ABSTRACT

Gemcitabine (2',2'-difluorodeoxycytidine) is a novel pyrimidine nucleoside drug with clinical efficacy in several common epithelial cancers. We have proposed that gemcitabine requires nucleoside transporter (NT) proteins to permeate the plasma membrane and to exhibit pharmacological activity. In humans, there are seven reported distinct NT activities varying in substrate specificity, sodium dependence, and sensitivity to inhibition by nitrobenzylthioguanosine (NBMPR) and dipyridamole. To determine which NTs are required for gemcitabine-dependent growth inhibition, cultures from a panel of 12 cell lines with defined plasma membrane NT activities were incubated with different concentrations of gemcitabine. Cell proliferation was assessed by the sulfonhomadamine B assay and cell enumeration to identify the concentrations of gemcitabine that inhibited cell replication by 50% (IC50). NT activity was a prerequisite for growth inhibition in vitro because: (a) the nucleoside transport-deficient cells were highly resistant to gemcitabine; and (b) treatment of cells that exhibited only equilibrative NT activity with NBMPR or dipyridamole increased resistance to gemcitabine by 10- to 1800-fold. These data suggested that the type of NT activities possessed by a cell may be an important determinant of its sensitivity to gemcitabine and that NT deficiency may confer significant gemcitabine resistance. We analyzed the uptake kinetics of [3H]gemcitabine by each of five human NT activities in cell lines that exhibited a single NT activity in isolation; transient transfection of the cDNAs encoding the human concentrative NT proteins (hCNT1 and hCNT2) was used to study the cit and cif activities, respectively. The efficiency of gemcitabine uptake varied markedly among the cell lines with single NTs: es = cit > ei > cif > db > df. The transportability of [3H]gemcitabine was demonstrated by reconstitution of the human es NT in proteoliposomes, confirming that gemcitabine permeation is a protein-mediated process.

INTRODUCTION

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC,

Gemzar) is a novel pyrimidine nucleoside anticancer drug with demonstrated clinical activity in common epithelial cancers. Unlike other nucleoside anticancer drugs used in clinical practice [cytarabine (1-β-D-arabinofuranosylimidazole), cladribine (chloroethylnucleosine), and fludarabine (fluorouracildehyde)], gemcitabine has demonstrated significant activity in Phase II and Phase III clinical trials in patients with advanced breast (1, 2), bladder (3), ovarian (4), non-small-cell lung (5, 6), pancreatic (7, 8), and head and neck cancers (9). The mechanism(s) accounting for the unique and broad spectrum of gemcitabine activity has not been fully determined. Although gemcitabine and cytarabine both require phosphorylation to produce the corresponding toxic triphosphates, dFdC and ara-C, the cellular pharmacology of gemcitabine differs from that of cytarabine, with relatively prolonged intracellular accumulation of gemcitabine triphosphate (10). dFdC can be incorporated into DNA, which is believed to be the main mechanism of cytotoxicity (11, 12). Gemcitabine is a substrate for deoxythidine deaminase, producing the noncytotoxic compound 2',2'-difluorodeoxuryridine (12).

For unknown reasons, some human tumors exhibit de novo gemcitabine resistance [as shown by low response rates in clinical trials of patients with advanced colon cancers (13)], whereas other initially sensitive cancers may develop resistance during therapy. The in vitro mechanisms of resistance to gemcitabine therapy are not defined, although several in vitro mechanisms of gemcitabine resistance have been identified. Gemcitabine resistance was induced by exposing A2780 human ovarian carcinoma cells to stepwise increases in gemcitabine concentration. The identified mechanism of resistance was a deficiency in deoxycytidine kinase, the enzyme necessary for the activation of gemcitabine to gemcitabine monophosphate (14). Transfection of CEM human leukemia cells with a vector that forced expression of cytidine deaminase conferred a 2.4-fold increase in gemcitabine resistance (15). Gemcitabine efficacy was decreased in fibrosarcoma cells induced by transfection to produce large quantities of heat shock protein 70 (16). Resistance to gemcitabine was not associated with increased P-glycoprotein expression and was not seen in cell lines resistant to alkylating agents and cisplatinum (17). Plasma membrane transport has been studied in Chinese hamster ovary cells, where membrane transport did not appear to be a major factor contributing to the more potent cytotoxicity of gemcitabine when compared with cytarabine (10).

