Nucleoside Transporter Profiles in Human Pancreatic Cancer Cells: Role of hCNT1 in 2′,2′-Difluorodeoxycytidine-Induced Cytotoxicity

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ABSTRACT

Purpose: **Concentrative nucleoside transporter (CNT) 1, CNT3, equilibrative nucleoside transporter (ENT) 1, and, to a lesser extent, ENT2, appear to be the transporters responsible for 2′,2′-difluorodeoxycytidine (gemcitabine; Gemzar) uptake into cells. Gemcitabine is used currently in the treatment of pancreatic cancer, but the role of specific nucleoside carrier proteins in gemcitabine cytotoxicity has not been elucidated. Indeed, it is not known which nucleoside transporters are expressed in human pancreas.**

Experimental Design: **In this study we have used four cell lines [pancreatic neoplasia (NP)9, NP18, NP29, and NP31] derived from human pancreatic adenocarcinomas to monitor the pattern of nucleoside transporter expression, and we have heterologously expressed the high-affinity gemcitabine transporter human orthologue (h) CNT1 to monitor its role in drug responsiveness.**

Results: **All of the cell lines take up gemcitabine mostly via the hENT1 transporter, which is expressed at high levels. Reverse transcription-PCR analysis of the other four cloned plasma membrane transporter mRNAs revealed very different expression patterns among NP cell lines, with apparent selective loss or decrease of hCNT mRNAs. NP cells transiently express hCNT1-type Na^+-dependent nucleoside transport activity at low/medium cell density but not in confluent cultures. Cells expressing hCNT1 in a more constitutive manner were cloned after stable transfection of hCNT1. Despite high constitutive hENT1 activity, this increased sensitivity to gemcitabine.**

Conclusion: **In summary, human pancreatic adenocarcinoma cells overexpress hENT1, although they retain the ability to express a functional hCNT1 transporter, an isoform that confers sensitivity to gemcitabine.**

INTRODUCTION

2′,2′-Difluorodeoxycytidine (gemcitabine) is a fluoropyrimidine that is used currently in the treatment of solid tumors, particularly pancreatic, bladder, and non-small cell lung cancers (1, 2). Moreover, gemcitabine similarly is highly active as a single agent in the treatment of relapsed or refractory low-grade non-Hodgkin's lymphoma and, in combination with other antimetabolites, in lymphatic and myeloid malignancies (3, 4). Promising activity of this drug against head and neck, ovarian, and breast carcinomas has been also reported (5–7).

Gemcitabine, like other nucleoside-derived drugs, needs to be metabolized to exert its clinical action. Cytotoxicity is associated with the formation of the triphosphate-nucleoside analogue and the subsequent inhibition of DNA synthesis, as well as with the ability of the diphosphate form of the molecule, which is a potent inhibitor of ribonucleotide reductase, to induce depletion of cellular dNTP pools. The initial phosphorylation step requires the presence of deoxycytidine kinase, which is the rate-limiting enzyme in gemcitabine activation (8), although transport across the plasma membrane is also essential for drug action (9).

Predicting and overcoming resistance of tumor cells to chemotherapy are major challenges in cancer treatment. Resistance to gemcitabine may be a result of multiple factors and, thus, according to *in vitro* studies, it has been suggested that deficiency in deoxycytidine kinase, increased deamination, increased dCTP pools, and decreased influx into the cell may contribute to impaired drug responsiveness (9–13). Indeed, the role of plasma membrane transporters in gemcitabine cytotoxicity has been addressed by monitoring drug uptake and sensitivity in a variety of established lymphoid cell lines with defined nucleoside transporter activities (9). Most nucleoside-derived drugs, such as gemcitabine, may be substrates of NTs[2] (14). There are two major families of NTs: CNT and ENT. Two ENT plasma membrane transporters have been cloned thus far, ENT1

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[2] The abbreviations used are: NT, nucleoside transporter; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; NP, pancreatic neoplasia; NBTI, nitrobenzylthioinosine; WT, wild-type; PC, transfected with vector alone; HC1, transfected with hCNT1; h, human orthologue; FBS, fetal bovine serum; poly(A)$^+$ RNA, polyadenylated RNA; RT-PCR, reverse transcription-PCR; FACS, fluorescence-activated cell sorter.

and ENT2. The former is inhibited by nanomolar concentrations of NBTI, whereas the latter is insensitive to this inhibitor. Similarly, three CNT isoforms have been cloned and functionally characterized: CNT1, which is pyrimidine-preferring, CNT2, which is purine-preferring, and CNT3, which shows broad specificity for both purines and pyrimidines. Gemcitabine is taken up by hCNT1 with high affinity (17–18 μM; Refs. 9, 15), whereas hENT1 also recognizes this fluoropyrimide as a substrate albeit at a much higher apparent K_m (0.33 mM; Ref. 9). hCNT3 also appears to accept gemcitabine as a substrate, although no kinetic parameters for this interaction have been reported (16).

Gemcitabine has shown efficacy against pancreatic cancer. The drug is administered i.v., reaching plasma concentrations in the low micromolar range (17, 18). At present, it is not known whether pancreatic tumors express low and/or high affinity nucleoside transporters, nor has the putative role of specific carrier isoforms in gemcitabine action been addressed.

