravite LM, Xiao G, Giacomini KM (2003) Localization of human equilibrative nucleoside transporters, hENT1 and hENT2, in renal epithelial cells. Am J Physiol Renal Physiol 284:F902–F910
36. Mangravite LM, Badagnani I, Giacomini KM (2003) Nucleoside transporters in the disposition and targeting of nucleoside analogs in the kidney. Eur J Pharmacol 479:269–281
37. Rao VA, Plunkett W (2003) Activation of a p53-mediated apoptotic pathway in quiescent lymphocytes after the inhibition of DNA repair by fludarabine. Clin Cancer Res 9:3204–3212
38. Ritzel MW, Yao SY, Huang MY, Elliott JF, Cass CE, Young JD (1997) Molecular cloning and functional expression of cDNAs encoding a human Na + -nucleoside cotransporter (hCNT1). Am J Physiol 272:C707–C714
39. Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE, Young JD (1998) Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Mol Membr Biol 15:203–211
40. Ritzel MW, Ng AM, Yao SY et al (2001) Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem 276:2914–2927
41. Smith KM, Slugoski MD, Cass CE, Baldwin SA, Karpinski E, Young JD (2007) Cation coupling properties of human concentrative nucleoside transporters hCNT1, hCNT2 and hCNT3. Mol Membr Biol 24:53–64
42. Spratlin J, Sangha R, Glubrecht D et al (2004) The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin Cancer Res 10:6956–6961
43. Terada T, Saito H, Mukai M, Inui KI (1996) Identification of the histidine residues involved in substrate recognition by a rat H^+/peptide cotransporter, PEPT1. FEBS Lett 394:196–200
44. Tobinai K, Watanabe T, Ogura M et al (2006) Phase II study of oral fludarabine phosphate in relapsed indolent B-Cell non-Hodgkin's lymphoma. J Clin Oncol 24:174–180
45. Tseng WC, Derse D, Cheng Y-C et al (1982) In vitro activity of 9-β-D-arabinofuranosyl-2-fluoroadenine and the biochemical actions of its triphosphate on DNA polymerases and ribonucleotide reductase from HeLa cells. Mol Pharmacol 21:474–477
46. Vickers MF, Zhang J, Visser F et al (2004) Uridine recognition motifs of human equilibrative nucleoside transporters 1 and 2 produced in Saccharomyces cerevisiae. Nucleosides Nucleotides Nucleic Acids 23:361–373
47. Wang J, Su SF, Dresser MJ, Schaner ME, Washington CB, Giacomini KM (1997) Na^+-dependent purine nucleoside transporter from human kidney: cloning and functional characterization. Am J Physiol Renal Physiol 273:F1058–F1065
48. Ward JL, Sherali A, Mo ZP, Tse CM (2000) Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. J Biol Chem 275:8375–8381
49. Xia L, Engel K, Zhou MY, Wang J (2007) Membrane localization and pH-dependent transport of a newly cloned organic cation transporter (PMAT) in kidney cells. Am J Physiol Renal Physiol 292:F682–F690
50. Zhang J, Smith KM, Tackaberry T et al (2005) Uridine binding and transportability determinants of human cocentrative nucleoside transporters. Mol Pharmacol 68:830–839
51. Zhang J, Visser F, Vickers MF et al (2003) Uridine binding motifs of human concentrative nucleoside transporters 1 and 3 produced in *Saccharomyces cerevisiae*. Mol Pharmacol 64:1512–1520