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Acquisition of Human Concentrative Nucleoside Transporter 2 (hCNT2) Activity by Gene Transfer Confers Sensitivity to Fluoropyrimidine Nucleosides in Drug-Resistant Leukemia Cells

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ABSTRACT

CEM-ARAC leukemia cells with resistance to cytarabine were shown to lack equilibrative transporter (hENT1) expression and activity. Stable transfer of hCNT2 cDNA into CEM-ARAC enabled Na^+-dependent transport of purine and pyrimidine nucleoside analogs and provided a unique in vitro model for studying hCNT2. Analysis of [^{3}H]uridine inhibitory activity by test substances in hCNT2 transfectant ARAC/D2 revealed structural requirements for interaction with hCNT2: 1) ribosyl and 2′-deoxyribosyl nucleosides were better inhibitors than 3′-deoxyribosyl, 2′,3′-dideoxyribosyl or arabinosyl nucleosides; 2) uridine analogs with halogens at position 5 were better inhibitors than 5-methyluridine or thymidine; 3) 2-chloroadenosine was a better inhibitor than 2-chloro-2′-deoxyadenosine (cladribine); and 4) cytosine-containing nucleosides, 7-deazaadenosine and nucleobases were not inhibitors. Quantification of inhibitory capacity yielded K_i values of 34–50 μM (5-halogenated uridine analogs, 2′-deoxyuridine), 82 μM (5-fluoro-2′-deoxyuridine), 197–246 μM (5-methyluridine < 5-bromo-2′-deoxyuridine < 5-iodo-2′-deoxyuridine), and 411 μM (5-fluoro-5′-deoxyuridine, capecitabine metabolite). Comparisons of hCNT2-mediated transport rates indicated halogenated uridine analogs were transported more rapidly than halogenated adenosine analogs, even though hCNT2 exhibited preference for physiologic purine nucleosides over uridine. Kinetics of hCNT2-mediated transport of 5-fluorouridine and uridine were similar (K_m values, 43–46 μM). The impact of hCNT2-mediated transport on chemosensitivity was assessed by comparing antiproliferative activity of nucleoside analogs against hCNT2-containing cells with transport-defective, drug-resistant cells. Chemosensitivity was restored partially for cladribine, completely for 5-fluorouridine and 5-fluoro-2′-deoxyuridine, whereas there was little effect on chemosensitivity for fludarabine, 7-deazaadenosine, or cytarabine. These studies, which demonstrated hCNT2 uptake of halogenated uridine analogs, suggested that hCNT2 is an important determinant of cytotoxicity of this class of compounds in vivo.

Fluoropyrimidine nucleosides have been used in the treatment of disseminated human cancers, especially of the gastrointestinal tract, breast, and ovary (Focan et al., 1999; Kim et al., 2001). The differences in metabolism, mechanism of action, and favorable pharmacokinetics of 5-fluorouridine compared with other fluoropyrimidine nucleosides have contibuted to its efficacy in the treatment of superficial bladder cancers, which represent about 80% of bladder cancers (Song et al., 1997). 5-Fluorouridine and 5-fluoro-2′-deoxyuridine are also useful radiopharmaceuticals in tumor imaging using positron emission tomography and in spectroscopic analysis with ^{19}F NMR to follow their intracellular metabolism in vitro and in vivo (Chen et al., 1999). Since serum levels of diagnostic radiopharmaceuticals are typically very low, active transport will influence their cellular and tissue distribution.

Human cells possess multiple nucleoside transporters that are either equilibrative or concentrative (Cass et al., 1998; Baldwin et al., 1999), of which five have been identified by molecular cloning.[1] The equilibrative nucleoside transporters (ENTs), which include the nitrobenzylthioinosine (NBMPR)-sensitive

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[1] The correspondence between human nucleoside transporter proteins (GenBank accession numbers given in parentheses) and their activities is: hENT1 (U81375), *es*; hENT2 (AF029358), *ei*; hCNT1 (U62966), *cit*; hCNT2 (AF036109), *cif*; hCNT3 (AF305210), *cib*.

ABBREVIATIONS: ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; h, human; r, rat; cytarabine, 1-β-D-arabinofuranosylcytosine (araC); NBMPR, nitrobenzylmercaptopurine ribonucleoside (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine); PBS, phosphate-buffered saline; kb, kilobase(s); MDR, multidrug-resistant; HIHS, heat-inactivated horse serum; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcriptase polymerase chain reaction; abbreviations used in transporter acronyms: *c*, concentrative; *e*, equilibrative; *s* and *i*, sensitive and insensitive to inhibition by NBMPR, respectively; *f*, formycin B (nonmetabolized purine nucleoside); *t*, thymidine.

and -insensitive transporters (hENT1, hENT2), exhibit broad permeant selectivities and appear to be widely distributed among cells and tissues (Griffiths et al., 1997a; Crawford et al., 1998b). The concentrative nucleoside transporters (CNTs) have been identified in specialized mammalian cells (e.g., intestinal and renal epithelia) and in several neoplastic cell types (Gutierrez et al., 1992; Belt et al., 1993; Roovers and Meckling-Gill 1996; Chandrasena et al., 1997; Flanagan and Meckling-Gill 1997; Patil and Unadkat, 1997). The cognate proteins (hCNT1, hCNT2, hCNT3) responsible for three (*cit*, *cif, cib*, respectively) of the human concentrative processes (Ritzel et al., 1997, 1998, 2000; Wang et al., 1997) have been recently identified by molecular cloning.

The multiplicity of nucleoside transporters and their overlapping substrate selectivities in human cells (Crawford et al., 1990b; Roovers and Meckling-Gill 1996; Boleti et al., 1997) have made the functional analysis of CNTs difficult. There are only a few examples of human cell types (e.g., erythrocytes and cultured CCRF-CEM leukemia cells) that naturally exhibit a single nucleoside-transport process (the prototypic equilibrative NBMPR-sensitive (*es*) process); these cell types have been used to study the *es* transport process in the absence of other processes, and much is known about its functionality (Cass 1995; Griffith and Jarvis 1996). A clonal derivative of the CEM cell line, CEM-ARAC, which was selected for resistance to cytarabine, exhibits deficiencies in transporting cytidine analogs and binding NBMPR and is cross-resistant to gemcitabine (Ullman et al., 1988; Mackey et al., 1998).

The emergence of drug-resistant cells following drug exposure mitigates their therapeutic potential and poses a major problem in chemotherapy. In the present study, CEM-ARAC cells were shown to be cross-resistant to purine and pyrimidine nucleoside drugs due to the lack of detectable hENT1 mRNA and the associated deficiency in nucleoside transport capability. CEM-ARAC cells were transfected with hCNT2 cDNA and a stable transfectant (ARAC/D2) was identified by screening cultures for acquisition of Na^+-dependent uptake of [^{3}H]uridine, thereby providing the first example of a cultured cell line with hCNT2 activity. The structural determinants for interaction with hCNT2 were assessed by comparing the ability of test compounds to inhibit transport of [^{3}H]uridine. The transportability and pharmacological properties of adenosine and uridine analogs were compared in cells containing hCNT2, hENT1, or no nucleoside transporters. CEM cells producing the broadly selective hENT1 were sensitive to both purine and pyrimidine nucleoside drugs, whereas ARAC/D2 cells producing the purine-nucleoside-selective hCNT2 were more sensitive to 5-fluorouridine and 5-fluoro-2′-deoxyuridine than either cladribine, fludarabine, or tubercidin. Chemosensitivity was correlated with the relative affinity of the nucleoside drugs for hCNT2. These results demonstrated that nucleoside drug resistance could be overcome by introduction of hCNT2 into nucleoside drug-resistant cells, and suggested the importance of hCNT2 in the transport of several fluoropyrimidine nucleoside drugs, but not that of 5-fluoro-5′-deoxyuridine.

