Gender Differences in Gemcitabine (Gemzar) Efficacy in Cancer Cells: Effect of Indole-3-Carbinol

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Abstract. *Pancreatic cancer has a poor prognosis, mainly due to lack of effective therapies. This study demonstrated the ability of dietary agent, indole-3-carbinol (I3C), to lower the LD_{50} of gemcitabine (Gemzar) in decreasing growth of both male (MiaPaca2) and female (SU86.86) pancreatic cancer cells. Female pancreatic cancer cells were more resistant to gemcitabine alone. Additionally, RT-PCR analysis of MiaPaca2 cells treated with 1, 10 or 100 μM of I3C showed that I3C reactivated the tumor suppressor gene p16INK4a in pancreatic cancer cells. Methylated-specific PCR analysis indicated that I3C demethylated the promoter region of p16 INK4a, which was methylated in the untreated cancer cells. p16INK4a inactivation through promoter hypermethylation is considered an early event in pancreatic carcinogenesis. A positive control using 5-azacytidine also reactivated p16INK4a. This study demonstrated the potential of I3C, a possible non-toxic hypomethylating agent, combined with the anticancer agent, gemcitabine, to be a powerful strategy for treating pancreatic cancer.*

Diagnosis of pancreatic cancer often results in a bleak prognosis, mainly due to the inability to detect early biomarkers and the lack of therapies available at later stages of the disease. Gemcitabine (2',2'-difluorodeoxycytidine) (Gemzar) is the gold standard for chemotherapeutic treatment of pancreatic cancer (1). This drug, being an analogue of deoxycytidine, when incorporated into DNA inhibits DNA synthesis and repair, resulting in apoptosis. However, treatment is hampered by drug resistance in approximately 75% of patients and only a modest increase in median survival over traditional treatment with 5-fluorouracil (5.65 months *versus* 4.41 months) (2, 3).

New drugs developed specifically for potential targets have shown little or no benefit as single agents over gemcitabine in clinical trails. These include farnesyl transferase inhibitors, matrix metalloproteinase inhibitors and vascular endothelial growth factor inhibitors (4-6). Attempting to focus on a single target in a disease where multiple pathways are simultaneously involved in pathogenesis may be an approach with limited effectiveness. In light of the shortcomings of current therapeutics for treatment of pancreatic cancer, new strategies must be found. One strategy gaining support is combinatorial treatment in order to improve overall effectiveness. This has been demonstrated with various gemcitabine-based combinations (7). However, these combination therapies often lead to severe toxicity and eventually patient death. Several dietary agents (for example, curcumin or resveratrol) have been reported to sensitize pancreatic cancer cells to standard chemotherapeutic drugs (for example, gemcitabine or erlotinib), which suggests that these agents could potentially be used as potentiators of standard chemotherapy (8-10).

Indole-3-carbinol (I3C), a compound found in Brassica vegetables such as cabbages and broccoli, has been shown to exert anticancer properties in various tumor cells (11-13). Moreover, I3C has inhibited spontaneous and chemical-induced tumorigenesis in mammary gland, liver, lung, cervix, and gastrointestinal tract in different animal model studies (14, 15). I3C is thought to exert its anticancer effects through numerous mechanisms (16-19). It was recently demonstrated that DNA methyltransferases may be a target for I3C (20). I3C decreased the expression of DNA methyltransferases 1 and 3b in pancreatic cancer cells. DNA methyltransferases are highly expressed in numerous cancers and are thought to be associated with silencing of tumor suppression genes through hypermethylation (21-24). Promoter hyper-methylation in critical genes such as $p16^{INK4a}$ is known to

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play a role in the progression of cancer (25). The *p16INK4a* pathway is inactivated in >95 of pancreatic carcinomas and recent studies have shown its inactivation in pancreatic secretion in inflammatory disease (26). The identification of genes targeted by hypermethylation is important for developing therapy for reactivation of important silenced genes.

The present study examined the ability of the non-toxic dietary agent I3C to enhance the anticancer effect of gemcitabine in pancreatic cancer cells. I3C effectively reduced the concentration of gemcitabine needed to significantly decrease the growth of male- and female-derived pancreatic cancer cells. The study also demonstrated that I3C can reactivate $p16^{INK4a}$ in pancreatic cancer cells through hypomethylation. These studies provide further rationale for combinational treatment of this cancer and specifically for the combined use of DNA methyltransferase inhibitors to target suppressor genes or other critical genes inactivated through this process.