The biochemical targets for gemcitabine are intracellular, and the obligatory first step in cytotoxicity is permeation across the plasma membrane. Because gemcitabine, like other anticancer nucleosides, is hydrophilic and would not be expected to readily permeate plasma membranes by passive diffusion, its cellular uptake requires the presence of specialized plasma membrane NT proteins (18). Seven distinct NT activities have been demonstrated in human cells. Nucleoside transport occurs by both sodium-independent, bidirectional equilibrative (ENT) and sodium-dependent, inwardly directed concentrative (CNT) processes: es (broad permeant selectivity); ei (broad selectivity), cif (purine-nucleoside selectivity); cs (concentrative, NBMPR-insensitive, guanosine selectivity); and cs (concentrative, NBMPR-insensitive, unknown selectivity). cDNAs encoding four human NTs have recently been cloned and functionally expressed: hENT1, which displays es activity,

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2 To whom requests for reprints should be addressed, at Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta, T6G 1Z2 Canada.

3 The abbreviations used are: dFdC, gemcitabine; CEM, CCRF-CEM; NT, nucleoside transporter; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; NBMPR, nitrobenzylmercaptopurine ribonucleoside (nitrobenzylthioguanosine); ei, equilibrative NBMPR-insensitive; es, equilibrative NBMPR-sensitive; cif, concentrative, insensitive to NBMPR and formycin B-selective; cib, concentrative, insensitive to NBMPR and thymidine selective; csg, concentrative, insensitive to NBMPR and broadly selective; OCTG, 2′,3′-dideoxyguanosine; h, human; NBTPG, nitrobenzylmercaptopurine guanine ribonucleoside (nitrobenzylthioguanosine).
GEMCITABINE CYTOTOXICITY AND NUCLEOSIDE TRANSPORT

(19); hENT2, which displays 
activity (20, 21); hCNT1, which displays 
activity (22); and hSPNT, or in our terminology, hCNT2, which displays 
activity (23).4 The equilibrative NTs appear to be widely distributed in different cell types and tissues, whereas the concentrative NTs appear to be limited to specialized cells such as intestinal and renal epithelia, liver, and leukemic cells. It is likely that different human tissues and cell lines vary widely in NT activities, due to variations in the number and type of NT proteins expressed on the plasma membrane (24).

To determine whether gemcitabine resistance could be correlated with nucleoside transport capabilities, we examined the gemcitabine sensitivity of 12 murine and human cell lines with defined nucleoside transport characteristics. To study the effect of pharmacological manipulation of nucleoside transport on gemcitabine-dependent inhibition of proliferation, the transport inhibitors NBMPR and dipyridamole were used to specifically inhibit, respectively, es NT activity or both es and ei NT activities. To further define the transportability of gemcitabine, we analyzed the uptake kinetics of [3H]gemcitabine by five human nucleoside transporters acting in cell lines exhibiting a single NT activity in isolation. To confirm the mediated character of [3H]gemcitabine permeation in a noncellular system, the human es NT was solubilized from membranes of cultured human BeWo choriocarcinoma cells and functionally reconstituted in proteoliposomes, which were used to study [3H]gemcitabine uptake.

MATERIALS AND METHODS

Materials. [3H]Gemcitabine was a kind gift of Eli Lilly, Inc. (Indianapolis, Indiana). [3H]NBMPR (22.5 Ci/mmol) and [5,6-3H]Uridine (40 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Radiolabeled nucleosides were purified by high-pressure liquid chromatography using water-methanol gradients on a C18 reverse phase column; repurification of [3H]gemcitabine was essential because some preparations contained 3H degradation products. [3C]Cholesterol oleate (0.1 mM/ml) was obtained from Amersham Canada, Ltd. (Oakville, Ontario, Canada). Asolectin (soybean phospholipids) was purchased from Associated Concentrates (Woodside, NY) and stored under N2. Other phospholipids and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). OCTG, adenosine, uridine, NBMPR, and dipyridamole were purchased from Sigma Chemical Co., and Sephadex G-50 (fine and medium) was from Pharmacia, Canada. Dilazep ([N1-N'-bis(3,4,5-trimethoxy-benzoyloxy)-propyl] homopiperazine) was a gift from F. Hoffman La Roche and Co. (Basel, Switzerland). All other chemicals were of analytical grade and commercially available.