Experimental Procedures

Plasmid Construction. The open reading frame of the cDNA encoding hCNT2 (Ritzel et al., 1998) was excised from the original cloning vector, pBluescript II KS(+) (Stratagene, La Jolla, CA) using *Eco*RI and *Xba*I restriction enzymes and was subcloned into the polylinker region downstream of the enhancer/promoter sequences of the immediate early gene of human cytomegalovirus of the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The pcDNA3/hCNT2 construct was transformed into electrocompetent JM109 *Escherichia coli* (Invitrogen) using a CELL-PORATOR (Invitrogen). Plasmid DNA for use as template in DNA sequencing and transfections was prepared using the Qiagen plasmid purification kit (Qiagen Corp., Mississauga, ON) according to the manufacturer's instructions. The structure of pcDNA3/hCNT2 was verified by restriction endonuclease mapping and DNA sequencing. DNA sequences were determined by *Taq* DyeDeoxy terminator cycle sequencing with an automated model 310 DNA sequencer (Applied Biosystems Inc., Foster City, CA). Sequence analysis used MacVector DNA analysis software (Oxford Molecular Ltd., Bethesda, MD).

RT-PCR Analysis. Poly(A)$^+$ RNA was isolated from actively proliferating cells using the FastTrack 2.0 isolation kit (Invitrogen) according to the manufacturer's instructions. mRNA concentrations were determined spectrophotometrically. For cDNA synthesis reactions, Superscript RT kits (Invitrogen) were used following the manufacturer's instructions. Oligonucleotide primers specific for hENT1 were ES2 and ES4, which corresponded, respectively, to hENT1 cDNA residues $^-$4–21 (sense, 5′-CACCATGACAACCAGTCACCAGCCT-3′, start codon underlined) and $^+$414–$^+$437 (antisense, 5′-GACCTGATATACTCCATTCTCC-3′) derived from the 3′-untranslated regions. Primers specific for hCNT2 were NT2D and JM23, which corresponded, respectively, to residues 1528–1549 (sense, 5′-GGAATGGAGGAGTGGATGAGG-3′) and 1949–1971 (antisense, 5′-GCTGTGGATTCTACAACAATACC-3′). Nested PCR was performed on "first-round" hCNT2 products using NT2KG25 and NT2KG26 internal primer sets, which corresponded, respectively, to residues 1562–1584 (sense, 5′-GGATTTCTGTGAGAGCTGAAATC-3′) and 1920–1939 (antisense, 5′-GAATTTCAATGCTATGGCCC-3′). Amplifications were performed on a Robocycler temperature cycler (Stratagene) as follows: 5 min at 94°C, 25 cycles (94°C for 1 min, 57°C for 2 min, 72°C for 2 min), and 20 min at 72°C. PCR products were subjected to electrophoresis on a 1% agarose gel with size markers (1-kb DNA ladder, Invitrogen) and bands that migrated with the expected mobilities of hENT1 and hCNT2 were excised, gel-purified, and sequenced in one direction for confirmation of identity.

Southern Blot Analysis. Genomic DNA was prepared from cells by the proteinase K-RNase method (Ausubel et al., 1997), cleaved with *Eco*RI (Invitrogen), subjected to electrophoresis on an 0.8% agarose gel with size markers (1-kb DNA ladder, Invitrogen) and transferred to Hybond-N$^+$ membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The hybridization probes were prepared by 1) PCR amplification of the full-length hCNT2 cDNA from pcDNA3/hCNT2; 2) separation on agarose gels; 3) purification on a G10 Sepharose column; 4) ^{32}P-radiolabeling using a Pharmacia random primer oligolabeling kit (5×10^8–10^9 dpm/μg of DNA); and 5) purification by gel filtration chromatography on a Nick column (Amersham Pharmacia Biotech). Hybridization was conducted under high stringency (65°C for 1 h) using Express Hybridization solution (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Autoradiography was carried out by exposure to Kodak X-ray films at −80°C.

Growth and Maintenance of Cell Lines. The CCRF-CEM (American Type Culture Collection, Manassas, VA; CCL-119) cell line (hereafter referred to as CEM) is a human T-lymphoblast line that was originally derived from a patient with acute lymphocytic leukemia and exhibits *es* nucleoside transport activity (Cass et al., 1992). CEM-ARAC-8C (hereafter referred to as CEM-ARAC) is a nucleoside transport-defective subline that was selected for resistance to cytarabine by Dr. B. Ullman (Oregon Health Sciences University, Portland, OR) (Ullman et al., 1988). CEM and its derivatives were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% fetal bovine serum (v/v) and, for CEM-ARAC cells, 0.25 μM

tubercidin and 0.5 μM cytarabine or, for hCNT2 transfectants, 0.25 μM cytarabine and 0.2 μg/μl G418; cells were subcultured every 3 to 4 days. HeLa S3 cells (American Type Culture Collection; CCL-2.2) were maintained as adherent cultures in RPMI 1640 medium supplemented with 10% calf serum and subcultured at weekly intervals. Cells were incubated at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air, and cell numbers were determined using a Coulter Z2 electronic particle counter equipped with a size analyzer (Coulter Electronics, Burlington, ON, Canada). The cell lines used in this study were periodically shown to be free of *Mycoplasma* by direct culture in agar/cell-free medium (Medical Microbiology Laboratory, Edmonton, AB, Canada).

Transfection and Selection of Transfectants. For transfections, pcDNA3 or pcDNA3/hCNT2 plasmids were 1) purified on a Qiagen Midi column from JM109 cultures that had been grown in the presence of 50 μg/ml ampicillin, 2) linearized with *Blg*II restriction enzyme, and 3) diluted to 0.25 μg/μl with RPMI 1640 that contained no glutamine (hereafter referred to as Gln-free RPMI). For production of transient transfectants, hCNT2/pcDNA3 was introduced into HeLa cells as described previously (Graham et al., 2000). Actively proliferating cultures (2×10^6 HeLa cells per 100-mm dish) were transfected with plasmid DNA (4 μg/dish) using DEAE-dextran (Amersham Pharmacia Biotech, Quebec, Canada) and uptake assays were performed 72 h later.

For production of stable transfectants, recipient cells were prepared from actively proliferating CEM-ARAC cultures by centrifugation (800*g*, 10 min), resuspension at 5×10^4 cells/ml in RPMI 1640 with 10% HIHS, and incubation at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air for 72 h to a concentration of about 4×10^5 cells/ml, after which they were collected by centrifugation (800*g*, 10 min), washed twice with Gln-free RPMI, and concentrated in Gln-free RPMI to 3×10^8 cells/ml. Electroporation was performed using a gene pulser equipped with a capacitance extender (Bio-Rad, Toronto, ON, Canada) and a 4-mm gap cuvette (Bio-Rad) in which 170 μl of cell suspension and 30 μl of the linearized pcDNA3/hCNT2 mixture was placed. After electroporation (190 V, 960 μF, 65–75 ms), cells were resuspended in RPMI 1640 supplemented with 15% HIHS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and incubated for 24 h at 37°C in a humidified (95%) atmosphere of 5% CO_2 and air, after which they were triturated, diluted to 2×10^5 cells/ml with RPMI 1640 supplemented with 10% HIHS, incubated at 37°C for an additional 24 h, collected by centrifugation (800*g*, 10 min), resuspended at 1×10^5 cells/ml in RPMI 1640 supplemented with 10% HIHS and 0.2 μg/μl G418 (geneticin, Invitrogen, Gaithersburg, MD), and incubated at 37°C in a humidified (95%) atmosphere of 5% CO_2 with medium changes at 3- to 4-day intervals for approximately 1 month.

