

The Role of Human Nucleoside Transporters in Cellular Uptake of 4'-Thio- β -D-arabinofuranosylcytosine and β -D-Arabinosylcytosine

Marilyn L. Clarke,¹ Vijaya L. Damaraju, Jing Zhang, Delores Mowles, Tracey Tackaberry, Thach Lang, Kyla M. Smith, James D. Young, Blake Tomkinson, and Carol E. Cass

Departments of Oncology (M.L.C., V.L.D., J.Z., D.M., T.T., T.L., C.E.C.) and Physiology (K.M.S., J.D.Y.) and the Membrane Protein Research Group, University of Alberta and the Cross Cancer Institute, Edmonton, Alberta, Canada (J.D.Y., C.E.C.); and Sanofi-Aventis, Parkway Malvern, Pennsylvania (B.T.)

Received December 6, 2005; accepted April 14, 2006

ABSTRACT

4'-Thio- β -D-arabinofuranosyl cytosine (TaraC) is in phase I development for treatment of cancer. In human equilibrative nucleoside transporter (hENT) 1-containing CEM cells, initial rates of uptake (10 μ M; picomoles per microliter of cell water per second) of [³H]TaraC and [³H]1- β -D-arabinofuranosyl cytosine (araC) were low (0.007 $\pm$ 0.003 and 0.034 $\pm$ 0.003, respectively) compared with that of [³H]uridine (0.317 $\pm$ 0.048), a high-activity hENT1 permeant. In hENT1- and hENT2-containing HeLa cells, initial rates of uptake (10 μ M; picomoles per cell per second) of [³H]TaraC, [³H]araC, and [³H]deoxycytidine were low (0.30 $\pm$ 0.003, 0.42 $\pm$ 0.03, and 0.51 $\pm$ 0.11, respectively) and mediated primarily by hENT1 (~74, ~65, and ~61%, respectively). In HeLa cells with recombinant human concentrative nucleoside transporter (hCNT) 1 or hCNT3 and pharmacologically blocked hENT1 and hENT2, transport of 10 μ M [³H]TaraC and [³H]araC was not detected. The apparent affinities of re-

combinant transporters (produced in yeast) for a panel of cytosine-containing nucleosides yielded results that were consistent with the observed low-permeant activities of TaraC and araC for hENT1/2 and negligible permeant activities for hCNT1/2/3. During prolonged drug exposures of CEM cells with hENT1 activity, araC was more cytotoxic than TaraC, whereas coexposures with nitrobenzylthioinosine (to pharmacologically block hENT1) yielded identical cytotoxicities for araC and TaraC. The introduction by gene transfer of hENT2 and hCNT1 activities, respectively, into nucleoside transport-defective CEM cells increased sensitivity to both drugs moderately and slightly. These results demonstrated that nucleoside transport capacity (primarily via hENT1, to a lesser extent by hENT2 and possibly by hCNT1) is a determinant of pharmacological activity of both drugs.

The synthesis of 4'-thio- β -D-arabinofuranosyl cytosine (TaraC) was reported more than 30 years ago (Whistler et al., 1971). In recent studies (Waud et al., 2003), TaraC and araC were evaluated for activity against a panel of human tumor cell lines. TaraC differs from araC in the replacement of the oxygen atom by a sulfur atom in the arabinose ring. Although

araC was more cytotoxic than TaraC in cell culture studies, TaraC showed superior antitumor activity in human tumor systems in mice compared with that of araC (Waud et al., 2003). AraC, which has shown promising activity in human leukemia xenograft models, was poorly active against human solid tumor xenografts (Gourdeau et al., 2001a).

Although previous reports indicated that araC is a better substrate than TaraC for deoxycytidine kinase (Parker et al., 2000; Someya et al., 2002), recent studies (Someya et al., 2003) suggest that the difference in phosphorylation rates at pharmacologically relevant concentrations is only 2- to 3-fold. The prolonged retention time of TaraC-5'-monophosphate resulting from its high rate of phosphorylation by UMP/CMP kinase has been suggested as a more important determinant for its activity against solid tumors than its resistance to cytidine and dCMP deaminase activities (Parker et al., 2000; Someya et al., 2005). Preliminary studies indicated that the

This work was supported by OSI Pharmaceuticals, a Canadian Cancer Society Grant to C.E.C. from the National Cancer Research Institute of Canada, and the Alberta Cancer Foundation of the Alberta Cancer Board. C.E.C. is Canada Research Chair in Oncology. J.D.Y. is a Heritage Scientist of the Alberta Heritage Foundation for Medical Research. J.Z. held studentships from the Canadian Institutes of Health Research and the Alberta Heritage Foundation for Medical Research. Portions of this work were presented in a preliminary report (Clarke et al., 2003).

M.L.C. and V.L.D. are co-first authors.

¹ Current affiliation: GTI Inc., Waukesha, WI.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.105.021543.

ABBREVIATIONS: TaraC, 4'-thio- β -D-arabinofuranosylcytosine; araC, 1- β -D-arabinofuranosylcytosine; NBMPR, nitrobenzylmercaptopurine riboside; hENT, human equilibrative nucleoside transporter; hCNT, human concentrative nucleoside transporter.

mechanism of TaraC cytotoxicity differs from that of either araC or gemcitabine, a difluoro-2'-deoxycytidine analog with activity against solid tumors (Blajeski et al., 2002). TaraC was a better substrate for nuclear DNA polymerase α and δ than either araC or gemcitabine, and its incorporation into DNA resulted in strong chain termination in comparison with that of araC and gemcitabine, which resulted in minimal termination (Richardson et al., 2004). In recent studies in which mice bearing human lung cancer xenografts (Calu-6, A549) were treated with graded doses of TaraC, TaraC was incorporated into internal nucleotide linkages in a manner that was dose- and time-dependent, leading to the conclusion that DNA synthesis may play a role in TaraC cytotoxicity (Richardson et al., 2005).

An area that has been neglected to date is the characterization of the uptake parameters of TaraC by plasma membrane nucleoside transporters. It is not known whether mediated uptake is required for TaraC cytotoxicity, as reported for araC (Ullman, 1989; Gati et al., 1997) and gemcitabine (Mackey et al., 1998a,b), or whether TaraC enters cells primarily by passive diffusion as reported for troxacitabine, a deoxycytidine derivative with an unusual dioxolane structure and non-natural L-configuration with activity against leukemic and solid tumor xenograft models (Gourdeau et al., 2001b).

In humans, seven functionally distinct nucleoside transport processes have been characterized in molecular terms through isolation and functional expression of cDNAs encoding the transporter proteins in *Xenopus laevis* oocytes, mammalian cells, or yeast (Griffiths et al., 1997a,b; Ritzel et al., 1997, 1998, 2001; Baldwin et al., 2005). The human (h) nucleoside transporters exhibit different permeant selectivities, and only hENT1 is inhibited by nanomolar concentrations of nitrobenzylmercaptapurine riboside (NBMPR). The proteins (and their permeant selectivities) are as follows: hENT1 (accepts purine and pyrimidine nucleosides), hENT2 (accepts purine and pyrimidine nucleosides and nucleobases), hENT3 (accepts purine and pyrimidine nucleosides), hENT4 (accepts adenosine), hCNT1 (accepts pyrimidine nucleosides and adenosine), hCNT2 (accepts purine nucleosides and uridine), and hCNT3 (accepts purine and pyrimidine nucleosides). Six of the seven transporters (hENT1/2/4, hCNT1/2/3) are found in plasma membranes, whereas hENT3 is found in lysosomal membranes.