Cell Culture. Murine cell lines were grown in RPMI and 10% horse serum, and human cell lines were cultured in RPMI 1640 and 10% fetal bovine serum as described elsewhere (25–27). Experiments were initiated with cells in exponential growth phase. S49 mouse lymphoma cells were used to study [3H]gemcitabine uptake selected using the de novo uridylate synthesis inhibitor Brequinar in the presence of the salvageable nucleoside uridine. The L1210/DU-5 cell line was cloned from this selection and had a single copy of cNT1 inserted into its genome (33). K562 is a human erythroid leukemia cell line that possesses es and ei transport activities (27). CEM is a human T-lymphoblast cell line with es transport activity originally derived from a patient with acute lymphocytic leukemia (34). The NT-defective ARAC-8C cell line is a clonal derivative of a hypoxanthine-guanine phosphoribosyltransferase-deficient CEM cell line originally isolated by mutagen exposure and selection for resistance to 8 µM cytarabine. For the present investigations, the absence of hypoxanthine-guanine phosphoribosyltransferase, a purine-metabolizing enzyme, was not pertinent to the metabolism or cytotoxicity of gemcitabine (35). CaCo-2 is a human colon carcinoma cell line that possesses es, ei, and cif activities (24). HeLa is a human cervical carcinoma cell line that possesses es and ei activities (36, 37).

Chemosensitivity Testing. The chemosensitivity of suspension cell lines was assessed using the sulforhodamine B assay, essentially as described previously (38, 39). Cells were seeded in multiples of eight wells in 96-well cell culture plates, with ~20,000 cells/well, then exposed to drug. Drug exposures were performed in growth media with 10% horse serum for murine cell lines and 10% fetal bovine serum for human cell lines. Gemcitabine was added to final concentrations that ranged from 10−6 to 10−9 M. Cells were enumerated with flow cytometry using a single cell counter after 72 h of drug exposure. Cytotoxicity was assessed by trypan blue exclusion and cell enumeration with a computer cell counter after 72 h of drug exposure. Chemosensitivity was expressed as the concentration of gemcitabine that inhibited cell proliferation by 50% (IC50), as determined by nonlinear regression analysis using GraphPad Prism 2.01 (GraphPad Software, San Diego, CA). All experiments were performed independently on two occasions, and mean IC50 values are reported.

Generation of Expression Constructs. For expression in HeLa cells, the full coding sequences of hCNT1 (22) and hCNT2 were removed from the original cloning vector pGEM3Z by AfrII/XbaI digestion. Subsequently, the 5' overhang was blunt-ended with the Klenow fragment and ligated into pCDNA-Amp and ligated into the vector at the XhoI site. The 3' ends of hCNT1 and hCNT2 were ligated into the vector at the XhoI site. The structures of pCNDNA/Amp-hCNT1 and pCNDNA/Amp-hCNT2 were confirmed by restriction mapping and sequence analysis using an ABI Prism 310 Genetic Analyser (Norwalk, CT).

Transient Expression of hCNT1 and hCNT2 in HeLa Cells. The transport characteristics of recombinant hCNT1 (cot activity) and hCNT2 (cif activity) were studied using a method described previously (40) for recombinant hCNT1 produced in COS-1 cells. HeLa cells were plated (5.0 × 10⁴ per 100-mm plate) and grown to 50% confluency in RPMI with 10% calf serum. Transfection was performed by a modification of the DEAE-dextran method (40) in which cells in DMEM and 10% NuSerum with 200 µM chloroquine (5 µM) were exposed to 100 µM of DEAE-dextran DNA mixture, using 5 µg of DNA per plate. Cells were incubated at 37°C in 5% CO2 for 2 h, then 2 ml of DMEM 10% solution were added. Plates were incubated for 2 min at room temperature, washed three times with PBS, and placed in growth media for 24 h. Cells were then trypsinized, pooled, and replated on 60-mm plates to determine any differences in transfection efficiencies among the individual cultures. Cells were used for uptake assays 72 h after transfection. Transfection efficiencies (determined by counting stained and unstained cells in cultures that had been independently transfected with β-galactosidase) ranged from 15% to 30%. Cells were treated with 10 µM dilazep to inhibit endogenous es- and ei-mediated NT activity prior to uptake assays.

