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# Adenoviral-mediated overexpression of human equilibrative nucleoside transporter 1 (hENT1) enhances gemcitabine response in human pancreatic cancer

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ABSTRACT

Nucleoside-derived anticancer agents must be transported across the plasma membrane as a preliminary step to their conversion into active drugs. Hence, modulation of a specific nucleoside transporter may affect bioavailability and contribute significantly to sensitizing tumor cells to these anticancer agents. We have generated and functionally characterized a new recombinant adenovirus (Ad-hENT1) that has allowed us to overexpress the equilibrative nucleoside transporter hENT1 and to analyze its effects in human pancreatic tumor cells. Overexpression of hENT1 is associated with changes in cell cycle profile, in a variable manner depending on the particular cell type, thus suggesting a metabolic link between hENT1-mediated transport processes and the enzymatic machinery responsible for intracellular nucleoside metabolism. When assayed *in vivo* in a human pancreatic adenocarcinoma xenograft, intratumoral Ad-hENT1 injection improved the therapeutic response to gemcitabine. In summary, hENT1 overexpression is associated with alterations in nucleoside enzymatic machinery and cell cycle progression in cultured cells and enhances gemcitabine action *in vivo*.



## 1. Introduction

An increasing number of nucleoside analogues are being used to treat a wide variety of cancers. They are antimetabolites that compete with natural nucleosides and interact with intracellular targets to induce cytotoxicity. Nucleoside analogues exert their effects in a three-stage process: uptake, metabolism into the active drug, and action on the pharmacological targets. Alterations in any of these steps can impair drug bioavailability and induce resistance to treatment [1]. Most of these molecules require specialised transporter proteins to enter into cells. These proteins include the

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*Abbreviations:* NT, nucleoside transporters; GE, gemcitabine; NBMPR, nitrobenzylmercaptopurine ribonucleoside; NBTI, nitrobenzylthioinosine; AU, arbitrary units.

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concentrative nucleoside transporters (hCNT) [2] and the equilibrative nucleoside transporters (hENT) [3], encoded by the gene families SLC28 and SLC29, respectively. hCNT carriers are secondary-active sodium-dependent transporters with selectivity for pyrimidine nucleosides (hCNT1), purine nucleosides plus uridine (hCNT2), and both pyrimidine and purine nucleosides (hCNT3) [4]. The equilibrative nucleoside transporters hENT1 and hENT2 are facilitative transporters found in plasma membranes, and exhibit similar nucleoside selectivity, although hENT2 is also able to transport some nucleobases. They can be functionally distinguished by either their sensitivity (hENT1) or resistance (hENT2) to inhibition by nitrobenzylmercaptopurine ribonucleoside (NBMPR).

Nucleoside transporter expression varies among different cell types and tissues and heterogeneity and selective loss of expression have been reported in neoplasias. Thus, hENT1 seems to be overexpressed in some tumors [5], while in rat hepatocarcinoma models CNT expression is impaired by transformation [6,7]. A high variability in transporter expression among patients has also been reported for solid tumors [8–10].

*In vitro* studies show that the uptake of most chemotherapeutic nucleoside analogues is dependent on the activity of nucleoside transporters. Moreover, transporter function may determine the response to these drugs [11]. Nucleoside transport-deficient variants of several cell lines exhibit a higher resistance to nucleoside analogues than parental cell lines [12,13] and the inhibition of equilibrative transporters confers resistance to gemcitabine [14]. In addition, a significant relationship between the levels of hENT1 mRNA and the $IC_{50}$ values for gemcitabine has been found in non-small cell lung cancer cell lines [15], and in human pancreatic adenocarcinoma and biliary tract carcinoma cell lines [16]. In a panel of sixty cell lines, a positive correlation between hENT1 expression and activity and potency of the nucleoside analogues azacytidine and inosine-glycodialdehyde has also been reported [17]. However, contradictory results have also been described. For example, no correlation between basal levels of cell surface hENT1 and gemcitabine sensitivity was found in four cancer cell lines [18], and the mRNA levels of all cloned nucleoside transporters, in fifty cell lines, were found to display no correlation with sensitivity to nucleoside analogues [19].

The role of nucleoside transporters *in vivo* is less known mainly due to the lack, until recently, of suitable antibodies. A positive correlation between cytarabine sensitivity and hENT1 expression has been found in acute myeloblastic and lymphoblastic leukaemias [20,21] and equilibrative transport activity correlates with *ex vivo* fludarabine cytotoxicity in chronic lymphocytic leukaemia cells [22], particularly with regard to hENT2 protein levels [23]. Patients with pancreatic adenocarcinoma with uniformly detectable hENT1 immunostaining and high hENT1-encoding mRNA levels have a significantly longer survival time after gemcitabine chemotherapy than those bearing tumors with low or undetectable hENT1 [24,25]; this relationship has recently also been suggested for non-small cell lung cancer [26].