This study was undertaken to characterize the transportability of TaraC by the human nucleoside transporters that are responsible for cellular uptake of exogenous nucleosides and to assess the importance of nucleoside transport in TaraC cytotoxicity by using cultured human cell lines with known nucleoside transporter phenotypes. The study was designed to compare TaraC and araC directly because of the structural similarities of the two compounds and the established role of araC in cancer chemotherapy. Transportability studies were conducted with well established model systems producing either native (cultured human cell lines) or recombinant human nucleoside transporters (yeast and *Xenopus laevis* oocyte expression systems) that enabled functional isolation of the transporter under investigation. Cytotoxicity studies were conducted in CEM cells with native hENT1 in the presence and absence of transport inhibitors or in a nucleoside transport-defective mutant line (CEM-ARAC) with either recombinant hENT2 or hCNT1 introduced by

stable transfection. The goal was to determine whether nucleoside transporter-mediated accumulation of TaraC contributes to its unique pharmacological profile, which includes prolonged intracellular retention and schedule independence.

Materials and Methods

Materials. TaraC was provided by OSI Pharmaceuticals, Inc. (Boulder, CO). [^3H]AraC (20.3 Ci/mmol), [^3H]TaraC (8.2 Ci/mmol), [^3H] (N)]2'-deoxycytidine (27 Ci/mmol), and [^3H]uridine (40 Ci/mmol) were purchased from Moravsek Biochemicals (Brea, CA). Research grade uridine, cytidine, 2'-deoxycytidine, 3'-deoxycytidine, 2',3'-dideoxycytidine, gemcitabine, and araC were from Sigma-Aldrich (St. Louis, MO). All other reagents used were analytical grade and from commercial sources.

Cell Culture. The human CCRF-CEM leukemia, hereafter termed CEM, and HeLa cervical carcinoma cell lines were obtained, respectively, from William T. Beck (formerly at St. Jude Children's Research Hospital, now at University of Illinois at Chicago, Chicago, IL) and the American Type Culture Collection (Manassas, VA). CEM/ARAC8C, a nucleoside transport-deficient derivative of CEM (Ullman et al., 1988), referred to as CEM-ARAC, was a gift from Dr. B. Ullman (Oregon Health and Science University, Portland, OR).

CEM-ARAC/hCNT1 was derived from CEM-ARAC by stable transfection with a pcDNA3 plasmid that contained the coding sequence for hCNT1 (Lang et al., 2004). hENT2 stable transfectants (CEM-ARAC/hENT2) were produced using procedures described previously (Lang et al., 2001, 2004). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada) as adherent (HeLa) or suspension cultures (CEM, CEM-ARAC, CEM-ARAC/hCNT1, and CEM-ARAC/hENT2). Cells were free of mycoplasma, maintained in the absence of antibiotics, incubated at 37°C in a humidified atmosphere (5% CO_2), and subcultured at 2- to 4-day intervals to maintain active proliferation. Cell numbers were determined with a Coulter Z2 electronic particle counter equipped with a size analyzer (Beckman Coulter Canada, Mississauga, ON, Canada).

Transient Expression of hCNT1 and hCNT3 in HeLa Cells. The cDNAs encoding the hCNT1 and hCNT3 proteins were subcloned into the mammalian expression vector, pcDNA3, to produce pcDNA3-hCNT1 (Graham et al., 2000) and pcDNA3-hCNT3 (this work). The plasmids were separately transfected into actively proliferating HeLa cells as described previously (Mackey et al., 1998b; Graham et al., 2000).

Nucleoside Uptake Assays in Cultured Cells. Nucleoside uptake assays were conducted at room temperature in transport buffer [20 mM Tris-HCl, 3 mM K_2HPO_4 , 1 mM $\text{MgCl}_2 \cdot (6\text{H}_2\text{O})$, 2 mM CaCl_2 , 5 mM glucose, and 130 mM NaCl, pH 7.4; 300 ± 15 mOsm] in CEM or HeLa cells, respectively, as described for suspension cultures (Boleti et al., 1997) or adherent cultures (Graham et al., 2000). Cells were lysed with 5% Triton X-100 and mixed with Ecolite scintillation fluid to measure cell-associated radioactivity (Beckman LS 6500 scintillation counter; Beckman Coulter Canada).

Transport Inhibition Assays in Yeast with Recombinant Transporters. *Saccharomyces cerevisiae* that were separately transformed with plasmids (pYPhENT1, pYPhENT2, pYPhCNT1, pYPhCNT2, pYPhCNT3) with cDNAs encoding, respectively, the hENT1, hENT2, hCNT1, hCNT2, or hCNT3 as described previously (Vickers et al., 2002; Visser et al., 2002; Zhang et al., 2003, 2005), were used to examine the ability of various cytosine-containing analogs to inhibit the uptake of 1 μM [^3H]uridine. Transport experiments were conducted in 96-well plates with a semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway) as described previously (Zhang et al., 2003). Yeast were incubated at room temperature with graded concentrations of test compounds in the presence of 1 μM [^3H]uridine for 20 min. Each

experiment was repeated at least three times. Data were subjected to nonlinear regression analysis using Prism software (version 3.0; GraphPad Software Inc., San Diego, CA) to obtain IC_{50} values that were used to calculate K_i values (Cheng and Prusoff, 1973).

Electrophysiological Studies in Frog Oocytes with Recombinant hCNT1. hCNT1 cDNA contained in the plasmid pGEM-HE (Ritzel et al., 1997) was linearized with NheI and transcribed with T3 or T7 polymerase using the mMESSAGE mMACHINE (Ambion, Austin, TX) transcription system. In vitro synthesized transcripts were injected into isolated mature stage VI oocytes from *X. laevis*, and oocyte membrane currents were measured using a GeneClamp 500B oocyte clamp (Molecular Devices, Sunnyvale, CA) in the two-electrode, voltage-clamp mode as described previously (Smith et al., 2004, 2005). Mock-injected oocytes were injected with water alone. All experiments were performed at room temperature (20°C) and oocytes were discarded if membrane potentials were unstable or more positive than -30 mV. The membrane potential was clamped at a holding potential of -50 mV and either 100 μ M uridine or 1 mM TaraC, araC, or 2'-deoxycytidine was added. The transport medium contained 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.5. Current values are presented as means $\pm$ S.E. of three or more oocytes.

Chemosensitivity Testing with Cultured CEM Cells. The relative cytotoxicities of TaraC and araC against CEM, CEM-ARAC, CEM-ARAC/hENT2, and CEM-ARAC/hCNT1 were assessed using the CellTiter 96 proliferation assay (Promega, Madison, WI) as described previously (Damaraju et al., 2005). This assay is based on the reduction of a tetrazolium compound to a soluble formazan derivative by the dehydrogenase enzymes of metabolically active cells. The absorbance (490 nm) is directly proportional to the number of living cells in culture. Cells were seeded in 96-well tissue culture plates (10^4 cells/well, six replicates/condition) and exposed to graded concentrations (0– 10^6 nM) of TaraC or araC for 24 or 48 h. Chemosensitivity was expressed as the effective drug concentration at which cell proliferation was inhibited by 50% (EC_{50} values) and was determined from concentration-effect relationships using GraphPad Prism.

