Enhanced Efficacy of Gemcitabine by Indole-3-carbinol in Pancreatic Cell Lines: The Role of Human Equilibrative Nucleoside Transporter 1

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Abstract. *Pancreatic cancer patients treated with gemcitabine (2',2'-difluorodeoxycytidine) can eventually develop resistance. Recently, published data from our laboratory demonstrated enhanced efficacy of gemcitabine with the dietary agent, indole-3-carbinol (I3C). The current study examined the possible mechanism for this I3C-enhanced efficacy. Several pancreatic cell lines (BxPC-3, Mia Paca-2, PL-45, AsPC-1 and PANC-1) were examined for modulation of human equilibrative nucleoside transporter 1 (hENT1) expression, the major transporter for gemcitabine, by I3C alone and combined with gemcitabine. I3C significantly ($p<0.01$) up-regulated hENT1 expression in several cell lines. Gemcitabine alone showed no effect on hENT1 expression. However, combining gemcitabine with I3C further increased hENT1 expression. Cell viability assays revealed no effect of I3C on normal cells, hTERT-HPNE. hENT1-specific inhibitor, nitrobenzylthioinosine, significantly abrogated I3C-induced gemcitabine cytotoxicity, further demonstrating its specificity. This study demonstrates that up-regulation of hENT1 expression may be a novel mechanism involved in the additive effect of I3C and gemcitabine.*

Pancreatic cancer is the fourth leading cause of cancer death in the US, with median survival of 6 months and a 5-year survival rate of 3–5% (1, 2). It remains a drug-resistant cancer and a silent killer, with most patients having poor outcomes due to its aggressive biology and lack of effective treatment.

Gemcitabine (Gemzar), a pyrimidine nucleoside analog, has broad antitumor activity in various solid tumors and is the single most effective agent approved by the US Food and Drug Administration as the first-line treatment for pancreatic cancer (3). This drug, as an analog of deoxycytidine, inhibits DNA synthesis and repair, resulting in apoptosis (4, 5). However, its efficacy is often reduced due to drug resistance and multiple adverse effects in large numbers of patients. Combinations of gemcitabine with other cancer drugs often add to its toxicity and reduces its clinical relevance (6-9). Therefore, there is a dire need to devise novel approaches to maximize the efficacy and minimize the toxicity of gemcitabine to improve the survival outcome for pancreatic cancer patients.

The pharmacological effect of gemcitabine is intracellular, requiring drug transporters for delivery across the plasma membrane; drug transporters play an important role in its conversion into its active compounds. The human equilibrative nucleoside transporter 1 (hENT1) is the most abundant and widely distributed plasma membrane nucleoside transporter in human cells and is the major transporter by which gemcitabine enters cells (10, 11). Limited intracellular uptake of gemcitabine through a decrease in hENT1 expression is an established resistance mechanism *in vitro* (12). In contrast, a high level of hENT1 expression represents an increase in positive predictive factors for patients' responses to gemcitabine, even those with very advanced cancer (12-15). A significant relationship between the levels of hENT1 mRNA and the half maximal inhibitory concentration (IC_{50}) values for gemcitabine has been found in human pancreatic adenocarcinoma and biliary tract carcinoma cell lines (16, 17). Although other candidate biomarkers of pancreatic cancer have been extensively investigated and demonstrated some clinical value, hENT1 expression has been shown to have the most important influence on gemcitabine sensitivity (14-16). Therefore, modulation of hENT1 expression may improve bioavailability of gemcitabine and promote its efficacy in pancreatic cancer therapeutics.

Recently, the uses of novel combinational treatments with conventional cancer therapies and dietary agents have received much attention for improvement in the quality of life for some cancer patients. One of the most important advantages of these agents is that they are derived from

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natural products found in our daily diet and often have demonstrated low or no toxicity. Studies have shown that dietary agents were able to improve the antitumor activity of cytotoxic drugs at reduced doses both *in vivo* and *in vitro*, hence minimize the systemic toxicity caused by chemotherapeutics (18-21). Indole-3-carbinol (I3C), a naturally occurring dietary compound, primarily found in some fruit and cruciferous vegetables, is one of such agents that have been investigated in detail for its putative anticancer properties. Earlier work from our laboratory and others has shown the marked synergistic cytotoxic effect of the combination of gemcitabine and I3C against pancreatic cancer cells, by targeting a wide spectrum of signaling pathways involving hormonal homeostasis, cell-cycle proliferation, cell proliferation and survival (20, 22-26). Additional relevant clinical studies have also reported that 3,3'-diindolylmethane (DIM), a major *in vivo* acid-catalyzed condensation product of I3C, reduces expression of epidermal growth factor receptor (EGFR), metastasis-associated protein 2 (MTA2), interleukin-1 receptor-associated kinase 1 (IRAK-1), and nuclear factor kappa B (NF-κB) in pancreatic cancer cells, which results in an inhibition of cell invasion (20, 27, 28). DIM has also been shown to function as a microRNA regulator leading to the reversal of epithelial-to-mesenchymal transition (EMT) phenotype, which is often associated with drug resistance and cancer cell metastasis (29-31). Interestingly, I3C reverses multiple drug resistance (MDR) by inhibiting the expression and activity of the drug efflux transporter, P-glycoprotein (19). These reports suggest that I3C might concomitantly target multiple signal pathways and transporters, and have importance as a potential therapeutic agent alone or in combination with other chemotherapeutic drugs. However, a number of these studies were conducted mainly on the effect of I3C on intracellular targets and on the efflux transporter of gemcitabine. In contrast, very little information is available on the regulation of cellular uptake of gemcitabine by I3C. The present study was performed to determine the mechanism by which I3C enhances efficacy of gemcitabine in pancreatic cancer cells, which had been shown in our earlier study (24). I3C effect on the modulation of hENT1 gene and protein expression, and function was examined in five pancreatic cancer cell lines and one normal pancreatic cell line.