Here we examine these issues using a panel of cell lines derived from human adenocarcinomas of the pancreas (19, 20). We show that these cells mostly expressed hENT1, and retained the ability to express hENT2 and some of the hCNT isoforms. Interestingly, CNT-type transport activity was transiently detected in all of the analyzed cell clones but only at low and medium cell density. Heterologous expression of hCNT1 in stably transfected cell clones resulted in hCNT1-type transport activity even in confluent monolayers, thus conferring sensitivity to gemcitabine.

MATERIALS AND METHODS

Pancreatic Cell Lines. Four cell lines, NP9, NP18, NP29, and NP31, derived from human adenocarcinomas of the pancreas (19, 21) were used. They were established and kindly provided by Dr. Gabriel Capellà (Institut Català d'Oncologia, Catalonia, Spain). Among many other cellular and genetic properties of these cell lines, NP9, NP18, and NP31 express a mutated p53, whereas NP18 is the only cell line to express WT p16 and K-*ras*. In contrast to healthy human pancreas, the four cell lines overexpress the epidermal growth factor receptor and transforming growth factor α (19). NP9 and NP29 were grown in DMEM, and NP18 and NP31 in RPMI 1640, both supplemented with 10% FBS and L-glutamine. Cells were maintained as monolayer cultures at 37°C in an atmosphere with 5% CO_2 and subcultured every 4–5 days. Cells were counted routinely using trypan blue staining (Sigma) and a Neubauer chamber, except for the drug sensitivity assay (see below).

Nucleoside Transport in Human Pancreatic Cell Lines. [^{3}H]uridine (Amersham, Buckinghamshire, United Kingdom) and [^{3}H]gemcitabine (Moravek, Brea, CA) were used as tracers in the uptake measurements. Transport was measured, as described (22), at substrate concentrations ranging from 0.5 μM to 1 mM for kinetic determinations. Other experiments were routinely performed using 1 μM uridine and gemcitabine, and putative inhibitors at 100 μM, except for NBTI, which was used at 1 μM to dissect both NBTI-sensitive and -insensitive components of transport (except for the kinetic experiments for which NBTI was used at 10 μM). Transport measurements were performed as indicated elsewhere (22) in either a 137 mM NaCl or a 137 mM choline medium [these media also contained 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4)]. When the desired incubation time had elapsed, the monolayers were washed three times in 2 ml of a cold buffer composed of 137 mM NaCl and 10 mM HEPES (pH 7.4). The cells were then dissolved in 0.5 ml of 0.5% Triton X-100, and 0.4 ml aliquots were sampled for radioactivity counting. Aliquots of 10–20 μl were also sampled for protein determination, according to the Bradford reaction (Bio-Rad Laboratories). To calculate the kinetic parameters of nucleoside uptake, rates were fitted to Michaelis-Menten kinetics using the software Grafit (Erithacus Software, Ltd.).

Total RNA Isolation, Poly(A)+Purification, cDNA Synthesis, and PCR. Total RNA was isolated from the pancreatic cell lines using the guanidinium thiocyanate method, as described (23). Poly(A)$^+$ RNA was purified from total RNA using the Poly(A)$^+$ tract mRNA isolation kit from Promega. cDNA was then synthesized using the PCR-related reverse transcription system (Promega, Madison, WI), and the cDNA/mRNA hybrids were treated with RNase H. Finally, the whole reaction was cleaned up by using the Wizard DNA clean-up system (Promega) before isoform-specific PCR amplification. The oligos used for hENT1, hENT2, hCNT1, and hCNT2 amplification were the following: hENT1: 5′-gcttgaaggacccggggagc-3′ and 5′-tggagaaggcaaaggcagcca-3′; hENT2: 5′-tcccaggcccaagctcagga-3′ and 5′-ggaaccgcaggcagaccagc-3′; hCNT1:5′-ctgtgtgggtcctcaccttcctg-3′ and 5′-ggagagggccaaggcacaaggg-3′; hCNT2: 5′-caaaggccagagcagctgatc-3′ and 5′-ctttacccccctcctcactctt-3′; and hCNT3: 5′-gaaacatgtttgactacccacag-3′ and 5′-gtggagttgaaggcattctctaaaacgt-3′, derived from the published cDNA sequences (24–27). The PCR reaction was set up by mixing (final concentrations) the following: 1× Taq Polymerase buffer, 1.5 mM $MgCl_2$, 2.2 mM each dNTP, 0.4 μM each F1/R1 oligo, the cDNA, and water to 50 μl of final volume. Fifty μl of mineral oil was added onto the reaction. The reaction mixture was heated to 94°C for 5 min and then cooled 80°C. Subsequently, 2.5 units of Taq Polymerase were added. The PCR conditions were as follows: 1 min, 94°C; 1 min 55°C (for hENT 1 and hENT 2 amplification) or 50°C (for hCNT1 and hCNT2 amplification) or 52°C (for hCNT3 amplification); and 3 min, 72°C for 40 cycles. Finally, the PCR was heated to 72°C for 15 min and cooled to 4°C. The samples were then run in a 1% agarose gel [45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.0)]. The expected sizes of the PCR products were 0.5 kb for hENT1, 0.43kb for hENT2, 0.8kb for hCNT1, 0.61kb for hCNT2, and 0.48kb for hCNT3.