Results

hENT1: Uptake Studies in CEM and CEM-ARAC Cells. The uptake of TaraC and araC was first examined in cell lines that were either nucleoside transport-competent by virtue of the presence of functional hENT1 (CEM) or were nucleoside transport-negative by virtue of genetic loss of functional hENT1 (CEM-ARAC). Uridine was included initially as a control for comparisons between experiments, because it exhibits high transportability by hENT1. Initial rates of uptake of [3 H]TaraC and [3 H]araC were measured and compared with that of [3 H]uridine at the pharmacologically relevant concentration of 10 μ M (Table 1). For CEM cells, uptake of [3 H]TaraC during the first 10 s was undetect-

TABLE 1

Initial uptake rates of 10 μ M [3 H]uridine, [3 H]TaraC, or [3 H]araC by CEM and CEM-ARAC cells

Uptake rates are expressed as picomoles per microliter of cell water per second $\pm$ S.E.M. and were calculated from the slopes of linear regression lines using Prism 3.02 (GraphPad Software Inc.). Each data point represents the mean of three experiments with three replicates per experiment.

Cell Line	Time Period	[3 H]Uridine	[3 H]TaraC	[3 H]AraC
	s			
CEM	0–10	0.317 $\pm$ 0.048	0	0.048 $\pm$ 0.020
CEM	0–60	N.A.	0.007 $\pm$ 0.003	0.034 $\pm$ 0.003
CEM-ARAC	0–60	0.007 $\pm$ 0.003	0.012 $\pm$ 0.004	0.013 $\pm$ 0.004

N.A., not applicable (uptake time course was not linear during 0- to 60-s uptake interval).

able, whereas uptake of [3 H]araC, although low, exhibited an initial rate of 0.048 $\pm$ 0.020 pmol/ μ L cell water/s, which was 6.6% of the rate observed for uptake of [3 H]uridine. Even after 60 s, uptake of [3 H]TaraC was barely detectable, with an initial rate value of only 0.007 $\pm$ 0.002 pmol/ μ L cell water/s. For CEM-ARAC cells, which lack nucleoside transport capacity because of a genetic loss of hENT1 activity (Ullman et al., 1988), the uptake rates for TaraC, araC, and uridine were also barely detectable (Table 1). These data suggested that TaraC might not be transported by hENT1. Higher concentrations of TaraC and longer uptake intervals (in the absence or presence of nucleoside transport inhibitors) were subsequently investigated, and because TaraC and araC are analogs of 2'-deoxycytidine, the latter compound was used instead of uridine in subsequent experiments as the control permeant.

Because NBMPR inhibits hENT1 at low concentrations and does not have other known metabolic targets, sensitivity of [3 H]nucleoside uptake to NBMPR inhibition is a well established diagnostic test for transportability via hENT1. Uptake of 10, 30, or 50 μ M [3 H]TaraC, [3 H]araC, and [3 H]2'-deoxycytidine after 1- and 4-h intervals was examined in the absence or presence of 100 nM NBMPR. Despite the low initial uptake rates for TaraC that had suggested that it may not be a permeant for hENT1 (Table 1), NBMPR inhibited accumulation of TaraC and its metabolites over both 1- and 4-h uptake intervals; the data are shown for 4 h in Fig. 1A. hENT1 accounted for >70, >52, and >55% of accumulated TaraC at 10, 30, and 50 μ M, respectively, at 4 h, and accumulation was dose-dependent. Although uptake of araC was dose-dependent at 1 h (data not shown), uptake at 4 h was independent of concentration (Fig. 1B), and the percentage of inhibition by NBMPR decreased with increasing araC concentrations, apparently because hENT1-mediated transport achieved intracellular concentrations of araC that exceeded the capacity for phosphorylation. Deoxycytidine accumulation was inhibited by NBMPR and, like that of TaraC, was dose-dependent (Fig. 1C). In a separate set of experiments designed to assess diffusional uptake (data not shown), time courses of uptake of 10 μ M [3 H]TaraC and [3 H]araC were determined in the presence or absence of 10 mM uridine over 5 min in pharmacologically blocked (in presence of 10 μ M NBMPR) CEM cells and in mutationally blocked CEM-ARAC cells. Uptake rates, which were negligible, were similar for all conditions, suggesting little, if any, diffusional uptake under the conditions used.

hENT1/2, hCNT1/3: Uptake Studies in Wild-Type and Transiently Transfected HeLa Cells. The transportability of TaraC and araC was also examined directly in HeLa cells, which naturally possess both hENT1 and hENT2. 2'-Deoxycytidine was included as a control to test for transporter functionality. The initial uptake rates measured over 2 to 10 s of 10 μ M [3 H]TaraC, [3 H]araC, and [3 H]2'-deoxycytidine were 0.30 $\pm$ 0.00, 0.42 $\pm$ 0.03, and 0.51 $\pm$ 0.11 pmol/ 10^6 cells/s, respectively (Table 2). The effects of dilazep, which inhibits both hENT1 and hENT2, and NBMPR, which inhibits only hENT1, implied that 1) the rates represented primarily transporter-mediated uptake because dilazep inhibition was 98 to 100%, and 2) hENT1 was the predominant transporter responsible for inward mediated fluxes of all three compounds because NBMPR inhibition was 61 to 74%. In the experiments of Table 2, the initial uptake rates ob-

served in the presence of dilazep, which presumably represented diffusional uptake, were negligible. At the longer time interval of 60 s (Fig. 2), time courses of uptake in the absence and presence of NBMPR or dilazep showed that both hENT1 and hENT2 contributed to accumulation of all three compounds.

hCNT1 and hCNT3 have previously been shown to accept cytidine and 2'-deoxycytidine as permeants in studies in which the recombinant proteins were produced in *X. laevis* oocytes or transiently transfected HeLa cells (Ritzel et al., 1997; Graham et al., 2000; Ritzel et al., 2001), and hCNT1 was recently shown to weakly transport araC in electrophysiological studies in the *X. laevis* oocytes (Smith et al., 2004). The transportability of [³H]TaraC and [³H]araC by hCNT1

and hCNT3 was first assessed in experiments that used recombinant hCNT1 or hCNT3 introduced into HeLa cells by transient transfection. Experiments were conducted in the presence of 100 μM dilazep to inhibit both hENT1 and hENT2 and functionality was shown by 1) demonstrating uptake of 10 μM [³H]2'-deoxycytidine and 2) its inhibition by 1 mM nonradioactive 2'-deoxycytidine. Sodium-dependent uptake was not observed with 10 or 100 μM [³H]TaraC and [³H]araC for either hCNT1 or hCNT3 over 3-min intervals or longer time intervals of 4 h (data not shown).

