0026-895X/06/6901-346–353$20.00
MOLECULAR PHARMACOLOGY
Vol. 69, No. 1
15768/3073296

Mol Pharmacol 69:346–353, 2006
Printed in U.S.A.

A Comparison of the Transportability, and Its Role in Cytotoxicity, of Clofarabine, Cladribine, and Fludarabine by Recombinant Human Nucleoside Transporters Produced in Three Model Expression Systems

Karen M. King, Vijaya L. Damaraju, Mark F. Vickers, Sylvia Y. Yao, Thach Lang, Tracey E. Tackaberry, Delores A. Mowles, Amy M. L. Ng, James D. Young, and Carol E. Cass

Departments of Oncology (K.M.K., V.L.D., M.F.V., T.E.T., T.L., D.A.M., C.E.C.), Biochemistry (K.M.K., C.E.C.), and Physiology (S.Y.Y., A.M.L.N., J.D.Y.), University of Alberta and the Cross Cancer Institute, Edmonton, Alberta, Canada

Received June 23, 2005; accepted October 18, 2005

ABSTRACT

2-Chloro-9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine (Cl-F-ara-A, clofarabine), a purine nucleoside analog with structural similarity to 2-chloro-2′-deoxyadenosine (Cl-dAdo, cladribine) and 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A, fludarabine), has activity in adult and pediatric leukemias. Mediated transport of the purine nucleoside analogs is believed to occur through the action of two structurally unrelated protein families, the equilibrative nucleoside transporters (ENTs) and the concentrative nucleoside transporters (CNTs). The current work assessed the transportability of Cl-F-ara-A, Cl-dAdo, and F-ara-A in cultured human leukemic CEM cells that were either nucleoside transport-defective or possessed individual human nucleoside transporter types and in *Xenopus laevis* oocytes and *Saccharomyces cerevisiae* yeast that produced individual recombinant human nucleoside transporter types. Cells producing hENT1 or hCNT3 exhibited the highest uptake of Cl-F-ara-A, whereas nucleoside transport-deficient cells and cells producing hCNT1 lacked uptake altogether. When Cl-F-ara-A transport rates by hENT1 were compared with those of Cl-dAdo and F-ara-A, Cl-dAdo had the highest efficiency of transport, although Cl-F-ara-A showed the greatest accumulation during 5-min exposures. In cytotoxicity studies with the CEM lines, Cl-F-ara-A was more cytotoxic to cells producing hENT1 than to the nucleoside transport-deficient cells. The efficiency of Cl-F-ara-A transport by oocytes with recombinant transporters was hCNT3 > hENT2 > hENT1 > hCNT2; no transport was observed with hCNT1. Affinity studies with recombinant transporters produced in yeast showed that hENT1, hENT2, and hCNT3 all had higher affinities for Cl-F-ara-A than for either Cl-dAdo or F-ara-A. These results suggest that the nature and activity of the plasma membrane proteins capable of inward transport of nucleosides are important determinants of Cl-F-ara-A activity in human cells.

Nucleoside analogs are one of the most common classes of drugs used to treat cancer. A limitation of one such drug, 9-β-D-arabinofuranosyladenine (ara-A), is its susceptibility to deamination to an inactive metabolite by adenosine deaminase (Plunkett et al., 1990), which led to the synthesis of adenosine-deaminase resistant analogs such as 2-chloro-2′-deoxyadenosine (Cl-dAdo, cladribine) and 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A, fludarabine) (Montgomery and Hewson, 1969; Christensen et al., 1972). Cl-dAdo is effective in the treatment of indolent lymphoid malignancies, including chronic lymphocytic leukemia, hairy-cell leukemia, low-grade non-Hodgkin's lymphoma, and acute myeloid leukemia (Saven and Piro, 1994; Hoffman, 1996; Piro, 1996). Once inside the cell, Cl-dAdo is phosphorylated by cytosolic deoxycytidine kinase (Carson et al., 1983) and mitochondrial deoxyguanosine kinase (Wang et al., 1993). The cytotoxicity of Cl-dAdo to proliferating cells is due to interruption of DNA

This work was supported by ILEX Products Inc., National Cancer Institute of Canada. C.E.C. holds the Canada Research Chair in Oncology, J.D.Y. is a Heritage Scientist of the Alberta Heritage Foundation for Medical Research, and K.M.K. was a recipient of an Alberta Cancer Board Research Fellowship.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.015768.

ABBREVIATIONS: ara-A, 9-β-D-arabinofuranosyladenine; Cl-dAdo, 2-chloro-2′-deoxyadenosine (cladribine); F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine); Cl-F-ara-A, 2-chloro-9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine (clofarabine); ENT, equilibrative nucleoside transporter family; CNT, concentrative nucleoside transporter family; NTD, nucleoside transport-deficient; NBMPR, nitrobenzylmercaptopurine ribonucleoside (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine); MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

synthesis by Cl-dATP inhibition of ribonucleotide reductase and DNA polymerases α and β with resultant dNTP pool imbalances and DNA strand breaks (Griffig et al., 1989; Hirota et al., 1989; Hentosh et al., 1991). The mechanism of cytotoxicity of Cl-dAdo to nondividing cells is by apoptosis (Carson et al., 1983; Seto et al., 1985; Robertson et al., 1993).

Clinical studies of F-ara-A monotherapy in chronic lymphocytic leukemia have demonstrated it to be at least as effective as standard therapies (Adkins et al., 1997). It has also shown activity in the treatment of chronic lymphocytic leukemias of T-cell origin and prolymphocytic leukemia and has been used for the treatment of acute leukemias and low-grade non-Hodgkins lymphoma. Mechanisms of action of F-ara-ATP include termination of DNA synthesis by incorporation into the elongating nucleic acid chain and inhibition of DNA primase, DNA ligase, DNA polymerases α, γ, and ϵ, and ribonucleotide reductase (Plunkett et al., 1990). In quiescent cells, F-ara-ATP induces apoptosis (Plunkett et al., 1990).