[3H]Gemcitabine and [3H]Uridine Cellular Uptake Assays. Nucleoside uptake assays were conducted at room temperature under zero-trans conditions in either sodium-containing transport buffer (20 mM Tris/HCl, 3 mM K2HPO4, 1 mM MgCl2,6H2O, 2 mM CaCl2, 5 mM glucose, and 130 mM NaCl, pH 7.4; 300 ± 15 µM) or sodium-free transport buffer (20 mM Tris/HCl, 3 mM K2HPO4, 1 mM MgCl2,6H2O, 2 mM CaCl2, 5 mM glucose, and 130 mM N-methyl-D-glucamine/HCl, pH 7.4; 300 ± 15 µM). Cells were washed once with the appropriate transport buffer and then processed immediately, or in some experiments, incubated with NBMPR or dilazep at room temperature for 30 min before the uptake assay. For suspension cell lines (27), each sample was processed individually, and permeant fluxes were terminated using the 3-phenylphosphono-1,2,4-oxadiazole-5-one (28).

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GEMCITABINE CYTOTOXICITY AND NUCLEOSIDE TRANSPORT

Table 1 Gemcitabine chemosensitivity in cell lines with defined NT activities

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>es</th>
<th>ei</th>
<th>cfi</th>
<th>cif</th>
<th>IC50 (μM)</th>
<th>Relative gemcitabine resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>S49</td>
<td>Murine lymphoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.013</td>
<td>1.0</td>
</tr>
<tr>
<td>AEI</td>
<td>S49 NT-deficient mutant</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.009</td>
<td>1.0</td>
</tr>
<tr>
<td>L1210</td>
<td>Murine leukemia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.0087</td>
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<td>L1210 mutant</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0.0058</td>
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<tr>
<td>DU-5</td>
<td>L1210 rCNT1 stable transfectant</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0.070</td>
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<td>1.0</td>
</tr>
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<td>L1210 NT-deficient mutant</td>
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<td>+</td>
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<td>K562</td>
<td>Erythroid leukemia</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>K562 + dipyridamole</td>
<td>Erythroid leukemia</td>
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<td>+</td>
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<td>1.0</td>
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<td>CEM</td>
<td>Lymphoblastic leukemia</td>
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<td>+</td>
<td>0.032</td>
<td>1.0</td>
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<tr>
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<td>Lymphoblastic leukemia</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>4.4</td>
<td>267</td>
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<tr>
<td>CEM-ARAC8C</td>
<td>NT-deficient CEM mutant</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>8.7</td>
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<tr>
<td>HeLa</td>
<td>Cervical cancer</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0.020</td>
<td>1.0</td>
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<tr>
<td>HeLa + NBMPR</td>
<td>Cervical cancer</td>
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<td>+</td>
<td>+</td>
<td>0.039</td>
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<tr>
<td>HeLa + dipyridamole</td>
<td>Cervical cancer</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0.023</td>
<td>39</td>
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<tr>
<td>CaCo-2</td>
<td>Colon cancer</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0.036</td>
<td>1.0</td>
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<td>CaCo-2 + NBMPR</td>
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<td>1.1</td>
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<tr>
<td>CaCo-2 + dipyridamole</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0.0041</td>
<td>1.3</td>
</tr>
</tbody>
</table>

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of reconstitution buffer with 20 μM 3H-labeled permeant. After specific incubation times, 100 μl of the reaction mixtures were layered on ice-cold Sephadex minicolumns (1-ml syringes fitted with a polyethylene filter), equilibrated in reconstitution buffer containing 10 μM dipyridamole and NBMPR, and centrifuged (45 s, 700 × g). The effluents were collected in preweighed tubes from which portions were removed for the determination of protein, 3H, and 14C contents. Estimates of zero-time uptake values were obtained by measuring the uptake of 3H at =2 s in the presence of ice-cold solutions containing 10 mM adenosine, 10 μM dipyridamole, and 10 μM NBTGR. Protein was measured using BSA as the standard with appropriate corrections for detergent interference (42, 45).

