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

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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-9-(2’-deoxy-2’-fluoroadenine (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 deoxyctydine 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 synthesis by inhibition of DNA polymerase α. The cytotoxicity of Fludarabine is due to inhibition of DNA polymerase α, with lesser effects on RNA polymerase II.

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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.

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synthesis by Cl-dATP inhibition of ribonucleotide reductase and DNA polymerases α and β with resultant dNTP pool imbalances and DNA strand breaks (Griffith 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-Hodgkin 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 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). [8-3H]Cl-dAdo, [8-3H]-F-ara-A, and [8-3H]-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). [1,2-14C]-Polyethylene glycol, [14C]-succrose, and [8-3H]-H2O 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 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 thiocytidine, 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% CO2/95% 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 K2HPO4, 144 mM NaCl, 1 mM MgCl2, and 1.2 mM CaCl2, pH 7.4) with 5 mM glucose. The assays were conducted at 22°C in microcentrifuge tubes. 3H-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 × 106 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 3H-labeled permeant was then removed by aspiration, and the tubes were washed twice with distilled H2O. 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 3H-permeant and immediate centrifugation of the cells through oil. Intracellular volumes of pellets were determined by subtraction of the extracellular volume (determined with [14C]polyethylene glycol or [14C]sucrose) from the total pellet volume (determined with [3H]H2O). Uptake values were then calculated as picomoles per microliter of cell H2O or picomoles per 106 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 [3H]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 CaCl2, 1 mM MgCl2, and 10 mM HEPES, pH 7.5. After incubation, extracellular radioactivity was removed by washes in ice-cold medium, and individual oocytes were dissolved in 5% SDS (w/v) for quantitation of oocyte-associated 3H 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 (Km and Vmax) parameters ± S.E. were determined using ENZFITTER software (Elsevier-Biosoft, Cambridge, UK).

**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/NMD 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 intracellular transport-deficient cells. Calculated IC50 values for CEM/hENT1 and CEM/NMD, 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 IC50 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\textsubscript{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 \mu M) 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.

**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 \mu M 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 \mu M 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

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

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**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\textsubscript{50} values were calculated from the resultant graphs.

**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 \mu M [\textsuperscript{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 \( \frac{V_{\text{max}}}{K_m} \) 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 \( \mu \)M \(^{3}H\)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 per- meant) 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 \( \mu \)M 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 \( V_{\text{max}} \) values for hENT2- and hCNT3-mediated transport of Cl-F-ara-A were identical (66 pmol/oocyte/min) whereas the \( V_{\text{max}} \) 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.

**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 \( \mu \)M \(^{3}H\)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. 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 \( \mu \)M \(^{3}H\)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 \( V_{\text{max}} \) 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_m ) (( \mu )M)</th>
<th>( V_{\text{max}} ) (pmol/10(^6) cells/s)</th>
<th>( V_{\text{max}}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-F-ara-A</td>
<td>108 ± 10</td>
<td>72 ± 4</td>
<td>0.7</td>
</tr>
<tr>
<td>Cl-dAdo</td>
<td>23 ± 6</td>
<td>42 ± 3</td>
<td>1.8</td>
</tr>
<tr>
<td>F-ara-A</td>
<td>107</td>
<td>84</td>
<td>0.8</td>
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</table>

**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 \( \mu \)M \(^{3}H\)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 calculated using ENZFITTER software. The 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 $\mu$M 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 $\mu$M. The efficiency of transport ($V_{\text{max}}/K_m$) 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 $[^3H]$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 $\mu$M.

**Table 3**

<table>
<thead>
<tr>
<th>Transport Process</th>
<th>$K_i$ ($\mu$M)</th>
<th>$V_{\text{max}}$ (pmol/oocyte/min)</th>
<th>$V_{\text{max}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hENT1</td>
<td>114 ± 12</td>
<td>12.3 ± 0.4</td>
<td>0.11</td>
</tr>
<tr>
<td>hENT2</td>
<td>328 ± 35</td>
<td>66.8 ± 2.8</td>
<td>0.20</td>
</tr>
<tr>
<td>hCNT2</td>
<td>81 ± 8</td>
<td>11.9 ± 0.3</td>
<td>0.15</td>
</tr>
<tr>
<td>hCNT3</td>
<td>52 ± 3</td>
<td>66.8 ± 0.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

$K_i$ values for adenosine transport by hENT1 and hENT2 ($18$ and $106$ $\mu$M, respectively) were determined previously (Visser et al., 2005), and the $K_m$ values for adenosine transport by hCNT3 ($53$ $\mu$M) was determined in this work (data not shown).

**Table 4**

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

<table>
<thead>
<tr>
<th>$K_i$ ($\mu$M)</th>
<th>hENT1</th>
<th>hENT2</th>
<th>hCNT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-F-ara-A</td>
<td>22</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>F-ara-A</td>
<td>61</td>
<td>168</td>
<td>353</td>
</tr>
<tr>
<td>Cl-dAdo</td>
<td>36</td>
<td>50</td>
<td>17</td>
</tr>
</tbody>
</table>

Recombinant transporters were produced in a transport-deficient strain of *S. cerevi-
siae* and the abilities of graded concentrations of Cl-F-ara-A, F-ara-A, and Cl-dAdo to inhibit transport of 1 $\mu$M $[^3H]$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 $\mu$M, respectively) were determined previously (Visser et al., 2005), and the $K_m$ value for adenosine transport by hCNT3 ($53$ $\mu$M) was determined in this work (data not shown).
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


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