Transfection efficiencies were assessed by modification of the β-galactosidase staining method. Parallel cultures were transfected with pcDNA3 or pcDNA3 containing the *Escherichia coli lacZ* gene (pcDNA3/*lacZ*) using either the DEAE-dextran (HeLa) or the electroporation procedure (CEM). Cultures were grown for 72 h; after which they were harvested as described above, fixed (0.5% glutaraldehyde in PBS) for 15 min at room temperature, and washed twice (PBS adjusted to pH 7.4) by centrifugation (800*g*, 5 min). The resulting cell pellets were incubated in 1-ml staining solution that consisted of 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 20 mM potassium ferricyanide/ferrocyanide, and 2 mM $MgCl_2$ in PBS for 24 h at 37°C. Transfection efficiencies were estimated by determination of the proportion of blue-stained cells using a hemacytometer and were about 30% for HeLa cells and 0.1 to 1% for CEM cells.

Cloning of Stable Transfectants in Semisolid Medium. G418-resistant cells were selected by growth in soft agarose cloning medium that consisted of equal volumes of fresh RPMI 1640 supplemented with 0.2 μg/μl G418, 20% HIHS, 1 mM α-ketoglutarate (Sigma, Oakville, ON, Canada), and 6 mM glutamine, and the same medium that had been "conditioned" by exposure to actively proliferating CEM-ARAC cells for 24 h. G418 selection cultures were suspended in cloning medium with 1% Seaplaque agarose (Mandel, Guelph, ON, Canada) to yield 5×10^3 to 1×10^4 cells/100-mm plate (Fisher Scientific, Ottawa, ON, Canada) and incubated for 3 weeks at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air. Surviving colonies were picked, expanded, and screened for nucleoside transport activity as described below.

Nucleoside Transport Assays. Initial rates of nucleoside uptake were measured under zero-*trans* conditions at room temperature with cells harvested from actively proliferating cultures (4×10^5 cells/ml). Briefly, cells were harvested by centrifugation (800*g*, 10 min), washed twice in either Na^+-containing transport buffer (5 mM D-glucose, 20 mM Tris-HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, and 130 mM NaCl, pH 7.4) or Na^+-free transport buffer (NaCl was substituted with *N*-methyl-D-glucammonium chloride), and then resuspended in the appropriate transport buffer. Time courses of uptake of ^{3}H-labeled nucleosides were determined using rapid sampling procedures in which the transport process was initiated by addition of cells to the ^{3}H-labeled nucleoside solution (1:1) and terminated by rapid addition of excess cold nucleoside solution followed by immediate centrifugation (16,000*g*, 30 s) through transport oil [mixture of paraffin oil (Fisher Scientific, Ottawa, ON, Canada) and silicone 550 oil (Dow Corning, Mississauga, ON, Canada) with final specific gravity of 1.03 g/ml] to separate the cells from the permeant solution. Replicate assay mixtures were exposed to either [^{14}C]polyethyleneglycol or ^3H_2O to determine trapped extracellular and intracellular water volumes. The cell pellets were solubilized in 0.5 ml of 5% Triton X-100, and cell-associated radioactivity was determined by liquid scintillation counting. Transport rates were derived from regression analysis of the linear component of uptake and kinetic parameters (apparent K_m and V_{max} values) were calculated using Prism (GraphPad Software Inc., San Diego, CA).

Materials. Radioisotopes were purchased from Moravek Biochemicals Inc. (Brea, CA) and ^{3}H-labeled nucleosides were purified by high-performance liquid chromatography using water-methanol gradients on a C_{18} reverse-phase column. Natural nucleosides, nucleoside analogs, and doxorubicin were purchased from either Sigma Chemical Corp. or Cross Cancer Institute Pharmacy (Edmonton, AB, Canada). Dilazep was a gift from F. Hoffman La Roche and Co. (Basel, Switzerland). All other chemicals were of analytical grade and obtained from commercial sources. Cell culture supplies were from Invitrogen.

Results and Discussion

Demonstration of the Absence of hENT1 mRNA in CEM-ARAC Cells. CEM-ARAC cells lack NBMPR-sensitive nucleoside transport activity and the capacity for high-affinity binding of NBMPR (Ullman et al., 1988). These characteristics suggested an absence of functional *es* transporters in the mutant cells. RT-PCR analysis with primers designed to detect full-length hENT1 transcripts was undertaken with RNA isolated from CEM and CEM-ARAC cells to determine whether the hENT1 gene was expressed in the mutant cells (Fig. 1A). A PCR product of the expected mobility was observed in reactions conducted with RNA from CEM cells whereas there was no such product in reactions with RNA from CEM-ARAC cells. Sequencing of the CEM-derived PCR product showed correspondence to the hENT1 coding sequence that was recently cloned from human placenta (Griffiths et al., 1997). A comparison of initial rates of uptake of 10 μM [^{3}H]uridine by CEM and CEM-ARAC cells is shown in Fig. 1B. The results shown in Fig. 1, A and B, established an association between the absence of hENT1 mRNA and the inability to transport uridine and other nucleosides in CEM-ARAC cells, indicating that the hENT1 protein was respon-

sible for mediating nucleoside uptake in CEM cells as predicted from its transport characteristics.

Production and Isolation of hCNT2-Producing Transfectants of CEM-ARAC Cells. Gene-transfer techniques were used to introduce the hCNT2 coding sequence into transport-deficient CEM-ARAC cells to assess the role of hCNT2 in the delivery of cytotoxic nucleosides. Isolation of hCNT2 stable transfectants with the introduced pcDNA3/hCNT2 construct was based on selection for neomycin resistance by growth in G418. Surviving G418-resistant clones were individually expanded and their nucleoside-transport activities were compared with those of CEM-ARAC cells by quantitating cellular uptake of radioactivity during 5-min exposures to 10 μM [^{3}H]uridine. Of the greater than 4000 clones that were produced and tested, most did not exhibit nucleoside transport activity, despite their resistance to G418 (results not shown). Several of the G418-resistant transfectants exhibited uridine-uptake activity. The clone (D2) with the highest activity was selected and recloned by limiting dilution. Subsequent uridine uptake measurements with the CEM-ARAC/hCNT2-D2 reclone (hereafter referred to as ARAC/D2) yielded no further enrichment in uridine transport activity. Values $\pm$ S.D. ranged from 0.11 $\pm$ 0.01 to 0.14 $\pm$ 0.008 pmol/μl cell water/s, indicating that the transport-competent phenotype was stable.

Genomic Integration and Expression of hCNT2 cDNA in ARAC/D2 Cells. Genomic DNA from ARAC/D2 cells was examined to determine whether the pcDNA3/hCNT2 construct was integrated into the host cell genome. High-stringency hybridization with full-length hCNT2 cDNA of *Eco*RI-digested genomic DNA revealed a single unique band of 3.4 kb in ARAC/D2 but not in CEM-ARAC preparations (data not shown), indicating integration of hCNT2 cDNA into host genomic DNA.

Poly(A)$^+$ RNA from actively proliferating cultures of ARAC/D2, CEM, and CEM-ARAC cells was analyzed by RT-PCR (Fig. 2), followed by nested PCR (data not shown). When pcDNA3/hCNT2 was used as the template, fragments with gel mobilities expected for the 458- and 425-base pair products of the external and nested hCNT2 primers were produced. Although PCR products that migrated with the same mobilities as those obtained with the hCNT2-plasmid preparations were detected in both rounds of PCR with the ARAC/D2 preparations, PCR products were not detected after either round (of 25 cycles each) conducted with the CEM and CEM/ARAC preparations.[2] Sequencing of the nested PCR product from the ARAC/D2 preparations demonstrated correspondence with hCNT2 DNA (data not shown). These results established that 1) CEM and CEM/ARAC cells lacked hCNT2 mRNA, indicating that the absence of endogenous hCNT2 activity was due to low (or no) expression of the hCNT2 gene; and 2) ARAC/D2 cells contained hCNT2 mRNA, evidently produced by expression of the introduced hCNT2 cDNA.