2-Chloro-9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine (Cl-F-ara-A, clofarabine), the 2′-arabino-fluoro derivative of Cl-dAdo, exhibits pharmacologic activity similar to that of Cl-dAdo and F-ara-A (Montgomery et al., 1992). It has cytotoxic activity in vivo against human colon and renal tumors, as well as human chronic lymphocytic leukemia cells transplanted into severe combined immunodeficient mice (Parker et al., 1991; Carson et al., 1992; Waud et al., 2000). Phase I and II studies have indicated that Cl-F-ara-A has antileukemic activity in pediatric acute lymphocytic leukemia and adult acute leukemias and myelodysplastic syndrome (Kantarjian et al., 2003; Jeha et al., 2004). The fluorine at the 2′ position of Cl-F-araA confers resistance to phosphorolytic cleavage (Montgomery et al., 1992) which, combined with its acid stability, suggests that it may be more available and less toxic than Cl-dAdo when given orally (Carson et al., 1992; Qian et al., 1994). Cl-F-ara-A is phosphorylated by deoxycytidine kinase and exhibits cytotoxicity to both proliferating and nonproliferating cells by mechanisms similar to those of Cl-dAdo. Cl-F-ara-A is also directly toxic to quiescent lymphocytes and macrophages, showing DNA damage characteristic of apoptosis (Carson et al., 1992).

Many of the purine nucleosides enter cells by via mediated transport processes (Plunkett and Saunders, 1991; Belt et al., 1993; Cass, 1995). These transport processes have been differentiated functionally on the basis of their equilibrative or concentrative properties, dependence on Na^+ gradients, sensitivities to nucleoside transport inhibitors, and preference of permeants (Cass, 1995). cDNAs encoding the corresponding nucleoside transporter proteins have been cloned and functionally expressed from humans and rodents (Griffiths et al., 1997; Ritzel et al., 1997, 2001; Wang et al., 1997; Crawford et al., 1998; Baldwin et al., 2005). In humans, these transporters include three members of the equilibrative nucleoside transporter (ENT) family (hENT1/2/3), and three members of the concentrative nucleoside transporter (CNT) family (hCNT1/2/3). hENT1 and hENT2 are facilitative transporters found in plasma membranes. They have broad and overlapping permeant selectivities that can be functionally distinguished by their sensitivity (hENT1) or resistance (hENT2) to inhibition by nanomolar concentrations of nitrobenzylmercaptopurine ribonucleoside (NBMPR). hENT3, a newly identified member of the ENT family, is associated with lysosomal/endosomal membranes and exhibits nucleoside transport activity that is NBMPR-insensitive, broadly selective, and proton-dependent (Baldwin et al., 2005). The three CNTs are secondary-active sodium-dependent transporters with selectivities for pyrimidine nucleosides (hCNT1), purine nucleosides plus uridine (hCNT2), and both pyrimidine and purine nucleosides (hCNT3).

Although the cellular uptake of Cl-dAdo and F-ara-A has been studied in model systems, less information is available for Cl-F-ara-A. The present study was undertaken to compare the relative importance of plasma membrane nucleoside transport in the cytotoxicities of Cl-dAdo, F-ara-A, and Cl-F-ara-A and to identify the human transporter proteins that accept Cl-F-ara-A as a permeant. The transportability and cytotoxicity of Cl-dAdo, Cl-F-ara-A, and F-ara-A were measured in a panel of cultured human leukemic cell lines that either lacked the capacity for nucleoside transport or exhibited a single nucleoside transport activity. The kinetics of transport of Cl-F-ara-A by human recombinant hENT1, hENT2, hCNT1, hCNT2, and hCNT3 were characterized in the *Xenopus laevis* oocyte expression system, a system that allows the transport processes to be studied in isolation (Huang et al., 1994). The relative affinities of the human transporters for the three nucleoside analogs were measured in *Saccharomyces cerevisiae* producing recombinant hENT1, hENT2, and hCNT3 using an inhibitor-sensitivity assay that allows the relative quantification of the inhibitory effects of test molecules on nucleoside transport (Zhang et al., 2003).

Materials and Methods

Chemicals. Cl-dAdo and F-ara-A were purchased from Sigma Chemical Co. (Mississauga, ON, Canada), and Cl-F-ara-A was provided by Drs. Dennis Carson, Carlos Carrera, and Howard Cottam (Scripps Clinic and Research Foundation, San Diego, CA) and ILEX Products Inc. (Boston, MA). [8-^{3}H]Cl-dAdo, [8-^{3}H]F-ara-A, and [8-^{3}H]Cl-F-ara-A were purchased from Moravek Biochemicals (Brea, CA) and purified by reversed-phase high-performance liquid chromatography on a Phenomenex Bondclone 10 C18 column (Phenomenex, Torrance, CA). [1,2-^{14}C]Polyethylene glycol, [^{14}C]sucrose, and [8-^{3}H]H_2O were purchased from PerkinElmer Life and Analytical Sciences (Woodbridge, ON, Canada) and GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. NBMPR, mineral oil, and unlabeled nucleosides were obtained from Sigma Chemical Co. (Mississauga, ON, Canada). Tissue culture (96-well) plates, tissue culture medium, fetal bovine serum, and horse serum were purchased from Invitrogen Canada (Burlington, ON, Canada). The CellTiter 96 Aqueous One Solution cell proliferation assay kit was obtained from Promega (Madison, Wisconsin). Ecolite was purchased from Valeant Canada Limitée (Saint-Laurent, QC, Canada) and 550 silicone oil from BC Bearings (Edmonton, AB, Canada). Dilazep was a gift from F. Hoffman La-Roche and Company (Basel, Switzerland).

Cell Culture. CCRF-CEM (transport competent, hereafter termed CEM/hENT1), CEM/ara-C (transport deficient, hereafter termed CEM/NTD), and stably transfected CEM/ara-C cell lines (CEM/hENT2, CEM/hCNT1, CEM/hCNT2) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Culture conditions for the transfected cell lines also included tubercidin, cytosine arabinoside, and G-418 (Geneticin), as described previously (Lang et al., 2001). The CEM/NTD line, which was derived from the CCRF-CEM line by selection for resistance to cytosine arabinoside (Ullman et al., 1988), exhibits cross-resistance to a variety of different cytotoxic nucleosides. The origin and characterization of the CEM/hCNT2 line, which was produced by stable transfection of CEM/NTD with a vector containing a cDNA encoding hCNT2, are described elsewhere (Lang et al., 2001), and generation

of the CEM/hENT2 and CEM/hCNT1 transfectants was by a similar protocol (Lang et al., 2004). Efforts to produce CEM/hCNT3 transfectants have thus far been unsuccessful. All cultures were kept at 37°C in 5% CO_2/95% air and subcultured every 2 to 3 days to maintain exponential growth. Transport and cytotoxicity experiments were performed with actively proliferating cells.