RESULTS

Growth Inhibition Studies. We have compared the ability of gemcitabine to inhibit proliferation of murine and human cancer cell lines exhibiting a spectrum of NT activities. In the experiments summarized in Table 1, cells were exposed to graded concentrations of gemcitabine: (a) for 48 h (suspension cultures) and quantitated using the sulforhodamine B assay (38); or (b) for 72 h (adherent cultures) and quantitated by trypsinization and electronic enumeration. Chemosensitivity was expressed as the concentration of gemcitabine that inhibited cell proliferation by 50% (IC50), and results are summarized in Table 1. We first compared chemosensitivity in pairs of cell lines that were either transport competent (parental line) or transport incompetent (mutant line) in experiments similar to the one shown in Fig. 1 for CEM, a human T-lymphoblast cell line with es transport activity (34), and ARAC-C8, a NT-incompetent clonal derivative that was isolated by mutagen exposure and selection for resistance to 8 μM cytarabine (35). The ARAC-C8 cell line was highly gemcitabine resistant with an IC50 exceeding 100 μM compared with an IC50 of 0.031 μM for the CEM line (Fig. 1). The other transport incompetent cell lines produced genetically by mutation (AE1, DNC-3) were also significantly less sensitive to gemcitabine than the matched parental cell lines (S49, L1210). The IC50 of gemcitabine in the transport-incompetent cell lines, when related to the IC50 in parental cell lines, increased by 118-fold to greater than 3200-fold. The high levels of resistance seen in transport-incompetent cell lines indicated that uptake of gemcitabine in the absence of functional NTs in the plasma membrane was inefficient and suggested that diffusional uptake of gemcitabine through the plasma membrane was capable of producing growth-inhibitory concentrations inside cells only in the presence of large transmembrane concentration gradients.

To determine the contributions of the two bidirectional equilibrative NT activities to the antiproliferative action of gemcitabine, we next examined the growth-inhibitory effects of gemcitabine in three human cancer cell lines (K562, CEM, and HeLa) in the presence and absence of pharmacological inhibitors of es and ei (Table 1). At the concentrations used in these studies, NBMPR (100 μM) selectively inhibits es-mediated transport, whereas dipyridamole (10 μM) inhibits both es- and ei-mediated transport. Although NBMPR exposures conferred 164-fold resistance to gemcitabine in CEM cells, which exhibit es NT activity in isolation, NBMPR was not capable of protecting HeLa cells, which exhibit both es and ei NT activities (26). In contrast, dipyridamole exposures conferred 39-fold resistance in HeLa cells, indicating that ei-mediated uptake alone was sufficient to achieve intracellular concentrations of gemcitabine that inhibited proliferation. Dipyridamole increased gemcitabine resistance by 39- to >1800-fold in cell lines with only equilibrative NT activities (K562, CEM, and HeLa), demonstrating that pharmacological inhibition of NT activities can significantly modulate the antiproliferative action of gemcitabine.
In addition to the equilibrative NTs described above, mammalian cells possess sodium-coupled inwardly directed concentrative NT activities (18). To determine the contributions of the concentrative NTs to gemcitabine activity, we examined gemcitabine-induced growth inhibition in cell lines that exhibited a single concentrative NT activity (cit, cuf, or cif; Table 1).

The cit NT exhibits sodium dependence, selectivity for pyrimidine-nucleosides, and insensitivity to inhibition by NBMPR, and cit NT activity has been observed in intestinal enterocytes, brush-border membrane vesicles of several species, and rabbit choroid plexus (18). Because cit activity has not yet been demonstrated in cultured cell lines, we used L1210/DU-5, a stable transfectant that produces recombinant rat CNTT1 (32) in the transport-incompetent background of L1210/DNC3. L1210/DU-5 cells exhibit sodium-dependent transport of thymidine that is inhibited by the pyrimidine nucleosides uridine and cytidine but not by the purine nucleosides inosine or guanosine (33). Gemcitabine inhibited proliferation of DU-5 cells (IC50 0.092 μM) in contrast to the parental, transport-incompetent DNC-3 cells (IC50 13 μM), suggesting that the cit transporter mediated gemcitabine influx.

The cif NT exhibits sodium dependence, selectivity for purine-nucleosides, and uridine, and insensitivity to inhibition by NBMPR, and cif NT activity has been observed in mouse splenocytes, macrophages, rat macrophages, and hepatocytes and in several cultured cell types, including mouse L1210 leukemia cells (18). In the experiment of Fig. 2, we examined gemcitabine inhibition of growth in CaCo-2 cells that were treated with dipyridamole to block endogenous es and ei activities. There were no differences in the responses to gemcitabine between untreated and inhibitor-treated cells. It has been shown previously that dipyridamole enhanced the toxicity of 9-β-D-arabinofuranosyladenine against the L1210/C2 cell line (47), which has es, ei, and cif NT activities (30). Although the initial rate of drug uptake was reduced by dipyridamole, there was an intracellular accumulation of drug above the steady-state intracellular concentration because of the elimination of drug efflux via the bidirectional equilibrative transporters. That dipyridamole treatment of CaCo-2 cells did not increase gemcitabine sensitivity suggested that release of gemcitabine back into the growth medium via the bidirectional equilibrative NTs was not a major component of net gemcitabine retention.