Materials and Methods

Cell culture and treatment. MiaPaca2 (male) and SU86.86 (female) cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). They were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin streptomycin. Gemcitabine was obtained from Eli Lilly and Co. (Indianapolis, IN, USA); I3C was obtained from Sigma. For toxicity studies cells were treated with various concentrations of gemcitabine for 24 h. In combination studies, either 100 μM or 200 μM I3C was included. For methylation analysis, cells were treated with I3C for 24 or 48 h at the following concentrations: 1, 10, or 100 μM. Cell pellets were frozen at −80˚C for molecular analysis.

MTT assay for cytotoxicity. Cell proliferation and cell toxicity assays were conducted using the MTT colorimetric assay. The activity of metabolically active cells was measured in an ELISA reader at optical density 570. All procedures were carried out according to the manufacturer's protocol, and the results were expressed as percent of cell growth of untreated cells.

DNA isolation and bisulfite modification. The QIAamp DNA kit (Qiagen; Valencia, CA, USA) was used to isolate genomic DNA from pancreatic cancer cells. For promoter hypermethylation, DNA was modified using a CpGenome™ DNA modification kit (Intergen Company; Purchase, NY, USA). The bisulfite treatment was carried out for 16 h at 50˚C on 1 μg of DNA according to the manufacturer's instructions. PCR annealing conditions were optimized to 95˚C (initial 15 min), followed by 40 cycles (1 min each) at 95, 54˚C (unmethylated) or 59˚C (methylated) and 72˚C, completing the reaction at 72˚C for 10 min. Resultant PCR products were analyzed by agarose gel electrophoresis. The following sense and antisense primers were used to detect unmethylated and methylated sequences: Unmethylated: forward, (F): 5'-TTATTAGAGGGTGGGGTGGATTGT-3', reverse (R): 5'-CAACCCCAAACCACA-ACCCATAA-3'; methylated, forward: 5'-TTATTAGAGGGTGGGGCGGATCGC-3', reverse: 5'-GACCCCGAA CCGCGACCGTAA-3'.

RNA isolation and RT-PCR analysis. A RNeasy isolation kit (Qiagen) was used to isolate total RNA. A Clontech cDNA synthesis kit (Palo Alto, CA, USA) was used for cDNA synthesis. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to determine the expression levels of $p16^{INK4a}$ and *GAPDH*. The primers used to detect the mRNA expression of these genes were synthesized by Sigma-Genosys (Woodlands, TX, USA). A 50μl sample consisted of 2.0 μl cDNA, 32.75 μl sterile distilled water, 5.0 μl 10X PCR buffer, 2.0 μl of each of the four DNTPs, 1.0 μl forward and reverse primer, and 0.25 μl taq polymerase was used to amplify the products by polymerase chain reaction (PCR). The PCR primers used in these studies were: $p16^{INK4a}$ F: 5'-CAACGCACCGAATAGTTACG-3' and R: 3'-ACCAGCGTGTCCAGGAA-5' (21). GAPDH primers were F: CCACCCATGGCA-AATTCCATGGCA and R: TCTAGACGG-CAGGTCAGGTCCACC. PCR for these genes was carried out by beginning with a hot start at 94˚C for two minutes. After completion, the cycle started at 94˚C for 30 s, 66˚C for one minute, and ended at 72˚C for one minute before the cycle repeated itself. Once the cycling was completed the reaction mixture underwent a seven minute extension for 30 cycles. The PCR products were separated using a 2.0% agarose gel. The PCR products were analyzed and quantified by an Alphaimager 2000 Documentation Analysis System (Alpha Innotech; San Leandro, CA) using *GAPDH* as a reference gene.

Statistical analysis. Prism IV software (GraphPAD Software; San Diego, CA, USA) was used for graphical analyses. Results for MTT toxicity studies are shown as % of control.

Results

I3C, in combination with the chemotherapeutic drug used to treat patients with pancreatic cancer, gemcitabine, has proven to be effective in inhibiting the growth of pancreatic cancer cells. Figure 1 shows that gemcitabine alone suppressed cell growth in the male cell line (MiaPaca2) at higher concentrations, indicating that alone it is a more effective anticancer agent at higher doses. I3C combination enhanced its anti-proliferative effect at lower doses, decreasing gemcitabine IC_{50} from 30 nM alone to 3 nM in combination, a ten-fold decrease in the amount of gemcitabine needed to kill a significant number of cancer cells. Pancreatic cancer cells from the female cell line (SU86.86) were highly resistant to gemcitabine alone and growth was not affected even at doses as high as 500μM compared to another powerful anti-cancer drug, doxorubicin (IC_{50}=2.47 μM) (Figure 2). However, I3C in combination with gemcitabine exerted powerful anti-cancer effects. The IC_{50} with 200 μM I3C was 0.388 μM and 1.13 μM with 100 μM I3C (Figure 3), which were lower than that of doxorubicin.