Materials and Methods

Cells and reagents. The human pancreatic cell lines, Mia PaCa-2, BxPC-3, AsPC-1, PANC-1, PL-45, and normal pancreatic ductal epithelial cells, hTERT-HPNE cells, were obtained from the American Type Culture Collection (Manassas, VA, USA). The human pancreatic cell lines were grown in Dullbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. The hTERT-HPNE cells were cultured in 75% DMEM without glucose and 25% medium M3 base supplemented with 5% FBS, 10 ng/ml human recombinant epidermal growth factor (EGF), 5.5 mM D-glucose (1 g/l) and 750 ng/ml puromycin. The cell lines were routinely maintained at 37˚C in a humidified 5% CO_2 atmosphere. Gemcitabine (Eli Lilly & Co., Indianapolis, IN, USA), I3C (Sigma, St. Louis, MO, USA) and hENT1 inhibitor, nitrobenzylthioinosine (NBMPR) (Sigma), were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and added to the media directly at different concentrations.

Cell culture treatments and whole cell lysate preparation. To examine the effect of I3C and gemcitabine on hENT1 protein and mRNA expression in hTERT-HPNE cells and pancreatic cancer cell lines, cells were first cultured for at least 48 h to achieve 60-70% confluence. The medium was then replaced with fresh medium containing gemcitabine or I3C at different concentrations. Cell culture was continued for an additional 48 h. For studies in which cells were treated with gemcitabine in combination with I3C, the cells were first pretreated with I3C at 50 μM for 24 h. The medium was then replaced with fresh medium, and the cells were further incubated with I3C at 50 μM in the presence of gemcitabine at different concentrations for 48 h. The cells were then used for viability assay or harvested for immunoblotting and mRNA isolation. The concentration of DMSO used in all experiments was 0.1% (vol/vol). No effects of the vehicle on cell viability and hENT1 protein or mRNA expression were observed at this concentration.

The cells were harvested by trypsin/EDTA solution. For whole-cell lysate preparation, cell pellet was resuspended in 200 μl of lysis buffer (1 M Tris·HCl, [pH 7.5], 10% SDS, 5 mg/ml DNase I, 1 M $MgCl_2$, 50 mg/ml phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail). The mixture was placed on ice for 1 h with vortexing every 15 min, and finally centrifuged at 15,100 × g for 15 min at 4˚C. The supernatant was immediately frozen in liquid N2 in aliquots and stored at –80˚C until use. Protein concentrations were determined by Nanodrop® Spectrophotometer (Wilmington, DE, USA).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting. The protein samples of whole-cell lysates (20 μg each lane) were separated in 12% SDS- polyacrylamide denaturing gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were subjected to immunoblotting by use of purified rabbit polyclonal antibody against SLC29A1 (hENT1) (OriGene Technologies, Inc. Rockville, MD, USA) as primary antibody at 1:50 dilution overnight at 4˚C, and then with goat anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) conjugate at 1:3000 dilution for 1 h. For detection of β-actin, membranes were incubated overnight with a β-actin mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA, USA) at 1:3000 dilution, and then with goat anti-mouse HRP-conjugated antibody (Bio-Rad, Hercules, CA, USA) at 1:6000 dilution for 1 h. The signal was detected using chemoluminescence reagents (Bio-Rad, Hercules, CA, USA). Relative hENT1 protein levels were determined by Molecular Imager Gel Doc™ XR System (Bio-Rad). β-Actin was used as an internal control.

Total RNA isolation and quantitative real-time TaqMan reverse transcription polymerase chain reaction (RT-PCR). After treatment of the pancreatic cell lines with I3C or gemcitabine as described above, total cellular RNA was isolated from the cells using the Qiagen RNeasy isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The concentration of RNA was determined by NanoDrop® Spectrophotometer (Bio-Rad). The