Transport Characteristics of ARAC/D2 Cells: Na$^+$ Dependence and *cif*-Type Activity. The nature of the acquired nucleoside-transport capability of ARAC/D2 cells was assessed by comparing time courses of uptake of 10 μM [^{3}H]uridine in the presence or absence of Na$^+$ by ARAC/D2

[2] Northern blot and RT-PCR analysis of mRNA from 10 other cultured human cancer cell lines of either hematopoietic or solid-tumor origin also gave negative results for hCNT2 transcripts (K. Graham, J. R. Mackey, D. Mowles, and C. E. Cass, unpublished results).

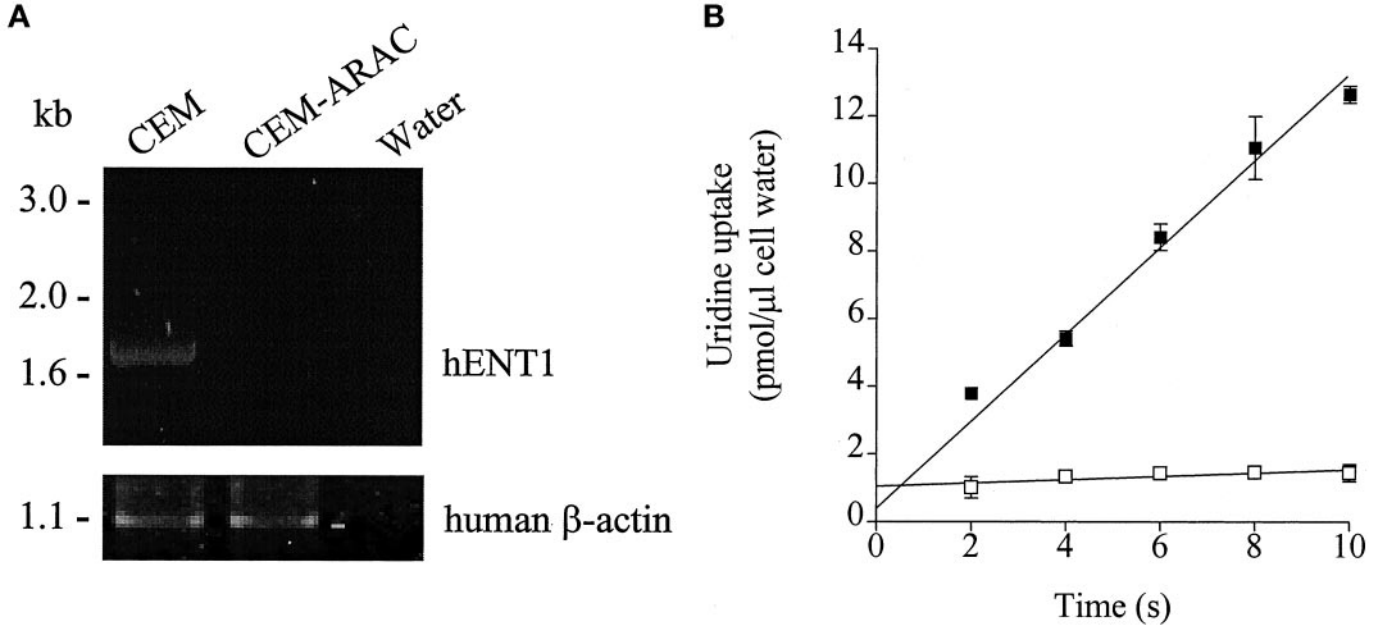

Fig. 1. Absence of hENT1 mRNA and uridine-transport activity in CEM-ARAC cells. Panel A, comparison of hENT1 gene expression in CEM and CEM-ARAC cells. Poly(A)$^+$ RNA was isolated from actively proliferating cultures, first-strand cDNA was synthesized by reverse transcription and then PCR-amplified with hENT1-specific primers; PCR products were separated on a 1% agarose gel, and the band that migrated with the expected hENT1 mobility was excised and sequenced as described under *Experimental Procedures*. PCR reactions in which DNA template was substituted with water served as negative controls. Panel B, comparison of uridine-transport activity in CEM and CEM-ARAC cells. Uptake of 10 μM [^{3}H]uridine by actively proliferating CEM (■) and CEM-ARAC (□) cells was measured at the time intervals shown in Na$^+$-containing transport buffer as described under *Experimental Procedures*. Each value represents the mean $\pm$ S.D. of three determinations, and error bars are not shown where S.D. values were smaller than that represented by the symbols.

cells with those of nucleoside transport-deficient CEM-ARAC cells and hENT1-containing CEM cells (Fig. 3A). In the presence of Na^+, ARAC/D2 cells exhibited initial uptake rates (i.e., transport) that were 25-fold greater than those of CEM-ARAC cells, whereas uptake rates were negligible in the absence of Na^+, indicating that the observed stimulation of uridine transport activity required the presence of an inwardly directed Na^+ gradient. Uridine uptake by CEM cells, which possessed only hENT1 activity, reached equilibrium at around 1 min and was not dependent on the Na^+ gradient. No measurable uridine uptake was observed in CEM-ARAC cells in either the presence or absence of Na^+. In the experiments shown in Fig. 3B, uptake of 35 μM [^{3}H]uridine by ARAC/D2 cells was completely inhibited by the presence of either 0.5 or 1 mM nonradioactive uridine. These results demonstrated that ARAC/D2 cells possessed functional recombinant hCNT2.

Natural nucleosides that are diagnostic for *cif*-type transport were also assessed for their ability to inhibit Na^+-dependent uridine transport in ARAC/D2 cells (Table 1). Na^+-stimulated uridine transport was completely inhibited by 1 mM physiological purine nucleosides. The other natural pyrimidine nucleosides and dilazep, a potent inhibitor of both hENT1 and hENT2, did not significantly reduce uridine transport when tested at 1 mM, indicating that the observed uptake was not due to either equilibrative nucleoside transporters (hENT1, hENT2) or to hCNT1. The inhibition characteristics of ARAC/D2 cells were consistent with those observed for naturally occurring *cif*-type transporters of human intestine and kidney epithelia (Gutierrez et al., 1992; Patil and Unadkat, 1997).

Structure-Activity Relationships of hCNT2: Ability of Purine and Pyrimidine Nucleosides and Nucleobases to Inhibit Uridine Transport. The experiments shown in Table 1 were undertaken to identify structural determinants for interaction with hCNT2. Rates of uptake of 10 μM [^{3}H]uridine were measured in the absence or presence of 1 mM concentrations of 1) physiologic purine and pyrimidine nucleosides, nucleobases, and ribose; 2) purine and pyrimidine arabinonucleosides and arabinose; and 3) analogs of adenosine and uridine. Studies involving adenosine and deaminase-sensitive adenosine analogs were conducted in the presence of 2 μM 2′-deoxycoformycin to prevent deamination (Barros et al., 1991). Deoxycoformycin did not inhibit hCNT2-mediated [^{3}H]uridine influx when tested at concentrations of 1 mM (data not shown). [^{3}H]Uridine influx by hCNT2 was inhibited completely by the physiologically occurring ribosyl and 2′-deoxyribosyl purine nucleosides, uridine, 2′-deoxyuridine, partially by thymidine and uracil, and not at all by ribose, cytidine, 2′-deoxycytidine or the other nucleobases that were tested. Although 2′-deoxyuridine and 2′-deoxyribosyl purine nucleosides inhibited uridine transport completely, 3′-deoxyuridine and 2′,3′-dideoxyribosyl derivatives of uridine and adenosine had no effect, suggesting a requirement of the hydroxyl group at position 3′ of the ribose moiety for interaction with hCNT2.

