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Characterization of equilibrative nucleoside transport of the pancreatic cancer cell line: Panc-1

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Abstract
Objectives: Gemcitabine is a nucleoside analog drug used as a first-line chemotherapeutic agent against pancreatic cancer. The characterization of cell lines is crucial for understanding drug resistance development in pancreatic cancer. In this study, we aimed to determine the nucleoside transport properties of Panc-1 cells, one of the commonly used pancreatic adenocarcinoma cell lines.

Materials and Methods: To assess the presence of Equilibrative nucleoside transporter 1 (ENT1) in Panc-1 cells, we performed immunofluorescence staining, western blot analysis, and S-(4-nitrobenzyl)-6-thioinosine (NBTI) binding assays. We also conducted standard uptake assays to measure the sodium-independent uptake of [3H]-labeled chloroadenosine, hypoxanthine, and uridine. Additionally, we determined the half-maximal inhibitory concentration (IC50) of gemcitabine. Statistical analyses were performed using GraphPad Prism version 8.0 for Windows.

Results: Sodium-independent uptake of [3H]-labeled chloroadenosine, hypoxanthine, and uridine was measured using standard uptake assays, and the transport rates were determined as 111.1 ± 3.4 pmol/mg protein/10 secs, 62.5 ± 4.8 pmol/mg protein/10 secs, and 101.3 ± 2.5 pmol/mg protein/10 secs, respectively. Furthermore, the presence of ENT1 protein was confirmed using S-(4-nitrobenzyl)-6-thioinosine (NBTI) binding assays (Bmax 1.52 ± 0.1 pmol/mg protein; Kd 0.42 ± 0.1 nM). Immunofluorescence assays and western blot analysis also showed the presence of ENT-1 in Panc-1 cells. The determined IC50 of gemcitabine in Panc-1 cells was 2 µM, indicating moderate sensitivity.

Conclusion: These results suggest that Panc-1 is a suitable preclinical cellular model for studying nucleoside analog drug transport properties and potential therapies in pancreatic cancer and pharmaceutical research.

Keywords: Panc-1, ENT-1, Gemcitabine, Transport, Pancreatic Cancer

Introduction

Gemcitabine, a nucleoside analog drug (NAD) and deoxycytidine nucleoside analog, is widely used as a chemotherapeutic agent in the treatment of various solid tumors, particularly pancreatic cancer, where it serves as a first-line therapy [1,2]. Gemcitabine is metabolized to an active triphosphate derivative that inhibits ribonucleotide reductase, leading to the arrest of de novo deoxyribonucleic acid (DNA) synthesis and induction of apoptosis [2]. Despite the initial sensitivity of pancreatic cancer cells to gemcitabine, most patients develop resistance within a few weeks of treatment initiation, resulting in poor survival rates [3]. Studies have shown that tumor cells deficient in nucleoside transporters are resistant to gemcitabine, highlighting the crucial role of these transport proteins in drug efficacy [4]. The levels of the predominant human nucleoside transporter, hENT1(human Equilibrative Nucleoside Transporter1), and the pyrimidine-preferring concentrative nucleoside transporter, hCNT1(human Concentrative Nucleoside Transporter 1) have been found to correlate with gemcitabine sensitivity [5,6]. Therefore,

understanding the role of nucleoside transporters in gemcitabine efficacy and developing novel approaches to enhance efficacy and overcome resistance are essential. Consequently, there is a need to better understand the role of nucleoside transporters in gemcitabine efficacy in pancreatic cells and to increase efficacy through the development of novel approaches that combine the established cytotoxicity of gemcitabine, taking into account the development of chemoresistance. Several different pancreatic tumor cell lines are used in research, which exhibit varying sensitivity to gemcitabine, but only a few have well-characterized transporter profiles. Panc-1, a preclinical cellular model of pancreatic cancer was cultured from a 56-year-old male with adenocarcinoma in the head of the pancreas that invaded the duodenal wall. Previous studies have reported a range of gemcitabine sensitivity (nM to mM) in Panc-1 [7, 8], but the transport characteristics remain unknown. Therefore, our objective was to characterize nucleoside transport activity in Panc-1 and investigate the presence of hENT1, which is both necessary and critical for gemcitabine efficacy [4]. Our results demonstrate that Panc-1 cells exhibit high levels of hENT1 protein expression and hENT1-dependent uptake. Moreover, Panc-1 cells demonstrate moderate sensitivity to gemcitabine, with an IC50 of 2 µM, suggesting that this cell line is a suitable preclinical cellular model for studying both the transport properties and NAD therapies.