hCNT1: Electrophysiological Studies in *X. laevis* Oocytes. The two-microelectrode voltage-clamp technique was used to test the relative hCNT1 transportabilities of TaraC, araC, and 2'-deoxycytidine at higher concentrations than could be used in the radiotracer flux experiments with HeLa cells. Oocytes were voltage clamped at a holding potential of −50 mV, and currents were measured at pH 7.5 in either

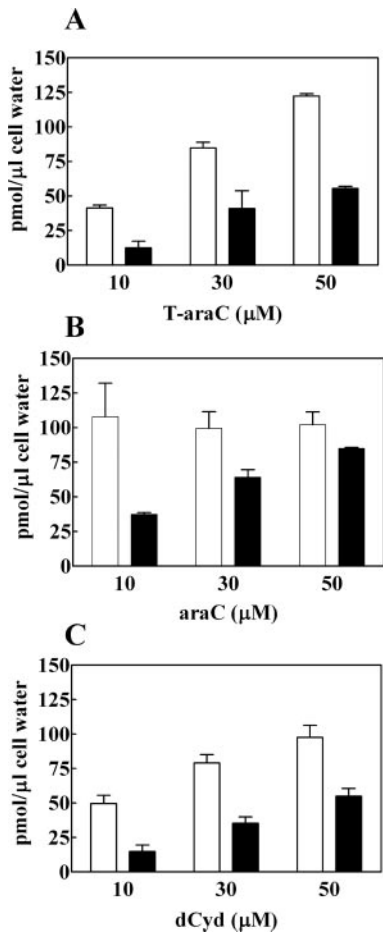


Fig. 1. Effects of the hENT1 inhibitor NBMPR on accumulation of TaraC (A), araC (B), and 2'-deoxycytidine (C) in CEM cells. CEM cells were exposed to 10, 30, or 50 μM [³H]TaraC, [³H]araC, or [³H]2'-deoxycytidine for 4 h in the absence (open columns) or presence (closed columns) of 100 nM NBMPR. Each data point represents the mean ± S.E.M. of two experiments (three replicates per experiment).

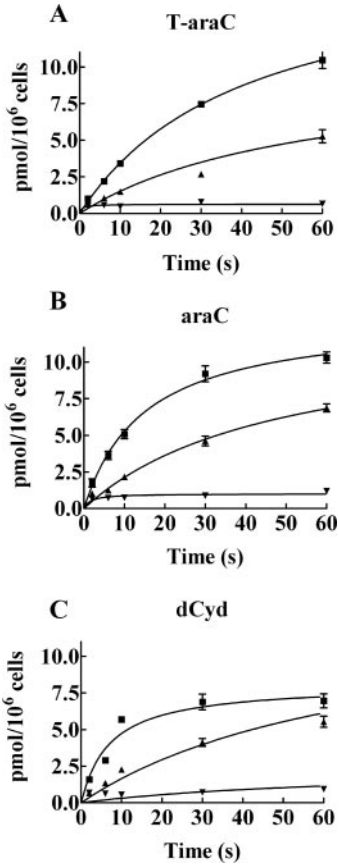


Fig. 2. Effects of transport inhibitors on the uptake of 10 μM [³H]TaraC, [³H]araC, and [³H]2'-deoxycytidine in HeLa cells. Cells were exposed to 10 μM ³H-nucleoside alone (■) or in the presence of 100 nM NBMPR (▲) or 100 μM dilazep (▼). Each value represents the mean ± S.D. of three determinations.

TABLE 2
Effects of transport inhibitors on the initial uptake rates of nucleosides in HeLa cells

HeLa cells were exposed to 10 μM concentrations of [³H]TaraC, [³H]araC, or [³H]2'-deoxycytidine in the absence and presence of 100 nM NBMPR or 100 μM dilazep. Initial uptake rates from two or more experiments each conducted with three replicates (2–10 s) are expressed as picomoles per 10⁶ cells per second ± S.E.

Treatment	2'-Deoxycytidine		AraC		TaraC	
	Initial Rate	% Inhibition	Initial Rate	% Inhibition	Initial Rate	% Inhibition
10 μM ³ H-Nucleoside	0.51 ± 0.11		0.42 ± 0.03		0.30 ± 0.00	
+ NBMPR	0.20 ± 0.02	61	0.14 ± 0.05	65	0.08 ± 0.02	74
+ Dilazep	0	100	0.01 ± 0.00	98	0	100

sodium-containing or sodium-free media that contained 1 mM TaraC, araC or 2'-deoxycytidine. The results indicated that there was little, if any, mediated transport of TaraC or araC. TaraC, araC, and 2'-deoxycytidine, respectively, induced inwardly directed Na^+ currents with mean $\pm$ S.E.M.

values ($n = 3$) of 2.7 ± 1.5 , 5.5 ± 0.5 , and 92 ± 10 nA (sodium-containing medium) and 0.0 ± 0.0 , 0.0 ± 0.0 , and 6.0 ± 2.3 (sodium-free medium). The values obtained with non-hCNT1-producing oocytes were negligible.

hENT1/2, hCNT1/2/3: Binding and Uptake Studies in Yeast. The concentration dependence of inhibition of uptake of 1 μM [^3H]uridine by recombinant human transporters produced in yeast was used to obtain a measure of the relative affinities of the transporters for TaraC and araC, together with other cytosine-containing nucleosides of interest (gemcitabine, cytidine, 2'-deoxycytidine, 3'-deoxycytidine, and 2',3'-dideoxycytidine). The concentration-effect curves for hENT1, hCNT1, and hCNT3 for inhibition of 1 μM [^3H]uridine uptake by various analogs of cytosine are shown in Fig. 3, and the EC_{50} values obtained from nonlinear regression analysis of the inhibition curves are shown in Table 3. The K_i values were calculated using K_m values for uridine obtained previously by the same procedures (Visser et al., 2002; Zhang et al., 2003).

For hENT1, relatively high concentrations of TaraC (>1 mM) or araC (788 μM) were required to inhibit uptake of [^3H]uridine by 50% (Table 3; Fig. 3). The uptake of [^3H]uridine was most sensitive to inhibition by cytidine ($\text{EC}_{50} = 171 \pm 46$ μM), whereas 2',3'-dideoxycytidine had no effect, even when present at a high concentration (10 mM). The order of inhibition was cytidine $>$ 2'-deoxycytidine $>$ gemcitabine $>$ araC $>$ T-araC $>$ 3'-deoxycytidine $\ggg$ 2',3'-dideoxycytidine. For hENT2, TaraC inhibited uptake of [^3H]uridine only at high concentrations (>3 mM), whereas cytidine, gemcitabine, and araC inhibited uptake with EC_{50} values of 650, 784, and 760 μM , respectively. For hCNT1, high concentrations of TaraC (>400 μM) and araC (>2 mM) were required to inhibit uptake of 1 μM [^3H]uridine by 50%, whereas 2'-deoxycytidine, gemcitabine, and cytidine exhibited EC_{50} values of 9, 13, and 17 μM , respectively. For hCNT2, high concentrations (>1 mM) of all cytidine analogs were required to inhibit uptake of [^3H]uridine by 50%. For hCNT3, high concentrations of TaraC (>2 mM) and araC (>10 mM) were required to inhibit uptake of 1 μM [^3H]uridine by 50%, whereas cytidine, 2'-deoxycytidine, and gemcitabine exhibited EC_{50} values of 5, 7, and 25 μM , respectively.