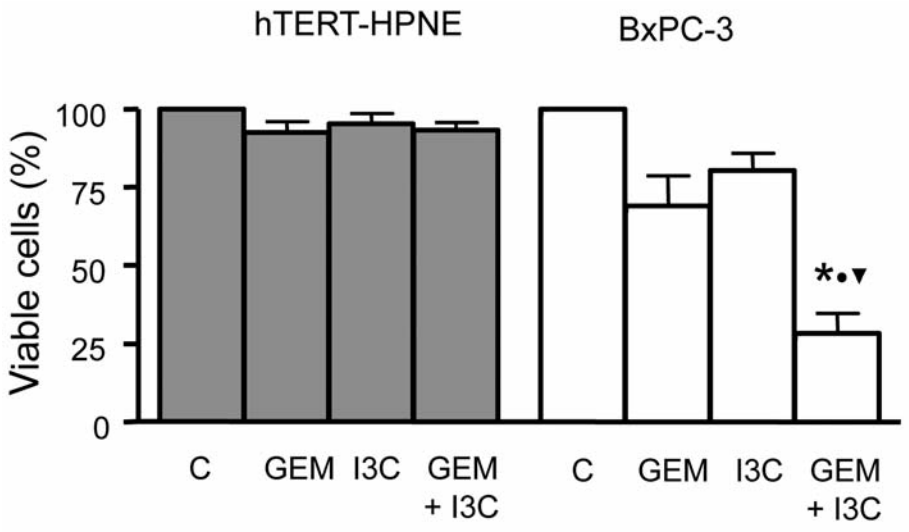

Figure 1. *Cytotoxicity of I3C and gemcitabine (GEM) at clinical relevant concentrations in hTERT-HPNE and BxPC-3 cells cells. Cell were pretreated with I3C (50 μM) for 24 h followed by co-incubation with gemcitabine (15 nM) in the presence or absence of I3C (50 μM) for 48 h. The half maximal inhibitory concentration (IC_{50}) was determined with MTS assay. The data represent the mean of three separate experiments (SD±SE). *p<0.01 compared with vehicle control (C). •p<0.01 compared with I3C treatment, ▼p<0.01 compared with gemcitabine treatment. GEM: gemcitabine.*

OD260/OD280 nm ratios of all RNA samples were determined to be between 1.7 and 2.0, ensuring that all RNA samples were highly pure. RNA integrity was verified by ExperionTM (Bio-Rad, Wilmington, NC, USA). Single-strand cDNA used for analysis of hENT1 was synthesized from 0.3 μg of purified total RNA using a TaqMan reverse transcription kit (Applied Biosystems, Branchberg, NJ, USA), Real-time PCR reactions were then performed using a TaqMan universal PCR master mix on iQTM5 Multicolor Real-time PCR Detection System (Bio-Rad). The primers and specific probes for hENT (cat. No.: Hs00191940_ml) and β-actin (cat. no.: Hs01085706) were synthesized by Applied Biosystems. Reactions were carried out in triplicates in a MicroAmp optical 96-well plate in a total volume of 20 μl. Each reaction mixture contained 10 μl of 2× TaqMan universal PCR master mix, 6.1 μl of sterile Millipore water, 0.47 μl of forward primer (235 nM), 0.47 μl of reverse primer (235 nM), 0.47 μl of probe (118 nM), and 2.5 μl of reverse-transcription products. PCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, 95˚C for 15 s and 60˚C for 1 min (40 cycles). Quantification of relative mRNA levels was carried out by determining the threshold cycle (CT), which is defined as the cycle at which the 6-carboxyfluorescein reporter fluorescence exceeds by 10 times the standard deviation of the mean baseline emission for cycles 3 to 10. β-Actin was used as an internal control. The mRNA levels of hENT1 were normalized to those of β-actin according to the following formula: CT (hENT1) – CT (β-actin) ΔCT. Thereafter, the relative mRNA levels of these genes after treatment were calculated using the ΔΔCT method: ΔCT (I3C treatment) – ΔCT (vehicle)=ΔΔCT (I3C treatment). The fold changes of mRNA levels of hENT1 in pancreatic cancer cells upon treatment with I3C were expressed as $2^{-\Delta\Delta CT}$.

Cell viability assay. Cells were seeded into 96-well plates (3000 cells/well) in triplicate. After overnight incubation, the medium was changed and cells were treated with I3C and/or NBMPR for 24 h. The medium was changed again and cells were cultured in medium containing different concentrations of gemcitabine in the presence or absence of the same concentrations of I3C and/or NBMPR for 48 h. The cells were then subjected to CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) as per the manufacturer's instructions (Promega, Madison, WI, USA). Absorbance at 490 nm was measured 2 h after the addition of 20 μl of MTS reagent/well.

Statistical analysis. Prism IV software (GraphPAD Software, San Diego, CA, USA) was used for graphical analyses. Data were analyzed for statistical significance using one-way ANOVA analysis and Student's *t*-test. Differences with *p*-values of <0.05 were considered statistically significant.

Results

Western blot analysis showed that the hENT1 protein expression in hTERT-HPNE cells was barely detectable (data not shown). MTS assay demonstrated that gemcitabine at 15 nM, I3C at 50 μM and the combination did not affect hTERT-HPNE cell viability. However, treatment with gemcitabine at 15 nM, I3C at 50 μM and, the combination resulted in 31%, 19% and 72% cell death of BxPC-3 cells, respectively (Figure 1).