Materials and Methods

Cell Culture

Panc-1 cells (ATCC + CRL-1469) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco™, Thermo Fisher Scientific, Milano, cat# LS11965092) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco™, Thermo Fisher Scientific, Milano, cat# 10437-036). The cells were maintained at 37 °C in a humidified incubator with 5% CO2 and were subcultured at a 1:4 ratio using 0.025% Trypsin-EDTA solution (Thermo Fisher Scientific, cat# 15090046). The authenticity of the Panc-1 cell line was confirmed by ATCC through RNA sequence analysis, which showed a 100% match to the original Panc-1 cell line.

Immunofluorescence Microscopy:

To determine the subcellular localization of hENT1, immunofluorescence assays were conducted with Panc-1 cells. The cells were seeded in 6-well plates on round, 18 mm poly-D-lysine pre-coated German glass coverslips (Electron Microscopy Sciences, cat# 72294-11) and were grown in a humidified incubator at 37°C with 5% CO2 for 20-24 hours until they reached a minimum of 60% confluency. The cells were then washed twice with pre-warmed phenol-free HBSS++ (Hanks' Balanced Salt Solution supplemented with calcium and magnesium, Thermo Fisher Scientific, cat# 14025076) for 5 minutes, fixed for 15 minutes in 4% (w/v) paraformaldehyde in calcium and magnesium-free Phosphate Buffer Saline (PBS), and rinsed. Following that, the cells were incubated with anti-hENT1 antibody (Santa Cruz, cat# sc-377283-AF488) for 1 hour at room temperature, and then rinsed. Subsequently, the cells were incubated with Wheat Germ Agglutinin 594 (WGA) at a concentration of 5.0 µg/mL and 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes at 1:30,000 dilution. After rinsing, the cells were mounted in calcium and magnesium-free PBS within a 35mm ChamlideTM dish-type magnetic chamber (Quorum Technologies, cat# CM-B-30). Images were obtained using a Yokogawa X1 head with a Borealis Spinning Disc Microscope.

Total Lysate and Cytosolic Protein Extractions:

To confirm the presence of hENT1 within the membranes of Panc-1 cells, immunohistochemistry assays were performed. Panc-1 cells were seeded in 10mm plates and grown in a humidified incubator at 37°C with 5% CO2 for 48 hours. The cells were then washed twice with room temperature PBS. For the total lysate collection, cells were harvested by scraping in NP-40 buffer (50mM NaF, 1mM Na2VO3) and protease inhibitor cocktail (Complete Mini, Roche, 11836153001), followed by membrane disruption by passing them through a 26-gauge needle attached to a 1mL syringe three times within 5-minute intervals on ice. Cell homogenate was fractionated by centrifugation at 8,000 x g for 5 minutes at 4ºC, and the supernatant containing protein extract was collected and stored on ice. The protein concentration was quantified using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s protocol. For the collection of cytosolic fractions, cells were harvested by scraping in PBS and collected using the Mem-PER Plus Membrane Protein Extraction Kit (ThermoFisher, Cat # 89842), and protein was collected following the manufacturer's protocol. Both cytosolic and membrane fractions were collected and stored on ice, and the protein concentration was quantified using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s protocol.

Immunoblot Analysis:

All lysates were prepared in Laemmli sample buffer (LSB; 0.5 M Tris, pH 6.8, glycerol, 10% Sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, and 5% bromophenol blue) and heated at 95ºC for 5 minutes. Proteins were resolved by glycine-Tris SDS-PAGE followed by transfer onto a nitrocellulose membrane (Bio-Rad). The membrane was then washed, blocked, and incubated overnight at 4ºC with a 1:1000 (v/v) dilution of anti-hENT1 antibody (Santa Cruz, cat# sc-377283). After three washes, proteins were detected using a 1:2000 (v/v) dilution of goat anti-mouse (IgG) secondary antibody conjugated to horseradish peroxidase for 2 hours at room temperature, followed by enhanced chemiluminescence (ECL) detection using the ECL Detection Kit (Bio-Rad, cat# 1705061). Primary and secondary antibodies were removed using Restore™ PLUS Western Blot Stripping Buffer according to the manufacturer's protocol (Thermo Fisher Scientific, cat# 46430). The blots were washed three times and probed to detect the loading control protein GAPDH using a 1:8000 (v/v) dilution of anti-GAPDH antibody (Santa Cruz, cat#