The particular role NT proteins play in nucleoside-derived drug uptake and chemosensitivity has been recently reviewed [27]. Nevertheless, this issue is still far from being completely elucidated, since nucleoside-metabolizing enzymes can also modulate chemosensitivity. For most nucleoside-derived drugs, the rate-limiting step in their metabolism is the phosphorylation step catalyzed by deoxycytidine kinase (dCK), and dCK activity has been shown to be decreased in various resistant cell lines [28]. The triphosphorylated forms are required for cytotoxic effects, either by direct incorporation into DNA and RNA or by interfering with enzymes involved in nucleic acid synthesis (DNA polymerases, ribonucleotide reductase), thus eliciting apoptotic cell death.

In this study, we have generated a recombinant adenovirus (Ad5CMV-hENT1) to overexpress hENT1, in an attempt to study in depth the specific role of hENT1 in nucleoside-derived cytotoxicity. This has allowed us to analyze *in vitro* the effect of hENT1 overexpression on nucleoside transport capacity, and on some enzymes involved in nucleic acid metabolism and the cell cycle, as well as to evaluate its impact on gemcitabine sensitivity in subcutaneous tumors established from human pancreatic cancer cells.

## 2. Materials and methods

### 2.1. Cell lines

NP-9, NP-18 and NP-29 cell lines were derived from human pancreatic adenocarcinomas, which had been perpetuated as xenografts in nude mice. NP-9 and NP-29 were maintained in Dulbecco's modified Eagle's medium and F12 mixture (1:1) and NP-18 in RPMI 1640 medium (Gibco, Grand Island, NY), both supplemented with 10% foetal bovine serum (FBS) and antibiotics at 37 °C, in a humidified atmosphere containing 5% $CO_2$ and subcultured every 3–4 days.

### 2.2. Construction of Ad5CMV-hENT1 (Ad-hENT1) and infection conditions

hENT1 cDNA was fused to a simian virus 40 polyadenylation signal and cloned downstream of the cytomegalovirus promoter in a pACCMVpLpA plasmid. Recombinant adenovirus was generated by cotransfection of this plasmid with pJM17 plasmid into subconfluent cultures of HEK293 cells. Individual viral plaques were isolated and amplified in HEK293 cells, and recombinant adenoviruses containing human ENT1 cDNA were identified by polymerase chain reaction amplification and restriction enzyme digestion. Virus without insert (Adctrol) was generated in the same way. Viral clones were propagated in HEK293 cells and recovered after 48 h by three cycles of freezing and thawing. Titers were determined by plaque assay on HEK293 cells.

NP cells were seeded 24 h before adenovirus infection. Viral stocks were diluted to reach the desired multiplicity of infection (MOI) in serum-free medium and added to the cell monolayer. Mock-infected cells were incubated with serum-free medium. After 4 h, cells were re-fed with medium containing 10% heat-inactivated FBS.

### 2.3. Cytotoxicity assays

Cytotoxicity assays were performed by seeding NP-9 and NP-29 cells at a density of 20,000 cells/well, and NP-18 at 12,000

cells/well in 24-well culture plates. Twenty-four hours after seeding, cultures were exposed for 24 h to increasing concentrations of gemcitabine (generously provided by Lilly S.A., Madrid, Spain). Viability was assessed 72 h after treatment by cell counting (Multisizer; Beckman Coulter, Inc., Fullerton, CA). Data were fitted to a dose–response curve by standard nonlinear regression using adapted Hill equation with Grafit software (Erithacus Software, Ltd.) to obtain the $IC_{50}$ value.

### 2.4. Nucleoside uptake measurements

[$^3$H]-Uridine (Amersham, Buckinghamshire, UK) was used as a tracer in the uptake measurements. Transport was measured by incubating cell monolayers in the presence of 1 μM uridine (specific activity 1 μCi/nmol) in 137 mM choline medium containing 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $Mg_2SO_4$ and 10 mM HEPES (pH 7.4). Transport was stopped by rapid aspiration of the uptake buffer followed by immediate washing with a cold stop solution, as previously described [29]. hENT1 activity was discriminated using 1 μM nitrobenzylthioinosine (NBTI). Cells were then lysed in 100 μl of 100 mM NaOH, 0.5% Triton X-100. Aliquots were used for radioactivity counting and protein determination using the BCA reaction (Pierce, Rockford, IL).

