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Cytotoxic activity of gemcitabine in cultured cell lines derived from histologically different types of bladder cancer: Role of thymidine kinase 2

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ABSTRACT

World wide incidence of bladder cancer is rising with nearly 13,760 deaths attributed to bladder cancer in 2007 in the USA. Tumor types of the urothelium include transitional cell carcinomas, squamous cell carcinomas, and adenocarcinomas. This study was undertaken to determine gemcitabine's efficacy against bladder cancer cell lines of different origins (HTB2, a papilloma; HTB3, a squamous cell carcinoma; and HTB4, a transitional cell carcinoma). Roles of nucleoside transporters and key enzymes in gemcitabine pharmacology were examined on the premise that cells originating from different types of bladder cancer exhibit different levels and/or types of nucleoside transporters and enzymes and thus may respond differently to gemcitabine. HTB2 cells had the highest transport efficiency and were also most responsive to gemcitabine. HTB3 and HTB4 cells had similar transport efficiencies, but exhibited different sensitivities to gemcitabine (HTB4 > HTB3). The highest accumulation of [^{3}H]gemcitabine was in HTB2 cells and the lowest was in HTB3 cells. Sequencing experiments revealed no mutations either in coding exons or intron–exon boundaries of the *hENT1* genes of the three cell lines. HTB3 cells exhibited high thymidine kinase 2 (TK2) activity whereas HTB2 and HTB4 cells lacked detectable TK2 activity and pretreatment of HTB3 but not of HTB2 and HTB4 cells with extracellular thymidine resulted in enhanced sensitivity to gemcitabine. Our results highlight the importance of hENT1 and TK2 activities in response to gemcitabine. Elevated TK expression in squamous cell carcinomas warrants further study and offers new insights into rational treatment strategies based on bladder cancer phenotype.

1. Introduction

World wide incidence of bladder cancer is continuing to rise and the prevalence in the USA is roughly 490,000 with nearly 13,760 deaths attributed to bladder cancer in 2007 [1]. Tumor types of the urothelium include transitional cell carcinomas, squamous cell carcinomas, and adenocarcinomas, and other less frequent lesions [2]. About 70% of low grade papillary tumors of the bladder are diagnosed as superficial (Ta, T1) at first presentation. Superficial bladder cancer makes up roughly 70–80% of newly diagnosed cases of bladder cancer in Canada [3]. A potential survival benefit for patients with bladder cancer has been demonstrated for adjuvant chemotherapy with cytotoxic drugs, including gemcitabine [2]. In studies of locally advanced and metastatic bladder cancers, gemcitabine has been evaluated in several phase I and II trials yielding response rates in chemonaive patients of 24–28% with complete response rates of 8–10% [4,5].

Gemcitabine is a hydrophilic antimetabolite drug that enters cells primarily via nucleoside transport proteins [6,7]. Human nucleoside transporters (hNTs), which exhibit broad permeant selectivities and wide tissue distributions [8], are sub-divided into equilibrative (hENTs), which mediate equilibrative transport of purine and pyrimidine nucleosides down their concentration gradients, and concentrative nucleoside transporters (hCNTs), which mediate cotransport of Na^+ down its concentration gradient and purine and pyrimidine nucleosides against their concentration gradients. Four of the five known human nucleoside transporters, hENT1, hENT2, hCNT1 and hCNT3 transport gemcitabine. hENT1 and hENT2 are distinguished by their relative sensitivities to low (nM) concentrations of nitrobenzylmercaptopurine ribonucleoside

Abbreviations: hENT, human equilibrative nucleoside transporters; NBMPR, nitrobenzylmercaptopurine riboside; IC_{50}, the concentration of drug that inhibited growth of treated cells by 50% relative to that of untreated cells; TK, thymidine kinase; dCK, deoxycytidine kinase; TK2, thymidine kinase 2.

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(NBMPR) as equilibrative-sensitive (hENT1) and equilibrative-insensitive (hENT2). hENT3 is found only in intracellular membranes and does not play a role in cellular uptake of external nucleosides [9], and hENT4 is a cell surface pH-dependent adenosine transporter [10]. Three isoforms, hCNT1, hCNT2, and hCNT3, which are found primarily in specialized tissues (*e.g.*, intestine, kidney, liver and choroid plexus) mediate, respectively, pyrimidine-nucleoside selective, purine-nucleoside selective and broadly selective nucleoside-transport activities.

Gemcitabine enters cells and is phosphorylated to its monophosphate form by deoxycytidine kinase (dCK) [11]. Deoxynucleoside kinases, such as dCK, two thymidine kinases (TK1 and TK2) and deoxyguanosine kinase (dGK), are involved in the salvage and *de novo* supply of deoxynucleotides for DNA synthesis [12]. dCK and TK1 are cytosolic, TK2 is present in both cytosol and mitochondria and dGK is present only in mitochondria [13]. Although TK1 is a cell-cycle regulated protein and does not accept deoxycytidine as a substrate, neither dCK nor TK2 is cell-cycle regulated and deoxycytidine is phosphorylated by both [14]. Unlike deoxycytidine, gemcitabine and cytarabine are poor substrates for TK2 [15]. TK2 has several fold higher capacity for deoxycytidine phosphorylation than dCK [16,17]. Increases in dCTP pools lead to feedback inhibition of dCK activity, which in cells exposed to gemcitabine, could result not only in decreased phosphorylation of deoxycytidine, but also of gemcitabine, thereby reducing gemcitabine toxicity. In some patients with bladder cancer, elevated total TK levels were associated with early recurrence [18] and positive correlations between total TK activity and disease stage and tumor grade were observed. Thymidine is an effective inhibitor of the deoxycytidine phosphorylating activity of TK2 and pretreatment of cultured cells with thymidine leads to lowered dCTP pools in cells and higher phosphorylation of anticancer nucleoside drugs [16].