Heterologous Expression of hCNT1 in NP-9 Cells. The hCNT1 clone was obtained by amplification of an oligodeoxythymidylic acid-primed cDNA library from human fetal liver (28). The cDNA was then subcloned into a pCDNA vector and transfected into NP-9 cells using Fugene (Sigma, St. Louis, MO). Putative hCNT1-expressing clones were selected using geneticin treatment, and several independent clones were checked for both activity and expression. On the basis of its sodium-dependent uridine transport, one clone of six was chosen for additional experiments (HC1). WT (nontransfected) NP-9 cells and cells transfected with the empty pCDNA (PC1) vector were used as controls in the activity experiments.

hCNT1-related activity was measured in several indepen-

dent clones by monitoring uptake of 10 μM [^{3}H]uridine (Amersham), as described above.

Expression of hCNT1 was also assessed by flow cytometry using a monospecific polyclonal antibody raised in our laboratory, showing specificity for the NH_2 terminus intracellular domain of the hCNT1 protein (28). Cell monolayers were grown on six-well plates and fixed with 0.5% *p*-formaldehyde solution for 5 min at 4°C, and then trypsinized 2′ at room temperature. After pelleting cells by centrifugation, cells were washed once with 1 ml PBS-1% FBS, again pelleted, and then blocked with 50 μl 3% BSA in PBS supplemented with 1% FBS at 4°C. Then cells were incubated for 45–60′ at 37°C with 50 μl of a dilution 1:50 of primary antibody in PBS-1% FBS-3% BSA, then washed by the addition of 1 ml of PBS-1% FBS and pelleted for incubation 45–60′ at 37°C with 50 μl of a dilution 1:40 of an antirabbit IgG coupled to fluorescein as second antibody (Progenetics). After one last wash in 1 ml of PBS-1% FBS, cells were resuspended in PBS- 1% FBS and ready to test for expression of hCNT1 using a FACS (Epics Elite Flow Cytometer).

Gemcitabine Cytotoxicity. NP-9, PC1, and HC1 cell lines were seeded at a density ranging from 5,000 to 10,000 cells/cm^2 on multiwell culture plates. Twenty four h after initiation of the culture, cells were exposed to increasing concentrations of gemcitabine (from 2 nM to 2 μM) for 90 min. Cultures were then allowed to proceed for 72 h, and remaining cells were counted (Coulter Multisizer). The relatively short exposure to gemcitabine (90 min) may be closer to clinical practice than routine cytotoxicity assays performed by incubating cells with drugs for 24 h or more. To improve the action of gemcitabine we designed a three consecutive dose experiment that might resemble a serial treatment. Cells were exposed for 90 min to gemcitabine, 24, 48, and 72 h after initiation of the culture, and then counted 72 h after the first dose of drug. Data were fitted to a dose-response curve, using the software Grafit (Erithacus Software, Ltd.), to obtain the IC_{50} values of the gemcitabine effect on cell viability.

RESULTS

Time Course and *Cis*-Inhibition of Uridine Uptake into the Tumor Pancreatic Cell Lines NP. One μM uridine uptake was monitored in the human tumor pancreatic cell lines NP9, NP18, NP29, and NP31, either in the presence or in the absence of sodium. At the cell densities used (3–4 × 10^4 cells/cm^2), uridine transport was mostly, if not exclusively, accounted for by a Na^+-independent component of transport (Fig. 1). Uridine transport activity was highly variable among cell lines: NP18 showed the fastest nucleoside uptake and NP31 showed the slowest. The high transport activity of NP18 cells was associated with a rapid loss of linearity of nucleoside incorporation. However, for the other cell lines tested, uptake was linear for several minutes.

To demonstrate carrier-mediated uptake of nucleosides and to discriminate between the NBTI-sensitive and -insensitive components of equilibrative nucleoside uptake (hENT1 and hENT2-mediated, respectively), 1 μM uridine transport into NP9, NP29, and NP31 cells was monitored either in the absence or in the presence of NBTI (1 μM), uridine, and formycin B (both at 100 μM). Results are shown in Fig. 2. Most of the

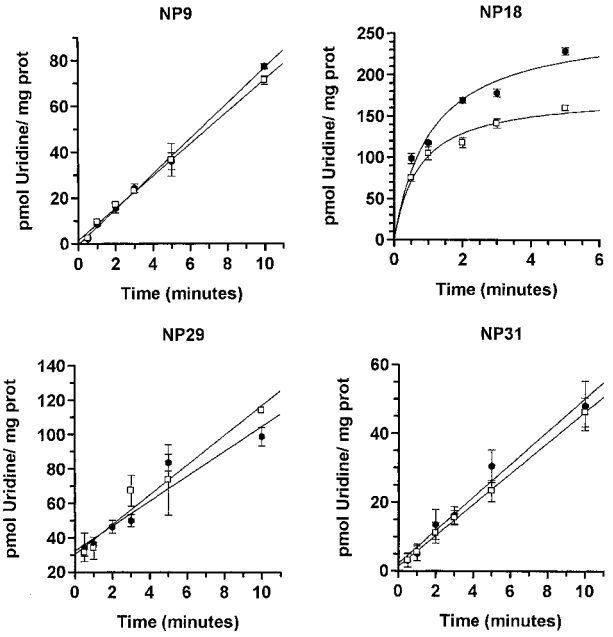

Fig. 1 Time course of 1 μM uridine uptake into NP cells. Cells were cultured as described under "Materials and Methods." Cells were incubated with 1 μM uridine either in a NaCl (●) or a choline chloride (□) medium. Results are the mean of triplicate measurements; *bars*, ±SE. *prot*, protein.