Cytotoxicity Assays. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used to determine the cytotoxicity of Cl-dAdo, F-ara-A, and Cl-F-ara-A in cultured CEM cell lines. This colorimetric assay employs the tetrazolium compound MTS, which is bioreduced into a colored formazan product by metabolically active cells. The resulting absorbance was read at 490 nm with a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

Nucleoside Uptake Assays in Cultured Cells. Uptake of nucleosides by cells in suspension culture was measured using the oil-stop transport method (Harley et al., 1982) with the following modifications. Cells were harvested from actively proliferating cultures and resuspended in "transport buffer" (20 mM Tris, 3 mM K_2HPO_4, 144 mM NaCl, 1 mM $MgCl_2$, and 1.2 mM $CaCl_2$, pH 7.4) with 5 mM glucose. The assays were conducted at 22°C in microcentrifuge tubes. ^{3}H-labeled nucleoside permeants (100 μl in transport buffer) were layered over 200 μl of oil (silicone/light mineral oil, 1.03 g/ml). Uptake intervals were initiated by the addition of 100 μl of cells (2×10^6 cells) and terminated by the addition of ice-cold dilazep (final concentration 100 μM), a potent inhibitor of ENT1 and ENT2-mediated transport, followed by centrifugation of the cells through the oil layer. The ^{3}H-labeled permeant was then removed by aspiration, and the tubes were washed twice with distilled H_2O. After the final wash of the samples, all water and most of the oil layers were removed, 5% Triton X-100 (250 μl) was added, and the pellets were solubilized for at least 2 h. The microcentrifuge tubes and contents were placed in scintillation vials, Ecolite (6 ml) was added, and the samples were assayed for radioactive content by liquid scintillation counting. Uptake at time 0 was determined by incubation of the cells with ice-cold transport buffer that contained 100 μM dilazep followed by addition of ^{3}H-permeant and immediate centrifugation of the cells through oil. Intracellular volumes of pellets were determined by subtraction of the extracellular volume (determined with [^{14}C]polyethylene glycol or [^{14}C]sucrose) from the total pellet volume (determined with [^{3}H]H_2O). Uptake values were then calculated as picomoles per microliter of cell H_2O or picomoles per 10^6 cells, and graphs were generated using the Prism software (GraphPad, San Diego, CA). Initial rates of uptake, which define transport rates, were calculated from the initial (linear) portions of uptake time courses.

Production of Recombinant Transporters and Measurement of Radioisotope Uptake in *Xenopus laevis* Oocytes. T3, T7, and SP6 polymerases were used to transcribe linearized plasmids, in the presence of the m7GpppG cap, using the MEGAscript transcription system (Ambion, Austin, TX). Remaining template was removed by DNAase 1 digestion. Oocytes, prepared as described previously (Yao et al., 2002), were then microinjected with either 20 nl of water alone (control) or 20 nl of water containing 20 ng of RNA transcripts. Unless otherwise indicated, uptake of [^{3}H]Cl-F-ara-A was measured 3 days after injection. Uptake assays were performed at room temperature on groups of 10 to 12 oocytes in medium containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.5. After incubation, extracellular radioactivity was removed by six washes in ice-cold medium, and individual oocytes were dissolved in 5% SDS (w/v) for quantitation of oocyte-associated ^{3}H by liquid scintillation counting (LS 6000 IC; Beckman Coulter, Fullerton, CA). Initial rates of uptake (transport) were obtained from the linear portions of uptake time courses. Kinetic (K_m and V_{max}) parameters ± S.E. were determined using ENZFITTER software (Elsevier-Biosoft, Cambridge, UK).

Production of Recombinant Transporters and Measurement of Uptake in *Saccharomyces cerevisiae*. hENT1, hENT2, and hCNT3 cDNAs were inserted into the yeast expression vector pYPGE15 and the resulting plasmids were transformed into fui1: TRP1 (MATα, gal, ura3–52, trp1, lys2, ade2, hisd2000, Δfui:TRP1) (Zhang et al, 2003; Vickers et al., 2004). Uptake of [^{3}H]Cl-F-ara-A and [^{3}H]adenosine into yeast was measured as described previously for uptake of [^{3}H]uridine (Zhang et al., 2003). Yeast cells were grown in CMM/GLU media to an A_{600} of 0.8 to 1.5. They were then washed three times with fresh media and resuspended to an A_{600} of 4, after which 100 μl of yeast suspension was 1) mixed with 100 μl of CMM/GLU containing the appropriate ^{3}H-labeled nucleoside to initiate uptake intervals, and 2) loaded into 96-well plates. The 96-well plates were placed (individually) in a semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway), and uptake intervals were terminated at timed intervals by harvesting the yeast cells onto binding-enhanced filtermats (Molecular Devices). Extracellular radioactivity was removed by washing with distilled, deionized water. The filter discs were then placed into individual scintillation counting vials (Fisher Scientific Co., Pittsburgh, PA), and 5 ml of scintillation counting fluid (EcoLite) was added. Samples were shaken at room temperature overnight before counting. Inhibition of [^{3}H]adenosine uptake by Cl-F-ara-A, Cl-dAdo, and F-ara-A was measured using a similar method. Yeast suspensions (100 μl) were mixed with 100 μl of CMM/GLU containing [^{3}H]adenosine and the inhibiting compound (Cl-F-ara-A, Cl-dAdo, or F-ara-A). Uptake intervals were terminated as described above. Experiments were performed at least in triplicate and kinetic parameters, and IC_{50} values were calculated using GraphPad Prism software.

Results

Cytotoxicity of Cl-dAdo, F-ara-A, and Cl-F-ara-A against Human Cells with a Single Equilibrative or Concentrative Nucleoside Transport Process. Because the cytotoxic actions of anticancer nucleoside drugs are intracellular, the presence or absence of mediated transport systems will affect the pharmacological activity of nucleoside drugs that do not penetrate cells readily by diffusion (Cass, 1995). Cultured cells that are nucleoside transport-deficient, either through mutation or pharmacological blockade by nucleoside transport inhibitors, show resistance to the actions of a variety of cytotoxic nucleoside analogs (Clarke et al., 2002).

Cytotoxicity experiments were first carried out with hENT1 to determine its impact on sensitivity of CEM cells to graded concentrations of Cl-dAdo, F-ara-A, or Cl-F-ara-A. The MTS cytotoxicity assay was used and cytotoxicity (calculated as percentage of control) was determined after 24-, 48-, and 72-h exposures. The percentage of control versus log micromolar concentration curves for 24-h exposures of CEM/hENT1 and CEM/NTD cells are depicted graphically in Fig. 1, where it is evident that the presence of hENT1 had a large impact on cytotoxicity compared with the nucleoside transport-deficient cells. Calculated IC_{50} values for CEM/hENT1 and CEM/NTD, respectively, for 24-h exposures were 1 and 39 μM for Cl-F-ara-A, 6 and >100 μM for Cl-dAdo, and 33 and >100 μM for F-ara-A. After 48- and 72-h incubations with Cl-F-ara-A, the IC_{50} values for all of the cell lines were in the range of 1 μM, suggesting that cellular uptake by passive diffusion was an important contributor to cytotoxicity during prolonged exposures.