This study was undertaken to determine gemcitabine's efficacy against three bladder cancer cell lines, HTB2 derived from a papilloma (superficial transitional carcinoma), HTB3 derived from a squamous cell carcinoma and HTB4 derived from an invasive transitional carcinoma (these cell lines are also known, respectively, as RT4, SCaBER and T24 or EJ28). Roles of nucleoside transporters and key enzymes in gemcitabine pharmacology were examined on the premise that cells originating from different types of bladder cancer exhibit different levels and/or types of nucleoside transporters and enzymes and thus may respond differently to gemcitabine. The three cell lines showed differences in NT, dCK and TK2 activities and the cell line derived from a superficial bladder cancer was most responsive to gemcitabine treatment whereas the cell line derived from a squamous cell cancer was least responsive. The latter cell line also had elevated TK2 activity and pretreatment with extracellular thymidine resulted in enhanced response to gemcitabine. The results suggested that bladder cancers with high TK activity could be sensitized to gemcitabine by pretreatment with excess thymidine prior to gemcitabine treatment.

2. Materials and methods

2.1. Chemicals

NBMPR, dilazep, unlabeled nucleosides and other chemicals were obtained from Sigma Chemical Company (Mississauga, ON). Tritiated nucleosides were purchased from Moravek Biochemicals (Brea, CA). Tissue culture (96- and 12-well) plates, cell culture media, and fetal bovine serum (FBS) were from Gibco BRL (Burlington, ON). Ecolite was from ICN Pharmaceuticals (Montreal, PQ). The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit was from Promega (Madison, WI). 5′-S-{2-(1-[(fluorescein-5-yl)thioureido]-hexanamido)ethyl}-6-N-(4-nitrobenzyl)-5′-thioadenosine (FTH-SAENTA) was a gift from Dr. Morris J. Robins (Brigham Young University, Provo, UT, U.S.A). Qiagen reagents and kits were used for genomic DNA and total RNA extraction and for cDNA synthesis from RNA (Qiagen, Mississauga, ON, Canada).

2.2. Cell culture

The human bladder cancer cell lines HTB-2, HTB-3 and HTB-4 were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 10% glucose. All cultures were kept at 37 °C in 5% CO_2/95% air and sub-cultured every 2–3 days to maintain exponential growth. Transport and cytotoxicity experiments were conducted with cells in exponential growth phase. Cells were free of mycoplasma contamination.

2.3. Confocal microscopy

Cells were seeded in glass bottom 35-mm dishes and allowed to grow for 48 h after which they were imaged by a laser scanning Zeiss Axioskop2 plus Microscope (LSM 510; Carl Zeiss Inc., Thornwood, NY).

2.4. Cytotoxicity assays

The CellTiter 96 proliferation assay kit was used to quantify drug-induced cytotoxicity. Unless otherwise noted, cells were seeded in 96-well plates at densities of 5000 per well and allowed to attach for 24 h. Cells were then exposed to graded gemcitabine concentrations in the absence or presence of 1 μM NMPR for 72 h and treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent for assessment of cytotoxicity. In some experiments, cells were pretreated with 2 mM thymidine for 16 h after which media was replaced with thymidine-free gemcitabine-containing media and further incubated for 72 h. IC_{50} values (the concentration of drug that inhibited growth of treated cells by 50% relative to that of untreated (*i.e.*, control) cells) were calculated from nonlinear regression analyses of values plotted as percentages of control values against the logarithm of drug concentrations.

2.5. Determination of hENT1 site densities

5′-S-{2-(1-[(fluorescein-5-yl)thioureido]-hexanamido)ethyl}-6-N-(4-nitrobenzyl)-5′-thioadenosine (FTH-SAENTA) is a structural analogue of NBMPR that binds tightly to the transport-inhibitory sites of the hENT1 protein and can be used as a fluorescent probe to detect hENT1 on cell surfaces [19,20]. hENT1 cell surface abundance in bladder cells was determined using FTH-SAENTA, a membrane-impermeable NBMPR analogue [20], that competes for binding to hENT1 at the extracellular side of plasma membranes, thus enabling measurement of extracellular and intracellular NBMPR binding sites. Adherent cells (approximately 100,000/well) in 12-well plates were incubated in triplicate at room temperature for 1 h with a saturating concentration (10 nM) of [^{3}H]NBMPR alone or with 100 nM FTH-SAENTA or 1 μM unlabeled NBMPR in 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, 150 mM NaCl and 5 mM glucose; pH 7.4. At the end of incubations, cultures were washed twice with the above buffer and solubilized with 0.5 ml of 5% Triton X-100, after which cell-associated [^{3}H]NBMPR was quantified by liquid scintillation counting. Specifically bound [^{3}H]NBMPR was calculated from the difference between [^{3}H]NBMPR bound in the absence (total bound) and presence (non-specifically bound) of 1 μM NBMPR. Extracellular specifically bound [^{3}H]NBMPR was calculated from the difference of [^{3}H]NBMPR bound without and with FTH-

SAENTA. The percentage of specifically bound extracellular [^{3}H]NBMPR was calculated as (extracellular/total bound [^{3}H]NBMPR) × 100.

2.6. Nucleoside uptake assays in bladder cancer cells

Cells (100,000/well) were seeded in 12-well plates and on the third day, rates of uptake of [^{3}H]nucleosides were measured. The transport buffers (pH 7.4) used were 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, and 5 mM glucose in either sodium-containing (NaCl) or sodium-free (KCl) salts at 144 mM. For uptake assays, cell growth medium was aspirated, cells were washed with sodium-containing or sodium-free buffers, [^{3}H]permeant (*i.e.*, uridine or gemcitabine) was added and uptake was measured for various time intervals (0–40 s or 0–120 s) in the presence or absence of potential inhibitors. ENT activities were determined by measuring transport of [^{3}H]uridine in the absence or presence of 0.1 μM NBMPR (inhibits hENT1 activity) or 10 μM dipyridamole (inhibits both hENT1 and hENT2 activities) to determine the relative contributions of hENT1 and hENT2-mediated processes. Total ENT activity was obtained by subtracting activity in the presence of 10 μM dipyridamole from that in absence of any inhibitors. hENT1 activity was obtained by subtracting activity in the presence of 0.1 μM NBMPR from total activity and hENT2 activity was determined from total-hENT1 activity. Kinetics of uridine uptake was determined at graded concentration of uridine (0–100 μM) and using 30-s incubations (shown in time-course studies to provide initial rates). At the end of uptake intervals, permeant-containing solutions were removed by aspiration; cells were quickly rinsed twice with either sodium-containing or sodium-free buffer and solubilized with 5% Triton X-100. Radioactivity in solubilized extracts was measured by liquid scintillation counting. Uptake values were expressed as pmol/10^6cells and graphs were generated using the software GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA). Each experiment was conducted two or more times with triplicate measurements.