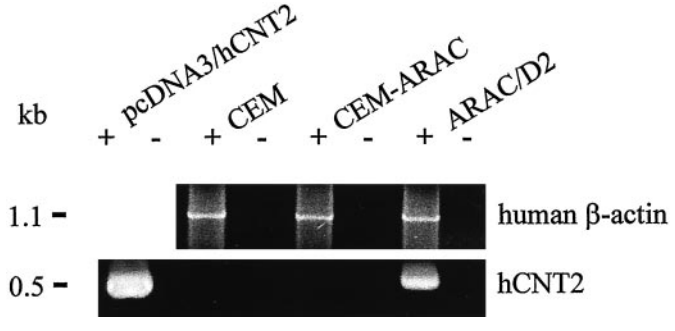

Fig. 2. Expression of hCNT2 mRNA in ARAC/D2 cells. Poly(A)$^+$ RNA was isolated from CEM, CEM-ARAC, and ARAC/D2 cells and reverse transcribed. The resulting cDNAs and pcDNA3/hCNT2 (as a positive control) were used as templates for PCR with hCNT2-specific primers (NT2D and JM23) as described under *Experimental Procedures*. The PCR products were separated on a 1% agarose gel with size markers (1-kb DNA ladder). For each set of PCR conditions, reactions were conducted with (+) or without (−) DNA template. The PCR products were sequenced to confirm their identity as described under *Experimental Procedures*.

The results of Table 1 showed that hCNT2-mediated [^{3}H]uridine uptake was markedly inhibited by purine arabinonucleosides (9-β-D-arabinofuranosyladenine, 9-β-D-arabinofuranosylhypoxanthine), whereas it was not inhibited or only partially inhibited by pyrimidine arabinonucleosides (1-β-D-arabinofuranosyluracil, 1-β-D-arabinofuranosylcytosine), 2-fluoro-9-β-D-arabinofuranosyladenine (fludarabine), and arabinose. Complete inhibition of hCNT2-mediated [^{3}H]uridine influx was observed for 2′-deoxyadenosine and 85% inhibition for 2-chloroadenosine, whereas 2-chloro-2′-deoxyadenosine (cladribine) exhibited 28% inhibition. The poor ability of 2-chloro-2′-deoxyadenosine and 2-fluoro-9-β-D-arabinofuranosyladenine, compared with that of 2′-deoxyadenosine and 9-β-D-arabinofuranosyladenine, to inhibit hCNT2-mediated uridine transport suggested a low tolerance for the presence of a halogen at position 2 of the purine base. Modifications of the purine ring to create 7-deazaadenosine (tubercidin) had no effect on hCNT2-mediated [^{3}H]uridine transport, which was consistent with previous reports demonstrating that 7-deazaadenosine was not a permeant of the murine *cif*-type transport process (Crawford et al., 1990).

Substitutions with a series of halogens with decreasing electronegativities at position 5 of uridine (5-fluorouridine, 5-bromouridine, 5-iodouridine) resulted in complete inhibition of hCNT2-mediated uridine transport. However, 5-methyluridine and thymidine were less potent inhibitors of [^{3}H]uridine transport (62 and 26% inhibition, respectively). The different 5-halogenated 2′-deoxyribosyl uridine analogs showed different magnitudes of inhibition of [^{3}H]uridine transport, with 5-fluoro-2′-deoxyuridine being the most effective (82% inhibition), followed by 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine (44% and 31% inhibition, respectively). 5-Fluoro-5′-deoxyuridine, an intermediate metabolite of the oral fluoropyrimidine capecitabine (Di Costanzo et al., 2000), showed only 20% inhibition of hCNT2-mediated [^{3}H]uridine transport. The combined results shown in Table 1 demonstrated that hCNT2 exhibited 1) tolerance for substitution of the hydroxyl group for a hydrogen in the 2′ but not 3′ position of the ribosyl moiety, 2) preference for the 2′-hydroxyl substituent in the α- rather than β-configuration, 3) low tolerance for modifications of position 2 on the purine base of adenosine analogs with halogen, and 4) higher tolerances for modifications of position 5 on the pyrimidine base of uridine analogs with halogen than with methyl substituents.

Interaction of hCNT2 with Halogenated Uridine Analogs. Since hCNT2 exhibited remarkably high tolerance for halogens at position 5 in several uridine analogs, inhibition experiments were performed with graded concentrations of

the halogenated analogs to quantify the relative effects of these modifications in ribosyl and deoxyribosyl derivatives of uridine. As shown in the representative dose-response curve of Fig. 4 comparing 5-fluorouridine, 5-fluoro-2′-deoxyuridine, and 5-fluoro-5′-deoxyuridine, hCNT2-mediated [^{3}H]uridine transport was inhibited by 5-halogenated uridine analogs to varying degrees. The calculated K_i values (Table 2) for 5-fluorouridine, 5-bromouridine, and 5-iodouridine (34, 46, and 50 μM, respectively) were close to the K_m value of 46 $\pm$ 4 μM observed for zero-*trans* influx of uridine. A much higher K_i value (197 μM) was obtained for 5-methyluridine, which was consistent with its poor ability to inhibit hCNT2-mediated [^{3}H]uridine transport in the inhibition studies of Table 1. Both thymidine and 1-β-D-arabinofuranosyluracil exhibited very high K_i values, indicating that they were likely not permeants of hCNT2. The halogen substituents in the pyrimidine ring of ribosyl analogs did not alter the apparent K_i values, whereas there was a progressive increase in K_i values that paralleled the increase in size and/or decrease in electronegativities of the halogen atoms in the 2′-deoxyribosyl analogs (5-fluoro-2′-deoxyuridine $<$ 5-bromo-2′-deoxyuridine $<$ 5-iodo-2′-deoxyuridine). A comparison of 5-fluoro-2′-deoxyuridine and 5-fluoro-5′-deoxyuridine demonstrated the importance of the hydroxyl group at the 5′-position for inhibitor-transporter interaction. Removal of the hydroxyl group at position 5′ in the ribosyl moiety of 5-fluorouridine increased the K_i value from 34 to 411 μM, whereas removal of the hydroxyl group at position 2′ increased the K_i value to 82 μM. These results indicated that 5-halogenated uridine analogs, 2′-deoxyuridine and 5-fluoro-2′-deoxyuridine bound well to hCNT2. Although inhibition of nucleoside influx indicated interaction between the inhibiting substance and hCNT2, it did not demonstrate that the inhibiting substance utilized the transporter to move through plasma membrane.

Transport Characteristics of ARAC/D2 Cells: hCNT2-Mediated Transport of Physiological and Modified Nucleosides. Uptake of several of the adenosine and uridine analogs used in this study was examined to determine whether these compounds were transported by hCNT2 and, if so, to compare their rates with those of the universal permeant uridine (Fig. 5). ARAC/D2 cells were included as a control to show the absence of mediated transport for several of the physiological and modified nucleosides. The results showed that the Na^+-dependent transport rates, which represented the hCNT2-mediated component, were generally higher for purine nucleosides than for pyrimidine nucleosides. Both adenosine and 2′-deoxyadenosine, which completely inhibited hCNT2-mediated [^{3}H]uridine transport (Table 1), exhibited approximately 2-fold higher transport rates than uridine (adenosine $>$ 2′-deoxyadenosine). Guanosine exhibited slightly higher rates of transport than 2′-deoxyguanosine.