The effect of I3C on hENT1 protein expression in the five human pancreatic cancer cell lines treated with I3C at different concentrations (5-100 μM) for 48 h was examined. I3C at 50 or 100 μM significantly increased hENT1 protein expression approximately 2.6-, 4- and 4.4-fold in BxPC-3, Mia Paca-2 and PANC-1 cells, respectively (Figure 2). However, PL-45 cells and AsPC-1 cells did not show up-regulation of hENT1 expression in response to I3C at the

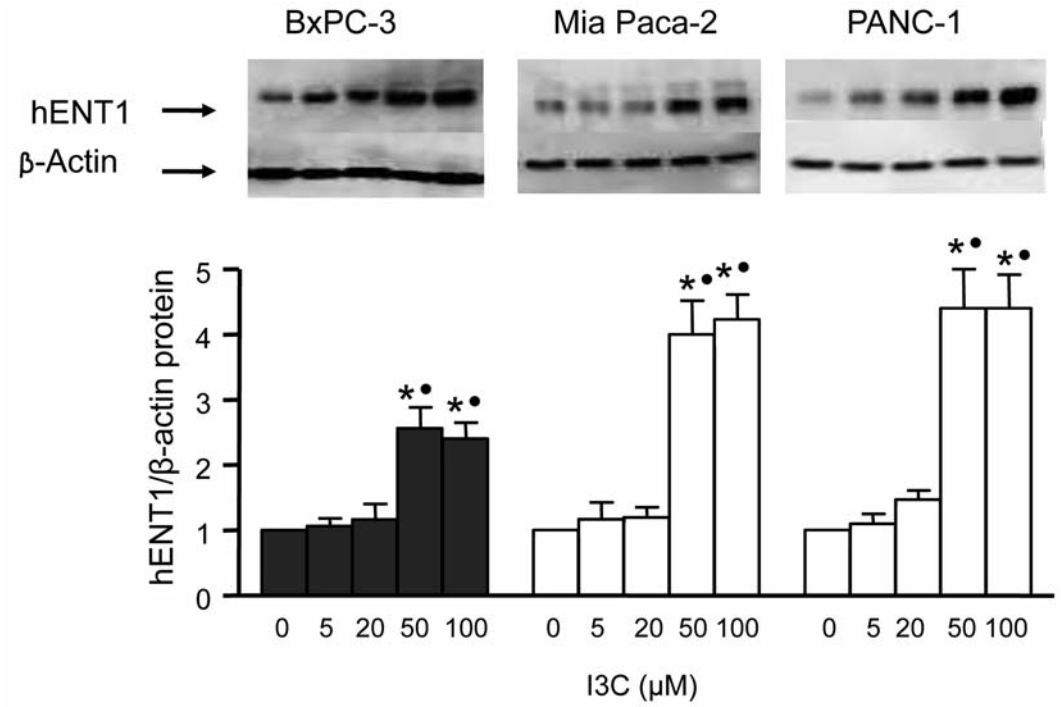

Figure 2. *Effect of I3C on hENT1 protein expression in pancreatic cancer cell lines. Relative hENT1 protein levels normalized to β-actin were determined as described in the Materials and Methods. Data shown are the means±SE from 3 independent experiments. Immunoblots shown are the representative results obtained in typical experiments. Differences in hENT1 protein levels are statistically significant: *p<0.01 compared with vehicle controls, •p<0.01 compared with I3C at 5 and 20 μM using one-way ANOVA. Relative hENT1 protein levels associated with vehicle controls are set as 1.*

concentrations tested (data not shown). We chose 50 μM of I3C in the following study because I3C at this concentration induced maximal hENT1 expression in all three pancreatic cancer cell lines, and were comparable with serum or tissue concentrations seen *in vivo*. Examination of the expression of hENT1 protein after I3C stimulation in BxPC-3, Mia Paca-2 and PANC-1 cells reflected of the change at the transcriptional level. Similarly, I3C at 50 μM significantly increased hENT1 mRNA expression approximately 2.8-, 4.1- and 4.9- fold in BxPC-3, Mia Paca-2 and PANC-1 cells, respectively (Figure 3).

The effect of gemcitabine (32, 33) on hENT1 protein expression in the five human pancreatic cancer and hTERT-HPNE cell lines was examined. No significant change in hENT1 expression was observed at the mRNA or protein level in any of the pancreatic cancer cells or the normal pancreatic cell line, hTERT-HPNE, at any concentration tested (data not shown).

Combination of I3C and gemcitabine treatment further increased hENT1 expression in BxPC-3 and PANC-1 cells compared with I3C alone treatment, and hENT1 expression was increased by combination of I3C and gemcitabine treatment in AsPC-1 cells. Pancreatic cancer and hTERT-HPNE cells were pretreated with I3C at 50 μM for 24 h followed by co-treatment with I3C at 50 μM and gemcitabine at 15 nM for 48 h. In BxPC-3 and PANC-1 cells, this combined treatment further increased hENT1 protein expression compared with I3C treatment alone (Figure 4). For example, in BxPC-3 cells, 50 μM of I3C alone stimulated hENT1 protein expression by 2.3-fold, whereas the combined treatment increased hENT1 protein by 4.1-fold (Figure 4). However, no further increase in hENT1 protein expression was observed in Mia Paca-2 cells. It was a novel finding that although I3C or gemcitabine alone at the above concentrations did not affect hENT1 expression in AsPC-1 cells, the combination of I3C and gemcitabine increased hENT1 expression to 3.4-fold in this cell line (Figure 4). Such an increase was not observed in PL-45 cells (data not shown).

I3C potentiated the cytotoxicity of gemcitabine in the pancreatic cancer cell lines. The growth-inhibitory effect of gemcitabine on different pancreatic cancer cell lines was assessed by MTS assay. A concentration-dependent inhibition of pancreatic cancer cell growth was observed. We also noted differential sensitivity of cells toward gemcitabine. The IC50 of gemcitabine was 37.6, 42.9, 92.7, 89.3 and 131.4 nM in BxPC-3, Mia Paca-2, PANC-1, PL-45 and AsPC-1 cells, respectively (Figure 5).