2.7. DNA isolation, cDNA synthesis and dideoxy sequencing

Qiagen reagents and kits were used for genomic DNA and total RNA extraction and for cDNA synthesis from RNA (Qiagen, Mississauga, ON, Canada). Genomic DNA from cell lines (HTB2, 3 and 4) was isolated with the QiaAmp kit. Exponentially growing cells were harvested, washed and lysed to isolate total RNA (RNeasy kit), which was subsequently used to generate cDNA using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Streetsville, ON), www.appliedbiosystems.com). Services of the Genome Quebec Innovation Centre (Montreal, PQ, Canada) were utilized for sequencing of both genomic DNA and cDNA. Primers were designed to sequence the *hENT1* gene from genomic DNA isolated from HTB2, 3 and 4. Primers covered up to 300 base pair (bp) sequences upstream of the ATG start codon and 300 bp beyond the 3′ untranslated region (UTR). The 50-bp flanking intron–exon boundaries were also sequenced to identify putative alternative splice sites. The genomic sequence (6p21.2-p21.1; contiguous position 44,295,371-44,309,856; goldenpath build hg18) for the *hENT1* gene and flanking exonic and intronic sequences were derived from the University of California Santa Cruz (UCSC) database http://genome.ucsc.edu/or http://snpper.chip.org/bio/find-gene. The National Center for Biotechnology Information (NCBI) reference sequence, NM_004955, corresponds to the full length hENT1 cDNA and includes the first non-coding exon described above (http://www.ncbi.nlm.nih.gov). To ensure full sequence coverage, primer pairs were designed to provide overlapping coverage for all amplicons for both genomic (data not shown) and cDNA sequencing. The primer pairs used for hENT1 (cDNA #01-04) for forward (F) and reverse (R) PCR reactions were as follows:

hENT1cDNA #01-F/R, ACTGAGTCGGCTCTGGTCTC/GATCAGAAACACCAGCAGGA; hENT1cDNA #02-F/R, GAGCGGAACTCTCTCAGTGC/GCAGACAGAGAAAGCCAGGA; hENT1cDNA #03-F/R, CAGGCAAAGAGGAATCTGGA/CCAGACCACTCAGGATCACC; hENT1cDNA #04-F/R, ATCATGGCCTTCTTCCTGTG/AAGAAAGGTTTATTTAAATCAGAGAGC.

2.8. Deoxycytidine (dCK) and thymidine kinase 2 (TK2) activities

Cell lysates were prepared as described [21] by repeated freeze-thaw cycles in an extraction buffer (pH 7.5) containing 50 mM Tris–HCl, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% NP40. Resulting lysates were clarified by centrifugation (11,000 × *g*) and were used either immediately for assays or stored at −70 °C. TK2 and dCK activities were measured in the absence or presence of excess thymidine to determine contributions of dCK (activity in the presence of excess thymidine) and TK2 (activity in the absence minus activity in the presence of excess thymidine). Reactions were measured at 37 °C in mixtures containing 10 μM [^{3}H]deoxycytidine, 5 mM ATP, 5 mM MgCl2, 50 mM Tris–HCl (pH 7.5), 10 mM NaF, 2 mM DTT, with or without 1.8 mM thymidine. Reactions were started by adding 35 μl of cell lysate containing 100 μg of protein. At different time intervals, 20-μl portions were pipetted onto Whatman DE-81 filter discs fitted to a vacuum device. Filters were then washed three times with 5 ml of cold 0.1 mM ammonium formate followed by one wash with 95% ethanol after which they were air dried. Radioactivity on filters was measured by liquid scintillation counting. Protein content was measured by Bio-Rad protein assay [22]. Each experiment was repeated at least three times with three replicates/time point. The specific activity is described as pmol/mg protein/min.

3. Results

3.1. Morphology of the cell lines

HTB2, HTB3 and HTB4 cells exhibited distinct morphologies (Fig. 1) and growth properties. Cells of the HTB2 line, which was derived from a transitional cell papilloma, grew as small rounded clusters (*i.e.*, papilloma-like) with an estimated doubling time of 56 h. The HTB2 line has frequently been used as a model of a superficial transitional carcinoma [23–26]. Cells of the HTB3 line, which was derived from a squamous cell carcinoma, were transparent and flat with an estimated doubling time of 26 h. Cells of the HTB4 line, which was derived from an invasive transitional cell carcinoma, grew as diffuse monolayer with an estimated doubling time of 19 h. The HTB4 line is widely used as a model of invasive bladder cancer [23–25].

3.2. Gemcitabine cytotoxicity

Gemcitabine resistance is frequently observed in bladder cancer patients and cytotoxicity experiments were conducted to determine if cell lines derived from the three phenotypes exhibited differences in gemcitabine sensitivity. Cells in 96-well plates were treated with graded gemcitabine concentrations (0–100 μM) for 72 h. IC_{50} values (mean ± S.E.M.) for HTB2, HTB3 and HTB4 cells (Fig. 2) were 4 ± 2, 210 ± 32 and 20 ± 5 nM, respectively, with a rank order of sensitivity of HTB2 > HTB4 ≫ HTB3. hENT1 is a major route of entry of gemcitabine into human cells and its absence in cultured cell lines confers resistance to gemcitabine [7,27]. To assess the impact of hENT1-mediated gemcitabine influx on cytotoxicity in the