Concentration-effect relationships were examined for CEM/hENT2, CEM/hCNT1, and CEM/hCNT2 to assess the relative effects of hENT2, hCNT1, and hCNT2 on cytotoxicity of Cl-F-ara-A, Cl-dAdo, and F-ara-A during 24-, 48-, and 72-h exposures; studies with hCNT3 were not undertaken because stable transfectants with only hCNT3 were not available.

Because the concentration-effect relationships for the transport-deficient cells (see Fig. 1) and several of the cell line-drug combinations (data not shown) exhibited <50% reductions in percentage of control values, IC_{50} values were difficult to compare among the cell lines. To allow a more accurate comparison of the cytotoxicity of the three drugs against the five cell lines, the results obtained for 24-h exposures to a single pharmacologically relevant concentration (i.e., 10 μM) of Cl-F-ara-A, Cl-dAdo, and F-ara-A are presented in Table 1. Of the three drugs, Cl-F-ara-A exhibited the greatest potency against the five cell lines, with percentage of control values for CEM/hENT1, /hCNT2, /hCNT1, /hENT2, and /NTD of 16, 24, 44, 55, and 61%, respectively. Overall, the cell line with hENT1 was the most sensitive to all three analogs, with percentage of control values for Cl-F-ara-A, Cl-dAdo, and F-ara-A, respectively, of 16, 30, and 82.

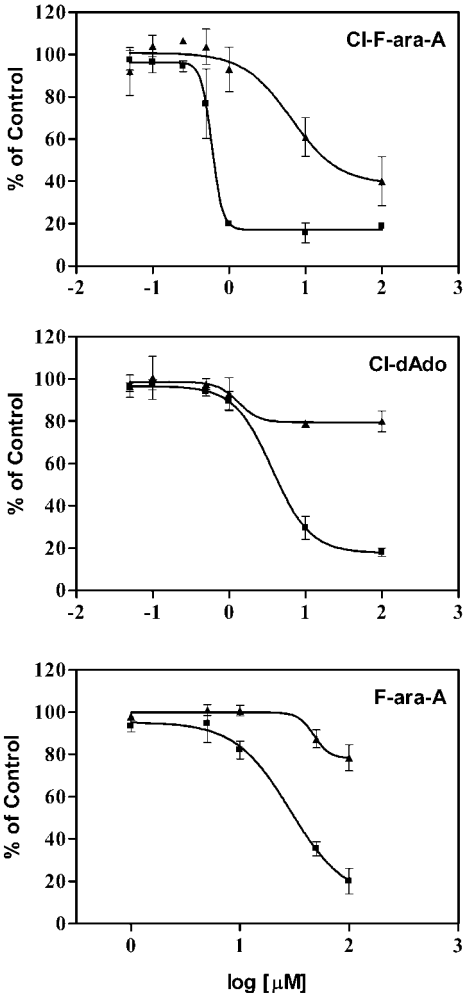

Fig. 1. Chemosensitivities of CEM cell lines (CEM/hENT1 and CEM/NTD) to Cl-F-ara-A, Cl-dAdo, and F-ara-A. Actively proliferating CEM/hENT1 cells, which possess a single nucleoside transport activity (■), and CEM/NTD cells, which lack nucleoside transport activity (▲), were exposed to graded concentrations of Cl-F-ara-A, Cl-dAdo, and F-ara-A for 24 h. The MTS assay, described under *Materials and Methods*, was used to determine cytotoxicity. Chemosensitivity was expressed as the absorbance of drug-treated cells compared with the absorbance of untreated (control) cells (measured by absorbance at 490 nm, which is directly proportional to the number of living cells in the culture). The values shown in the graphs are the means of at least three separate experiments and IC_{50} values were calculated from the resultant graphs.

Transportability of Cl-F-ara-A, Cl-dAdo, and F-ara-A by the Human Equilibrative and Concentrative Nucleoside Transporters. The wild-type CEM cell line (CEM/hENT1), which exhibits only hENT1-mediated transport activity, is one of the few examples of a human cell type in which there is a single endogenous nucleoside transport activity; most cell types examined thus far possess multiple nucleoside-transport activities (Crawford et al., 1990). The other transport-competent CEM lines used in this study were produced by stable transfection of CEM/NTD with the cDNAs of either hENT2, hCNT1, or hCNT2 (Lang et al., 2001, 2004), thereby producing cell lines that each had a single transporter type. A stable transfectant with hCNT3 has not yet been produced.

The initial rates of uptake of 10 μM radiolabeled Cl-F-ara-A by the individual human transporters are shown in Fig. 2. CEM/hENT1 and CEM/hCNT2 were the only cell lines that showed an initial rate of uptake above background. The uptake of Cl-F-ara-A was abolished by addition of excess unlabeled Cl-F-ara-A in these two cell lines, indicating that uptake was mediated (data not shown).

hENT1-mediated uptake of 10 μM Cl-F-ara-A, Cl-dAdo, and F-ara-A was measured in CEM/hENT1 cells over 5 min (Fig. 3), where it is evident that Cl-F-ara-A accumulation was higher than that of either Cl-dAdo or F-ara-A. Initial rates of uptake of graded concentrations of Cl-F-ara-A, Cl-dAdo, and F-ara-A by CEM/hENT1 cells were also determined and the

TABLE 1

Cytotoxicity of Cl-F-ara-A, Cl-dAdo and F-ara-A in CEM/hENT1, CEM/hENT2, CEM/hCNT1, CEM/hCNT2, and CEM/NTD

Cells were incubated with 10 μM Cl-F-ara-A, Cl-dAdo, and F-ara-A for 24 h, and the MTS assay described under *Materials and Methods* was used to determine cytotoxicity (expressed as the percentage of control (untreated) cells remaining after drug exposures). Values are the means ± S.D. of eight replicates within a representative experiment.