I3C is known to affect a number of biomarkers associated with highly aggressive cancers, including the expression of DNA methyltransferases. Inactivation of a number of genes through epigenetic mechanisms is known in several cancer types, including pancreatic cancer. Experiments were

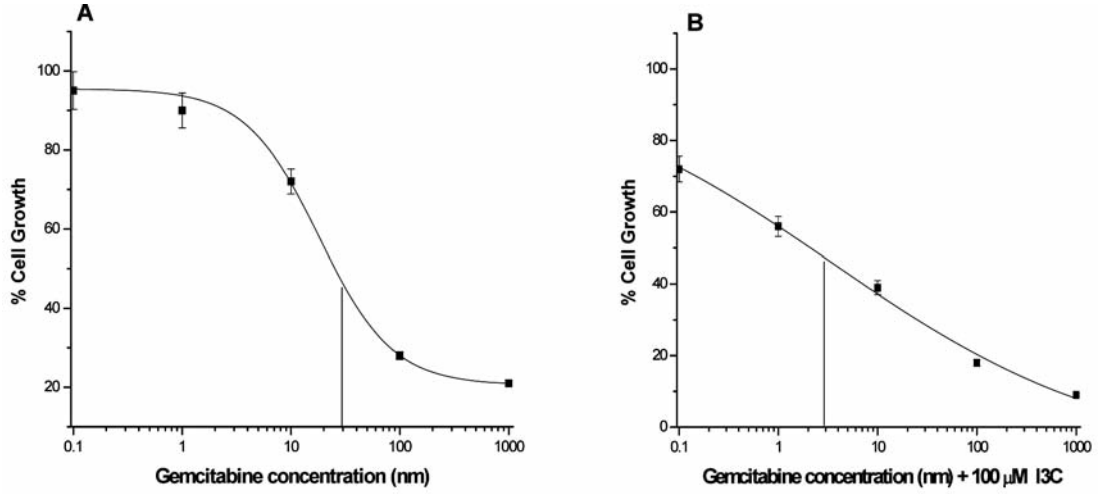

Figure 1. *Cytotoxicity of gemcitabine singly and in combination with I3C in pancreatic cancer cells by MTT assay. MiaPaCa2 cells (male) were treated with 0.1, 1, 10, 100 or 1000 nM of gemcitabine in the absence (A) or the presence of 100 μM indole-3-carbinol (B). Each data point is the average of at least three determinations.*