equilibrative nucleoside uptake found in these three cell lines was NBTI-sensitive, which is consistent, as will be discussed below, with high expression of the hENT1 isoform. The highest uridine uptake rate was found in NP18 cells. In this clone a broader inhibitory analysis than that performed in NP9, NP29, and NP31 cells was undertaken. In agreement with the expression of a broad-specificity transporter all of the nucleosides and nucleoside analogues tested inhibited transport (Fig. 2). Moreover, NP18 cells showed the highest ENT1-mediated transport activity of the four clones analyzed, as deduced from the inhibition by NBTI, which, in contrast to its effect in the other cell lines, abolished uridine transport almost completely.

Characteristics of Gemcitabine Uptake into NP31 Cells. To determine the major routes involved in gemcitabine transport in NP cells, we monitored the uptake of 1 μM gemcitabine into NP31 cells (Fig. 3*A*). At the densities tested (routinely close to confluence), the transport of this drug was almost exclusively mediated by equilibrative Na^+-independent transport systems. The uptake was linear for at least 3 min, as for the natural substrate uridine (Fig. 3*A*), and it was inhibited by a variety of natural nucleosides, being also highly sensitive to inhibition by NBTI (Fig. 3*B*). Indeed, NBTI at 1 μM almost completely abolished gemcitabine uptake. This inhibitory pattern is consistent with hENT1 being the major mediator of gemcitabine transport in NP31 cells. This feature is also in agreement with the evidence that hENT2, when heterologously expressed in *Xenopus laevis* oocytes, can transport gemcitabine with a much lower affinity than that reported for hENT1 (29).

The concentration dependence of hENT1-mediated uridine and gemcitabine uptake into NP31 cells is shown in Fig. 4.

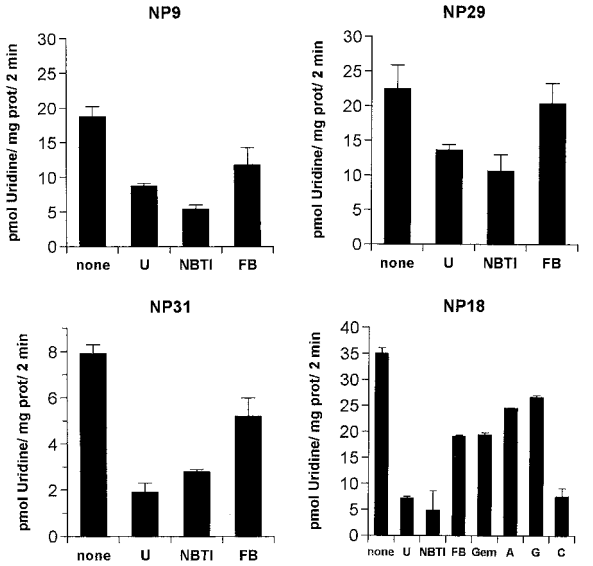

Fig. 2 Effect of different nucleosides and analogues on equilibrative uridine uptake. NP cells were incubated, as indicated under "Materials and Methods," in the presence of 1 μM uridine for 2 min, either in the absence or presence of selected nucleosides or nucleoside analogues at a concentration of 100 μM (except for NBTI, which was used at 1 μM). Measurements were performed in a choline chloride medium to determine equilibrative uridine uptake. Results are the mean of triplicate measurements; *bars*, ±SE. *prot*, protein. *U*, uridine; *FB*, formycin B.

Kinetic parameters derived from these data are given in Table 1. Gemcitabine was transported into the NP31 cell line at significantly lower rates than those reported for uridine. Nevertheless, the apparent affinities for both substrates were similar and closely resembled those values derived from the functional analysis of cloned hENT1 transporters expressed in oocytes (29).

RT-PCR Analysis of Nucleoside Transporter Expression in Human Tumor Pancreatic Cells. Although the analysis of nucleoside transport in NP cells showed that a hENT1-type transporter is expressed and functional in these cell lines, some NBTI-resistant uptake could be ascribed to hENT2 expression. Nor could we rule out the possibility that CNT transporters might be expressed in NP cells, because NP18 cells appeared to have some Na-dependent nucleoside transport activity (Fig. 1). Thus, we decided to address this issue by determining the presence of mRNA for the five nucleoside plasma membrane transporters cloned thus far (hENT1, hENT2, hCNT1, hCNT2, and hCNT3). A representative result obtained from confluent cell cultures is shown in Fig. 5. Although this experiment was designed as a qualitative approach to NT expression, the data may reflect to some extent differences in mRNA abundance among cell lines. Indeed, the four human pancreatic cell lines used, NP9, NP18, NP29, and NP31, showed high levels of hENT1 mRNA after RT-PCR amplification, which is consistent with a NBTI-sensitive transport activity being a major component of nucleoside transport in tumor pancreatic cells. Similarly, hENT2 was detected in all of the cell lines, although less than the NBTI-sensitive transporter. An interesting finding was that, despite the negligible or very low Na-dependent transport activity found in NP cells, they all retained, to some extent, the ability to express one or more hCNT genes (Fig. 5). Indeed, NP18, NP29, and NP31 appeared to express significant amounts of hCNT1 mRNA, whereas this particular isoform was not easily detected in NP9 cells by routine RT-PCR analysis (Fig. 5).