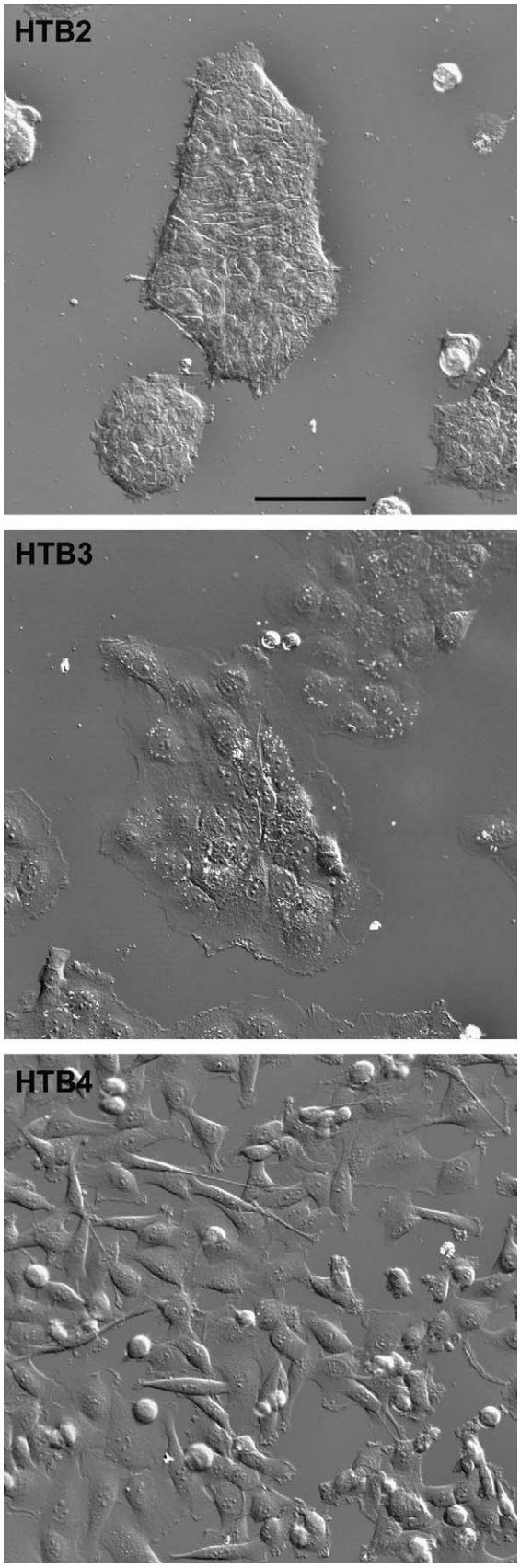

Fig. 1. Morphology of HTB2, HTB3 and HTB4 cells. Phase contrast images of exponentially growing cells were obtained with a 10× lens using a laser scanning confocal microscope. For all panels scale bar shown was 100 μm.

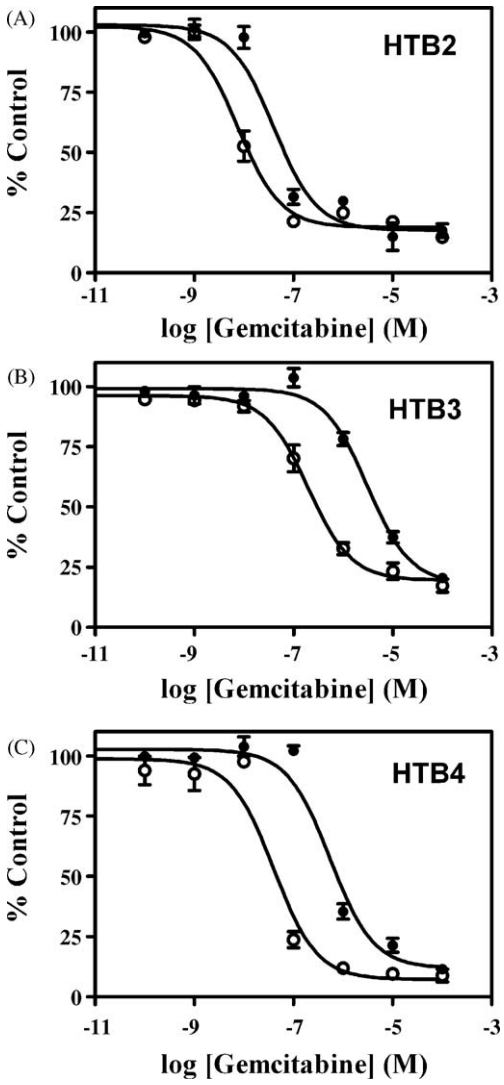

Fig. 2. Gemcitabine toxicity in HTB2, HTB3 and HTB4 cells. Cells were exposed to graded gemcitabine concentrations (0–100 μM) for 72 h in the absence (○) or presence (●) of 1 μM NBMPR. Values, expressed as % control (untreated cells) shown in the graph are the means (±S.E.M.) of three separate experiments, each conducted with 6 replicates and where error bars are not shown, they were smaller than the symbols.

bladder cancer cell lines, the ability of 1 μM NBMPR, a non-toxic high-affinity inhibitor of hENT1, to protect HTB2, HTB3 and HTB4 cells from gemcitabine cytotoxicity was examined (Fig. 2). The pharmacologic protection conferred by NBMPR (filled symbols) as evidenced by higher IC_{50} values of 40, 3100 and 550 Nm, respectively for HTB2, HTB3 and HTB4 cells suggested involvement of hENT1 in gemcitabine uptake in the three cell lines.

3.3. Site-specific binding of [^{3}H]NBMPR

To assess potential contributions of hENT1-mediated transport to cellular gemcitabine uptake, binding of [^{3}H]NBMPR was measured in the three cell lines to determine hENT1 densities. To determine the proportion of hENT1 on intracellular and cell surface plasma membranes, binding assays were performed using a single concentration of 10 nM [^{3}H]NBMPR that fully saturated high-affinity sites in the presence or absence of excess FTH-SAENTA. FTH-SAENTA, a tight-binding, impermeant inhibitor of hENT1 that is structurally related to NBMPR, binds hENT1 on extracellular surfaces of plasma membranes, thereby enabling

Table 1
Binding of [^{3}H]NBMPR in three human bladder cancer cell lines. Specific binding of 10 nM [^{3}H]NBMPR was measured in three bladder cancer cell lines in absence or presence of FTH-SAENTA or excess NBMPR as described in Section 2 to quantitate the total, intracellular and extracellular number of hENT1 sites/cell. Values shown are means (±S.E.M.) of three experiments, each performed with three or more replicates.