4H]Gemcitabine and 4H]Uridine Cellular Uptake Assays. To maximize the potential clinical relevance of this work, gemcitabine transport by each of the human NTs was examined individually, either in transport-competent cell lines or in human cell lines transiently transfected with cloned human NT cDNAs. The transport-incompetent CEM-Ara-C-8C cell line was studied first to establish whether gemcitabine was capable of entry into cells in the absence of a functional nucleoside transporter (i.e., by diffusion). No significant 4H]gemcitabine uptake occurred (Fig. 3), because uptake rates did not significantly vary from zero. The parental cell line CEM, from which CEM-AraC8C was derived, possesses only es activity, with
4.3 ±1.3 pmol/s/10^6 cells (mean ± SE of three independent experiments)

...transfection of the hCNT2 cDNA into HeLa cells had no effect on uptake of [^3H]gemcitabine, although the expected [^3H]uridine uptake was observed (Fig. 5B). This result was consistent with the observed gemcitabine resistance in L1210/MA27 cells, which exhibit only the purine-nucleoside selective cfp NT.

Analysis of cip-dependent transport of gemcitabine was difficult. A human cDNA encoding a sodium-dependent NT with broad permeant selectivity has not yet been reported, and the CaCo-2 cell line exhibits relatively low levels of endogenous cib activity in addition to es and ei activities (24). Uptake of [^3H]gemcitabine was examined (not shown) in CaCo-2 cells in the presence of 10 μM dilazep to block es and ei activities. Gemcitabine entry was mediated by the cip transporter, but uptake rates were too low for kinetic analysis. The demonstration of cip-mediated gemcitabine uptake explains our earlier observation (Fig. 2) of high-level gemcitabine activity in CaCo-2 despite the presence of inhibitors of equilibrative NT.

Transport efficiency can be expressed as K_m/V_max, where low values indicate efficient transporter-mediated permeation. The values of Table 2 indicate that the es (K_m/V_max, 20.1) and ei (K_m/V_max, 22.8) NT activities were the most efficient mediators of gemcitabine influx, followed by the ei (K_m/V_max, 215) NT activity. The cip NT did not mediate permeation of gemcitabine across plasma membranes.

Because uridine is accepted as a permeant by all of the reported human NTs, it is considered to be a "universal permeant" and was chosen as a reference nucleoside with which to compare gemcitabine uptake rates. To allow an assessment of efficiency of gemcitabine uptake among the human NTs at the therapeutically and physiologically relevant concentration of 10 μM, uptake rates of both gemcitabine and uridine were determined for each of the human NTs (Table 3). Relative uptake rates (gemcitabine/uridine) were 0.12 for es, 0.26 for ei, 0.10 for cif, and 0.58 for cib (there was no gemcitabine uptake for cip). Thus, although gemcitabine was transported by four of the five NTs, the physiological substrate uridine was transported by all five with much greater efficiency. In the HeLa cell line, known to possess both es and ei activities (26), the relative contributions to initial [^3H]gemcitabine uptake at a concentration of 10 μM were 72.4 and 27.6%, respectively (Table 4), indicating that the major component of gemcitabine influx was mediated by the es NT.