Effect of Cell Density on CNT- and ENT-Related Nucleoside Transport Activities in Human Tumor Pancreatic Cell Lines. To examine the effect of CNT mRNAs, we monitored nucleoside transport properties in these cell lines from the initiation of the culture until confluence. Results from two of the cell lines (NP29 and NP31) expressing CNT-1 transporter mRNA but no related functional activity at confluence are shown in Fig. 6. Na^+-dependent and -independent 1 μM uridine uptake was monitored for 5 days, starting 24 h after 10^5 cells on 8-cm^2 tissue culture dishes. Equilibrative Na^+-independent uridine uptake was detected at every time point assayed, but showed a slight decrease when cell density increased exponentially (Fig. 6). Interestingly, Na^+-dependent nucleoside transport activity was transiently detected at low/medium densities, before the exponential increase in cell growth occurred. This activity appeared to be related to hCNT1 expression, because, first, Na^+-dependent cytidine but not guanosine transport was detected at these time points after initiation of the culture, and second, immunocytochemistry using an anti-hCNT1 antibody (28) revealed that the protein was expressed at these cell densities (data not shown). This transient appearance of CNT-type transport activities was also found in NP9 cells although at a much lower scale (data not shown) than in NP29 and NP31 cell lines.

Heterologous Expression of hCNT1 in NP-9 Cells. The NP9 cell line was chosen as a model in which cloned hCNT1 cDNA was expressed heterologously, as reported previously (28), because of its negligible hCNT1 mRNA levels at confluence. NP cells were not easy to transfect, but we isolated a clone that retained some Na^+-dependent nucleoside transport activity even when this was measured at confluence (Fig. 7*A*). Immunofluorescence coupled to FACS analysis of permeabilized cells revealed that hCNT1 protein was expressed in WT NP9 confluent cells, whereas the clone expressing a hCNT1-type transport activity had a higher hCNT1 protein amount than the WT or a representative clone transfected with the empty vector (Fig. 7*B*). These observations taken together would favor the view that hCNT1 protein is mostly located intracellularly in the WT cells, in accordance with the lack of Na^+-dependent transport activity, whereas the hCNT1 transfected clone expresses some excess protein at the plasma membrane, thus resulting in a low but significant Na^+-dependent nucleoside transport activity.

Effect of hCNT1 Expression on Cell Sensitivity to Gemcitabine. The ability of the hCNT1 cDNA-transfected clone to express a hCNT1 activity in a more constitutive manner, irrespective of cell density, was thereafter used to determine the putative role of this transporter in gemcitabine cytotoxicity. As shown in Fig. 8, cells expressing hCNT1-related activity were more sensitive to gemcitabine treatment than the WT and empty vector-transfected cells. Similar results were obtained when cells had been exposed three times consecutively to gemcitabine for the same period of time every 24 h, although in these conditions the IC_{50} values were approximately four times lower

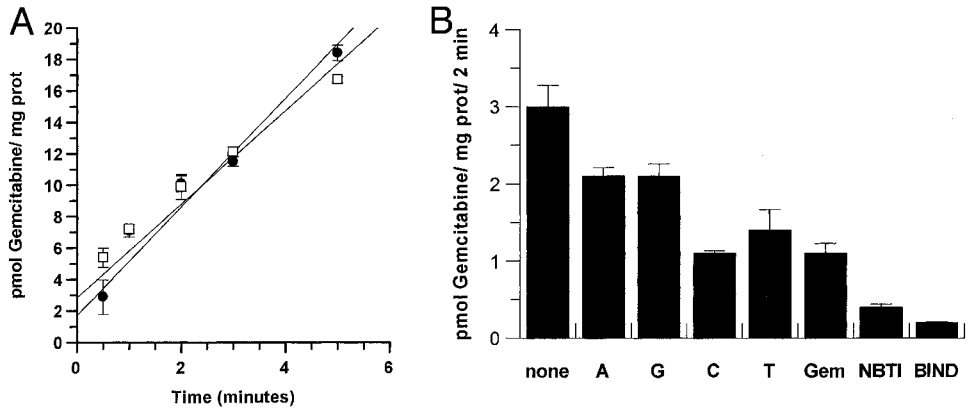

Fig. 3 *A*, time course of gemcitabine uptake into the tumor pancreatic cell line NP-31. Cells were incubated with 1 μM gemcitabine, either in a NaCl medium (●) or in a choline chloride (□) medium. Incubation was stopped at the indicated times. Results represent the mean of triplicate measurements; *bars*, ±SE. *prot*, protein. *B*, gemcitabine equilibrative uptake was measured either in the absence or in the presence of 100 μM (1 μM NBTI) of selected nucleosides and gemcitabine itself. Nonspecific binding was measured by incubating cells in ice-cold medium containing 1 μM gemcitabine, and immediately removing it and washing the cells three times with 2 ml of ice-cold stop solution, as described under "Materials and Methods." Results are the mean of triplicate measurements; *bars*, ±SE. *prot*, protein; *A*, adenosine; *G*, guanosine; *C*, cytidine; *T*, thymidine; *Gem*, gemcitabine.