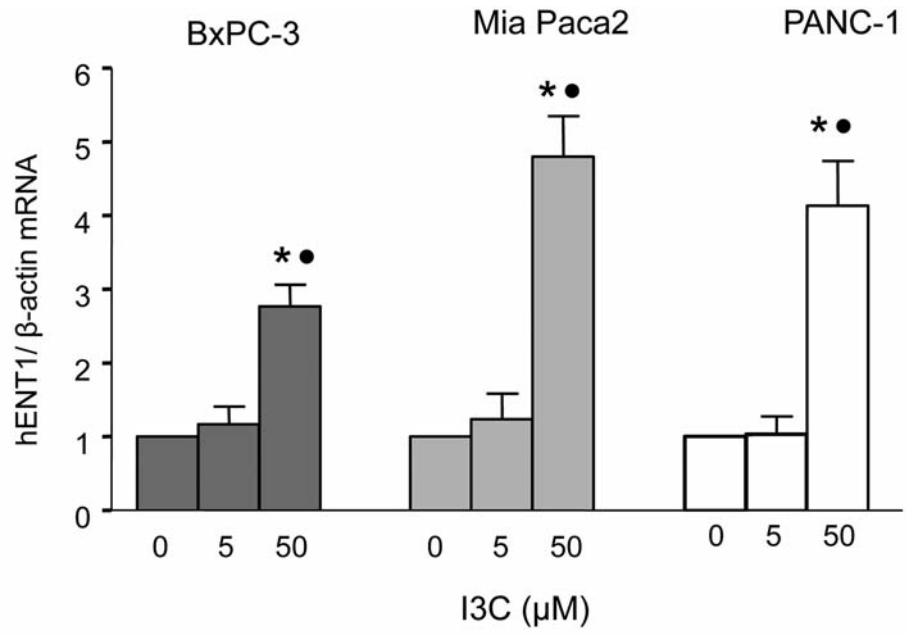

Figure 3. *Effects of I3C on hENT1 mRNA expression in pancreatic cancer cell lines. Total RNA was isolated from cells, and relative hENT1 mRNA levels were determined by real-time RT-PCR as described in the Materials and Methods. Relative hENT1 mRNA levels normalized to β-actin are presented, with vehicle control levels set as 1. Data shown are the means±SE from 3 independent experiments. Differences in hENT1 mRNA levels are statistically significant: •p<0.01 compared with I3C at 5 μM treatment; *p<0.01 compared with vehicle controls.*