Experiments (data not shown) to examine uptake of 10 and 100 μM [^3H]TaraC directly were conducted in yeast producing either hCNT1 or hCNT3 in the absence or presence of a high concentration (10 mM) of cytidine or uridine over 90-

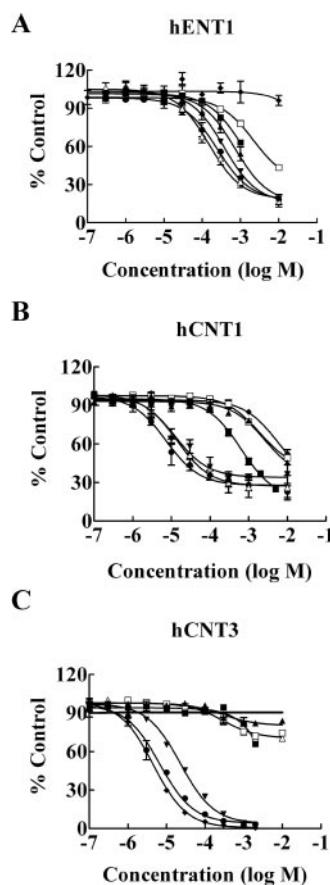


Fig. 3. Inhibition of uridine uptake by cytosine-containing nucleosides in *S. cerevisiae* transformed with plasmids pYPhENT1, pYPhCNT1, or pYPhCNT3. Yeast cells were exposed to graded concentrations (0.01 μM –10 mM) of cytidine, 2'-deoxycytidine, gemcitabine, araC, TaraC, 3'-deoxycytidine, or 2',3'-dideoxycytidine to examine the ability of each analog to inhibit the uptake of 1 μM [^3H]uridine. The data represents the mean $\pm$ S.E.M. of three separate experiments for each analog. Each data point within an individual experiment represents the mean of three to four replicates/concentration. ■, TaraC; ▲, araC; ▼, gemcitabine; ●, cytidine; ◆, 2'-deoxycytidine; ♦, 3'-deoxycytidine; and □, 2',3'-dideoxycytidine.

TABLE 3

Effects of various cytidine analogs on the uptake of uridine in *S. cerevisiae* producing pYPhENT1, pYPhENT2, pYPhCNT1, pYPhCNT2, or pYPhCNT3

Presented are EC_{50} values, which represent the effective concentrations at which the uptake of 1 μM [^3H]uridine was inhibited by 50% over a 20-min time period as determined by computer-generated concentration-effect curves (see Fig. 4). The corresponding K_i values are given in parentheses, where $K_i = \text{EC}_{50}/1 + ([\text{S}]/K_m)$ (Cheng and Prusoff 1973). In these calculations, $[\text{S}] = 1.0$ μM and K_m (uridine/hENT1) = 44.2 μM ; K_m (uridine/hENT2) = 195 μM ; K_m (uridine/hCNT1) = 55.4 μM ; and K_m (uridine/hCNT2) = 29.1 μM and K_m (uridine/hCNT3) = 8.7 μM .

Compound	EC_{50} (K_i)				
	hENT1	hENT2	hCNT1	hCNT2	hCNT3
			μM		
Cytidine	171 (167)	650 (647)	17 (16.4)	1445 (1397)	5 (4)
2'-Deoxycytidine	288 (282)	1035 (1030)	9 (8.6)	1714 (1657)	7 (6.3)
Gemcitabine	362 (354)	784 (780)	13.4 (13)	1365 (1320)	25 (22.3)
AraC	788 (771)	760 (756)	$>2000^a$	$>10,000^a$	$>10,000^a$
TaraC	$>1000^a$	$>3000^a$	459 (451)	$>3000^a$	$>2000^a$
3'-Deoxycytidine	$>2000^a$	$>10,000^a$	$>2000^a$	$>20,000^a$	$>10,000^a$
2',3'-Dideoxycytidine	$>10,000^a$	$>20,000^a$	$>5000^a$	$>20,000^a$	$>10,000^a$

^a K_i values were not calculated due to high EC_{50} values.

min time intervals; hCNT2 was not included because it was previously shown to lack the capacity to transport cytosine-containing nucleosides (Ritzel et al., 1998). There was no evidence of mediated transport of [³H]TaraC by either hCNT1 or hCNT3.

Chemosensitivity Testing. The results of the transportability and binding studies indicated that TaraC and araC exhibited similar profiles, having weak activities with hENT1 (araC > TaraC), marginal activities with hENT2, and negligible activities with hCNT1, hCNT2, and hCNT3. To assess the importance of nucleoside transporters in TaraC and araC cytotoxicity, cell proliferation assays were conducted with CEM cells that possessed either functional hENT1, hENT2, or hCNT1 or that lacked nucleoside transport capability either pharmacologically by treatment with NBMPR or genetically by previous selection for resistance to araC (Ullman et al., 1988). Cells were exposed to TaraC and araC for 24- and/or 48-h intervals, proliferation was measured using a commercial cytotoxicity assay, and the results are summarized in Table 4.

For CEM cells, which naturally possess hENT1, araC was more toxic than TaraC. For example, the concentrations of araC and TaraC at which proliferation was inhibited by 50% (EC₅₀ values) during 48-h exposures were 0.02 and 0.2 μM, respectively. However, when hENT1 activity was blocked by treatment of CEM cells with 1 μM NBMPR, the differences in cytotoxicity for both 24- and 48-h exposures were eliminated, indicating that the two drugs were pharmacologically equivalent in CEM cells in the absence of transport activity. AraC cytotoxicity was more dependent on transport than TaraC because strong protection by NBMPR against araC cytotoxicity was observed at both 24 and 48 h, with 16- and 25-fold increases in EC₅₀ values, respectively, whereas weak protection was observed for TaraC only at 24 h, with a 4.5-fold increase in EC₅₀ value. The minor differences in the EC₅₀ values for TaraC cytotoxicity at 48 h in the presence and absence of NBMPR (Table 4) were within experimental error. The cytotoxicities of TaraC and araC to CEM cells in the presence of NBMPR suggested that, at higher concentrations, both drugs enter cells passively in sufficient quantities to cause cell death.

Because hENT2 exhibited some capacity for transport of

TaraC and araC, cytotoxicity studies were undertaken with a stable transfectant that produces hENT2 (CEM-ARAC/hENT2) generated from a nucleoside transport defective mutant (CEM-ARAC). Functionality of hENT2 was established by demonstration of dilazep-sensitive transport of [³H]uridine (data not shown). The impact of acquisition of functional hENT2 seemed to be time-dependent; during 24-h exposures, there was no apparent effect on cytotoxicity of TaraC and araC, whereas during 48-h exposures, increased cytotoxicity was observed for both drugs. These results demonstrated that the presence of functional hENT2 modestly enhanced sensitivity of CEM cells to both drugs during prolonged exposures.