The adenosine analogs, fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine) and cladribine (2-chloro-2′-deoxyadenosine), exhibited low transport rates (Fig. 5), which was consistent with their poor ability to inhibit hCNT2-mediated [^{3}H]uridine transport in the inhibition experiments of Table 1. These results are in contrast to previous reports showing that cladribine was a permeant of the rat homolog of hCNT2 (also termed SPNT) in transiently transfected cervical cancer cell lines (Schaner et al., 1997). This difference in cladribine transportability suggested species differences in substrate selectivities between the rat and human CNT2 proteins.

Since hCNT2-mediated uridine transport was inhibited by

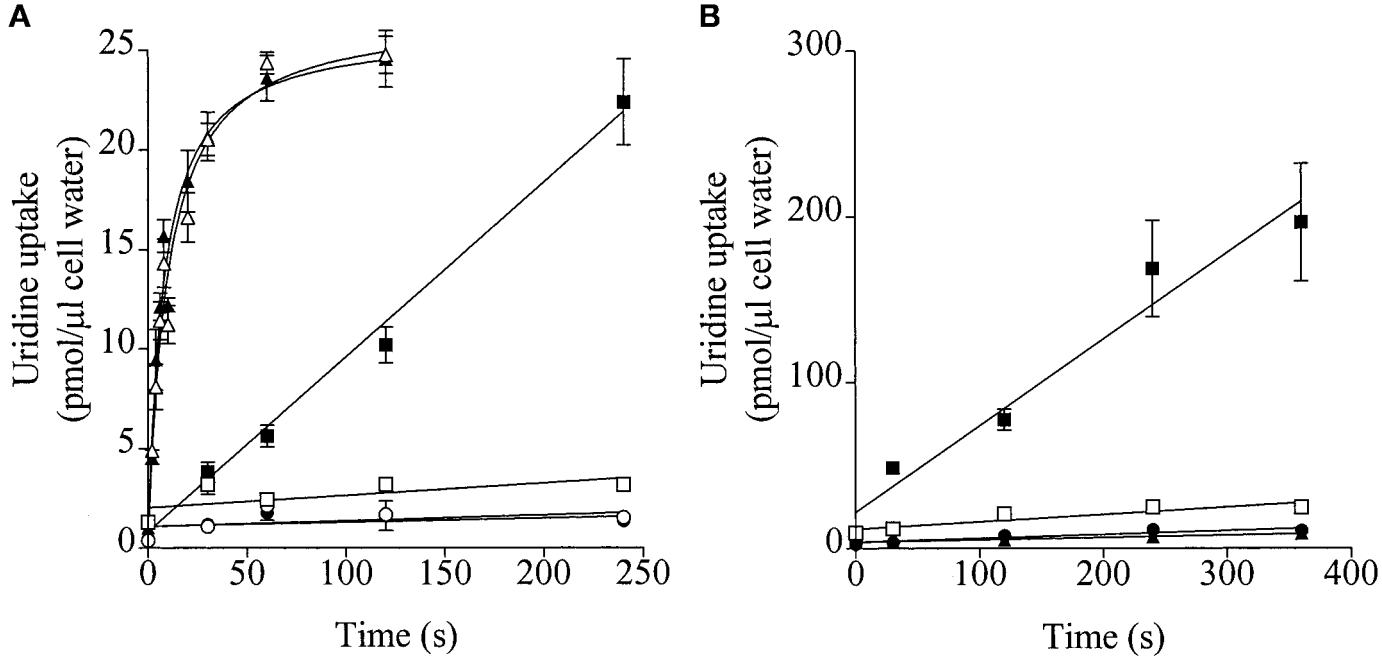

Fig. 3. Demonstration of Na^+-dependent, carrier-mediated uptake by ARAC/D2 cells. Panel A, measurement of uridine uptake in the different cell lines. Uptake of 10 μM [^{3}H]uridine was measured at the time intervals shown in the presence (closed symbols) or absence (open symbols) of Na^+ in ARAC/D2 (squares), untransfected CEM-ARAC (circles), or CEM (triangles) cells. Panel B, inhibition of [^{3}H]uridine uptake by excess nonradioactive nucleosides. Uptake of 35 μM [^{3}H]uridine was determined in Na^+-containing (closed symbols) or Na^+-free (open symbols) transport buffer in the absence (squares) or presence of either 500 μM (circles) or 1 mM (triangles) nonradioactive uridine. Each value represents the mean $\pm$ S.D. of three determinations, and error bars are not shown where S.D. values were smaller than that represented by the symbols.

several modified uridine analogs, a series of pyrimidine nucleosides and nucleobases were also examined for transportability in the experiments of Fig. 5. The Na^+-dependent component of the transport rate for 5-fluoruridine (0.132 ± 0.003 pmol/μl cell water/s) was rapid and higher than that observed for uridine (0.097 ± 0.009 pmol/μl cell water/s). Although uracil and thymidine partially inhibited hCNT2-mediated [^{3}H]uridine transport (Table 1), direct measurement of [^{3}H]uracil and [^{3}H]thymidine influx into ARAC/D2 cells (Fig. 5) indicated that these inhibitors were not permeants of hCNT2. The relatively high rates of uptake observed for [^{3}H]uracil in the presence and absence of extracellular sodium gradient may have been due to uptake by a nucleobase transporter.

TABLE 1

Inhibition of uridine uptake in ARAC/D2 cells by ribonucleosides, arabinonucleosides, and nucleobases: identification of candidate permeants and inhibitors of hCNT2

Rates of transport of 10 μM [^{3}H]uridine obtained from 3 to 4-min time courses similar to those shown in Fig. 3 were conducted in Na^+-containing and Na^+-free transport buffer in the absence and presence of 1 mM test compounds listed. Uridine transport values in the absence of additives (controls) ranged from 0.1 ± 0.005 pmol/μl cell water/s to 0.12 ± 0.013 pmol/μl cell water/s. Net transport rates are the Na^+-dependent component expressed as percentage of control values and are means ± S.D. of triplicate determinations.

Additive (Test Compound)	Net [^{3}H]Uridine Transport
	% control
Pyrimidine nucleosides, nucleobases, ribose and dilazep	
None	100
Uridine	2 ± 0.8
5-Fluorouridine	1 ± 0.6
5-Bromouridine	7 ± 5
5-Iodouridine	9 ± 7
5-Methyluridine	38 ± 3
2′-Deoxyuridine	3 ± 1.4
3′-Deoxyuridine	99 ± 7
2′,3′-Dideoxyuridine	101 ± 5
5-Fluoro-2′-deoxyuridine	18 ± 2
5-Bromo-2′-deoxyuridine	56 ± 3
5-Iodo-2′-deoxyuridine	69 ± 5
5-Fluoro-5′-deoxyuridine	80 ± 5
Thymidine	74 ± 3
Cytidine	94 ± 7
2′-Deoxycytidine	96 ± 6
Uracil	68 ± 3
5-Fluorouracil	82 ± 3
Thymine	85 ± 2
Cytosine	99 ± 5
Ribose	97 ± 4
Dilazep	88 ± 3
Purine nucleosides and nucleobases	
Inosine	4 ± 2.8
2′-Deoxyinosine	5 ± 4
Guanosine	1 ± 0.8
2′-Deoxyguanosine	3 ± 2
Adenosine	2 ± 1.7
2′-Deoxyadenosine	2 ± 1.3
2′,3′-Dideoxyadenosine	96 ± 3
2-Chloroadenosine	15 ± 3
2-Chloro-2′-deoxyadenosine	72 ± 4
7-Deazaadenosine	100 ± 2
Adenine	98 ± 3
Hypoxanthine	96 ± 5
Guanine	101 ± 2
Arabinonucleosides and arabinose	
1-β-D-Arabinofuranosyluracil	70 ± 4
1-β-D-Arabinofuranosylcytosine	102 ± 6
9-β-D-Arabinofuranosyladenine	31 ± 2
2-Fluoro-9-β-D-arabinofuranosyladenine	84 ± 3
9-β-D-Arabinofuranosylhypoxanthine	20 ± 2
Arabinose	99 ± 3