	Cl-F-ara-A	Cl-dAdo	F-ara-A
		%	
CEM/hENT1	16 ± 8	30 ± 8	82 ± 7
CEM/hENT2	55 ± 8	87 ± 8	82 ± 19
CEM/hCNT1	44 ± 2	61 ± 3	74 ± 14
CEM/hCNT2	24 ± 6	42 ± 9	94 ± 6
CEM/NTD	61 ± 16	79 ± 3	100 ± 4

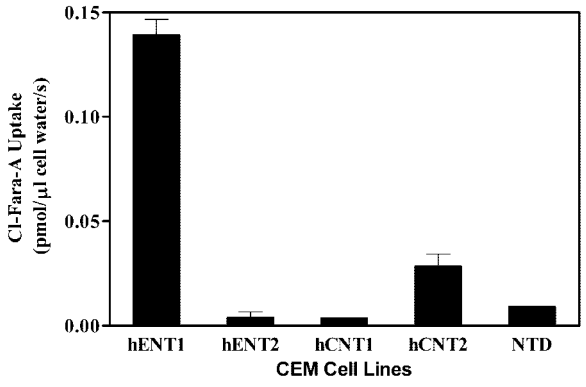

Fig. 2. Uptake of Cl-F-ara-A into CEM/hENT1, CEM/hENT2, CEM/hCNT1, CEM/hCNT2, and CEM/NTD cells. Cells were harvested from actively proliferating cultures, centrifuged and resuspended in transport buffer and uptake of 10 μM [^{3}H]Cl-F-ara-A over 5 min was determined using the oil-stop procedure as described under *Materials and Methods*. Initial rates were calculated from the linear portion of the time courses (up to 10 s). Data shown are the means of at least two separate experiments for each cell line.

resultant kinetic parameters were calculated (Table 2). The efficiency of transport (V_{max}/K_m) of Cl-dAdo was greater than that of either Cl-F-ara-A or F-ara-A (1.8 compared with 0.7 and 0.8).

The *X. laevis* oocyte expression system was used to compare inward fluxes of 10 μM [^{3}H]Cl-F-ara-A by recombinant hENT1, hENT2, hCNT1, hCNT2, and hCNT3. Uptake of both [^{3}H]Cl-F-ara-A and [^{3}H]uridine (used as a control permeant) was observed over a 30-min period (Fig. 4) into hENT1-, hENT2-, hCNT1-, hCNT2-, and hCNT3-producing oocytes. The highest accumulation of Cl-F-ara-A was observed in the hCNT3-producing oocytes. Negligible amounts of Cl-F-ara-A accumulated in hCNT1-producing oocytes, consistent with the pyrimidine-nucleoside selectivity of hCNT1. Additional experiments were therefore conducted with the hENT1-, hENT2-, hCNT2-, and hCNT3-producing oocytes to determine kinetic constants for transport of Cl-F-ara-A (Fig. 5 and Table 3). The K_m value of 52 μM for hCNT3-mediated transport of Cl-F-ara-A was lower than those for all other transporters, indicating that hCNT3 has higher affinity for Cl-F-ara-A than the other transporter types. The V_{max} values for hENT2- and hCNT3-mediated transport of Cl-F-ara-A were identical (66 pmol/oocyte/min) whereas the V_{max} values for hENT1- and hCNT2-mediated transport of Cl-F-ara-A were close to 12 pmol/oocyte/min. hCNT3 displayed the highest efficiency of transport of Cl-F-ara-A.

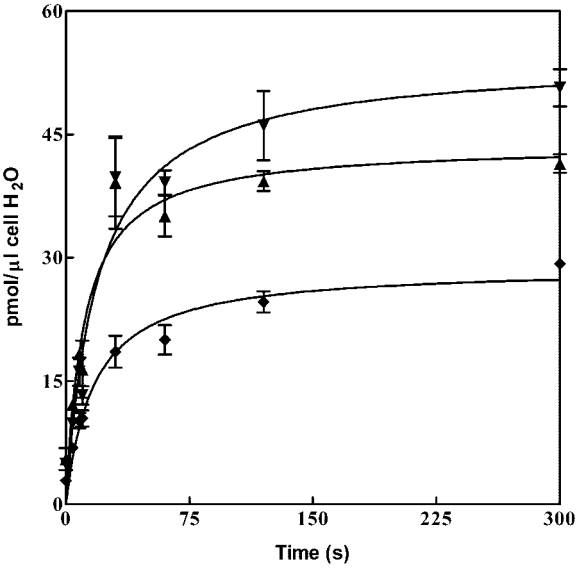

Fig. 3. Comparison of uptake of Cl-F-ara-A, Cl-dAdo, and F-ara-A into CEM/hENT1 cells. CEM/hENT1 cells were harvested from actively proliferating cultures, centrifuged, and resuspended in transport buffer. Uptake of 10 μM [^{3}H]Cl-F-ara-A (▼), [^{3}H]Cl-dAdo (▲), and [^{3}H]F-ara-A (♦) over 5 min was determined at the indicated times using the oil-stop procedure described under *Materials and Methods*. Values (mean ± S.D) are from one representative experiment.

TABLE 2

Kinetics of Cl-F-ara-A, Cl-dAdo, and F-ara-A transport by CEM/hENT1

Cells were harvested from actively proliferating cultures by centrifugation. Transport assays were conducted using the oil-stop transport procedure as described under *Materials and Methods*. Initial transport rates were calculated for up to six substrate concentrations and the data were subjected to Lineweaver-Burk kinetic analyses (GraphPad Prism) to obtain kinetic constants. The K_m and V_{max} values for Cl-F-ara-A and Cl-dAdo (means ± S.D.) shown are from at least three experiments. The values for F-ara-A are from two experiments.

Compound	K_m	V_{max}	V_{max}/K_m
	μM	*pmol/10^6 cells/s*	
Cl-F-ara-A	108 ± 10	72 ± 4	0.7
Cl-dAdo	23 ± 6	42 ± 3	1.8
F-ara-A	107	84	0.8

Comparison of Transportability and Binding of Cl-F-ara-A to Human Equilibrative and Concentrative Transporters Produced in Yeast. Interaction of Cl-F-ara-A with the human transporters was assessed in a strain of *S. cerevisiae* deficient in nucleoside transport into which cDNAs encoding the nucleoside transporters were introduced. Transport was demonstrated in yeast producing either hENT1, hENT2, or hCNT3 by incubating the yeast strains with 1 μM [^{3}H]Cl-F-ara-A and measuring uptake over 30 min using the semiautomated cell harvester method described under *Materials and Methods*. The initial trans-

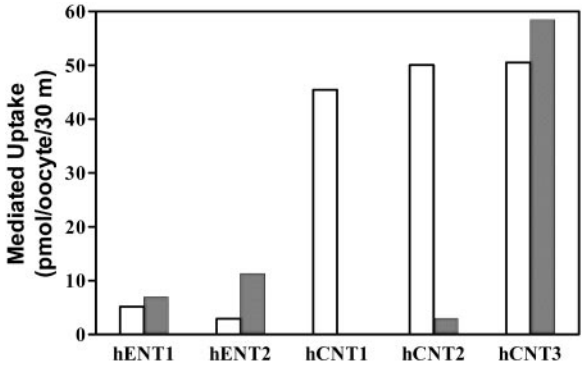

Fig. 4. Uptake of Cl-F-ara-A and uridine into *X. laevis* oocytes producing recombinant transporters hENT1, hENT2, hCNT1, hCNT2, and hCNT3. Oocytes were microinjected with 20 nl of water alone or with the mRNA transcripts of either CEM/hENT1, hENT2, hCNT1, hCNT2, or hCNT3 and then incubated at 18°C for 72 h. Uptake of 10 μM [^{3}H]Cl-F-ara-A (■) and [^{3}H]uridine (□) was measured in oocytes over 30 min and plotted as picomoles per oocyte per 30 min.