Also shown in Table 4 are results for cytotoxicity studies that were conducted with CEM-ARAC/hCNT1 cells. During 48-h exposures, TaraC and araC exhibited slightly greater cytotoxicity against hCNT1-containing cells than against nucleoside transport-deficient cells, suggesting that hCNT1 may have some capacity for transport of TaraC and araC, despite the absence of detectable transport of TaraC and araC in isotope flux studies with recombinant hCNT1 in HeLa cells and oocytes.

CEM-ARAC, the nucleoside transport-defective mutant used as the host for production of transporter-producing stable transfectants, exhibited substantial resistance to both T-araC and araC (Table 4) compared with "wild-type" CEM cells in either the absence or presence of NBMPR. This was not unexpected, because CEM-ARAC cells were originally isolated by prolonged exposure of CEM cells to 8 μM araC (Ullman et al., 1988), and additional, uncharacterized mutations that affect a common step in the metabolism of T-araC and araC might be present.

Discussion

TaraC is a novel nucleoside analog of araC that was developed in a search for nucleoside analogs with enhanced activity and decreased susceptibility to nucleoside catabolizing enzymes. Although the mechanism of action of TaraC is similar to that of araC, these two drugs exhibit major differences in metabolism (Parker et al., 2000): the rate of phosphorylation of TaraC is much less than that of araC (~1%), inhibition of DNA synthesis by TaraCTP is 10- to 20-fold greater than that by araCTP, TaraCTP has a 10-fold longer half-life than araCTP, deamination of TaraC by cytidine deaminase is only 10% of that of araC, and dCMP deaminase degrades araCMP better than TaraCMP. The cumulative effect of these differences leads to prolonged retention of TaraCTP in comparison with that of araCTP. In addition, the activity of TaraC has been shown to be independent of administration schedule in contrast to that of araC, which is highly schedule-dependent (Waud et al., 2003). When TaraC and araC were evaluated for activity against human solid tumor xenograft models, TaraC exhibited superior antitumor activity (Waud et al., 2003). AraC, which is used clinically against hematological malignancies, is known to require mediated transporters for efficient cellular uptake (Wiley et al., 1985; Jamieson et al., 1990; Gati et al., 1997), whereas there is no information on the transportability of TaraC. Information on the mechanism(s) by which TaraC gets into cells, in addition to the existing information on its enhanced retention and schedule independence of administration in pa-

TABLE 4

Chemosensitivities of CEM, CEM-ARAC/hENT2, CEM-ARAC/hCNT1, and CEM-ARAC cells to AraC and TaraC

Actively proliferating CEM cell lines were exposed to graded concentrations of araC or TaraC in 96-well plates for 24 and 48 h as described under *Materials and Methods*. In protection experiments, CEM cells were treated with 1 μM NBMPR 1 h before addition of araC or TaraC. An EC₅₀ value of 0.22 ± 0.01 μM was obtained for TaraC in presence of NBMPR at 48 h in an independent experiment. EC₅₀ values were obtained from the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, cyto-cytotoxicity relationships, and are means (±S.E.) of at least two or more experiments, each conducted with six replicates.

Cell Line	EC ₅₀			
	araC		TaraC	
	24 h	48 h	24 h	48 h
	μM			
CEM	0.05 ± 0.01	0.02 ± 0.01	0.2 ± 0.03	0.2 ± 0.03
+ NBMPR	0.8 ± 0.1	0.5 ± 0.2	0.9 ± 0.2	0.4 ± 0.1
CEM-ARAC/hENT2	>10	1.3 ± 0.3	40 ± 35	1.6 ± 0.4
CEM-ARAC/hCNT1	N.D.	21 ± 4	N.D.	5 ± 1
CEM-ARAC	>10	68 ± 27	>100	10 ± 2

N.D., not determined.

tients, would further help in design of clinical trials with TaraC.

The current study was undertaken to examine the role of human nucleoside transporters in the uptake and cytotoxicity of TaraC; araC was included for comparison. Compared with uridine, both araC and TaraC were poor permeants for hENT1 in CEM cells. The initial rates of mediated uptake of araC were higher than those of TaraC in both CEM (4.9-fold) and HeLa (1.4-fold) cells. At longer time intervals (60 s), the accumulation of TaraC, araC, and 2'-deoxycytidine in HeLa cells was dependent on both hENT1 and hENT2, based on results of inhibition studies with NBMPR and dilazep. In CEM cells, which exhibit only hENT1 activity, accumulation of 10 μ M araC was much greater than that of 10 μ M TaraC, and this was consistent with the 1) lower transportability of TaraC by hENT1 (this study), 2) lower specificity of CEM-derived deoxycytidine kinase for TaraC (Parker et al., 2000; Someya et al., 2002, 2003), and 3) lack of deaminase-related degradation of araC in CEM cells (Parker et al., 2000; Gourdeau et al., 2001b). HeLa cells are reported to have both cytidine deaminase (Meyers et al., 1973) and dCMP deaminase (Gelbard et al., 1969) activities. The presence of cytidine deaminase in HeLa cells and its apparent higher activity ($\sim$ 10-fold) for araC than for TaraC (Parker et al., 2000) may explain why araC did not accumulate in HeLa cells to a greater extent than TaraC as was observed in CEM cells.

The ability of araC to inhibit the uptake of radiolabeled uridine by hCNT1 in transiently transfected HeLa cells was demonstrated in a previous study (Graham et al., 2000) in which high araC concentrations (e.g., 0.5 mM) were required to partially inhibit the uptake of uridine. This result led to the suggestion that the presence of hCNT1 in human cells would probably have no impact on cytotoxicity because of its low affinity for araC (Graham et al., 2000). The results of the current study were consistent with these findings in that direct uptake of radiolabeled araC by hCNT1 was not observed. In addition, there was no demonstrable uptake of radiolabeled TaraC or araC by the broadly specific hCNT3 in either HeLa cells or yeast. hCNT2 was not examined because it does not transport cytosine-containing nucleosides (Ritzel et al., 1998). Recent electrophysiological studies (Smith et al., 2004) with recombinant hCNT1 produced in *X. laevis* oocytes demonstrated that 1 mM araC produced small inward sodium currents, indicating that araC is a low-affinity hCNT1 permeant.

The relative affinities of the human transporter proteins for various deoxycytidine analogs were investigated in yeast using an assay that assumed competitive inhibition of uridine transport and yielded K_i values for the inhibitory analogs. This assay was used previously to assess interactions of uridine analogs with the human nucleoside transporters (Visser et al., 2002; Zhang et al., 2003; Vickers et al., 2004). Studies with recombinant hENT1 and hENT2 (Vickers et al., 2002) showed that both transporters exhibited relatively low apparent affinities for cytidine and its analogs (hENT1 > hENT2). In a separate study (Zhang et al., 2003), it was shown that hCNT1 and hCNT3 exhibited little tolerance for modification at C(3') or inversion in the configuration at C(2') or C(3'). In this study, yeast strains producing hENT1, hENT2, hCNT1, hCNT2, or hCNT3 were examined for their ability to transport [3 H]uridine in the presence of increasing concentrations of nonradiolabeled TaraC, araC, and 2'-de-

oxycytidine as well as several other cytosine-containing nucleosides (gemcitabine, cytidine, 3'-deoxycytidine, and 2',3'-dideoxycytidine). In comparison with cytidine and 2'-deoxycytidine, araC exhibited much lower ability to inhibit hCNT1- and hCNT3-mediated transport, indicating that the presence of the 2'-hydroxyl group above the plane of the sugar ring hindered binding to the transporters. Consistent with previous studies with uridine analogs, the presence of a hydroxyl group at the 3'-position was important for recognition by all transporters examined because 3'-deoxycytidine and 2',3'-dideoxycytidine did not inhibit uridine transport. Although the yeast inhibitor assay indicated that TaraC was bound more tightly by hCNT1 than by hENT1 (compare EC_{50} values in Table 3), direct measurements of radiolabeled uptake in transiently transfected HeLa cells suggested that TaraC was not a permeant for hCNT1. Further evaluation with higher concentrations of TaraC and araC in electrophysiological studies of hCNT1 produced in *X. laevis* oocytes showed little, if any, transport by hCNT1.