### 2.5. Real-time quantitative RT-PCR

Total RNA was isolated from cell lines using the SV Total RNA Isolation System (Promega, Madison, WI). In total, 1 μg of RNA was retro-transcribed to cDNA and the analysis of hENT1, hENT2 and GAPDH was performed by real time quantitative reverse transcriptase-polymerase chain reaction as previously described by Molina-Arcas et al. [22]. Expression of deoxycytidine kinase and ribonucleotide reductase (R2) was analyzed using a pre-designed Assay-on-demand (Applied Biosystems, Foster City, CA). Relative quantification of gene expression was assessed as described in the *Taq*Man user's manual using GAPDH as an internal control. The amounts of mRNA were given as arbitrary units using the ΔΔCT method (User Bulletin #2, Applied Biosystems).

### 2.6. Cell cycle analysis

Cells (250,000) were seeded in 60 mm culture plates and infected with Ad-hENT1 or Adctrol 24 h later. At 48 h post-infection, cells were harvested and fixed with 70% ethanol (v/v). Cells were centrifuged at 4000 rpm for 4 min and were stained in PBS containing 10 μg/ml RNAse A and 100 μg/ml propidium iodide for 1 h at 4 °C. All measurements of cell cycle distribution were performed on an EPICS-XL flow cytometer (Coulter, Villepinte, France). Data from ≥10,000 cells were collected and analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

### 2.7. Western blot analysis

Total cell lysates were obtained by incubation in lysis buffer (40 mM NaCl, 5 mM NaF, 10% glycerol, 1 mM EDTA, 1 mM $NaVO_4$, 0.5% Igepal CA-630, 4 mM dithiothreitol, and a protease inhibitor cocktail (Roche, Mannheim, Germany) in 10 mM Tris–HCl, pH 7.4) for one hour at 4 °C and then were centrifuged at 14,000 × *g*. Whole protein extracts (30 μg/lane as assessed by the Bradford assay (Bio-Rad, Hercules, CA)), were electrophoretically separated on 8% polyacrylamide-sodium dodecyl sulphate gels and transferred (180 mA, 70 min) to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Membranes were incubated with α-hENT1 (raised and characterized in our laboratory), α-PARP (Pharmingen, San Diego, CA), α-cyclin E (Santa Cruz, Santa Cruz, CA), α-cyclin B (Santa Cruz, Santa Cruz, CA) or α-actin (Sigma, St Louis, MO) and the corresponding HRP-conjugated secondary antibodies (Amersham, Buckinghamshire, UK). Membranes were developed according to Amersham's ECL protocol (Amersham, Buckinghamshire, UK).

### 2.8. Animals

Female BALB/c nude mice were used in *in vivo* experiments. Mice were fed *ad libitum* and maintained under a 12-h light:12-h dark cycle. All the animal procedures were previously approved by the Animal Ethics Committee of the Autonomous Government of Catalonia, and performed accordingly with recommendations for the proper care and use of laboratory animals.

Tumor xenografts were developed after subcutaneous injection of $6 \times 10^6$ NP-9 cells into the flanks of BALB/c nude mice. Tumor volume was measured twice a week and was calculated according to the equation $V\ (mm^3) = \pi/6 \times W \times L^2$, where *L* and *W* are length and width of the tumor, respectively. Once tumors reached 160–180 mm$^3$, mice were randomized ($n$ = 5–6 per group) and cycles were performed once a week for three weeks of Ad-hENT1 intratumoral injection ($2 \times 10^8$ pfu/20 μl in PBS at 4 injection sites/tumor) followed by 150 mg/kg of gemcitabine administered intraperitoneally 36 h later.

### 2.9. Statistical analysis

Multivariant ANOVA test was used to compare experimental data. Significance was set at $P < 0.05$. This analysis has been carried out using Statgraphics software (version 5.1).

## 3. Results

### 3.1. Uridine uptake versus gemcitabine sensitivity into tumor pancreatic cell lines

In order to examine the relationship between gemcitabine sensitivity and hENT1 transport, 1 μM uridine uptake was determined at 1 min, either in the presence or in the absence of NBTI (1 μM), to assess the NBTI-sensitive component of equilibrative nucleoside transport (hENT1). Fig. 1 shows hENT1-mediated uridine uptake into cells of the three cell lines, and basal hENT1 mRNA levels together with $IC_{50}$ values for the cytotoxicity triggered by 24-hour gemcitabine treatment determined by MTT assays 72 h after the end of treatment. Interestingly, there is a strong association between uridine uptake and basal hENT1 mRNA levels in all the cell lines examined, and the expression levels correlate positively