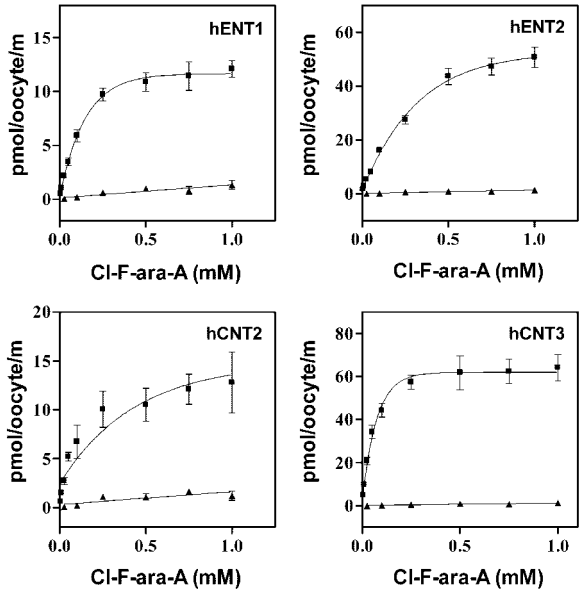

Fig. 5. Kinetics of Cl-F-ara-A uptake by human nucleoside transporters produced in *X. laevis* oocytes. Oocytes were microinjected with 20 nl of water alone or with mRNA transcripts of either CEM/hENT1, hENT2, hCNT2, or hCNT3 and then incubated at 18°C for 72 h. Flux measurements of [^{3}H]Cl-F-ara-A (0 to 1 mM) were performed over 5 min, and the oocytes were processed as described under *Materials and Methods*. The graphs show the influx of Cl-F-ara-A in either RNA-injected oocytes (■) or water-injected oocytes (▲). The corresponding kinetic parameters are given in Table 3.

port rates obtained were 0.4, 0.3, and 5.1 pmol/mg of protein/min for hENT1, hENT2, and hCNT3, respectively. Transport rates for recombinant hCNT2 were not obtained because the expression level of hCNT2 cDNA in the yeast strain employed for these experiments was very low. Studies were not conducted with hCNT1 because this transporter is selective for pyrimidine nucleosides.

The yeast expression system was also used to provide a measure of the relative binding affinities of the transporters for the three drugs in the experiments of Table 4, in which inhibition of recombinant hENT1-mediated adenosine transport in yeast by graded concentrations of Cl-F-ara-A, Cl-dAdo, and F-ara-A was determined. The K_i values for Cl-F-ara-A, Cl-dAdo, and F-ara-A were estimated from the resulting IC_{50} values and K_m values for adenosine transport by hENT1, hENT2, and hCNT3 produced in the yeast expression system by the Cheng and Prusoff (1973) equation. The transporters exhibited the highest affinity for Cl-F-ara-A, with K_i values of 22, 17, and 1 μM for hENT1, hENT2, and hCNT3, respectively. To ensure that deamination of adenosine was not a problem in these experiments, adenosine was incubated with the yeast for 40 min, and the resulting metabolites were resolved by high-performance liquid chromatography on a C-18 column; deamination was not observed (data not shown).

Discussion

Nucleoside analogs have applications in the treatment of both cancers and viral diseases. Cl-F-ara-A is a purine nucleoside that has activity in adult and pediatric leukemias. It is structurally related to Cl-dAdo and F-ara-A, two purine nucleosides that have generated interest because of their activity in the chemotherapy of the indolent lymphoid malignancies. Cl-F-ara-A shows cytotoxicity to proliferating and nonproliferating cells through the same mechanisms as Cl-dAdo and F-ara-A. Because the pharmacological targets of these analogs are intracellular, the presence of nucleoside transport processes in plasma membranes, as well as the permeant selectivities of these processes, may be important determinants of their cytotoxicity. This study was undertaken to define the role of nucleoside transport in the cytotoxicity of Cl-F-ara-A and to compare Cl-F-ara-A transport to Cl-dAdo and F-ara-A transport.

The first system examined was the panel of cultured human cell lines possessing a single nucleoside transport process either naturally (CEM/hENT1) or through stable transfection of transporter cDNAs into a nucleoside transport-deficient cell line (CEM/hENT2, CEM/hCNT1, and CEM/hCNT2). A cell line with only hCNT3 activity was not available. Cytotoxicity experiments showed that cells possessing only hENT1 were more sensitive to Cl-F-ara-A than to Cl-dAdo or F-ara-A. As well, the cells possessing hENT1 were more sensitive to all three compounds than the cells that were nucleoside transport-deficient (CEM/NTD), demonstrating the importance of transporter activity for cytotoxicity, at least during 24-h exposures. At higher drug concentrations and longer exposure times, the differences in cytotoxicity among the cell lines were much less apparent, suggesting that there was some contribution of diffusion to drug accumulation and cytotoxicity. However, because Cl-F-ara-A is administered clinically as a 1-h infusion daily for 5 days (Cooper et al., 2004) and plasma pharmacokinetic studies performed in rats showed three phases of elimination, with half-lives of 0.3, 1.3, and 12.8 h (Bonate et al., 2005), it is likely that transport activity is an important determinant of cytotoxicity in vivo.