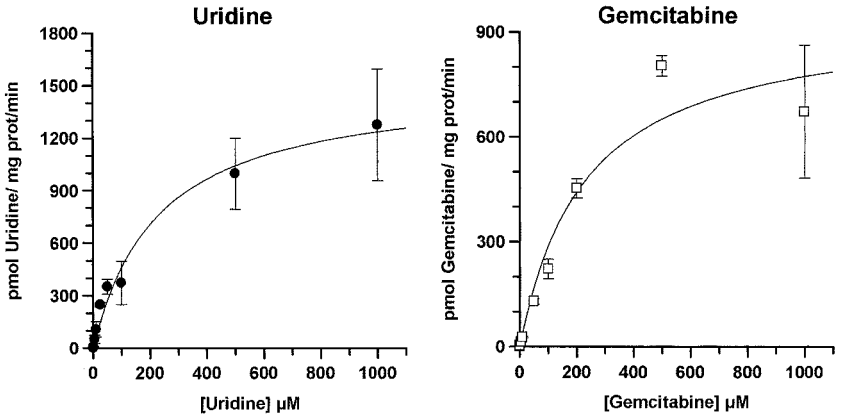

Fig. 4 Concentration dependence of hENT1-mediated uridine and gemcitabine uptake into NP-31 cells. Uridine and gemcitabine uptake was analyzed either in the absence or in the presence of 10 μM NBTI over a range of different substrate concentrations from 0.5 μM to 1 mM. hENT1-related transport was calculated by subtracting those rates measured in the presence of NBTI from those measured in the absence of NBTI. Results are the mean of triplicate measurements; *bars*, ±SE. *prot*, protein.

Table 1 Kinetic parameters of equilibrative sensitive hENT1-related uridine and gemcitabine uptake in NP31 cells

	K_m (μM)	V_{max} (pmol/mg prot/2 min)
Uridine	229 ± 51	1520 ± 115
Gemcitabine	230 ± 87	950 ± 132

than in the initial experiments in which cells had been exposed to the drug just once. IC_{50} values derived from these two protocols are shown in Table 2.

DISCUSSION

Gemcitabine is a fluoropyrimidine that shows variable cytotoxic responses in lymphoid-derived cell lines known to express different patterns of nucleoside transporter expression (9). High-affinity uptake of gemcitabine is basically mediated by the pyrimidine-preferring nucleoside transporter hCNT1. Using an electrophysiological approach it has been shown that the apparent K_m of the transporter for the drug is low and in the range of the pharmacological concentrations reached during chemotherapy (15). hENT1, despite its apparent higher K_m values for gemcitabine, also plays a role in drug uptake. This might be relevant considering that this particular isoform seems to be overexpressed in some tumors, as deduced from labeled NBTI-specific binding assays (30), and has been implicated in the channeling of nucleosides into DNA in murine bone marrow macrophages (31). More recently, hENT1 activity has been correlated with *ex vivo* fludarabine cytotoxicity in chronic lymphocytic leukemia (32) cells.

On the basis of these considerations, the possibility that nucleoside transporters would significantly contribute to gemcitabine cytotoxicity deserved additional analysis, especially in tumor types in which this drug is currently used, such as pancreatic cancer. Moreover, no information was available regarding the types of nucleoside transporters expressed in human pancreas.