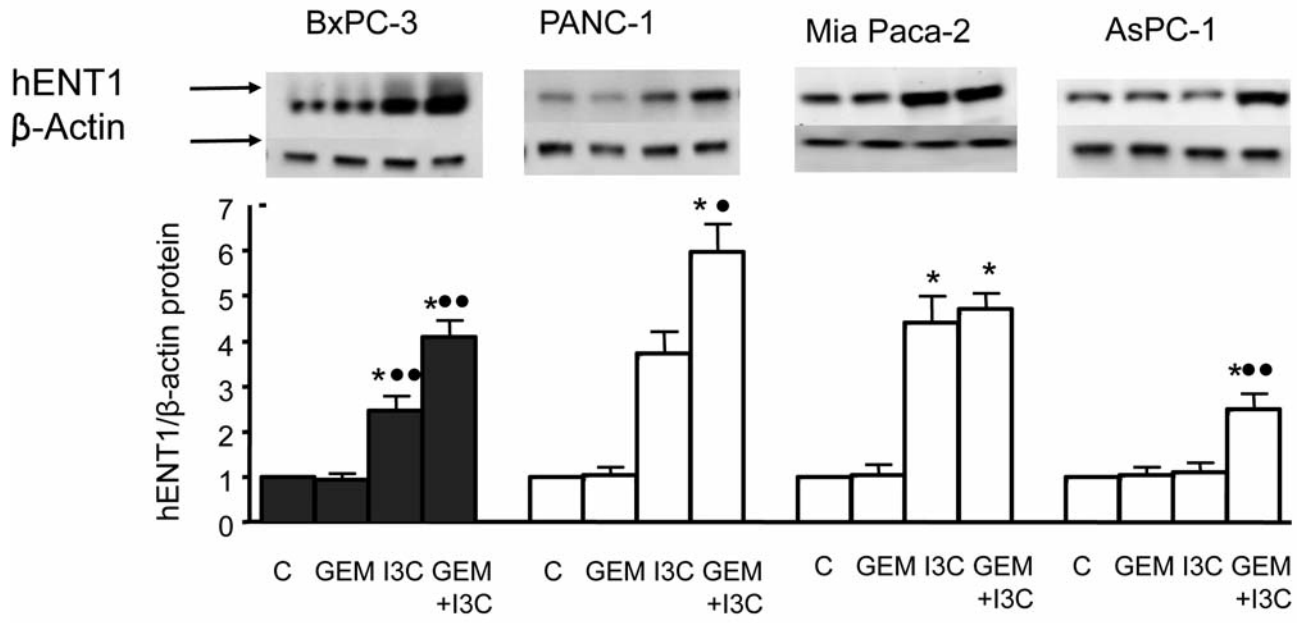

Figure 4. *Effect of I3C in combination with gemcitabine (GEM) on hENT1 protein expression in pancreatic cancer cell lines. Cell were pretreated with I3C (50 μM) for 24 h followed by co-incubation with gemcitabine (15 nM) in the presence or absence of I3C (50 μM) for 48 h. Cells were harvested and subjected to immunoblotting. Relative hENT1 protein levels normalized to β-actin were determined as described in Materials and Methods. Data shown are the means±SE from 3 independent experiments. Immunoblots shown are the representative results obtained in typical experiments. Differences in hENT1 protein levels are statistically significant: *p<0.01 compared with vehicle control (C). •p<0.05, ••p<0.01 compared with I3C only. Relative hENT1 protein levels associated with vehicle controls are set as 1.*

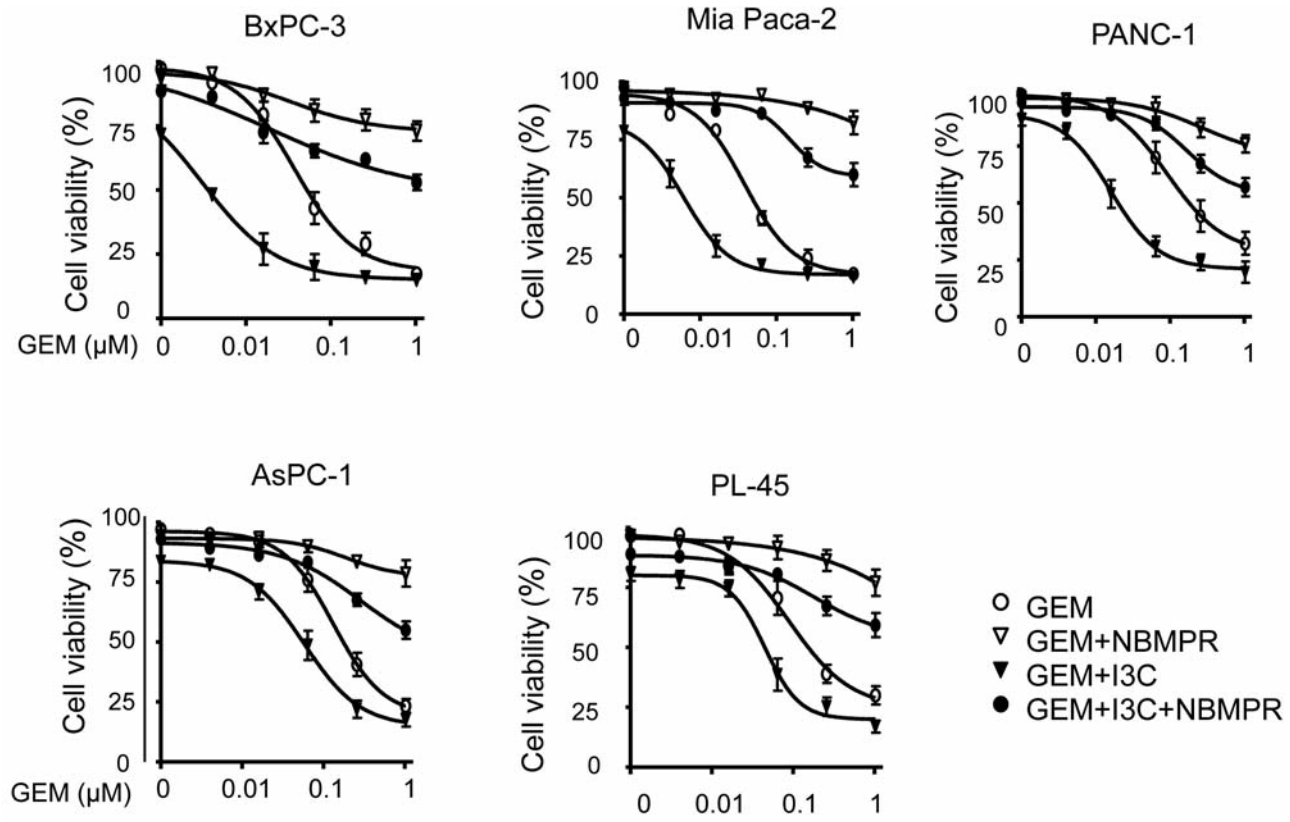

Figure 5. *Concentration dependent cytotoxicity of gemcitabine (GEM) and GEM in combination with I3C in pancreatic cancer cell lines. Cells were seeded into 96-well plates (3000 cells/well) in triplicate. After overnight incubation, cells were treated as described in the Materials and Methods. The IC_{50} was determined with MTS assay and the concentration curve generated using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The data represent the mean of three separate experiments (mean±SE); bars are not shown where values were small and obscured by data points. NBMPR: Nitrobenzylthioinosine.*

We also wanted to determine whether I3C would potentate gemcitabine-induced cell death in the five cell lines in this study. Pretreatment of cells with I3C at 50 μM for 24 h followed by treatment with gemcitabine for 48 h resulted in a significant loss of cell viability in all the cell lines tested compared with gemcitabine treatment alone. The ratio of IC_{50} values between treatment with gemcitabine only and gemcitabine in combination with I3C in the same cell lines was used to assess the cell death induced by I3C. Interestingly, in the three cell lines (BXPC-3, Mia Paca-2 and PANC-1), in which I3C increased hENT1 expression, the IC50 decreased by 12.2-, 7.1- and, 5.8- fold, respectively. However, in AsPC-1 and PL-45 cells, in which I3C does not increase hENT1 expression, the IC_{50} decreased by only 2.7- and 2- fold, respectively. MTS assay also showed that NBMPR significantly, but not completely, abrogated the pancreatic cancer cell growth inhibition caused by gemcitabine alone and by the combination of I3C and gemcitabine.