Cell lines	Total	Bound [^{3}H]NBMPR	
		Intracellular	Extracellular
		(Sites/Cell) × 10^{-5}	
HTB2	8.3 ± 0.5	2.0 ± 0.5	6.3 ± 1.0
HTB3	2.1 ± 0.25	0.38 ± 0.1	1.7 ± 0.3
HTB4	2.0 ± 0.3	0.84 ± 0.3	1.1 ± 0.4

quantification of extracellular and intracellular NBMPR binding sites. HTB2 cells had the highest number of total and extracellular binding sites followed by HTB3 and HTB4 cells, which had similar number of total NBMPR binding sites although the intra and extracellular distributions differed between the two cell lines (Table 1).

3.4. [^{3}H]gemcitabine uptake and accumulation

Since gemcitabine cytotoxicity differed in the three cell lines, uptake (0–120 s incubations) and accumulation (2-h incubations) of [^{3}H]gemcitabine were monitored in the three cell lines to determine if either process resembled the observed cytotoxicity pattern. In initial rate studies of [^{3}H]gemcitabine uptake, HTB2 cells exhibited the highest rate followed by HTB4 and HTB3 (Fig. 3A). In accumulation studies, HTB2 cells had the highest accumulation of gemcitabine with the rank order being HTB2 > HTB4 > HTB3 (Fig. 3B). These results showed that gemcitabine initial uptake rates and accumulation after 2-h exposures paralleled the cell lines cytotoxicity responses.

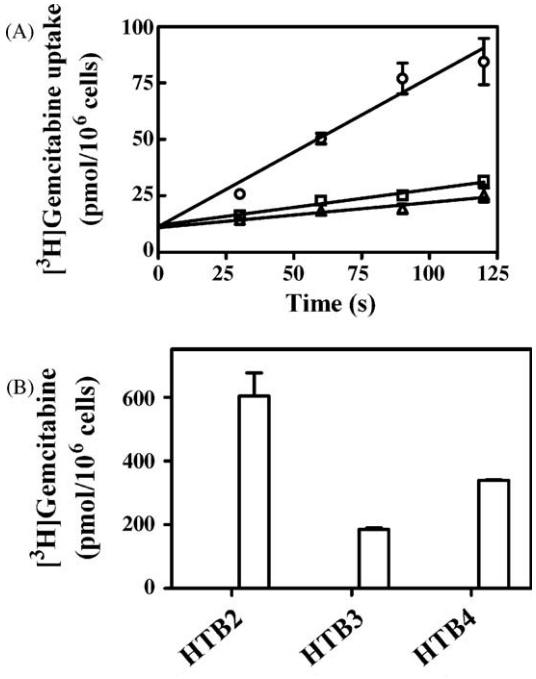

Fig. 3. [^{3}H]Gemcitabine uptake and accumulation: Uptake (0–120 s) of 10 μM [^{3}H]gemcitabine in HTB2 (○), HTB3 (Δ) and HTB4 (□) cells are shown in panel A. Results presented are means (±S.E.M.) of two experiments, each conducted with three replicates. Panel B shows accumulation (2 h) at room temperature of 10 μM [^{3}H]gemcitabine by HTB2, HTB3 and HTB4 cells. Values presented are mean (±S.D.) of one representative experiment (conducted twice with three replicates per condition), and where error bars are not visible, they were smaller than the symbols.

3.5. Evaluation of nucleoside transport processes in HTB cells

Since gemcitabine transport and accumulation are known to be dependent on nucleoside transport activity in other cell types [28], experiments were undertaken to determine which nucleoside transporters are present in HTB cells using uridine, which is a permeant for the four hNTs known to also accept gemcitabine as a permeant [7]. Initial rates (0–40 s) of uptake of 10 μM [^{3}H]uridine were measured in the three cell lines (Fig. 4A); HTB 2 had the highest transport activity followed by HTB3 and HTB4. Uptake of [^{3}H]uridine was similar in both sodium-containing and sodium-free buffers (data not shown), indicating the absence of sodium-dependent nucleoside transport activity in HTB cell lines thereby excluding hCNT1 and hCNT3 from further consideration. Sodium-independent activity was further dissected by measuring uptake of [^{3}H]uridine in the absence or presence of 0.1 μM NBMPR (inhibits hENT1 activity) or 10 μM dipyridamole (inhibits both hENT1 and hENT2 activities) to determine relative contributions of hENT1 and hENT2-mediated processes to uridine uptake. Mediated transport of 10 μM [^{3}H]uridine was highest in HTB2 cells, which exhibited both hENT1 and hENT2 activities, whereas it was lower in HTB3 and HTB4 cells, which exhibited only hENT1 activity (Table 2).

The rank order of uridine transport activities (HTB2 > HTB3 > HTB4) differed from that observed for gemcitabine accumulation (HTB2 > HTB4 > HTB3). The concentration-dependence of initial rates of uptake (30-s incubations) of [^{3}H]uridine was determined to further characterize uridine transport activity in HTB cells, recognizing that uptake values represented combined activities of hENT1 and hENT2 in HTB2 cells. Uptake rates exhibited saturation as the uridine concentration was

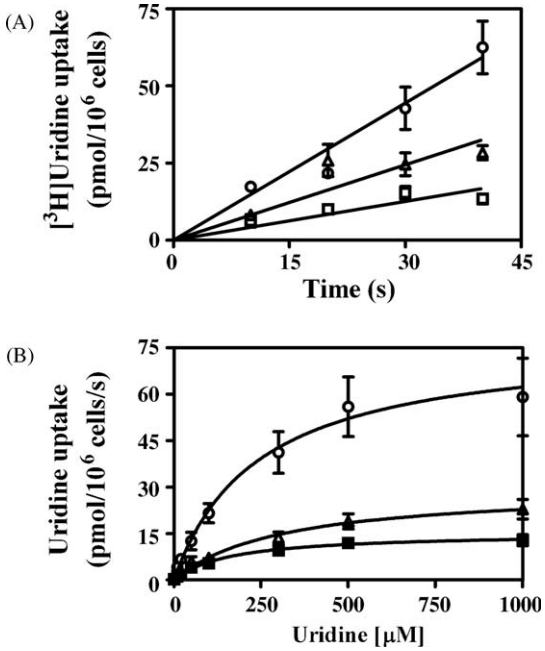

Fig. 4. Kinetics of [^{3}H]uridine uptake by HTB2, HTB3 and HTB4 cells. Panel A shows time courses for HTB2 (○), HTB3 (Δ) and HTB4 (□) cells that were exposed from 0 to 40 s to 10 μM [^{3}H]uridine. Results presented are mean (±S.E.M.) of two experiments (each conducted with three replicates per condition). The concentration-dependence of [^{3}H]uridine transport (*i.e.*, initial rates of uptake for 30 s) was measured (panel B) in HTB2 (○), HTB3 (Δ) and HTB4 (□) cells as described in Section 2. Kinetic parameters K_m and V_{max} obtained from Michaelis–Menten analysis after correction for non-saturable components of uptake rates are summarized in Table 2. Values represent the mean (±S.E.M.) of three experiments (three replicates/measurement) and where error bars are not shown, they were smaller than the symbols.