(0411): sc-47724) for 2 hours at room temperature. Proteins were detected using a 1:2000 (v/v) dilution of goat anti-mouse (IgG) secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature, followed by enhanced chemiluminescence (ECL) detection using the ECL Detection Kit (Bio-Rad, cat# 1705061). Western blot assays were performed three times, and the data from each experiment were pooled. The signals corresponding to the intensity of the hENT1 protein were obtained by analyzing the original .scn files using ImageJ software to determine the area under the peak in the appropriate lane and band for each cell type. The area values collected for Replicate 3 samples were adjusted to show the corresponding value of expression in 10 µg of protein. The area values were normalized to the loading control (e.g., GAPDH) signal obtained after reblotting. The data are expressed as mean values that have been normalized.

Transport Assay:

The transport characteristics of chloroadenosine (a purine analogue), uridine (a pyrimidine analogue), and hypoxanthine (a nucleobase) were determined using standard assays and [3H]-labeled substrates. Panc-1 cells were seeded into three 6-well plates at a density of approximately 300,000 cells per well and were grown in a humidified incubator at 37°C with 5% (v/v) CO2 for 24-48 hours until they reached 80% confluency. Uptake was measured in sodium-free buffer (20 mM Tris-HCl, 3 mM K2HPO4, 1 mM MgCl2, 2 mM CaCl2.2H2O, 5 mM glucose, and 130 mM N-methyl-D-glucamine, pH 7.4) containing 10 µM cold substrate and 1 x 106 cpm/ml [3H]-labeled substrate. The cells were washed with sodium-free transport buffer and incubated for 10 seconds in 1.25 ml of permeant solution (sodium-free buffer containing either chloroadenosine, hypoxanthine, or uridine as the substrate). The uptake was stopped by rapid aspiration of the permeant solution and rapid washing of cells three times with sodium-free, ice-cold stop buffer (100 nM NBTI plus 30 µM dipyridamole). Cells in each well were lysed in 1 ml 2 M NaOH overnight at 4°C. Aliquots were taken to measure the protein content using a modified Lowry protein assay (Bio-Rad), and the [3H]-labeled substrate uptake was measured by conducting standard liquid scintillation counting. Substrate uptake is expressed as picomoles per milligram of protein.

NBTI Binding

To confirm the presence and correct orientation of the hENT1 protein on the cell surface, the binding of NBTI was measured. NBTI is a high-affinity, non-transportable nucleoside analog that tightly binds to hENT1 and inhibits ENT1-mediated transport at nanomolar concentrations. Panc-1 cells were seeded in 10 cm plates and grown to 90-100% confluency. The cells were washed and collected by scraping, followed by resuspension in binding buffer (10 mM Tris-HCl, 100 mM KCl, 0.1 mM MgCl2, and 0.1 mM CaCl2, pH 7.4). NBTI binding experiments were performed by incubating the cells in the presence of increasing concentrations of [3H] NBTI (0.186 - 7.45 nM). Binding was calculated as the difference between binding in the absence (total binding) and the presence (nonspecific binding) of 10 µM unlabeled NBTI. The cells were incubated at room temperature with increasing concentrations of [3H] NBTI for 50 minutes to reach equilibrium, and the reaction was stopped by adding ice-cold binding buffer. The samples were then subjected to scintillation counting to measure the accumulated radioactivity. The [3H] NBTI binding constants, Kd (binding affinity), and Bmax (maximum specific binding) were obtained by non-linear regression analysis using GraphPad Software (PRISM version 8.0 for Windows).

Determination of IC_{50}

To determine the IC50 of gemcitabine in Panc-1 cells, the doubling time of Panc-1 was first established to be 40 hours. The cells were then treated with a range of concentrations of gemcitabine (500 nM to 100 µM) for 48 hours, and 50% inhibition of cell growth was determined using a trypan exclusion viability assay measured with the Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter, USA). The experiments were repeated three times, and statistical analyses were performed using GraphPad Prism version 8.0 for Windows.

Data analysis

Nucleoside and nucleobase uptake and NBTI binding assays were performed three times, and the data from each experiment were pooled. The data are expressed as means ± standard error of the mean (SEM). Uptake data were compared using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison test to determine statistical significance at $p < 0.05$. The Kd and Bmax values for [3H]-NBTI binding were calculated using non-linear regression analysis. All statistical analyses were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, 2018).