Transport of 5-Fluorouridine by hCNT2. The kinetics of transport of 5-fluorouridine by hCNT2- and hENT1-containing cells were compared with those of the naturally occurring nucleoside uridine (Table 3). Initial rates of uptake by hCNT2-containing ARAC/D2 cells were determined as a function of concentration and the relationship so obtained conformed to Michaelis-Menten kinetics, with apparent K_m and V_{max} values (mean ± S.D.) of 43 ± 7 μM and 0.38 ± 0.07 pmol/μl cell water/s, respectively. The apparent K_m value for 5-fluorouridine was comparable to the K_i value determined from the inhibition experiments of Table 2. The K_m and V_{max} values for 5-fluorouridine were almost identical to those obtained for uridine, indicating that hCNT2 indiscriminately transports either substance with equal efficiency. Higher apparent K_m values were observed for the transport mecha-

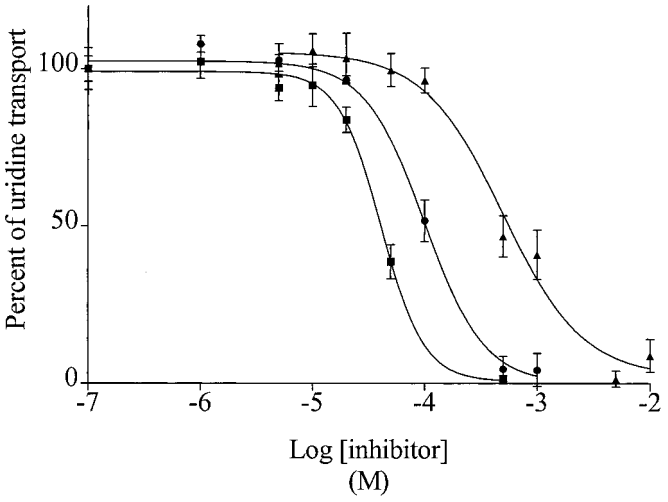

Fig. 4. Dose-response inhibition of Na^+-dependent uridine transport in ARAC/D2 cells by fluorinated uridine analogs. [^{3}H]Uridine uptake was measured alone or with graded concentrations of either 5-fluorouridine (■) (0.1–500 μM), 5-fluoro-2′-deoxyuridine (●) (0.1–1000 μM), or 5-fluoro-5′-deoxyuridine (▲) (5 μM–10 mM). Na^+-dependent transport rates were calculated from the uptake measurements performed in both Na^+-containing and Na^+-free transport buffer. Results are presented as the fraction of uridine transport remaining as a function of the logarithm of the concentration of inhibitor and are the means ±S.D. of data obtained from triplicate determinations in two experiments. The IC_{50} values for 5-fluorouridine, 5-fluoro-2′-deoxyuridine, and 5-fluoro-5′-deoxyuridine inhibition of uridine transport were 41, 100, and 500 μM, respectively.

TABLE 2

Inhibitor constants estimated from influx competition experiments

IC_{50} values from dose-response relationships similar to those shown in Fig. 4 were used to calculate the K_i values for the different nucleosides listed according to the equation of Cheng and Prusoff (1973). Kinetic constants obtained in the absence of inhibitor for zero-*trans* influx of uridine were K_m = 46 ± 4 μM and V_{max} = 0.42 ± 0.05 pmol/μl cell water/s (values are means ± S.D. from seven experiments).

Nucleoside	K_i
	μM
5-Fluorouridine	34
5-Iodouridine	50
5-Bromouridine	46
5-Methyluridine	197
Thymidine	>500
2′-Deoxyuridine	39
1-β-D-arabinofuranosyluracil	>500
5-Fluoro-2′-deoxyuridine	82
5-Bromo-2′-deoxyuridine	219
5-Iodo-2′-deoxyuridine	246
5-Fluoro-5′-deoxyuridine	411

nism mediated by hENT1, with the 5-fluorouridine values being slightly higher than those for uridine.

Enhanced Sensitivity to Purine and Pyrimidine Nucleoside Drugs in hCNT2-Producing Cells. To assess relationships between efficiency of transport and cellular toxicity, several cytotoxic nucleoside drugs were compared for their ability to inhibit cell proliferation in cancer cell lines that possessed either hCNT2, hENT1, or no nucleoside transporters (Table 4). Doxorubicin was included to demonstrate that the resistance of CEM-ARAC cells was not associated with membrane transport-associated multidrug-resistant (MDR) proteins (van den Heuvel-Eibrink et al., 2000), since all the cell lines tested were equally sensitive. CEM cells were sensitive, whereas CEM-ARAC cells were highly resistant to all of the nucleoside drugs tested, indicating a requirement for mediated permeation across the plasma membrane to achieve cytotoxicity. Cladribine and fludarabine were less effective against ARAC/D2 cells than CEM cells, which was consistent with the apparent low-affinity interaction with hCNT2 and the very low transport rates observed in isotopic flux analyses. Their relatively high cytotoxicities against CEM cells were probably influenced by the greater capacity for uptake via hENT1.

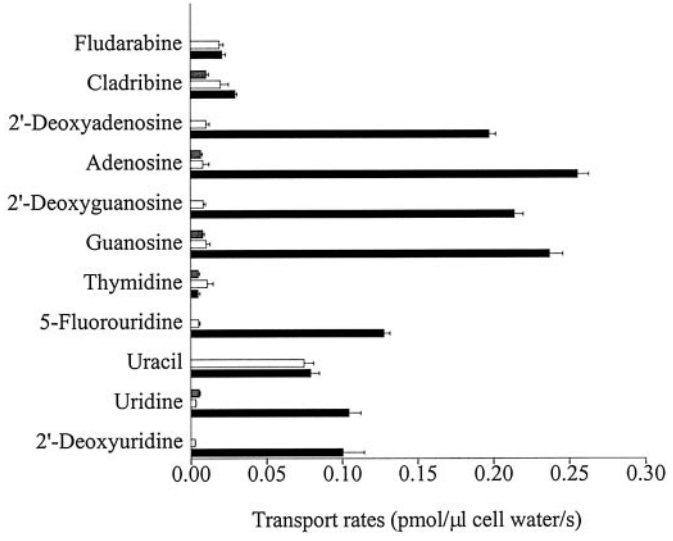

Fig. 5. Transport of purine and pyrimidine nucleosides into ARAC/D2 cells. Uptake of 10 μM ^{3}H-labeled nucleoside into ARAC/D2 cells was performed as described under *Experimental Procedures*, and initial rates were determined from the linear portions of time courses of isotopic fluxes in Na^+-containing (filled bars) and Na^+-free (open bars) transport buffer. Uptake was also determined in CEM-ARAC cells for some test compounds (shaded bars) in Na^+-containing transport buffer. Each bar represents the mean ± S.D. of three determinations, and error bars are not shown where S.D. values were smaller than those represented by the symbols.

ARAC/D2 cells exhibited greater sensitivity to cladribine than fludarabine, although the differences in their transport rates were small. This may have been due to differences in events downstream from transport, which would likely influence the accumulation of triphosphate metabolites in ARAC/D2 cells. It has been shown that cladribine is a better substrate for deoxycytidine kinase than fludarabine (Arner and Eriksson, 1995; Johansson and Karlsson, 1995). The unique ability of cladribine compared with fludarabine to affect mitochondrial function upon cell entry may also be a factor in the greater cytotoxic effects observed against ARAC/D2 cells (Genini et al., 2000).