In summary, TaraC and araC exhibited similar transportability profiles. At the pharmacologically relevant concentration of 10 μ M, both drugs were transported, albeit weakly, by the hENTs (araC > TaraC), and neither was transported by the hCNTs. Efforts to quantitate diffusional uptake of TaraC and araC by CEM cells that were transport defective either pharmacologically or mutationally were unsuccessful, evidently because the rates of diffusion were below the limits of experimental detection. The cytotoxicity studies provided evidence that transportability was a determinant of pharmacological activity of both drugs (araC > TaraC). First, treatment of CEM cells with NBMPR strongly protected against araC and weakly protected against TaraC cytotoxicity. Second, because TaraC and araC were equivalent pharmacologically in NBMPR-treated CEM cells (IC_{50} values, respectively, of 0.9 and 0.8 μ M at 24 h and 0.5 and 0.4 μ M at 48 h), the explanation for the greater cytotoxicity of araC than TaraC in the absence of NBMPR was the greater influx of araC via hENT1. Third, the introduction of hENT2 (and possibly also hCNT1) into CEM/ARAC by gene transfer sensitized cells to both drugs.

It was shown earlier that TaraC was markedly more cytotoxic than araC when high concentrations of the compounds were given for short periods (Someya et al., 2005), presumably because of the longer intracellular half-life of TaraCTP than araCTP. Once TaraC enters cells by mediated transport, its metabolites are trapped inside, resulting in prolonged cytotoxic action; in contrast, araC cytotoxicity requires continued influx of araC because of its susceptibility to degradation. These results support the use of TaraC over araC because at clinically achievable drug concentrations its accumulation in tumors is sustained (Parker et al., 2000) and independent of the drug administration schedule (Waud et al., 2003). In addition, recent studies (Thottassery et al., 2006) have shown that TaraC, but not araC, is a potent inducer of apoptosis by stabilization of p73 in both p53-positive and p53-negative cells, providing evidence favoring use of TaraC in treatment of tumors.

In conclusion, isotope flux studies demonstrated that cellular uptake of TaraC was mediated, albeit poorly, by hENT1 and hENT2, thereby resulting in sufficient cellular retention of TaraC metabolites to achieve pharmacological activity. Longer intracellular retention of TaraC metabolites may

have been an important contributor to manifestation of cytotoxicity during prolonged exposures. Although TaraC exhibited lower transportability than araC, once inside cells, its characteristics (greater inhibition of DNA synthesis, longer half-life, and decreased susceptibility to degradation) may give rise to therapeutic advantages over araC in therapy of tumors.