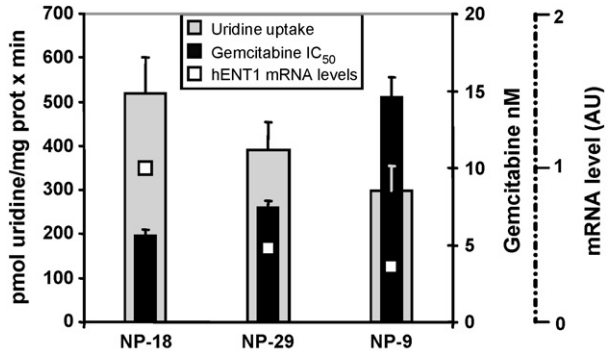


**Fig. 1 – Characterization of pancreatic cancer cell lines for hENT1 mRNA levels, uridine uptake and gemcitabine cytotoxicity. $IC_{50}$ values for gemcitabine obtained from cytotoxicity assays performed 48 h after treatment (black bars), basal hENT1-mediated uridine uptake activity (grey bars) and relative amounts of hENT1 mRNA (white squares) in each cell line. Bars represent the mean ± S.E.M. ($n$ = 4).**

with their sensitivity to gemcitabine treatment. In this sense, NP-9, the most resistant cell line, has the lowest levels of hENT1-mediated uptake. Moreover, NP-18 behaves in the opposite manner, since this cell line shows the highest gemcitabine sensitivity of all the three cell lines along with a high uptake capacity. Accordingly, NP-29 displays an intermediate pattern for both parameters. Similar results were obtained in all cell lines after a 90 min treatment with the drug.

### 3.2. *Validation of Ad-hENT1 function*

The NP-9 and NP-18 cell lines were chosen to analyze the effects of Ad-hENT1 infection because of their opposing characteristics regarding basal hENT1 expression, uridine uptake and gemcitabine sensitivity, as shown in Fig. 1. To examine the effectiveness of Ad-hENT1 infection, NP-9 cells were infected at different MOIs with Adctrol and Ad-hENT1, and 48 h after infection the levels of hENT1 mRNA were determined by Real Time RT-PCR. hENT1 mRNA levels increase in a dose-dependent manner after cDNA transduction (Fig. 2A), whereas no corresponding changes were observed with Adctrol infection at the same MOI (insert in Fig. 2A). Similar results were obtained in NP-18 cells, although the total increase in hENT1 mRNA was found to be from twenty- to seventy-fold lower than in NP-9 cells, depending on the adenoviral dose (Supplementary data). No significant changes in hENT2 mRNA levels were observed under any conditions, in either cell line (data not shown). Moreover, slight increases in hENT1 levels were only detected in NP-9 cells when protein expression was analyzed by western blot (Fig. 2 and Supplementary data).

Once ectopic hENT1 expression was confirmed, uridine uptake was analyzed to assess the functionality of this nucleoside transporter. At the tested MOIs, uridine uptake in NP-9 cells 48 h post-infection increased at an