Results

ENT1 is a key transporter involved in the uptake of NAD drugs like gemcitabine. [11] In this study, we characterized the ENT-dependent uptake and presence of hENT1 in Panc-1 cells. Co-localization analysis with the membrane marker WGA confirmed the presence of hENT1 at the cellular membrane (Figure 1A), supporting previous findings. [12, 13, 14] We also observed the presence of hENT1 intracellularly, consistent with previous reports of ENT1 localization in internal structures such as membrane-bound vesicles, endosomes, and lysosomes in other cell lines. [15]

To compare the levels of hENT1 protein in Panc-1 cells with a well-established cell model, we examined HEK-293 cells (Figure 1B). Immunoblot analysis revealed that Panc-1 cells have approximately 13% higher levels of hENT1 protein compared to HEK-293 cells (Figure 1C). However, the presence of ENT1 protein alone does not confirm its functional uptake. Therefore, we measured nucleoside and nucleobase uptake in the absence of sodium to exclude uptake via CNT. Our results showed that chloroadenosine, uridine, and hypoxanthine can all be transported into Panc-1 cells, with varying rates. Chloroadenosine and uridine exhibited higher uptake compared to hypoxanthine (Figure 2A), consistent with the preference of ENT1 for nucleosides over nucleobases. The measurable uptake of hypoxanthine is likely attributed to ENT2, which can transport both nucleosides and nucleobases. [16]

To confirm that ENT1 is responsible for the majority of chloroadenosine and uridine transport, we performed inhibition assays using NBTI, a specific inhibitor of ENT1 (Figure 2B). The effective inhibition of nucleoside uptake by NBTI further supported the involvement of ENT1 in the transport process. NBTI binding assays were also performed to confirm the presence of hENT1 on the cell surface membrane (Figure 2C). The binding capacity, represented by Bmax (maximum binding capacity), of Panc-1 cells (Bmax = 1.52 pmol/mg protein, Kd = 0.43 nM) suggested a higher number of hENT1 proteins with high affinity compared to other cell lines, such as MCF-7(B_{max} 1.01 pmol/mg protein), HL-1 (B_{max} =0.58 pmol/mg protein), and HEK293 (0.45 pmol/mg protein) cells. [17,18,19] The higher Bmax in Panc-1 cells (111.18 ± 3.45 pmol/mg protein) correlated with the significantly greater uptake of chloroadenosine compared to HEK293 cells (60±2 pmol/mg protein). [20] The affinity (Kd) of NBTI binding in Panc-1 cells fell within the range of published values for other cell lines, indicating similar binding site characteristics. [17, 21, 22] Lastly, we determined the IC50 of gemcitabine in Panc-1 cells, which was found to be 2 µM (Figure 3). This value represents the concentration of gemcitabine required to inhibit 50% of cell growth after a 48-hour gemcitabine treatment.

Overall, these findings provide insights into the ENT-dependent uptake of nucleosides and nucleobases in Panc-1 cells, confirm the presence of hENT1 at the cellular membrane, and demonstrate the functional relevance of ENT1 in gemcitabine uptake.

Discussion: Based on our findings, we can confirm the significant hENT1-dependent uptake of gemcitabine in Panc-1 cells, which is consistent with hENT1 playing a major role in determining the response to gemcitabine in pancreatic cancer patients. [6, 23] This reinforces the use of Panc-1 cells as a suitable model for studying the susceptibility of pancreatic tumors to gemcitabine treatment, either alone or in combination with other therapies.