Discussion

The present study represents the first *in vitro* study on the effect of the dietary agent, I3C, on expression and activity of hENT1 in pancreatic cancer cell lines and a normal pancreatic cell line. During the progression of tumor, signaling pathways and transporters relevant to cell progression and survival are constitutively modulated to overcome genomic instability and acquire a resistance phenotype to anticancer drugs. In light of the heterogeneity of genetic and molecular profiles in human cancer, five established pancreatic cell lines were used in this study. In the five cell lines tested, I3C at a clinically achievable concentration up-regulated hENT1 expression in BxPC-3, Mia Paca-2 and PANC-1 pancreatic cancer cells. A good correlation was found between protein and mRNA levels of hENT1 in these three cell lines, suggesting that I3C regulates hENT1 expression, at least in part, by a transcriptional mechanism. In contrast, hENT1 expression was not increased

by exposure to I3C in PL-45 and AsPC-1 cells. Variation in response to drug treatment has also been reported in other cancer cell lines, which is believed to be caused by the heterogeneity of molecular aberrations among pancreatic cancer cell lines (28, 34, 35).

MTS assay results showed that pretreatment of cells with I3C significantly reduced the IC_{50} of gemcitabine in all PC cell lines tested. These results affirm our hypothesis and are in line with previous reports that I3C improves gemcitabine efficacy by achieving the maximal effect at lower doses and thereby minimizing toxicity on normal cells (20, 28). Interestingly, I3C elicited a relatively greater increase of hENT1 protein expression in Mia Paca-2 and PANC-1 cells (4- and 4.4- fold, respectively) compared with that in BxPC-3 cells (2.6-fold); however, the combination of I3C and gemcitabine caused a 12.2-fold decrease in IC_{50} compared with gemcitabine treatment alone in BxPC-3 cells, but only a 7.1- and 5.8- fold decrease in Mia Paca-2 and PANC-1 cells, respectively. These results support the previous report that mechanisms independent of hENT1 up-regulation may also be involved in the sensitization of pancreatic cancer cells to gemcitabine by I3C (28). Indeed, it has been shown that constitutive activation of cancer cell survival pathways such as cyclooxygenase (COX-2), NF-κB and epidermal growth factor receptor (EGFR) signaling play critical role in cancer cell growth and the development of chemoresisitance. Agents to suppress the above cancer cell survival pathways, as potential therapeutic approaches for pancreatic cancer, are being exploited. However, inactivation of single survival pathways was not sufficient to suppress cancer cell growth and metastasis (19, 20, 26, 27). A gene profile study revealed the overactivation/overexpression of COX-2, NF-κB and EGFR, in BxPC-3, but not in Mia Paca-2 and PANC-1 cells. I3C has of pleiotropic modes of action and is able to inhibit all COX-2, NF-κB and EGFR pathways (18-20, 28). The highly activated anti-apoptotic pathways, COX-2, NF-κB, and EGFR in BxPC-3 cells may underlie the vulnerability of BxPC-3 cells to I3C treatment (36-38). Further study on the genetic constitution of individual patients may provide key information in determining individual variation in response and tolerance to I3C and I3C and gemcitabine combination treatment.

In vivo and *in vitro* studies have demonstrated that anticancer drugs have differential effects on cancer cells and normal cells (20). In light of this knowledge, we examined the effect of gemcitabine and I3C on normal pancreatic hTERT-HPNE cells. Although the low level of hENT1 expression in hTERT-HPNE cells impeded the observation of expression change of this transporter in response to I3C and gemcitabine treatment, MTS assay found that I3C does not enhance gemcitabine cytotoxicity in hTERT-HPNE cells. This result revealed that I3C has minimal effect on normal human pancreatic ductal epithelial cells with regard to hENT1 expression and activity. Our result confirmed the safety of this dietary agent and is in line with previous studies showing that tumor cells are more sensitive to chemotherapeutic drugs than are normal cells (20, 39, 40). Studies have shown that approximately thirty genes were identified as being significantly up-regulated in pancreatic cancer. These genes include transcription or translation-related genes, transcriptional or translational machinery-related genes, cell adhesion and migration-related genes, and DNA replication and mitosis-related genes (41-44). Therefore, it is reasonable to postulate that the differential effects of these genes in cancer cells underlie higher sensitivity to I3C.