Table 2
Uptake of 10 μM [^{3}H]uridine in three human bladder cancer cell lines. Uptake 10 μM [^{3}H]uridine was measured in sodium-containing medium in the absence or presence of 100 nM NBMPR or 10 μM dipyridamole to distinguish between hENT1 and hENT2 mediated transport, respectively as described in Section 2. Initial rates (pmol/10^{6}cells/s) presented in the table below were obtained from two experiments each conducted with three replicates per measurement and values shown represent means (±S.E.M.).

Cell lines	[^{3}H]Uridine	
	hENT1-mediated	hENT2-mediated
	(pmol/10^{6}cells/s)	
HTB2	0.88 ± 0.08	0.23 ± 0.06
HTB3	0.50 ± 0.04	0.03 ± 0.02
HTB4	0.30 ± 0.04	0.02 ± 0.01

increased from 0 to 1000 μM in all three cell lines (Fig. 4B). Apparent kinetic parameters obtained from Michaelis–Menten analysis after correction of uptake values for non-saturable components are summarized in Table 3. Although the three cell lines exhibited different K_m and V_{max} values, V_{max}:K_m ratios (indicator of transporter efficiencies) were 0.31, 0.10 and 0.11, respectively for HTB2, HTB3 and HTB4.

3.6. Mutations in hENT1 gene

To determine if mutations in the *hENT1* gene were responsible for the observed differences in uridine transport activity in the three HTB cell lines, full length *hENT1* cDNAs were sequenced using four primer pairs to ensure sequence overlap between all four amplicons (data not shown). The sequence containing the ATG start codon was designated as exon 1 as described earlier [29]. A single mutation (C > T) was found in the HTB4 cDNA in the non-coding upstream sequence at nucleotide position −185 from the ATG start site. The region upstream of the ATG codon from HTB2 and HTB3 could not be sequenced despite several attempts, therefore genomic DNA from the three cell lines spanning all 12 exons and the upstream region was sequenced to confirm the −185 C > T mutation in HTB4, and to determine if similar or other mutations were present in the *hENT1* gene of HTB2 and HTB3 cells. The C > T mutation was confirmed for HTB4 whereas the corresponding region from HTB3 genomic DNA showed no mutation and the sequence information for HTB2 cells was inconclusive. There were no mutations in the flanking 50 bp regions of the coding exons, indicating the absence of splice site variants in the *hENT1* gene in these cell lines (data not shown).

3.7. Deoxycytidine kinase (dCK) and thymidine kinase 2 (TK2) assays

In addition to its dependence on hENT1-mediated transport activity, gemcitabine cytotoxicity is also dependent on phosphorylation by dCK to its active form. The rank order of dCK activities in the three cell lines was HTB4 > HTB3 > HTB2 with specific activities (mean ± S.E.M.) of 2.6 ± 0.2, 1.7 ± 0.1 and 1.1 ± 0.1 pmol/mg protein/

Table 3
Kinetic parameters of [^{3}H]uridine influx mediated by hNTs in HTB cells. Uridine transport kinetics were determined in the three cell lines by incubating with graded [^{3}H]uridine concentrations (0–1000 μM) for 30 s at room temperature. Mean K_m and V_{max} values (±S.E.M., n = 3) were determined from data shown in Fig. 4B by nonlinear regression analysis using Graph Pad Prism version 4.03 software.

Cells	Apparent K_m (μM)	Apparent V_{max} (pmol/10^6 cells/s)	V_{max}:K_m ratio (pmol/10^6 cells/s/μM)
HTB2	234 ± 46	74 ± 23	0.32
HTB3	126 ± 11	13 ± 2	0.10
HTB4	214 ± 73	24 ± 4	0.11

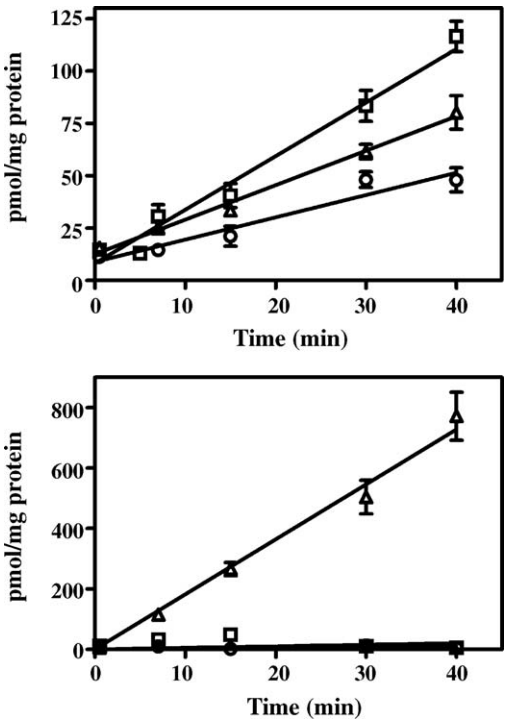

Fig. 5. Activities of dCK and TK2 in HTB2, HTB3 and HTB4 cells: Time courses (0–40 min) of dCK (Panel A) and TK2 (Panel B) activities in HTB2 (○), HTB3 (Δ) and HTB4 (□) cells were measured as described in Section 2. Values represent the mean (±S.E.M.) of three experiments (three replicates/measurement) and where error bars are not shown, they were smaller than the symbols.

min, respectively (Fig. 5A). Although HTB2 cells had the lowest dCK activity, they were most sensitive to gemcitabine, whereas HTB4 cells had roughly 1.5-fold higher dCK activity than HTB3 cells and were 5-fold more sensitive to gemcitabine toxicity than HTB3 cells.