It is worth noting that the IC50 level of gemcitabine in our study differed from previous reports, which have reported IC50 values ranging from nanomolar to millimolar levels. [7, 8]. The reasons for these discrepancies are not clear, but they emphasize the importance of establishing the sensitivity of a cell line to a drug before using it for further research. Gemcitabine sensitivity of tumor cell lines can vary under different experimental conditions, including drug concentration, exposure time, and the assay used for evaluation. In a study examining the cytotoxic effect of gemcitabine, various cell cultures were assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell viability assay. The cells included primary pancreatic tumor cells derived from ductal adenocarcinoma, pancreatic stellate cells, and established pancreatic ductal adenocarcinoma cell lines (BxPC-3, Mia PaCa-2, and Panc-1). Results indicated that gemcitabine exhibited a dose-dependent inhibition of cell viability in all primary tumor cell cultures and the established cell lines. However, none of the pancreatic stellate cells displayed sensitivity to the cytotoxic effects of gemcitabine. At a specific concentration of gemcitabine (10µM), the viability of primary cancer cells decreased by 58-70%, while the pancreatic ductal adenocarcinoma cell lines (BxPC-3, Mia PaCa-2, and Panc-1) exhibited a reduction in viability ranging from 40-56% (BxPC-3 the most and Panc-1 the least sensitive). Primary pancreatic cancer cells exhibited higher chemosensitivity compared to the pancreatic ductal adenocarcinoma cell lines. The resistance of satellite cells to gemcitabine was attributed to the absence of membrane transporters. [28] The low to moderate sensitivity of Panc-1 cells to gemcitabine provides an opportunity to explore combination therapies, such as ultrasound and microbubbles (USMB) in conjunction with gemcitabine. [24, 25] Studies have shown that this combination can enhance cytotoxicity in pancreatic cancer therapy compared to either treatment alone.

The development of novel approaches is crucial for pancreatic cancer treatment, considering that the disease often presents at an advanced stage and tumor cells rapidly develop resistance. [26] Incubation of cells with NADs, such as gemcitabine, may lead to down-regulation of transporter expression and the selection of transporter-deficient cells, contributing to clinical resistance to gemcitabine chemotherapy. [10] These aspects of gemcitabine resistance can be investigated in Panc-1 cells, as they reflect cellular behavior in response to drug treatment. For example, studies have demonstrated that gemcitabine resistance induced by the epithelial-to-mesenchymal transition in pancreatic cancer cells involves the functional loss of hENT1. [14]

Tumors typically consist of heterogeneous populations of cells, each potentially having varying NT expression profiles. [27] High expression of ENT1 in pancreatic cancer has been associated with increased patient survival in those receiving gemcitabine treatment, highlighting the significance of ENT1 in therapeutic response. [6, 23] Apart from ENT1, hENT-2 (human Equilibrative nucleoside transporter 2) was identified to transport gemcitabine into cells however it is not as effective as ENT1 in drug uptake.[29] However, hENT2 when aberrantly expressed is thought to contribute to gemcitabine resistance.[2] There has been an inverse proportion showed between hCNT3 (human Concentrative Nucleoside Transporter 3) amount and gemcitabine toxicity.[30] CNT-3 is considered a potential therapeutic target for addressing resistance to toxic nucleoside analog treatments. While much research has focused on hENT1 as a target for overcoming gemcitabine resistance in pancreatic cancer patients, it was found that hCNT3-transfected cells with functional hENTs exhibited an 8-fold increase in gemcitabine uptake and cells without functional ENTs had much higher uptake pointing out that functional hENTs present in cells result in a decrease in gemcitabine uptake. [31] In another study, it was found that patients with high expression levels of ENT1 had notably longer disease-free survival and overall survival compared to patients with low expression levels. High expression levels of hCNT3 were associated with longer overall survival but not with disease-free survival. However, pancreatic cancer patients who exhibited both high expression levels of hENT1 and hCNT3 experienced a remarkable five-fold longer overall survival compared to other patients. This suggests that the combined presence of high expression levels of both hENT1 and hCNT3 may lead to improved survival outcomes in pancreatic cancer patients. [32]

These findings suggest that the role of hCNT3 in gemcitabine treatment response and patient prognosis is complex. While increased hCNT3 expression may lead to decreased gemcitabine uptake, the presence of both high hENT1 and hCNT3 expression levels appears to be associated with improved survival outcomes in pancreatic cancer patients.

In summary, our data support the role of hENT1-dependent uptake in Panc-1 cells, which has implications for gemcitabine response in pancreatic cancer patients. Panc-1 cells provide a valuable model for studying the efficacy of gemcitabine and exploring combination therapies.

Conclusion: The significant expression of hENT1, the observed hENT-dependent uptake, and the sensitivity to gemcitabine in Panc-1 cells indicate that this cell line serves as a valuable model for investigating NAD therapies in combination with other strategies for pancreatic cancer treatment. Understanding the mechanisms of gemcitabine efficacy in Panc-1 cells can contribute to the development of improved treatment strategies for pancreatic cancer. Our findings contribute to the understanding of the molecular mechanisms underlying gemcitabine response and resistance in pancreatic cancer and pave the way for future studies aimed at improving therapeutic outcomes and exploring novel treatment approaches for this challenging disease.