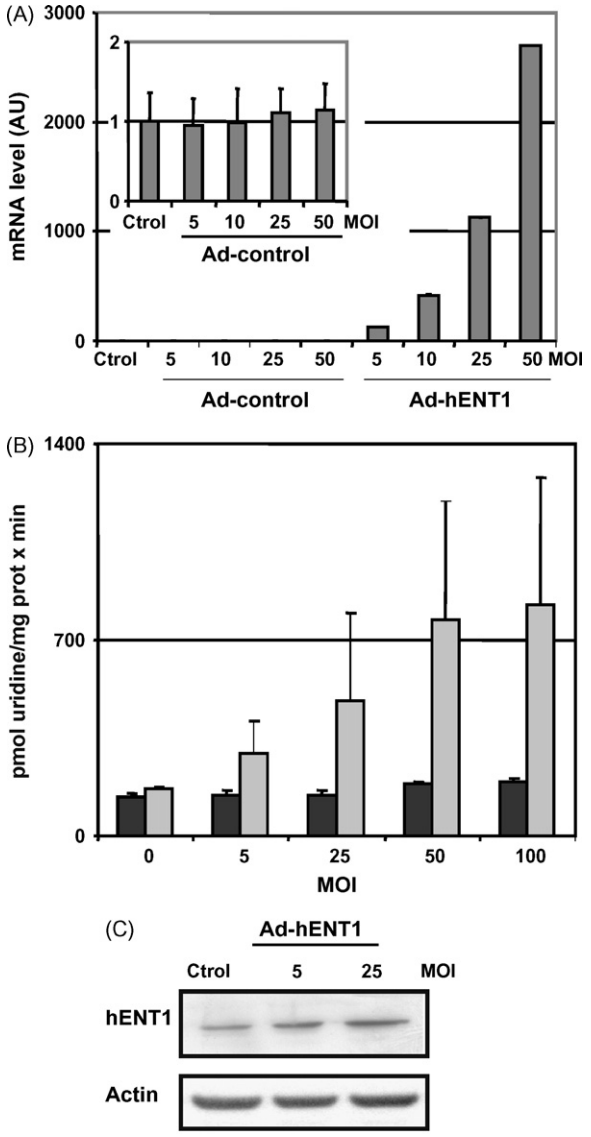


**Fig. 2 – Ad-hENT1 infection effectiveness in NP-9 cells. (A) Relative amounts of hENT1 mRNA were determined 48 h after infection with different MOIs of Ad-hENT1 (grey bars) or Adctrol (black bars). Results are represented as mean values ± S.E.M. ($n$ = 3). (B) hENT1 transport activity at 48 h post-infection. Bars represent the mean ± S.E.M. ($n$ = 4). Statistical comparisons were done by a multivariant ANOVA test. hENT1 mRNA, AdhENT1 vs. Adctrol: $F$ = 112.22, $p$ = 0.0018. hENT1 transport activity, AdhENT1 vs. Adctrol: $F$ = 14.49, $p$ = 0.0318. (C) Western-blot analysis of hENT1 expression at 48 h post-infection.**

MOI of 5 and above (Fig. 2B), in accordance with the rise in mRNA levels (Fig. 2A). However, no significant increases in uridine uptake were found in NP-18 cells. Finally, similar experiments were addressed in other cell line expressing basal uridine uptake similar to those in NP-9, the colon cancer cell line CaCo-2. Significant increases in transport activity were achieved in a dose-responsive manner (Supplementary data).

### 3.3. *Effect of hENT1 expression on enzymes involved in nucleic acid metabolism*

To analyze the impact of Ad-hENT1 infection on enzymes involved in nucleic acid metabolism, dCK and R2 ribonucleotide reductase mRNA levels were examined. Total mRNA from hENT1-transduced NP-9 and NP-18 cells was extracted 48 h after infection at 5, 10, 25 and 50 MOI. NP-9 cells showed a marked decrease in dCK and R2 mRNA levels at an MOI of 5 and above, whereas in NP-18 cells no significant changes were observed (Fig. 3).

### 3.4. *Effect of hENT1 expression on cell cycle and apoptosis*

In order to elucidate the differences between NP-9 and NP-18 cells exerted by Ad-hENT1 infection, the cell cycle progression in both cell lines was analyzed. Cell cycle profiles were determined after 48 h-infection, using 5 and 10 MOI of the adenovirus (Fig. 4A). No clear alterations were observed in NP-18 cell cycle profiles. In NP-9 cells, overexpression of ectopic hENT1 leads to an accumulation of cells in $G_2$ in a dose-responsive manner (30.1% at 5 MOI versus 20.7% in the control) (Fig. 4B), which correlates with an increase in the cyclin B/cyclin E ratio (Fig. 4C). Moreover, concomitant sub-G1 population increases are observed as the Ad-hENT1 dose rises (6.2% at 10 MOI versus 1.7% in the control). To characterize this sub-G1 population, caspase activation was checked by PARP analysis. No PARP cleavage appeared under any infection condition discarding apoptosis (Fig. 4C).

### 3.5. *Effect of hENT1 overexpression in subcutaneous NP-9 tumors*

Intratumoral Ad-hENT1 infection was performed to determine the effects of hENT1 overexpression on tumor growth and gemcitabine sensitivity. NP-9 cells were injected into the subcutaneous tissue of nude mice and 5 weeks thereafter animals were randomized in groups and treated as described in Section 2. Gemcitabine alone (150 mg/kg, i.p.) or Ad-hENT1 overexpression induced a slight tumor growth inhibition when compared to Adctrol-infected tumors. In contrast, when tumors were treated with gemcitabine (150 mg/kg), hENT1 overexpression increased drug sensitivity, and this group achieved statistical significant differences with respect to all the other experimental groups (Fig. 5).

## 4. Discussion

Human equilibrative nucleoside transporter 1 (hENT1) is broadly expressed in human tissues, probably playing a major role in nucleoside salvage processes, particularly following cell proliferation [30]. Under selected physiological conditions, hENT1 can mediate the unidirectional uptake of nucleosides whenever tight coupling between their transport across the