X. laevis oocytes lack the capacity for endogenous transport of nucleosides (Huang et al., 1994) and individual recombinant nucleoside transporters can therefore be studied in the absence of background activity. Transport of Cl-F-ara-A was assessed in *X. laevis* oocytes producing each of the five human transporters. Initial rates of uptake of Cl-F-ara-A by hENT1, hENT2, hCNT2, and hCNT3 were saturable and conformed to Michaelis-Menten kinetics, with apparent K_m values, respectively, of 114, 328, 81, and 52 μM. The efficiency of transport (V_{max}/K_m) was the highest for recombinant hCNT3 (1.3). Although hENT1 demonstrated a poor efficiency of transport (0.11) in the oocyte studies, its presence conferred sensitivity of cultured CEM cells to Cl-F-ara-A, Cl-dAdo, and F-ara-A. Mediated uptake of Cl-F-ara-A was not observed in oocytes producing hCNT1, consistent with the known selectivity of this transporter for pyrimidine nucleosides.

The yeast expression system, which allows quantitative analysis of interactions of nucleoside analogs of interest with recombinant nucleoside transporters (Zhang et al., 2003), was used to determine the relative binding affinities of hENT1, hENT2, and hCNT3 for Cl-F-ara-A, Cl-dAdo, and F-ara-A through their ability to inhibit [^{3}H]adenosine transport. The demonstrated EC_{50} and calculated K_i values indicated that all three transporters exhibited higher affinities for Cl-F-ara-A than for either Cl-dAdo or F-ara-A. hCNT3 exhibited the highest affinity of all the transporters for Cl-F-ara-A, with a K_i value of 1 μM.

TABLE 3

Kinetics of Cl-F-ara-A transport by recombinant human nucleoside transporters expressed in *X. laevis* oocytes

X. laevis oocytes producing recombinant hENT1, hENT2, hCNT2, or hCNT3 were prepared and inward fluxes of graded concentrations of [^{3}H]Cl-F-ara-A were measured and analyzed as described under *Materials and Methods*. The kinetic parameters were calculated using ENZFITTER software.

Transport Process	K_m	V_{max}	V_{max}/K_m
	μM	*pmol/oocyte/min*	
hENT1	114 ± 12	12.3 ± 0.4	0.11
hENT2	328 ± 35	66.8 ± 2.8	0.20
hCNT2	81 ± 8	11.9 ± 0.3	0.15
hCNT3	52 ± 3	66.8 ± 0.8	1.3

TABLE 4

Inhibition of adenosine transport by Cl-F-ara-A, F-ara-A, and Cl-dAdo in *S. cerevisiae* producing recombinant human transporters

Recombinant transporters were produced in a transport-deficient strain of *S. cerevisiae* and the abilities of graded concentrations of Cl-F-ara-A, F-ara-A, and Cl-dAdo to inhibit transport of 1 μM [^{3}H]adenosine were determined, and the resulting IC_{50} values were used to calculate K_i values as described under *Materials and Methods*. K_m values for adenosine transport by hENT1 and hENT2 (18 and 106 μM, respectively) were determined previously (Visser et al. 2005), and the K_m value for adenosine transport by hCNT3 (3 μM) was determined in this work (data not shown).

	K_i		
	hENT1	hENT2	hCNT3
		μM	
Cl-F-ara-A	22	17	1
F-ara-A	61	168	353
Cl-dAdo	36	50	17

Although the current study suggested a potential relationship between the presence of functional nucleoside transport processes and the pharmacological activity of Cl-F-ara-A, F-ara-A, and Cl-dAdo, the exact extent of this relationship will be difficult to determine. Cellular accumulation of these compounds represents the combined effects of permeation across plasma membranes (mediated transport plus diffusion) and subsequent metabolism. Cl-F-ara-A, F-ara-A, and Cl-dAdo are all activated by deoxycytidine kinase, the kinetic parameters of which have been previously compared (Parker et al., 1999). The efficiencies of phosphorylation, relative to that of deoxycytidine, were 87, 23, and 3.4 for Cl-F-ara-A, Cl-dAdo, and F-ara-A, respectively. Therefore, the relative cytotoxicity values obtained in this study reflected differences in both transport and metabolic activation of the three compounds.

Cl-F-ara-A was shown to be an efficient permeant of two of the transporters (hENT1 and hCNT3) and was more cytotoxic to cells having the hENT1 transport process than either Cl-dAdo or F-ara-A. Immunohistochemistry studies have been performed in human tumor tissue to determine the abundance of the various nucleoside transport proteins. hENT1 and hCNT3 seem to be the most abundant transporters and thus are likely to contribute importantly to the transport and cytotoxicity of nucleoside analogs. hENT3, the most recently identified member of the ENT family, has been shown to be associated with lysosomal/endosomal membranes (Baldwin et al., 2005). There is also evidence of the presence of nucleoside transport activity in the mitochondrial membrane (K. M. King, unpublished data). The association of nucleoside transporters with organellar membranes may also contribute to the cytotoxicity/toxicity of Cl-F-ara-A.

This study demonstrated that the presence of functional nucleoside transporters in cells is an important contributor to the pharmacological activity of Cl-F-ara-A, Cl-dAdo, and F-ara-A. Future studies are needed to define the relative importance of transport processes and metabolism in determining the anticancer effects of these compounds as well as the toxicities associated with them.

References

Adkins JC, Peters DH, and Markham A (1997) Fludarabine. An update of its pharmacology and use in the treatment of haematological malignancies. *Drugs* **53:**1005–1037.

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.

Belt JA, Marina NM, Phelps DA, and Crawford CR (1993) Nucleoside transport in normal and neoplastic cells. *Adv Enzyme Regul* **33:**235–252.

Bonate PL, Arthaud L, Stuhler J, Yerino P, Press RJ, and Rose JQ (2005) The distribution, metabolism and elimination of clofarabine in rats. *Drug Metab Dispos* **33:**739–748.

Carson DA, Wasson DB, Esparza LM, Carrera CJ, Kipps TJ, and Cottam HB (1992) Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine. *Proc Natl Acad Sci USA* **89:**2970–2974.

Carson DA, Wasson DB, Taetle R, and Yu A (1983) Specific toxicity of 2-chlorodeoxyadenosine toward resting and proliferating human lymphocytes. *Blood* **62:**737–743.

Cass CE (1995) Nucleoside transport, in *Drug Transport in Antimicrobial and Anticancer Chemotherapy* (Georgopapadakou NH ed) pp 403–451, Marcel Dekker, New York, NY.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Christensen LF, Broom AD, Robins MJ, and Bloch A (1972) Synthesis and biological activity of selected 2,6-disubstituted-(2-deoxy- and -D-erythro-pentofuranosyl)purines. *J Med Chem* **15:**735–739.

Clarke ML, Mackey JR, Baldwin SA, Young JD, and Cass CE (2002) The role of membrane transporters in cellular resistance to anticancer nucleoside drugs. *Cancer Treat Res* **112:**27–47.