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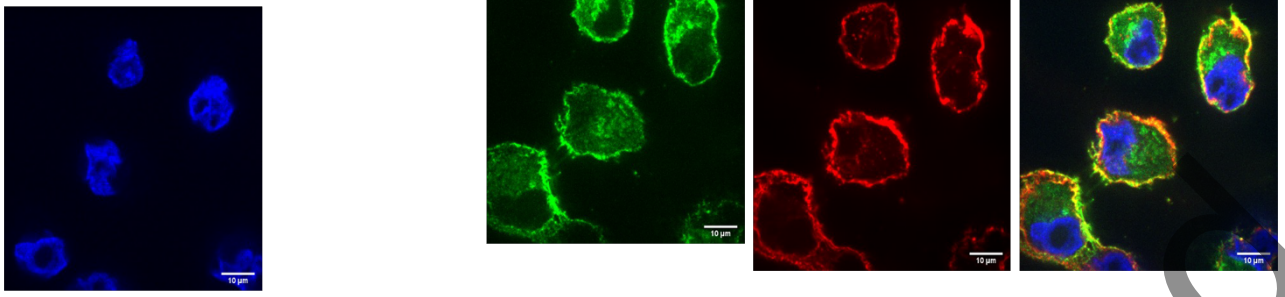
Table 1: Sensitivity of Pancreatic Adenocarcinoma Cell Lines against Gemcitabine

Cell Line	**Sensitivity to Gemcitabine**
AsPc-1	Low to Moderate [33,35]
BXPC-3	High to Moderate [28,34]
Capan-1	High to Moderate [35,35]
MiaPaca-2	High to Moderate [28,35]
Panc-1	Low to Moderate [28,8]