[^3H]Gemcitabine and [^3H]Uridine Uptake by Proteoliposomes Prepared from Detergent-solubilized Membrane Fractions Enriched in NBMPR-binding Activity. The representative experiment shown in Fig. 6 examined the initial rates of uptake of 20 μM[^3H]gemcitabine by proteoliposomes prepared from BeWo cells by
sucreose-density centrifugation and examined in the presence and absence of a mixture of inhibitors (adenosine, NBTGR, and dilazep) of es- and ei-mediated transport. We have shown previously (41) that proteoliposomes so prepared exhibit only es NT activity, despite the presence of both es and ei activities in BeWo crude membranes. The value from four separate experiments for the uninhibited initial rate of gemcitabine uptake was 20 ± 2 pmol/mg protein/s, and preincubation of proteoliposome with the mixture of transport inhibitors decreased this rate to 13 ± 2 pmol/mg proteins/s, or 65% of total uptake. The mediated component of [3H]gemcitabine uptake, which was obtained by subtracting the inhibited value from the total value, was 7 ± 2 pmol/mg proteins/s. In a separate study conducted similarly, the initial rate of uptake of 20 μM [3H]uridine (six separate experiments) was 45.0 ± 5.0 pmol/mg proteins/s (41). The ratio (0.15) of [3H]gemcitabine to [3H]uridine uptake rates by the reconstituted BeWo es NT was very similar to that (0.12) for the native es NT of CEM cells. These results support the use of functional reconstitution of individual human NT proteins as an appropriate means of studying human NT activities in isolation; this is the first report of gemcitabine uptake mediated by a reconstituted NT.

**DISCUSSION**

Although resistance to gemcitabine because of decreased drug influx has not been reported previously, there is evidence that nucleoside-transport deficiency may be a mechanism of resistance to anticancer nucleosides both in vitro and in vivo. Decreased es NT activity correlated with in vivo cytarabine resistance in human acute myelogenous leukemia (49). Resistance to cytarabine therapy has also been shown to be associated with decreased es activity in freshly isolated human leukemia cells (50, 51). Estimates of the numbers of es transporters have been inferred from quantitative analysis of cellular NBMPR binding. Decreases in NBMPR-binding sites have been observed during treatment of leukemia; in a patient with T-cell acute lymphoblastic leukemia, the leukemic blasts examined at relapse exhibited a 75% reduction in NBMPR binding when compared with blasts examined before initiation of cytarabine treatment (52). Murine erythroleukemia cells that were selected in vitro for resistance to cytotoxic purine nucleosides exhibited decreased levels of the es transporter, as indicated by a reduction in NBMPR-binding sites (53). Similarly, CEM T lymphoblasts exhibited a 75% decrease in 2',3'-dideoxycytidine sensitivity when transmembrane nucleoside transport activity was decreased by 80% (35).

The mechanisms of gemcitabine permeation have not been defined. The present study demonstrated that transport-incompetent cells, produced either by mutation or by pharmacological inhibition of NT activity, were resistant to gemcitabine inhibition, and in some cell lines, the lack of mediated uptake conferred greater than 1000-fold resistance to the antiproliferative action of gemcitabine. These observations suggested that passive diffusion of gemcitabine through plasma membranes was a relatively minor component of gemcitabine uptake. By studying gemcitabine-dependent inhibition of proliferation in a panel of murine and human cell lines with defined NT activities, we were able to infer that gemcitabine uptake was mediated by the es, ei, cit, and cif NTs but not by the cif NT. The es, ei, and cif NTs exhibit broad permeant selectivity, accepting both purine and pyrimidine nucleosides, whereas the cit and cif NTs accept uridine and exhibit selectivity, respectively, for pyrimidine and purine nucleosides (18).

To confirm the transportability of gemcitabine suggested by the growth-inhibition studies, we performed detailed kinetic studies of [3H]gemcitabine uptake in human cells. Initial rates of uptake were measured in cells in which NT activity was absent or in which only a single NT activity was present (either naturally or by transient transfection with the relevant cDNA). The results of the transport studies were entirely concordant with those of the growth-inhibition studies. Diffusion was not a measurable component of gemcitabine cellular influx, and gemcitabine uptake was mediated by several NT activities. However, the NT activities exhibited by the various cell lines varied markedly in their apparent efficiencies of gemcitabine permeation: es ≫ cit > ei > cif. Because the absolute numbers of functional transporters (which probably differ among the various cell types) could not be determined, it was not possible to directly compare the intrinsic transportability of gemcitabine by the different NTs.

Uridine is accepted as a permeant by all of the known human NTs and was therefore used as a standard with which to compare NT-mediated gemcitabine uptake. Initial uptake rates for a concentration (10 μM) that is therapeutically relevant for gemcitabine and physiologically relevant for uridine were determined for the five NT activities (see Table 3). The ratios of gemcitabine:uridine uptake rate varied among the NTs, and gemcitabine rates were consistently less than uridine rates for the es, ei, cit, and cif NTs. The cif NT, which mediates transport of uridine and purine nucleosides, including cladribine (54), did not mediate gemcitabine influx.