TK2, which is present in both cytosol and mitochondria [13], phosphorylates deoxycytidine and thymidine [16], giving rise, after subsequent phosphorylations, to dCTP, the major feedback inhibitor of dCK. An increase in dCTP pools as a result of higher TK2 activity will decrease gemcitabine cytotoxicity because of decreased gemcitabine phosphorylation by dCK [30]. Activities of TK2 were determined for the three cell lines, with HTB3 cells exhibiting an activity of 19 pmol/mg protein and HTB2 and HTB4 cells exhibiting negligible activities (Fig. 5B).

3.8. Effect of pretreatment with 2 mM thymidine on gemcitabine toxicity

HTB3 cells exhibited the lowest sensitivity to gemcitabine cytotoxicity (see Fig. 2). Since HTB3 cells exhibited high TK2 activity, it was possible that high intracellular levels of dCTP could inhibit dCK activity thereby leading to low levels of phosphorylated gemcitabine compared to HTB2 and HTB4 cells. To determine if the low gemcitabine sensitivity of HTB3 cells, relative to that of HTB2 and HTB4 cells, was related to high TK2 activity, the three cell lines were first exposed to 2 mM thymidine for 16 h to inhibit the deoxycytidine-phosphorylating activity of TK2 [16] and then exposed to graded gemcitabine concentrations for 72 h in the absence of thymidine. Cell growth was unaffected in cells exposed to 2 mM thymidine (data not shown). Cell viability was measured by the MTS assay and results are presented in Fig. 6. HTB3 cells were sensitized to gemcitabine toxicity by pretreatment with thymidine (IC_{50} values of 35 nM compared to 250 nM in untreated cells) whereas HTB2 or HTB4 cells with no detectable TK2 activities were unaffected (IC_{50} values in treated vs. untreated cells,

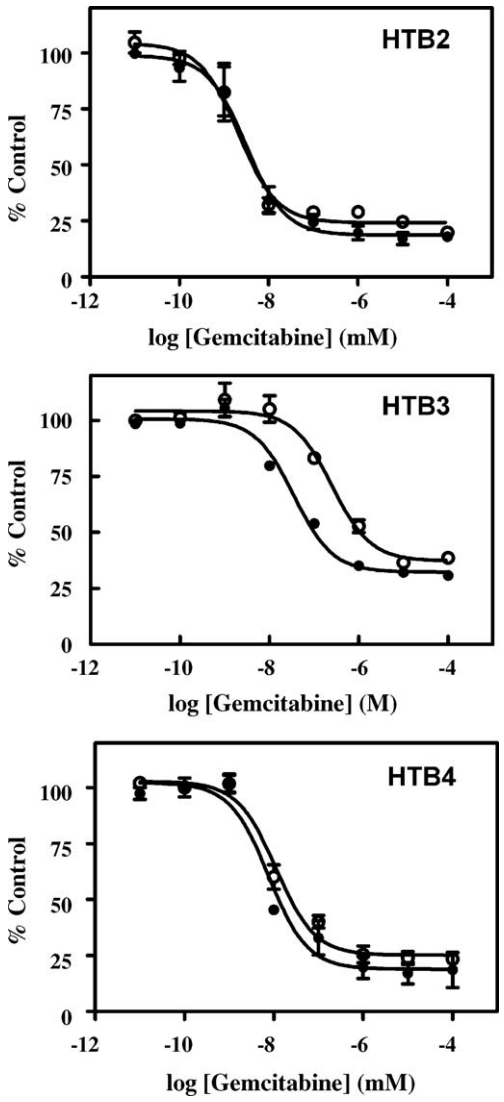

Fig. 6. Effects of thymidine on gemcitabine toxicity in HTB2, HTB3 and HTB4 cells. Cells were grown in 96-well plates, and after 24 h, medium with (●) or without (○) 2 mM thymidine was added. After an additional 16 h, media was replaced with fresh thymidine-free media containing graded gemcitabine concentrations (0–100 μM) and cultures were further incubated for 72 h. Values represent means (±S.E.M.) of three separate experiments, each conducted with 6 replicates and where error bars are not shown, they were smaller than the symbols.

respectively, were 3 nM vs. 2 nM (HTB2) and 9 nM vs. 11 nM (HTB4)).

4. Discussion

The antimetabolite gemcitabine with its favorable toxicity profile has become the standard treatment for bladder cancer either alone or in combination. Gemcitabine is a prodrug and is metabolized intracellularly to its mono-, di- and tri-phosphate forms. In this study three bladder cancer cell lines originating from bladder cancers that exhibited different histologies showed differences in sensitivity to gemcitabine. Multiple mechanisms contribute to gemcitabine resistance. Among these, alterations in transport activity, including mutations in transporters, changes in expression of drug-metabolizing enzymes and/or alterations in molecular pathways that control apoptosis, are likely to alter sensitivity to gemcitabine [31].

Gemcitabine has been used in bladder cancers at different stages of the disease. Intravesicle instillation of gemcitabine in patients with superficial bladder cancer achieved 50% complete responses (CR) [32]. In locally advanced and metastatic bladder cancer gemcitabine has been evaluated in Phase I and II trials in both untreated and previously treated patients [4,5,33]. Subsequently von der Maase et al. [34] undertook a phase III study comparing gemcitabine and cisplatin vs. methotrexate, vinblastine, doxorubicin and cisplatin (MVAC); in that study gemcitabine and cisplatin showed comparable efficacy to MVAC with much less toxicity leading to adoption of gemcitabine and cisplatin as the standard treatment of metastatic bladder cancer.