Tubercidin (7-deazaadenosine), a known permeant of *es* processes (Cass et al., 1992), exhibited markedly different cytotoxicities against hENT1-containing CEM and hCNT2-containing ARAC/D2 cells, with IC_{50} values of 0.08 and 16.7 μM, respectively, a 200-fold difference. A qualitatively similar result was obtained with cytarabine, which is a known *es* permeant (Wiley et al., 1982); IC_{50} values of 0.10 and > 100 μM, respectively, were obtained with CEM and ARAC/D2 cells. The relative insensitivities of hCNT2-containing cells to tubercidin and cytarabine were consistent with the conclusion that these drugs are not permeants of hCNT2. Cytarabine and tubercidin are also poor permeants of the human and rat pyrimidine-nucleoside selective (CNT1) transporters (Crawford et al., 1998a; Graham et al., 2000).

5-Fluorouridine, which was shown in the present study to be a high-affinity hCNT2 permeant, was considerably more toxic to CEM and ARAC/D2 cells (IC_{50} values of 0.06 and 0.05 μM, respectively) than to CEM-ARAC cells ($IC_{50} > 50$ μM). For ARAC/D2 cells, the acquired sensitivity to 5-fluorouridine was associated with, and most likely due to, the acquisition of hCNT2 activity, whereas for CEM cells, the intrinsic sensitivity to 5-fluorouridine was attributed to hENT1-mediated uptake. 5-Fluorouridine was equally cytotoxic to ARAC/D2 and CEM cells, although the V_{max}/K_m ratio was greater for CEM cells as shown in Table 3. The similar chemosensitivities, despite the different kinetic characteristics, were attributed to differences in the intrinsic properties of the transporters in the two cell lines. ARAC/D2 cells possess hCNT2, which is unidirectional and capable of concentrating nucleoside drugs inside cells, whereas CEM cells possess hENT1, which is bidirectional and transports nucleoside drugs in either direction, depending on the concentration gradient. Thus, hCNT2-containing ARAC/D2 cells could not lose drug by outward transport whereas hENT1-containing CEM cells could, if intracellular trapping of drug via

TABLE 3

Michaelis-Menten constants for zero-*trans* influx of nucleosides in ARAC/D2 and CEM cells

Initial rates of uptake were determined for each of the graded concentrations of [^{3}H]uridine (ARAC/D2, 0–100 μM; CEM, 0–400 μM) or [^{3}H]5-fluorouridine (ARAC/D2, 0–100 μM; CEM, 0–400 μM). The K_m and V_{max} values are the means ± S.D. of individual values determined from Woolf, Eadie-Hofstee, and Lineweaver-Burke plots for each of the three independent experiments. Uridine and 5-fluorouridine were not transported by CEM-ARAC cells (data not shown).

Nucleoside	ARAC/D2			CEM		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
	μM	*pmol/μl cell water/s*			*pmol/μl cell water/s*	
Uridine	46 ± 4	0.42 ± 0.05	0.009	192 ± 10	27 ± 11	0.14
5-Fluorouridine	43 ± 7	0.38 ± 0.07	0.009	217 ± 16	22 ± 9	0.10

[a] Expressed as μM.

phosphorylation was slower than inward transport. Studies with murine leukemia L1210 cells (which possess two equilibrative and one concentrative nucleoside transporter) demonstrated that treatment of cells with inhibitors of equilibrative transport reduced initial rates of nucleoside drug uptake but enhanced net accumulation of nucleoside drug and therefore toxicity by blocking drug efflux (Dagnino and Paterson 1990).

ARAC/D2 cells were sensitive to 5-fluoro-2′-deoxyuridine, albeit moderate compared with 5-fluorouridine, whereas CEM-ARAC cells were resistant. These results suggested that 5-fluoro-2′-deoxyuridine utilized hCNT2 to cross the cell membrane, since transport-deficient cells were far less sensitive to this analog than hCNT2-containing cells. In contrast, 5-fluoro-5′-deoxyuridine was equally ineffective against ARAC/D2 and CEM-ARAC cells as compared with CEM cells, indicating that its entry was not mediated by hCNT2.

Conclusion. For many cell types, the presence of heterogeneous nucleoside transporters and complex overlapping substrate selectivities poses difficulty in the interpretation of flux measurements and determination of mechanism of drug sensitivity. The studies presented here provide the first demonstration of the transport properties of hCNT2 in structure-activity and structure-cytotoxicity relationships in a human cancer cell line that possessed only hCNT2-mediated transport. The results demonstrated that the resistance of CEM-ARAC cells to purine and pyrimidine nucleoside drugs was due to the absence of expression of the hENT1 gene leading to a loss of nucleoside transport activity. Introduction of hCNT2 by gene transfer conferred the capacity for nucleoside transport and drug sensitivity to the resistant cells. The 3′- and 5′-hydroxyl groups of the ribosyl moiety were important for interaction of uridine analogs with hCNT2, whereas removal of the hydroxyl at position 2′ did not significantly affect interaction with hCNT2. The transporter exhibited a preference for the 2′-hydroxyl group in the α- rather than β-configuration and a tolerance for modifications of the 5 but not the 3 position of the base with halogen substituents. The transport activity of some adenosine analogs such as cladribine, which is a known substrate for CNT2 from rat or mouse species (Crawford et al., 1990a; Schaner et al., 1997), and fludarabine were low compared with several halogenated uridine analogs. 5-Fluorouridine, 5-fluoro-2′-deoxyuridine, and 5-fluoro-5′-deoxyuridine exhibited progressively decreasing affinities for hCNT2, and this was reflected in the degree of their cytotoxicity against hCNT2-containing cells. The high transportability of 5-fluorouridine and 5-fluoro-2′-deoxyuridine by hCNT2 suggests a role for this transporter for fluoropyrimidine nucleoside chemotherapy and radiopharmaceutical imaging.

TABLE 4

Inhibition of cell proliferation in CEM, CEM-ARAC, and ARAC/D2 cells by exposures to various purine and pyrimidine nucleoside drugs

Cultures (1×10^5 cells/ml) of ARAC/D2, CEM, and CEM-ARAC cells were incubated for 24 h (in triplicates), after which they were exposed continuously to graded concentrations of various cytotoxic nucleosides for 48 h. Cell concentrations were quantitated at 24 and 48 h by electronic particle counting. Chemosensitivity was expressed as the concentration of drug (in micromolar values) that inhibited cell proliferation by 50% (IC_{50}), as determined by linear regression analysis of growth rates and plotted as a function of the drug concentrations. Resistance factors (in brackets) were normalized against IC_{50} values of CEM cells. The values reported are the means of duplicate experiments.

Nucleoside Drug	IC_{50} for Inhibition of Cell Proliferation		
	CEM (hENT1)	CEM-ARAC (NT-deficient)	ARAC/D2 (hCNT2)
Cladribine	0.05 (1)	1.96 (39)	0.19 (3.8)
Fludarabine	1.5 (1)	40 (26.7)	30 (20)
Tubercidin	0.08 (1)	17.9 (224)	16.7 (209)
Cytarabine	0.10 (1)	>100 (>1000)	>100 (>1000)
5-Fluorouridine	0.06 (1)	>50 (>833)	0.05 (0.8)
5-Fluoro-2′-deoxyuridine	0.11 (1)	22.7 (206)	0.25 (2.3)
5-Fluoro-5′-deoxyuridine	0.98 (1)	>50 (>51)	47 (48)
Doxorubicin	0.04 (1)	0.09 (2.3)	0.12 (3)

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