In this study we chose a panel of cell lines derived from

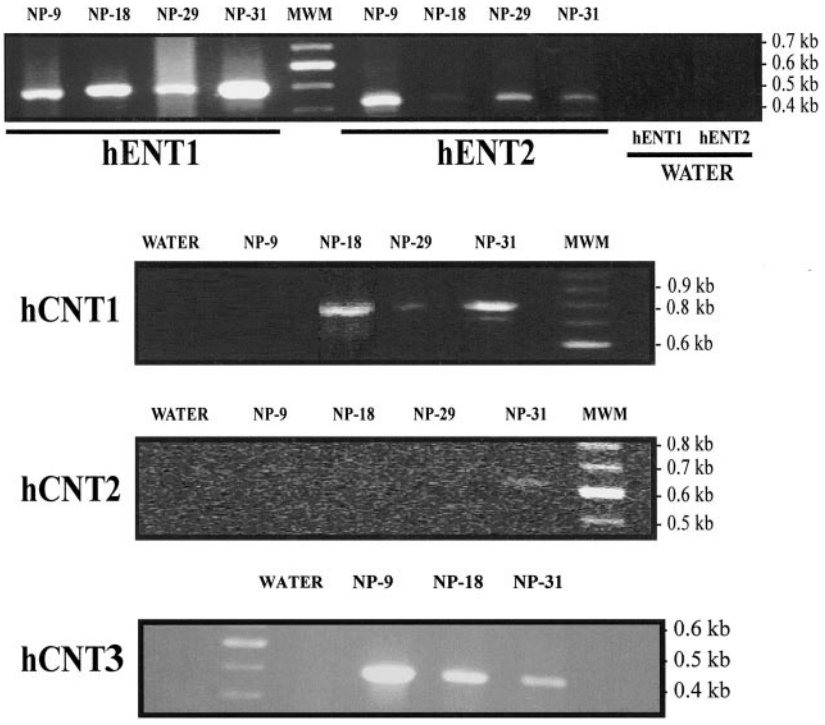

Fig. 5 Qualitative RT-PCR analysis of hENT1, hENT2, hCNT1, hCNT2, and hCNT3 expression in NP cell lines. Total RNA was extracted and processed as described under "Materials and Methods." *MWM,* molecular weight marker (Roche). The expected size of the PCR products were 0.5 kb for hENT1, 0.43 kb for hENT2, 0.8 kb for hCNT1, 0.61 kb for hCNT2, and 0.48 kb for hCNT3.

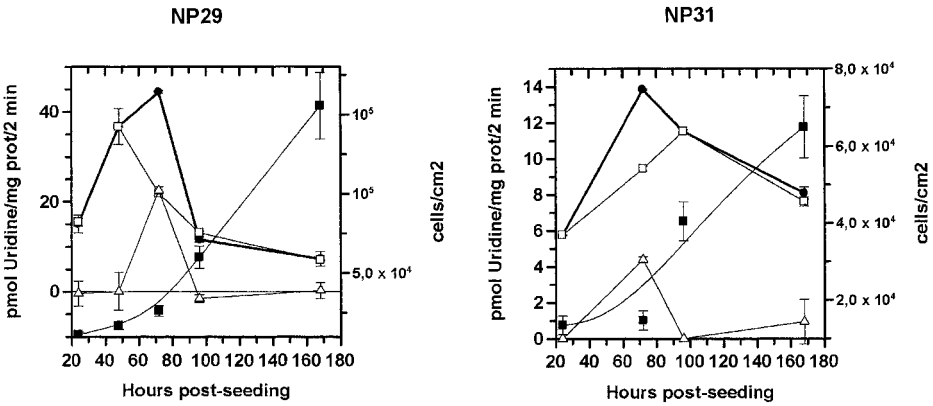

Fig. 6 *A,* effect of cell density on CNT- and ENT-related nucleoside transport activities in NP29 and NP31 cells. Cells (10^5) were seeded in 8-cm^2 culture plates, and uridine uptake was measured either in a NaCl medium (●) or in a choline chloride (□) medium 24, 48, 72, 96, and 168 h after initiation of the culture. Na^+-dependent transport (△) was calculated by subtracting those rates measured in the choline chloride medium from those measured in the NaCl medium. Cell density (■) was calculated by cell counting of plates run in parallel. Results are the mean of triplicate measurements; *bars,* ±SE.

human pancreatic adenocarcinomas to determine nucleoside transporter isoform expression and to establish the role of the high-affinity gemcitabine transporter hCNT1 in drug-induced cytotoxicity. The four cell lines analyzed showed high ENT1-type transport activity in accordance with high apparent mRNA levels, as suggested from qualitative RT-PCR determinations. Moreover, kinetic analysis of gemcitabine uptake via the NBTI-sensitive transport system (hENT1-related) revealed an apparent K_m value for the drug that closely resembled that found in heterologous systems expressing the hENT1 isoform. Thus, hENT1 is the major, to some extent constitutive, gemcitabine transporter in these human pancreatic adenocarcinoma cells.

Human ENT2, CNT1, CNT2, and CNT3 transporters appeared to be differentially expressed in the four cell lines analyzed. Previous evidence obtained in *in vitro* and *in vivo* rat hepatocarcinoma models suggests that CNT isoform expression is impaired by transformation (33), CNT1 and CNT2 being features of differentiated hepatocytes (34). Consistent with this view, CNT expression seems to be specifically lost or reduced in these pancreatic adenocarcinoma cell lines. Indeed, although rat pancreas shows high CNT2 expression (35), this particular isoform is absent or expressed at very low amounts in all four of the cell lines.