The current study was undertaken to evaluate the *in vitro* response to gemcitabine in three human bladder cancer cell lines, HTB2 derived from a papilloma (superficial transitional carcinoma), HTB3 derived from a squamous cell carcinoma and HTB4 derived from an invasive transitional carcinoma, to determine if there are differences in sensitivity to gemcitabine and to identify factors responsible for these differences in sensitivity. Cell lines differed in their sensitivities to gemcitabine with the rank order of sensitivity HTB2 > HTB4 > HTB3.

hENT1-mediated transport was the major route of gemcitabine entry in the three cell lines. Binding of NBMPR (a high-affinity and specific inhibitor used to quantify hENT1 molecules) and uptake of uridine (widely used to characterize nucleoside transporters) were measured in all three cell lines. HTB2 cells exhibited the highest number of NBMPR binding sites and uridine transport rates followed by HTB3 and HTB4 cells. Although transport and binding sites exhibited the same rank order in the three cell lines (HTB2 > HTB3 > HTB4), the differences in response to gemcitabine could not be explained based on the transport results. Accumulation of gemcitabine (HTB2 > HTB4 > HTB3) paralleled the cytotoxicity results (HTB2 > HTB4 > HTB3) wherein HTB2 cells were the most sensitive and had the highest accumulation of gemcitabine. Kinetic studies of uridine transport yielded V_{max}:K_m ratios of 0.31, 0.10 and 0.11 for HTB2, HTB3 and HTB4 cells, respectively. Thus, although nucleoside transport efficiencies in HTB3 and HTB4 cells were similar, overall accumulation of gemcitabine was lower in HTB3 cells than in HTB4 cells.

To determine if observed differences in kinetics of uridine transport were due to mutations in the *hENT1* gene, full length *hENT1* cDNAs from HTB2, HTB3 and HTB4 cells were sequenced, using the ATG start site to designate the upstream region instead of the transcription start site to describe the −185 C > T mutation as reported previously [29]. There were no mutations in the intron–exon boundaries, ruling out the possibility of alternate splice variants as a mechanism to explain the differences in transport kinetics. It is possible that further upstream regions of the *hENT1* gene may be involved in differential regulation. Precise mechanisms governing *hENT1* gene regulation are currently unclear. Recent evidence indicated down regulation of *hENT1* gene expression by hypoxia and nitric oxide in gestational diabetic conditions [29,35]. Several polymorphisms (frequency in populations at >1%) in the *hENT1* gene have been reported for Caucasian, African American and Japanese populations, although two non-synonymous polymorphic variants Ile216Thr and Glu391Lys that were identified showed no functional alterations in ENT1 [36]. Mutational analysis of the *hENT1* genes in cultured cell lines resistant to cytarabine (CEM/4xAraC and CEM/20AraC) has yielded important insights into hENT1- and dCK-dependent mechanism of resistance to cytarabine [19]. Point mutations in exon or exon–intron boundaries led to aberrant *hENT1* and *dCK* gene expression. A point mutation in exon 4 of the *hENT1* gene resulted in a truncation of the protein due to a stop codon and another point mutation resulted in disruption of splicing of the last exon, whereas a mutation in the *dCK* gene affected splicing of exons 2 and 3.

Evidence presented thus far suggested that HTB2 cells, which exhibited the highest NT activity, were also the most sensitive to

gemcitabine. Although HTB3 and HTB4 cells had similar transport efficiencies, they responded differently to gemcitabine. To find out if this could have been due to differences in cellular levels of deoxynucleotide pools, dCK and TK2 activities were measured in the three cell lines. Although HTB2 cells had lower dCK activity than HTB3 or HTB4 cells, they had the highest accumulation of gemcitabine probably due to high hENT1 activity. In addition, the role of cytoplasmic 5′ nucleotidase, which brings about the dephosphorylation of dFdCMP and the deactivation of gemcitabine via deamination to cytidine catalysed by the enzyme cytidine deaminase, cannot be ruled out in the differential response of HTB2, HTB3 and HTB4 cells to gemcitabine.

Both dCK and TK2 phosphorylate deoxycytidine and neither enzyme is cell-cycle regulated [14]. TK2 is present in both cytosol and mitochondria whereas dCK is present only in cytosol [13]. Human TK2 and dCK phosphorylate deoxycytidine with V_{max} values, respectively, of 900 and 560 nmol/min/mg, consistent with the suggestion that TK2 has higher capacity for phosphorylation of deoxycytidine than dCK [16,37]. Increased levels of cellular dCTP lead to decreased phosphorylation of gemcitabine since dCTP is a feedback inhibitor of dCK [38]. High TK2 levels could decrease the response to gemcitabine, since TK2 phosphorylates deoxycytidine more efficiently than gemcitabine, resulting in higher dCTP pools than gemcitabine triphosphate pools, which would lead to reduced incorporation of the analog into DNA and less toxicity. Correlations between gemcitabine sensitivity and dCK/TK2 ratios were reported earlier [39,40]. High ratios of dCK/TK2 activity in tumor cells result in increased phosphorylation of several nucleoside analogs leading to enhanced cytotoxicity [40]. Similarly a decrease in ratios of dCK/TK2 could lead to decrease in gemcitabine toxicity. HTB3 cells had high TK2 activity compared to HTB2 or HTB4 cells. When HTB3 cells were pretreated with thymidine to deplete intracellular dCTP pools, toxicity of gemcitabine was enhanced, consistent with a role of TK2 in their resistance to gemcitabine. When the prognostic significance of total TK activity was assessed in bladder cancer specimens using [^{3}H]thymidine as a substrate, TK activity was higher in muscle invasive cancer than in superficial bladder carcinoma and positive correlations were established between disease stage and tumor grade [18].

In summary, the differences in response to gemcitabine were explored using bladder cancer cell lines of different origins (HTB2, a papilloma; HTB3, a squamous cell carcinoma; and HTB4, a transitional cell carcinoma). HTB2 cells had the highest transport efficiency and were also most responsive to gemcitabine. HTB3 and HTB4 cells had similar transport efficiencies, but exhibited different responses to gemcitabine. The highest accumulation of [^{3}H]gemcitabine was in HTB2 cells and the lowest was in HTB3 cells. Sequencing experiments revealed no mutations either in coding exons or intron–exon boundaries of the *hENT1* genes of the three cell lines. Using cytoplasmic extracts and [^{3}H]deoxycytidine with or without excess non-radioactive thymidine, dCK activity was highest in HTB4 > HTB3 > HTB2 cells. In addition, HTB3 cells exhibited high TK2 activity whereas HTB2 and HTB4 cells lacked detectable TK2 activity. Our results highlight the importance of hENT1 and TK2 activities in response to gemcitabine. Elevated TK expression in squamous cell carcinomas warrants further study and offers new insights into rational treatment strategies based on bladder cancer phenotype.

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