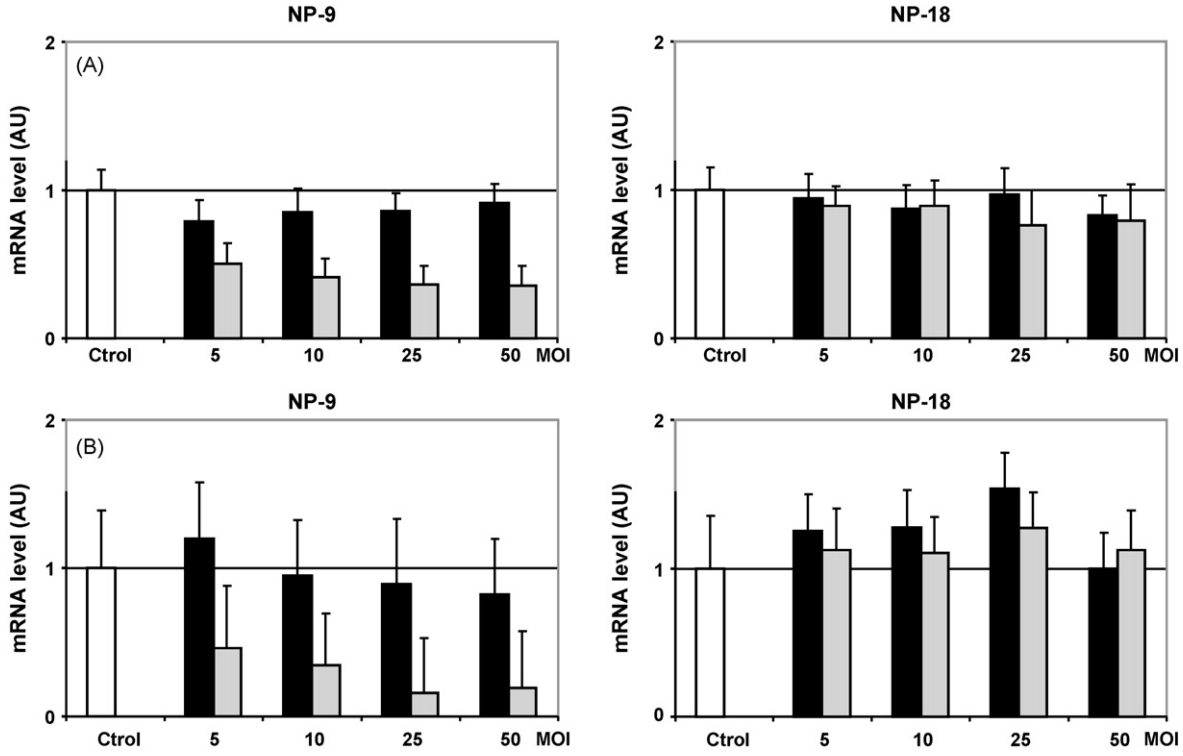


**Fig. 3 – Deoxycytidine kinase (dCK) and ribonucleotide reductase (R2) expression. Relative amounts of dCK and R2 mRNA were determined 48 h after infection with different MOIs of Ad-hENT1 (grey bars) or Adctrol (black bars). Results are represented as mean values ± S.E.M. ($n$ = 3). (A) Deoxycytidine kinase; (B) ribonucleotide reductase. Statistical comparisons were done by a multivariant ANOVA test. dCK mRNA, AdhENT1 vs. Adctrol: $F$ = 62,37 $p$ = 0.0042. R2 mRNA, AdhENT1 vs. Adctrol: $F$ = 408,76 $p$ = 0.003.**