An interesting finding of this contribution is that, to

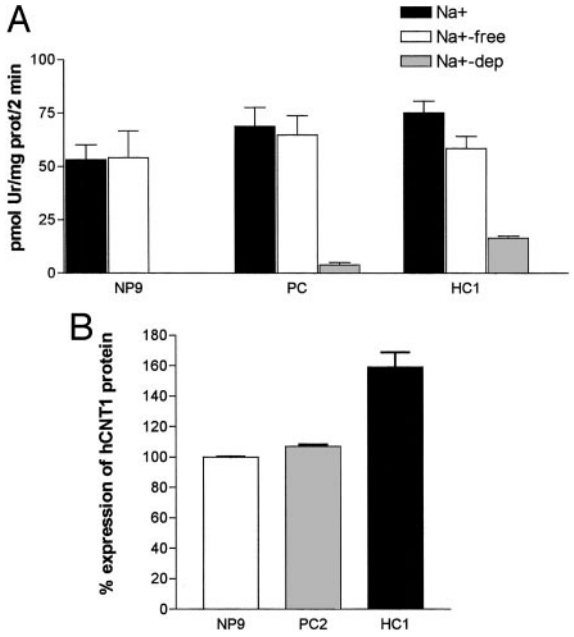

Fig. 7 *A*, characterization of uridine uptake activity in NP9 cells stably transfected with hCNT1 gene. NP9 cell types cultured as described under experimental procedures were grown until confluence and incubated with 10 μM uridine either in the absence or presence of Na^+ for 2 min. The transfection of hCNT1 gene into NP9 cells induced a Na^+-dependent activity even in confluent cultures, not seen either in WT or in the empty vector-transfected cells. Values are the mean of six independent experiments; *bars*, ±SE. The emergence of this hCNT1-type activity was statistically validated by comparing uptake rates in the choline chloride medium with those measured in the NaCl medium (paired *t* test; $P < 0.001$). *B*, expression of hCNT1 protein in WT, PC, and HC1 confluent cells by immunofluorescence coupled to FACS analysis. Cells were cultured as described under "Material and Methods" and analyzed for hCNT1 expression by immunocytochemistry. The results are the percentage of hCNT1 protein expression related to WT hCNT1 expression and represent the mean of three independent experiments; *bars*, ±SE. In this result a slight increase in hCNT1 protein is detected, consistent with a Na^+-dependent nucleoside transport activity.

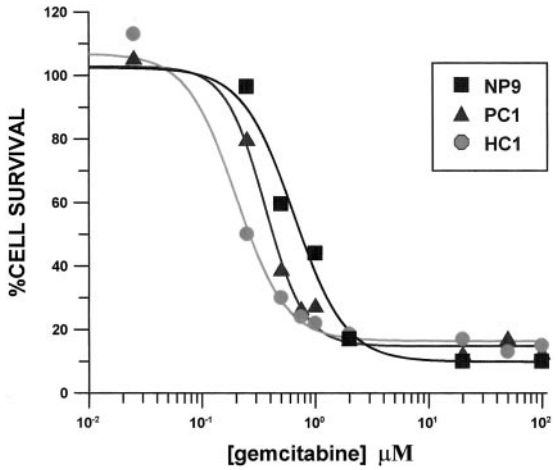

Fig. 8 Cytotoxicity of gemcitabine in the three different clones of NP9 cells. Cell viability was assessed by cell counting 72 h after 90-min exposure to the drug. This dose-response curve is one representative experiment. IC_{50} values derived from three independent experiments are shown in Table 2.

Table 2 Relationship between hCNT1 expression and cytotoxic effect of gemcitabine

Data obtained in either a single or triple treatment derived from the cytotoxicity assay shown in Fig 8. These results are the mean of three independent experiments. IC_{50} values for gemcitabine cytotoxicity assay in a triple treatment are a summary of data not shown. IC_{50} values are expressed in nanomolar

	Gemcitabine IC_{50}	
	Single	Triple
NP9	411 ± 99	100 ± 25
PC1	315 ± 23	86 ± 12
HC1	168 ± 30	36 ± 3

some extent, NP cells have the ability to express CNT-type transport activity at some point during cell growth, although the increase in cell density is associated with the disappearance of this Na^+-dependent transport component. In the cases analyzed, this transport activity is presumably the result of hCNT1 expression, because Na^+-dependent cytidine uptake emerged when little or negligible guanosine transport was detected. If broad-specificity Na^+-dependent nucleoside transporter isoform CNT3 is responsible for this transient change in transport activity, both nucleosides should be effectively taken up in a concentrative manner.

CNT1 expression is clearly a feature of differentiated hepatocytes. CNT1 is mostly located in transcytotic structures, thus suggesting that trafficking and insertion into the plasma membrane may be new elements of CNT1 regulation (36). The possibility that, besides selectively losing CNT1 expression, insertion into membrane is also affected in tumors should then be analyzed. In this context, overexpression of hCNT1 in these pancreatic adenocarcinoma cell lines was envisaged as a way to promote transporter synthesis driven by a powerful constitutive promotor, thus contributing to the saturation of the system and resulting in some constitutive hCNT1-type transport activity independent of the cell proliferation status. Despite having the hCNT1 cDNA cloned in a vector that results in high expression levels in other cell systems, such as Chinese hamster ovary cells (28), in NP9 cells, hCNT1 activity was relatively low compared with the endogenous ENT1 transporter. However, absolute nucleoside uptake rates were in the range of endogenous CNT activities found in other human cell lines (37). The relative overexpression of hCNT1 in NP9 cells, as measured by flow cytometry, was enough to yield functional activity and significantly sensitize cells to gemcitabine action. These data are consistent with the view that expression of a high-affinity nucleoside transporter, like hCNT1, determines an advantage in therapeutic response over the abundant hENT1-related low-affinity drug transport activity. This study also anticipates that hCNTs are present in human pancreas, and their expression is expected to be highly variable in tumors, although this will require additional study. Moreover, the abil-

ity of pancreatic adenocarcinoma cells to express CNT-type activities depending on cell growth conditions, suggests that a putative apparent loss of functional nucleoside transporter expression may not always be related to a complete inability to express these genes. Thus, it would be possible, theoretically, to induce either dormant transporter genes or promote plasma membrane insertion by pharmacological means, thus contributing to increased drug bioavailability.

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