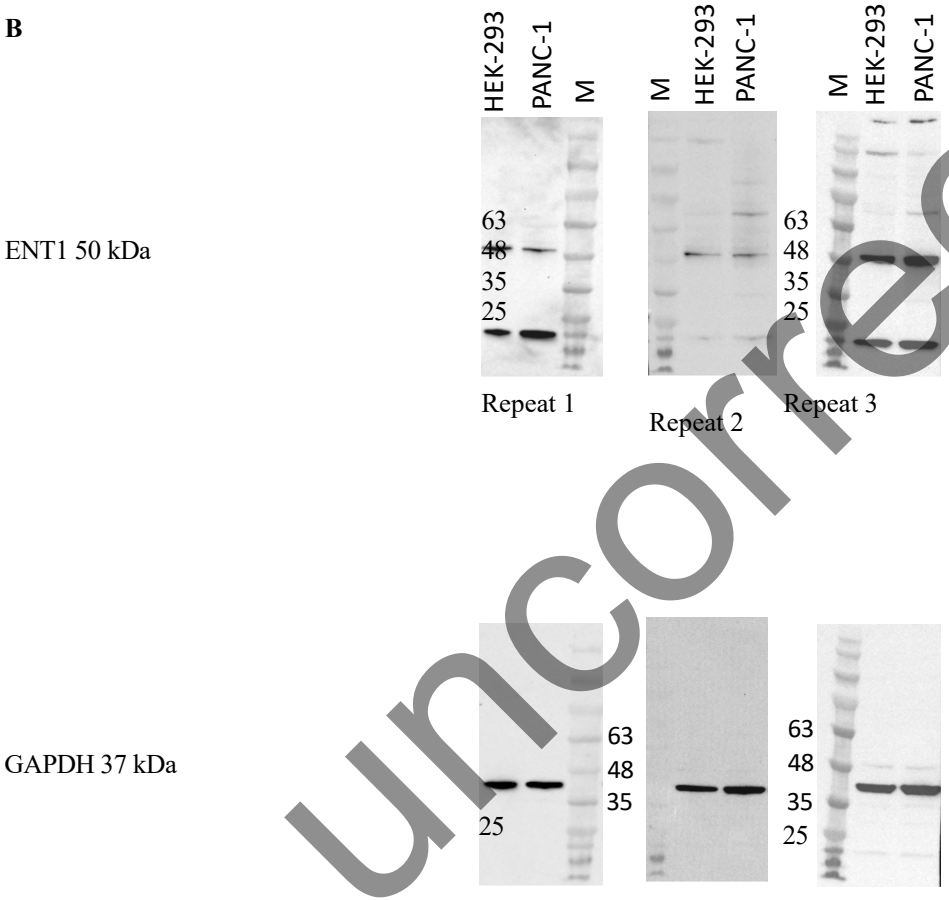
Figure 1

A

DAPI **hENT1** **WGA** **MERGE**



B



C

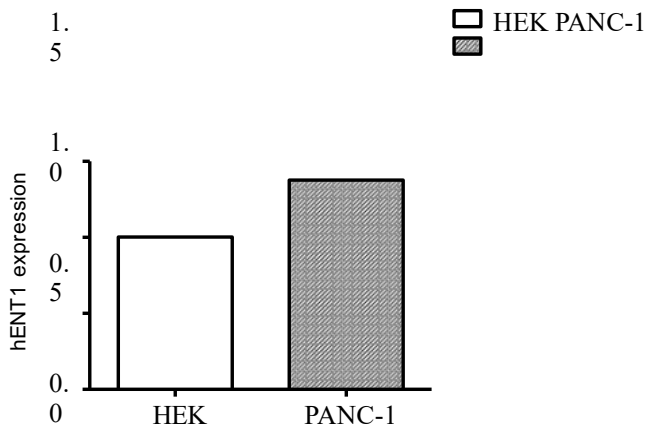
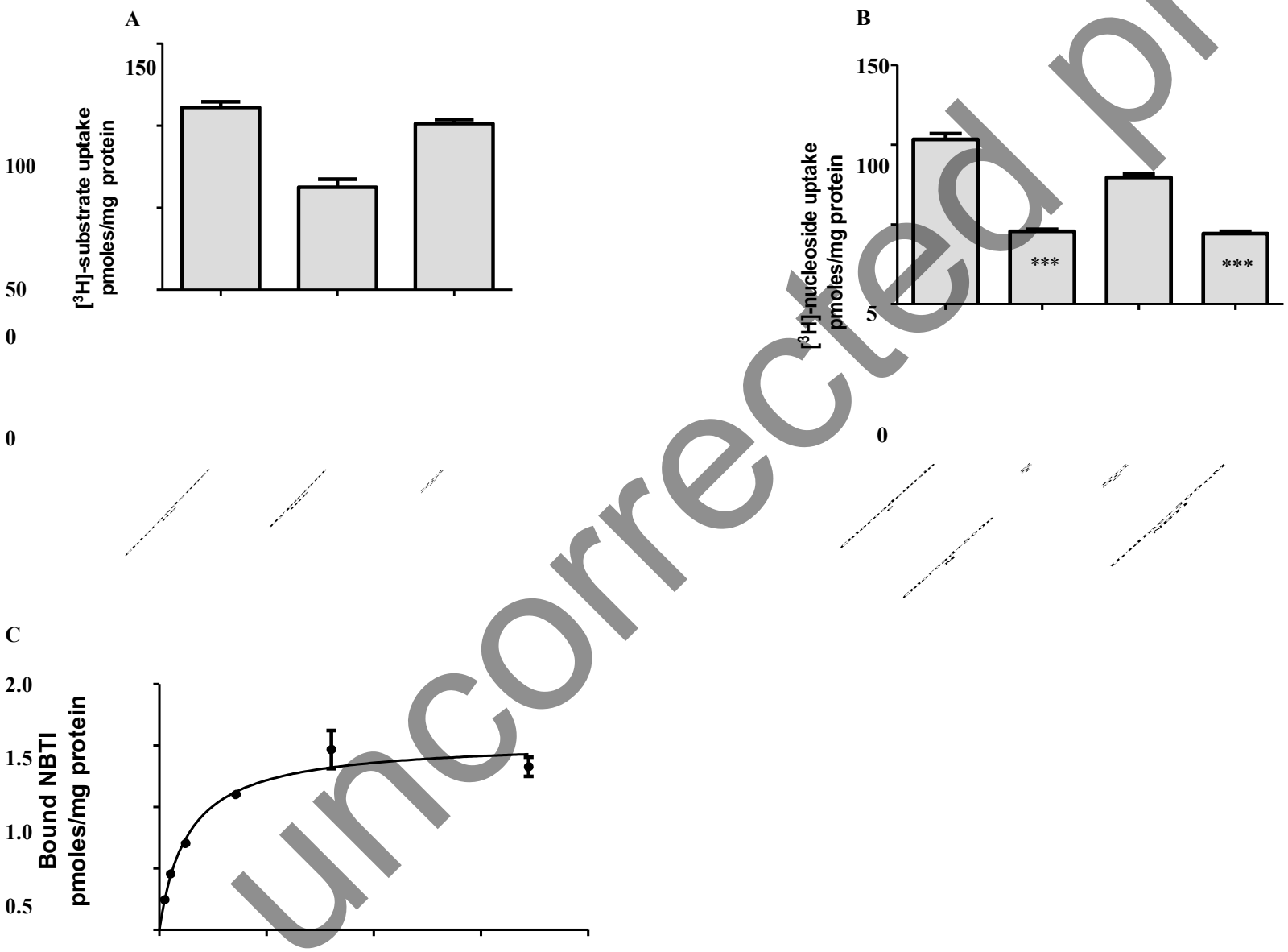