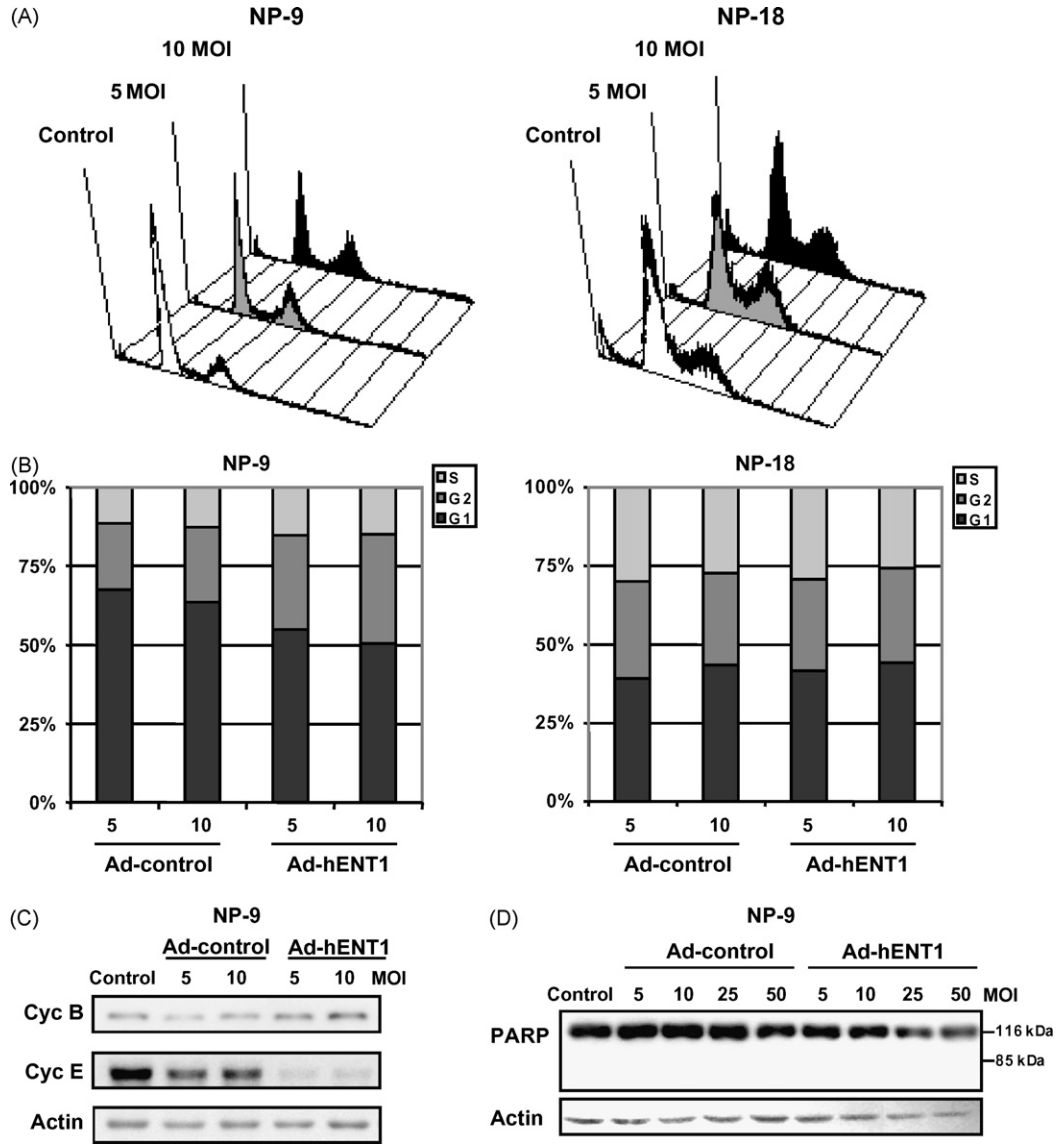


**Fig. 4 – Effects on cell cycle and apoptosis in NP-18 and NP-9 cells. Cell cycle profiles (A) and quantification of cell cycle phases (B) determined by flow cytometry analysis of propidium iodide-stained cells. (C) Western-blot analysis of cyclin B and cyclin E levels in NP-9 cells. (D) Immunoblots to detect Poly (ADP-ribose) polymerase (PARP) cleavage in Adctrol- or Ad-hENT1-transduced NP-9 cells. All experiments were performed 48 h after infection. The graphics correspond to a representative experiment ($n = 3$).**

plasma membrane and their subsequent metabolism occurs. hENT1-mediated channelling of nucleosides into DNA [30] might be relevant in chemotherapy because most fluoropyrimidines used in anticancer treatment, such as gemcitabine, are hENT1 substrates [27]. Moreover, it is also possible that hENT1 significantly contributes to maintaining intracellular nucleotide pools, since its own presence at the plasma membrane (as determined by high-affinity NBMPR binding site numbers) is regulated by nucleotide levels [31,32].

In this paper we report the generation of a recombinant adenoviral vector suitable for the heterologous overexpression of hENT1. Ad-hENT1 infection consistently induced a marked increase in hENT1 mRNA levels, which was routinely followed by moderate, or even negligible, induction of hENT1-mediated transport activity. This might be dependent on the cell background in which hENT1 is expressed. Thus, NP-18 cells which show high hENT1-related biological activity only displayed modest increases in transport uptake rates after Ad-hENT1 transduction, whereas NP-9 cells, which show the lowest hENT1-related activity and gemcitabine-induced cytotoxicity, do show a consistent increase in hENT1-mediated uridine uptake after adenoviral transduction with Ad-hENT1.