NBMPR at 100 nM significantly abrogated the growth inhibitory effect of gemcitabine alone and in combination with I3C, which affirmed that promoting cellular entry of gemcitabine by up-regulating hENT1 expression is an important mechanism by which I3C enhances the efficacy of gemcitabine. NBMPR at 100 nM was used in this study because NBMPR selectively inhibits hENT1 at nanomolar concentrations and can be used to functionally distinguish hENT1 from other nucleoside transporters (45). Cell viability assay in the present study also showed that NBMPR was unable to completely block growth inhibition induced by gemcitabine and combination of gemcitabine with I3C. For example, co-incubation with 100 nM of NBMPR blocked only about 70% of cell growth inhibition induced by the combination of I3C and gemcitabine in BxPC-3 cells. This result indicates that NBMPR may not be able to completely block hENT1 activity. It is also possible that gemcitabine could be transported by other nucleoside transporters, such as human equilibrative nucleoside transporter 2 (hENT2) and human concentrative nucleoside transporters (hCNTs) (46). Indeed, the contributions of other transporters to total cellular gemcitabine uptake can be readily detected in *in vitro* studies, particularly when NBMPR is used to inhibit hENT1 activity (46, 47).

Notably, though pretreatment of I3C did not up-regulate hENT1 expression in PL-45 and AsPC-1 cells, it significantly reduced the IC50 of gemcitabine in these two cell lines. This result further supports the notion that the antitumor activity of I3C is also mediated by hENT1-independent mechanisms. Nevertheless, the fact that pretreatment of I3C reduced IC_{50} of gemcitabine by only 2- and 2.7-fold in PL-45 and AsPC-1 cells, respectively, but by 7.1-, 5.8- and 12.2-fold in PANC-1, Mia Paca-2 and BxPC-3 cells respectively, argues for the critical role of hENT1 in gemcitabine cellular uptake and the value of I3C in the enhancement of gemcitabine-induced pancreatic cancer cell death. In our study, NBMPR also significantly abrogated gemcitabine cytotoxicity in PL-45 and AsPC-1 cells. This may be caused by the inhibition of endogenous hENT1 activity by NBMPR.

It remains unclear how gemcitabine influences its own transporter in the treatment of pancreatic cancer. The present study showed no significant change of hENT1 expression in any of the tested pancreatic cancer cell lines after 48 h incubation with gemcitabine ranging from 2-200 nM. This result is in line with a recent study in which clinically achievable concentrations of gemcitabine were used. However, in the same study, it was also shown that high dosages of gemcitabine (20 μM for 1 h) elicited up-regulation of hENT1 in a few pancreatic cancer cell lines (48). Nakano and co-workers reported increased hENT1 expression in gemcitabine-resistant pancreatic cancer cells generated by exposing to incrementally increasing gemcitabine concentration starting at 3 nM after 6 months' incubation (35). Therefore, the discrepancy between our study and those of others may result from the low dose of gemcitabine used and shorter incubation time in our study.

Our data are in line with previous clinical trials demonstrating greater anticancer activity of gemcitabine and I3C combination treatment than single agent, which foster gemcitabine and I3C combination-based therapeutic strategy in pancreatic cancer treatment (20, 24, 28). Therefore, we were interested in determining the combined effect of I3C and gemcitabine on hENT1 expression in pancreatic cancer cell lines. Our data showed that the combination of I3C and gemcitabine at clinically achievable concentrations further increased I3C-mediated up-regulation of hENT1 in PANC-1 and BxPC-3 cells compared with treatment with I3C only, but not in Mia Paca-2 cells. Interestingly, though AsPC-1 cells showed no response to either gemcitabine or I3C treatment with regard to hENT1 expression, the combination elicited 3.4 fold increase of hENT1 expression in this cell line. Up-regulation of hENT1 expression by gemcitabine in combination with other therapeutic agents has also been reported in pancreatic cancer cell lines (48). For example, in a recent study, gemcitabine at 20 μM or 5-fluorouracil up-regulated hENT1 mRNA expression in Mia Paca-2, PANC-1 and Capan-1 cells, and the combination of 5-fluorouracil and gemcitabine caused further increase in hENT1 expression. However, 20 μM of gemcitabine is greater than the concentration achievable *in vivo* (49, 50). Our data may be of greater clinical significance because I3C and gemcitabine were used at *in vivo*-relevant concentrations. The up-regulation of hENT1 is likely to be a compensatory response of cells to I3C and gemcitabine treatment. Improved uptake of gemcitabine by I3C causes enhanced termination of DNA and RNA elongation, which promotes cell survival by uptake of more physiologic nucleobases and nucleosides through increasing hENT1 expression. But increased hENT1 expression can also promote the cellular uptake of gemcitabine, which resulted in further enhanced gemcitabine-induced cell death. This interpretation is in line with previous reports of anticancer properties of I3C (20, 24, 25, 28). Further studies will investigate the molecular mechanisms of I3C-evoked up-regulation of hENT1 expression.

In summary, we showed that I3C in combination with clinically relevant concentrations of gemcitabine enhances pancreatic cancer cell death through up-regulation of hENT1. Our results contribute to further understanding of the mechanisms of I3C treatment of pancreatic cancer. In addition, our results suggest that variations in hENT1 expression in pancreatic cancer cells may account for, at least partially, the interindividual differences in the clinical effect of gemcitabine. Increasing hENT1 expression by I3C may be a promising strategy to improve the effectiveness of gemcitabine. Moreover, understanding the genetic variations that contribute to the interindividual variability in response to I3C may be useful in predicting the additive effect of I3C in pancreatic cancer treatment. Therefore, further hENT1-related pharmacogenetic studies will provide useful information required for 'personalized' therapy.

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