Figure 1. hENT1 is present at the plasma membrane of Panc-1 cells.
(A) Endogenous hENT1 is found at the plasma membrane of Panc-1 cells based on co-localization with WGA which localizes to the plasma membrane. Some hENT1 was observed in intracellular structures distributed in a punctate pattern.
(B) Three representative western blot analyses show the presence of hENT1 at the predicted size of ~50 kDa in Panc-1 cells in comparison to HEK293 cells. Cytosolic fractions (repeat 1: 10ug) or whole-cell lysates (repeats 2-3: 10ug and 15ug, respectively) were resolved by immunoblotting and probed with antibodies targeting hENT1 (top) or loading control GAPDH (bottom). M represents the marker lane.
(C) Quantification of the western blots show 13% higher expression of hENT1 in Panc-1 cells compared to HEK-293

Figure 2



0.0

0 2 4 6 8

Free NBTI (nM)

Figure 2. Panc-1 cells exhibit hENT-dependent nucleoside transport.
(A) Pam 1 cells exhibit higher levels of uptake of the nucleosides chloroadenosine (1) and uridine (3) compared to the nucleobase hypoxanthine (2). Data are pooled from 3 independent experiments with 6 replicates for each substrate. Error bars represent mean ± SEM. (B) hENT1 was confirmed to contribute to chloroadenosine (1) and uridine (3) uptake in Panc-1 cells since it was reduced in the presence of the hENT1 specific inhibitor NBTI (500 nM), chloroadenosine + NBTI (2), uridine + NBTI (4).
Data are pooled from 3 independent experiment with 6 replicates for each substrate.
Error bars represent mean ± SEM. ***P < 0.001 (C) Presence of hENT1 protein in Panc-1 cells was confirmed by NBTI binding. Representative experiment shown repeated 3 times with similar results. Each point is a mean of two replicates ± SD.

Figure 3

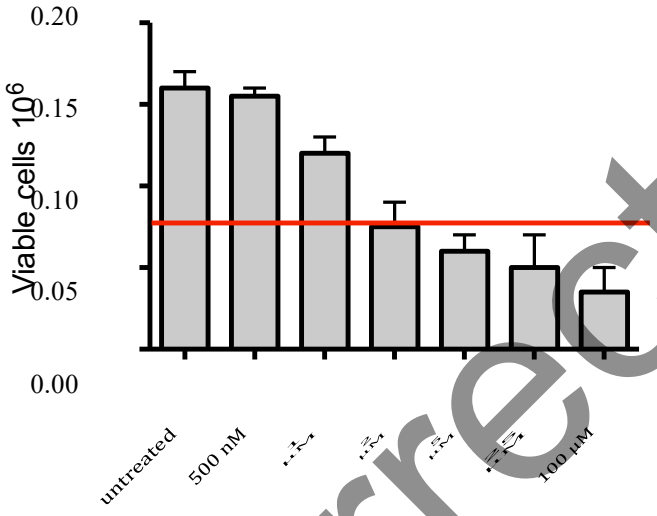


Figure 3 The IC_{50} of gemcitabine in Panc-1 cells.
IC_{50} of Panc-1 cells to gemcitabine was determined to be 2 µM by incubation in range of concentrations of the drug (500nM-100 µM) and measurement of viability after 48 hours. (0 = no treatment, 1 = 500nM, 2= 1 µM, 3= 2 µM, 4= 5 µM, 5= 25 µM, 6= 100 µM). Error bars represent mean ± SEM. **P < 0.005. The experiment was repeated 3 times with similar results and each treatment was done in duplicates.