Overexpression of hENT1 in a relatively low transporter activity background resulted in a dramatic decrease in the amounts of mRNA encoding key nucleotide metabolism enzymes such as dCK and RR. These data suggest that overexpression of hENT1 on a relatively low hENT1-expressing background, triggered by non-physiological means such as that

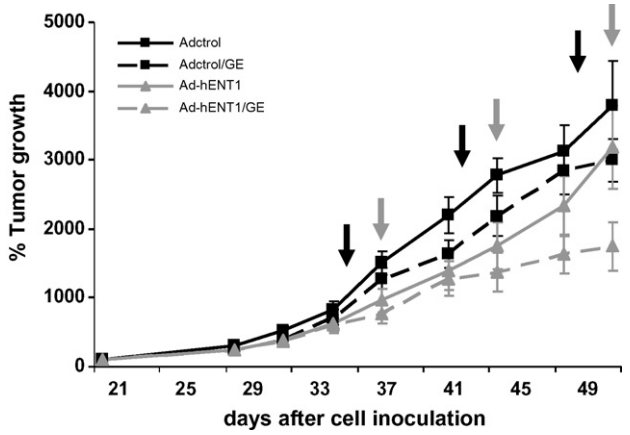


**Fig. 5 – *In vivo* antitumoral efficacy of the combination of Ad-hENT1 and gemcitabine in subcutaneous NP-9 tumors. Percentage of tumor growth curves. Established tumors were treated after reaching 160–180 mm$^3$. Black arrows indicate intratumoral injection of Ad-hENT1 ($2 \times 10^8$ pfu/injection) or Adctrol. Grey arrows indicate intraperitoneal administration of gemcitabine (GE) at 150 mg/Kg or saline. Groups injected with Adctrol and saline (■), Adctrol and GE (■), Ad-hENT1 and saline (▲) and Ad-hENT1 and GE (▲). Data are expressed as the mean of the percentage of growth of each group ($n$ = 5–6). Error bars, S.E.M. All statistical comparisons were done by a multivariant ANOVA test; only effects of the various treatments are compared. Gemcitabine treatment vs. Adctrol: $F$ = 8.46, $p$ = 0.0042; Ad-hENT1 vs. Adctrol: $F$ = 12.55, $p$ = 0.0005; Ad-hENT1/GE vs. gemcitabine: $F$ = 23.63, $p$ < 0.0001; Ad-hENT1/GE vs. Ad-hENT1: $F$ = 7.60, $p$ = 0.0066.**

resulting from the transduction of Ad-hENT1, results in a down-regulation of intracellular metabolism, which strongly supports the view of a need for a coordinate supply of nucleosides/nucleotides for salvage purposes probably balanced from extracellular sources and endogenous synthesis. In agreement with this point of view, this study shows that cell cycle progression is also affected by overexpression of hENT1. Forty eight hours after Ad-hENT1 infection, NP-9 cells displayed an altered cell cycle profile caused by an accumulation of cells at the $G_2$ phase, even at low viral doses, whereas in NP-18 cells this accumulation is not apparent until high viral doses are used. Thus, a sustained and aberrant hENT1 activity during $G_2$ phase could induce cells to arrest, in an attempt to balance deviations in nucleotide pools resulting from an altered nucleoside transporter profile and cell division needs. In fact, a combined expression index including the mRNA levels of hENT1, dCK, and RR subunits M1 and M2 has recently been correlated with gemcitabine responsiveness in human pancreatic cancer cells [33]. Moreover, a putative relationship between nucleoside transporter expression and nucleotide metabolism enzymes has also been identified in breast cancer patients [34]. Although all these data point to a tight link between nucleoside transport mechanisms and intracellular nucleotide metabolism, the chronological sequence of the events here reported, which leads to cell accumulation at $G_2$ as well as to other cellular consequences (altered enzyme activities and cell death through non-apoptotic mechanisms) deserves further research.

Correlations are not in themselves proofs of concept of a particular pharmacological interaction and, in this context, the apparent relationship established between hENT1 expression and responsiveness to nucleoside-derived drugs has not been unequivocally proven. Here NP-9 cells were used to develop a human xenograft model due to their relatively low hENT1 activity and chemosensitivity to gemcitabine. In this *in vivo* model, Ad-hENT1 intratumoral injection improved gemcitabine antitumor effects, which suggests that overexpression of hENT1 in a low endogenous activity background allows a better response to this nucleoside-derived drug. Therefore, pharmacological modulation of hENT1 in the clinical set should result in improved responsiveness to the drug.

Since this observation is in good agreement with the reported correlations between high hENT1 expression in pancreatic adenocarcinomas and longer survival times in patients treated by gemcitabine monotherapy [24,25], our data strongly support a mechanistic link between transport processes and therapeutic action of gemcitabine in pancreatic adenocarcinoma and, it is highly likely, in many other solid tumors in which this nucleoside analogue exerts its pharmacological action.

In summary, the generation of a recombinant adenovirus allowing overexpression of hENT1 mRNA in pancreatic adenocarcinoma cells has enabled us to unveil a tight relationship between nucleoside transporter function and some enzymes of nucleoside metabolism. This putative metabolic link may depend on the genetic background, and it also raises the possibility that transporter function is actually related to cell cycle progression. Moreover, overexpression of hENT1 significantly contributes to an enhanced response to gemcitabine treatment *in vivo*.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.05.011.

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