Cooper T, Kantarjian H, Plunkett W, and Gandhi V (2004) Clofarabine in adult acute leukemias: clinical success and pharmacokinetics. *Nucleosides Nucleotides Nucleic Acids* **23:**1417–1423.

Crawford CR, Ng CY, Ullman B, and Belt JA (1990) Identification and reconstitution of the nucleoside transporter of CEM human leukemia cells. *Biochim Biophys Acta* **1024:**289–297.

Crawford CR, Patel DH, Naeve C, and Belt JA (1998) Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter ei by functional expression in a transport-deficient cell line. *J Biol Chem* **273:**5288–5293.

Griffig J, Koob R, and Blakley RL (1989) Mechanisms of inhibition of DNA synthesis by 2-chlorodeoxyadenosine in human lymphoblastic cells. *Cancer Res* **49:**6923–6928.

Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, and Baldwin SA (1997) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. *Biochem J* **328:**739–743.

Harley ER, Paterson AR, and Cass CE (1982) Initial rate kinetics of the transport of adenosine and 4-amino-7-(beta-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (tubercidin) in cultured cells. *Cancer Res* **42:**1289–1295.

Hentosh P, McCastlain JC, and Blakley RL (1991) Effects of 2-chloro-2′-deoxyadenosine 5′-triphosphate on DNA synthesis in vitro by purified bacterial and viral DNA polymerases. *Biochemistry* **30:**547–554.

Hirota Y, Yoshioka A, Tanaka S, Watanabe K, Otani T, Minowada J, Matsuda A, Ueda T, and Wataya Y (1989) Imbalance of deoxyribonucleoside triphosphates, DNA double-strand breaks and cell death caused by 2-chlorodeoxyadenosine in mouse FM3A cells. *Cancer Res* **49:**915–919.

Hoffman MA (1996) Cladribine for the treatment of indolent non-Hodgkin's lymphomas. *Semin Hematol* **33:**40–44.

Huang QQ, Yao SY, Ritzel MW, Paterson AR, Cass CE, and Young JD (1994) Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. *J Biol Chem* **269:**17757–17760.

Jeha S, Gandhi V, Chan KW, McDonald L, Ramirez I, Madden R, Rytting M, Brandt M, Keating M, Plunkett W, et al. (2004) Clofarabine, a novel nucleoside analog, is active in pediatric patients with advanced leukemia. *Blood* **103:**784–789.

Kantarjian H, Gandhi V, Cortes J, Verstovsek S, Du M, Garcia-Manero G, Giles F, Faderl S, O'Brien S, Jeha S, et al. (2003) Phase 2 clinical and pharmacologic study of clofarabine in patients with refractory or relapsed acute leukemia. *Blood* **102:**2379–2386.

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.

Montgomery JA and Hewson K (1969) Nucleosides of 2-fluoroadenine. *J Med Chem* **12:**498–504.

Montgomery JA, Shortnacy-Fowler AT, Clayton SD, Riordan JM, and Secrist JA 3rd (1992) Synthesis and biologic activity of 2′-fluoro-2-halo derivatives of 9-beta-D-arabinofuranosyladenine. *J Med Chem* **35:**397–401.

Parker WB, Shaddix SC, Chang CH, White EL, Rose LM, Brockman RW, Shortnacy AT, Montgomery JA, Secrist JA 3rd, and Bennett LL Jr (1991) Effects of 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)adenine on K562 cellular metabolism and the inhibition of human ribonucleotide reductase and DNA polymerases by its 5′-triphosphate. *Cancer Res* **51:**2386–2394.

Parker WB, Shaddix SC, Rose LM, Shewach DS, Hertel LW, Secrist JA 3rd, Montgomery JA, and Bennett LL Jr (1999) Comparison of the mechanism of cytotoxicity of 2-chloro-9-(2-deoxy-2- fluoro-β-D-arabinofuranosyl)adenine, 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine, and 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl)adenine in CEM cells. *Mol Pharmacol* **55:**515–520.

Piro LD (1996) Cladribine in the treatment of low-grade non-Hodgkin's lymphoma. *Semin Hematol* **33:**34–39.

Plunkett W, Huang P, and Gandhi V (1990) Metabolism and action of fludarabine phosphate. *Semin Oncol* **17:**3–17.

Plunkett W and Saunders PP (1991) Metabolism and action of purine nucleoside analogs. *Pharmacol Ther* **49:**239–268.

Qian M, Wang X, Shanmuganathan K, Chu CK, and Gallo JM (1994) Pharmacokinetics of the anticancer agent 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine in rats. *Cancer Chemother Pharmacol* **33:**484–488.

Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, Ritzel RG, Mowles DA, Carpenter P, Chen XZ, 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 MW, 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.

Robertson LE, Chubb S, Meyn RE, Story M, Ford R, Hittelman WN, and Plunkett W (1993) Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2′-deoxyadenosine and 9-beta-D-arabinosyl-2-fluoroadenine. *Blood* **81:**143–150.

Saven A and Piro LD (1994) 2-Chlorodeoxyadenosine: a newer purine analog active in the treatment of indolent lymphoid malignancies. *Ann Intern Med* **120:**784–791.

Seto S, Carrera CJ, Kubota M, Wasson DB, and Carson DA (1985) Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to nondividing human lymphocytes. *J Clin Investig* **75:**377–383.

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, 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.

Wang J, Su SF, Dresser MJ, Schaner ME, Washington CB, and Giacomini KM (1997) Na^+-dependent purine nucleoside transporter from human kidney: cloning and functional characterization. *Am J Physiol* **273:**F1058–F1065.

Wang L, Karlsson A, Arner ES, and Eriksson S (1993) Substrate specificity of mitochondrial 2′-deoxyguanosine kinase. Efficient phosphorylation of 2-chlorodeoxyadenosine. *J Biol Chem* **268:**22847–22852.

Waud WR, Schmid SM, Montgomery JA, and Secrist JA 3rd (2000) Preclinical antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)adenine (Cl-F-ara-A). *Nucleosides Nucleotides Nucleic Acids* **19:**447–460.

Yao SY, Ng AM, Vickers MF, Sundaram M, Cass CE, Baldwin SA, and Young JD (2002) Functional and molecular characterization of nucleobase transport by recombinant human and rat equilibrative nucleoside transporters 1 and 2. Chimeric constructs reveal a role for the ENT2 helix 5–6 region in nucleobase translocation. *J Biol Chem* **277:**24938–24948.

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 Avenue, Edmonton, AB, Canada, T6G 1Z2. E-mail: carol.cass@cancerboard.ab.ca