Because the equilibrative NTs are bidirectional, net gemcitabine uptake would be expected to represent the combined contributions of NT-mediated influx and efflux. Therefore, the antiproliferative action of gemcitabine might be potentiated by inhibitors of equilibrative transport, if a concentrative NT was also present. This possibility was explored in the CaCo-2 cell line, which possesses es, ei, and cif; however, dipyridamole exposure did not potentiate gemcitabine cytotoxicity, suggesting that drug efflux by the equilibrative NTs was not a major component of net gemcitabine retention.

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**Table 4** [3H]Gemcitabine uptake initial rates by HeLa cells in the presence and absence of inhibitors of nucleoside transport

<table>
<thead>
<tr>
<th>Uptake (no inhibitors)</th>
<th>Uptake (NBMPR)</th>
<th>Uptake (dilazep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.504</td>
<td>0.110</td>
<td>0</td>
</tr>
<tr>
<td>0.214</td>
<td>0.078</td>
<td>0</td>
</tr>
<tr>
<td>0.331</td>
<td>0.045</td>
<td>0</td>
</tr>
<tr>
<td>0.387</td>
<td>0.161</td>
<td>0</td>
</tr>
<tr>
<td>0.359 ± 0.060</td>
<td>0.090 ± 0.024</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 6. Time course of [3H]gemcitabine uptake by proteoliposomes prepared from detergent-solubilized membrane fractions from BeWo cells. Proteoliposomes were prepared from OCTG-solubilized membrane fractions that were isolated by density-gradient centrifugation in 25% sucrose as described in "Materials and Methods." The proteoliposomes were incubated with 20 μM [3H]gemcitabine for the indicated times in the presence (C, non-mediated uptake) or absence (B, total uptake) of 10 μM NBTGR, and 10 μM dipyridamole. Values for mediated uptake (●) were calculated from the difference between total and nonmediated uptake. Assays were conducted in duplicate and plots from one of four separate experiments are shown.

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The pharmacokinetic properties of gemcitabine suggest that efficient uptake of gemcitabine may be required for anticancer efficacy in vivo. Gemcitabine is typically administered to humans as an i.v. bolus infusion lasting 30 min, in doses ranging from 800 to 1200 mg/m². The most common dosage schedule is weekly administration on the exposed to significant gemcitabine concentrations for only short periods and raise the possibility that inefficient cellular uptake may be a mechanism underlying the observed resistance to gemcitabine in some solid tumors. Although gemcitabine was shown to be a substrate for four NTs, the major mediators of gemcitabine uptake in human tissues are probably the equilibrative NTs, because human cit activity has, thus far, only been demonstrated in kidney, liver, and intestinal epithelium (22, 57), and human cib activity has only been demonstrated in human myeloid leukemia cell lines, freshly isolated myeloblasts (46), and the colon cancer cell line used in this report, CaCo-2 (24). By implication, deficiency of equilibrative NT in tumor cells may be a mechanism of gemcitabine resistance in vivo. Given that both deoxycytidine kinase deficiency and NT deficiency confer high-level resistance to gemcitabine cytotoxicity in vitro, either mechanism could be the basis of the acquired or de novo resistance to gemcitabine observed in clinical trials.

In summary, we have demonstrated that functional NTs are required for manifestation of gemcitabine toxicity in vitro. Conversely, a deficiency in nucleoside-transport activity was capable of conferring high-level resistance to the toxicity of gemcitabine. We have performed detailed kinetic studies of gemcitabine uptake in human cell lines exhibiting a range of NT activities and have demonstrated marked variability in the capacity of the different NTs to mediate gemcitabine influx. Finally, we have demonstrated mediated uptake of gemcitabine by the human ex NT reconstituted in proteoliposomes. Although the potential for clinical application of these observations is high, efforts to rationally modulate the antitumor and normal tissue toxicities of gemcitabine must await a clearer understanding of the tumor and tissue distribution of the NT proteins. The recent cloning of cDNAs encoding the four major human NTs (19-23) has led to new molecular and immunological probes to study NT distribution which, in turn, may lead to transport-based strategies capable of improving the therapeutic indices of anticancer nucleosides.

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REFERENCES

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Functional Nucleoside Transporters Are Required for Gemcitabine Influx and Manifestation of Toxicity in Cancer Cell Lines

John R. Mackey, Rajam S. Mani, Milada Selner, et al.


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