References

- Baldwin SA, Yao SY, Hyde RJ, Ng AM, Foppolo S, Barnes K, Ritzel MW, Cass CE, and Young JD (2005) Functional characterisation of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. *J Biol Chem* **280**:15880–15887.
- Blajeski AL, Richardson KA, Emerson DL, Richardson FC, and Fridland A (2002) OSI-7836 (4'-thio- β -D-arabinofuranosylcytosine): a novel anticancer nucleoside analog that inhibits DNA polymerases and induces G₂ arrest. *Proc Am Assoc Cancer Res* **43**:abstract 4779.
- Boleti H, Coe IR, Baldwin SA, Young JD and Cass CE (1997) Molecular identification of the equilibrative NBMPR-sensitive (es) nucleoside transporter and demonstration of an equilibrative NBMPR-insensitive (ei) transport activity in human erythroleukemia (K562) cells. *Neuropharmacology* **36**:1167–1179.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Clarke ML, Mowles D, Vickers MF, Zhang J, Young JD, Tomkinson B, and Cass CE (2003) Uptake of 4'-thio- β -D-arabinofuranosylcytosine and β -D-arabinosylcytosine by human equilibrative and concentrative nucleoside transporters. *Proc Am Assoc Cancer Res* **44**:abstract 3699.
- Damaraju VL, Visser F, Zhang J, Mowles D, Ng AM, Young JD, Jayaram HN, and Cass CE (2005) Role of human nucleoside transporters in the cellular uptake of two inhibitors of IMP dehydrogenase, tiazofurin and benzamide riboside. *Mol Pharmacol* **67**:273–279.
- Gati WP, Paterson AR, Larratt LM, Turner AR, and Belch AR (1997) Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular es nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. *Blood* **90**:346–353.
- Gelbard AS, Kim JH, and Perez AG (1969) Fluctuations in deoxycytidine monophosphate deaminase activity during the cell cycle in synchronous populations of HeLa cells. *Biochim Biophys Acta* **182**:564–566.
- Gourdeau H, Bibeau L, Ouellet F, Custeau D, Bernier L, and Bowlin T (2001a) Comparative study of a novel nucleoside analogue (troxatyl, troxacitabine, BCH-4556) and AraC against leukemic human tumor xenografts expressing high or low cytidine deaminase activity. *Cancer Chemother Pharmacol* **47**:236–240.
- Gourdeau H, Clarke ML, Ouellet F, Mowles D, Selner M, Richard A, Lee N, Mackey JR, Young JD, Jolivet J, et al. (2001b) Mechanisms of uptake and resistance to troxacitabine, a novel deoxycytidine nucleoside analogue, in human leukemic and solid tumor cell lines. *Cancer Res* **61**:7217–7224.
- Graham KA, Leithoff J, Coe IR, Mowles D, Mackey JR, Young JD, and Cass CE (2000) Differential transport of cytosine-containing nucleosides by recombinant human concentrative nucleoside transporter protein hCNT1. *Nucleosides Nucleotides* **19**:415–434.
- Griffiths M, Beaumont N, Yao SY, Sundaram M, Boumah CE, Davies A, Kwong FY, Coe I, Cass CE, Young JD, et al. (1997a) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat Med* **3**:89–93.
- Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, and Baldwin SA (1997b) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. *Biochem J* **328**:739–743.
- Jamieson GP, Snook MB, and Wiley JS (1990) Saturation of intracellular cytosine arabinoside triphosphate accumulation in human leukemic blast cells. *Leuk Res* **14**:475–479.
- Lang TT, Selner M, Young JD, and Cass CE (2001) Acquisition of human concentrative nucleoside transporter 2 (hcnt2) activity by gene transfer confers sensitivity to fluoropyrimidine nucleosides in drug-resistant leukemia cells. *Mol Pharmacol* **60**:1143–1152.
- Lang TT, Young JD, and Cass CE (2004) Interactions of nucleoside analogs, caffeine and nicotine with human concentrative nucleoside transporters 1 and 2 stably produced in a transport-defective human cell line. *Mol Pharmacol* **65**:925–933.
- Mackey JR, Baldwin SA, Young JD, and Cass CE (1998a) Nucleoside transport and its significance for anticancer drug resistance. *Drug Resist Updat* **1**:310–324.
- Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, Crawford CR, and Cass CE (1998b) Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res* **58**:4349–4357.
- Meyers R, Malathi VG, Cox RP, and Silber R (1973) Studies on nucleoside deaminase. Increase in activity in HeLa cell cultures caused by cytosine arabinoside. *J Biol Chem* **248**:5909–5913.
- Parker WB, Shaddix SC, Rose LM, Waud WR, Shewach DS, Tiwari KN, and Secrist JA (2000) Metabolism of 4'-thio-beta-D-arabinofuranosylcytosine in CEM cells. *Biochem Pharmacol* **60**:1925–1932.
- Richardson F, Black C, Richardson K, Franks A, Wells E, Karimi S, Sennello G, Hart K, Meyer D, Emerson D, et al. (2005) Incorporation of OSI-7836 into DNA of Calu-6 and H460 xenograft tumors. *Cancer Chemother Pharmacol* **55**:213–221.
- Richardson KA, Vega TP, Richardson FC, Moore CL, Rohloff JC, Tomkinson B, Bendele RA, and Kuchta RD (2004) Polymerization of the triphosphates of AraC, 2',2'-difluoro-deoxycytidine (dFdC) and OSI-7836 (T-araC) by human DNA polymerase alpha and DNA primase. *Biochem Pharmacol* **68**:2337–2346.
- Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE, and 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.
- Ritzel MWL, Ng AM, Yao SY, Graham KA, Loewen SK, Smith KM, Ritzel GW, Mowles DA, Carpenter P, Chen X-Z, 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.
- Ritzel MWL, Yao SY, Huang MY, Elliott JF, Cass CE, and Young JD (1997) Molecular cloning and functional expression of cDNAs encoding a human Na⁺-nucleoside cotransporter (hCNT1). *Am J Physiol* **272**:C707–C714.
- Smith KM, Ng AM, Yao SY, Labedz KA, Knaus EE, Wiebe LI, Cass CE, Baldwin SA, Chen XZ, Karpinski E, et al. (2004) Electrophysiological characterization of a recombinant human Na⁺-coupled nucleoside transporter (hCNT1) produced in *Xenopus* oocytes. *J Physiol (Lond)* **558**:807–823.
- Smith KM, Slugoski MD, Loewen SK, Ng AM, Yao SY, Chen XZ, Karpinski E, Cass CE, Baldwin SA, and Young JD (2005) The broadly selective human Na⁺/nucleoside cotransporter (hCNT3) exhibits novel cation-coupled nucleoside transport characteristics. *J Biol Chem* **280**:25436–24439.
- Someya H, Shaddix SC, Tiwari KN, Secrist JA 3rd, and Parker WB (2003) Phosphorylation of 4'-thio- β -D-arabinofuranosylcytosine and its analogs by human deoxycytidine kinase. *J Pharmacol Exp Ther* **304**:1314–1322.
- Someya H, Tiwari KN, Secrist JA, and Parker WB (2002) Structure-activity relationship of 4'-thionucleoside analogs with deoxycytidine kinase (dCK) from CEM cells. *Proc Am Assoc Cancer Res* **43**:abstract 3674.
- Someya H, Waud WR, and Parker WB (2005) Long intracellular retention of 4'-thio-arabinofuranosylcytosine 5'-triphosphate as a critical factor for the anti-solid tumor activity of 4'-thio-arabinofuranosylcytosine. *Cancer Chemother Pharmacol* **57**:772–780.
- Thottassery JV, Westbrook L, Someya H, and Parker WB (2006) c-Abl-independent p73 stabilization during gemcitabine- or 4'-thio-(beta)-D-arabinofuranosylcytosine-induced apoptosis in wild-type and p53-null colorectal cancer cells. *Mol Cancer Ther* **5**:400–410.
- Ullman B (1989) Dideoxycytidine metabolism in wild type and mutant CEM cells deficient in nucleoside transport or deoxycytidine kinase. *Adv Exp Med Biol* **253B**:415–420.
- Ullman B, Coons T, Rockwell S, and McCartan K (1988) Genetic analysis of 2',3'-dideoxycytidine incorporation into cultured human T lymphoblasts. *J Biol Chem* **263**:12391–12396.
- Vickers MF, Kumar R, Visser F, Zhang J, Charania J, Raborn RT, Baldwin SA, Young JD, and Cass CE (2002) Comparison of the interaction of uridine, cytidine and other pyrimidine nucleoside analogues with recombinant human equilibrative nucleoside transporter 2 (hENT2) produced in *Saccharomyces cerevisiae*. *Biochem Cell Biol* **80**:639–644.
- Vickers MF, Zhang J, Visser F, Tackaberry T, Robins MJ, Nielsen LP, Nowak I, Baldwin SA, Young JD, and Cass CE (2004) Uridine recognition motifs of human equilibrative nucleoside transporters 1 and 2 produced in *Saccharomyces cerevisiae*. *Nucleosides Nucleotides Nucleic Acids* **23**:361–373.
- Visser F, Vickers MF, Ng AM, Baldwin SA, Young JD, and Cass CE (2002) Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyrdamole. *J Biol Chem* **277**:395–401.
- Waud WR, Gilbert KS, Shepherd RV, Montgomery JA, and Secrist JA 3rd (2003) Preclinical antitumor activity of 4'-thio-beta-D-arabinofuranosylcytosine (4'-thio-ara-C). *Cancer Chemother Pharmacol* **51**:422–426.
- Whistler RL, Doner LW, and Nayak UG (1971) 4-Thio-D-arabinofuranosylpyrimidine nucleosides. *J Org Chem* **36**:108–110.
- Wiley JS, Taupin J, Jamieson GP, Snook MB, Sawyer WH, and Finch LR (1985) Cytosine arabinoside transport and metabolism in acute leukemias and T cell lymphoblastic lymphoma. *J Clin Invest* **75**:632–642.
- Zhang J, Smith KM, Tackaberry T, Visser F, Robins MJ, Nielsen LP, Nowak I, Karpinski E, Baldwin SA, Young JD, et al. (2005) Uridine binding and transportability determinants of human concentrative nucleoside transporters. *Mol Pharmacol* **68**:830–839.
- Zhang J, Visser F, Vickers MF, Lang T, Robins MJ, Nielsen LP, Nowak I, Baldwin SA, Young JD, and Cass CE (2003) Uridine binding motifs of human concentrative nucleoside transporters 1 and 3 produced in *Saccharomyces cerevisiae*. *Mol Pharmacol* **64**:1512–1520.

Address correspondence to: Dr. Carol E. Cass, Department of Oncology, Cross Cancer Institute, 11560 University Ave., Edmonton, AB, T6G 1Z2 Canada. E-mail: carol.cass@cancerboard.ab.ca