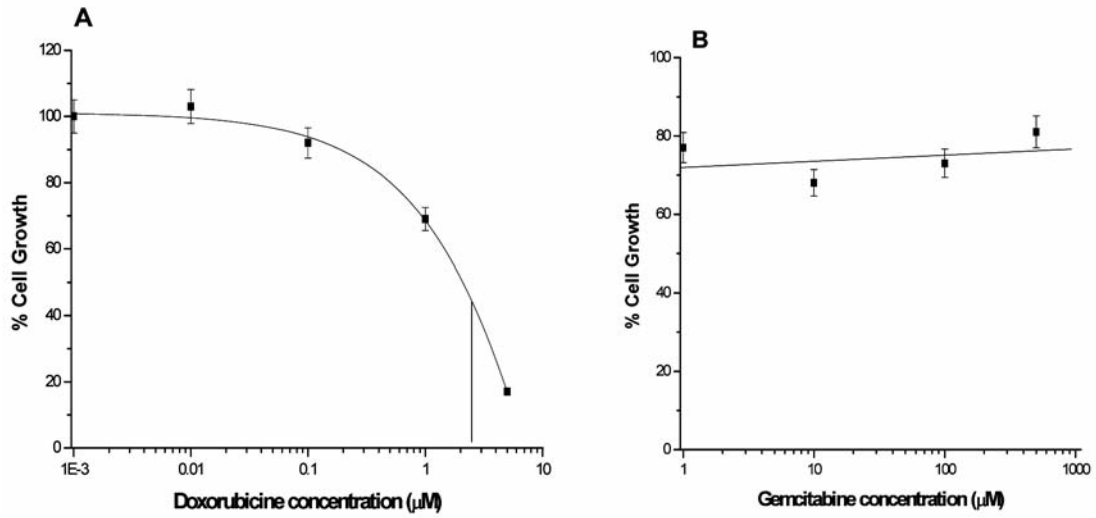

Figure 2. *Cytotoxicity of doxorubicin (A) and gemcitabine (B) in pancreatic cancer cells by MTT assay. SU86.86 cells (female) were treated with 0.001, 0.01, 0.1, 1 or 5 μM of doxorubicin or with 0.1,1, 10, 100 or 500 μM gemcitabine. Each data point is the average of at least three determinations.*

conducted to explore possible mechanisms underlying the effect of I3C in the pancreatic cancer cells shown in this study. I3C was shown to reactivate $p16^{INK4a}$ as demonstrated by increased expression in MiaPaca2 cells (Figure 4). Furthermore, this effect was through demethylation of the $p16^{INK4a}$ promoter, after 24 h (Figure 5). Treating pancreatic cancer cells with a known hypomethylating agent, 5-azacytidine, was found to also reactivate $p16^{INK4a}$ after 48 h (Figure 6).

Discussion

Combinational therapy is a promising strategy for the treatment of pancreatic cancer. This study showed that combining the non-toxic dietary agent I3C with gemcitabine greatly enhances its anticancer effect in male- and female-derived pancreatic cancer cells. Positive effects have been found with dietary agents in several other studies. Curcumin has been reported to sensitize pancreatic cancer cells to

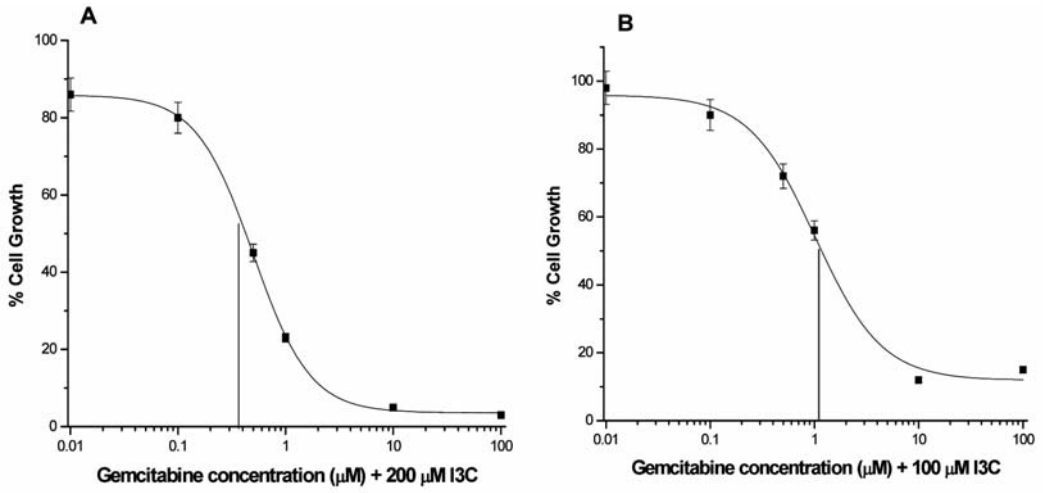

Figure 3. *Cytotoxicity of gemcitabine singly and in combination with I3C in pancreatic cancer cells by MTT assay. SU86.86 cells (female) were treated with 0.01, 0.1, 0.5, 1, 10 or 100 μM of gemcitabine in the presence of 200 μM indole-3-carbinol (A) or in the presence of 100 μM indole-3-carbinol (B). Each point is the average of at least three determinations.*

gemcitabine (8, 9), celecoxib (27), and paclitaxel (28). In an orthotopic animal model in which human pancreatic cancer cells (MiaPaCa2) were implanted in the pancreas of athymic nude mice, curcumin (1 g/kg, once daily by mouth) enhanced the antitumor activity of gemcitabine (25 mg/kg, twice weekly, intraperitoneal injection) by inhibiting cell proliferation, angiogenesis and NFκB-regulated gene products (cyclin D1, BCL2, BCL-xL, c-myc, COX2, and surviving) (8). Genistein, an isoflavonoid found in soy products, has been shown to induce apoptosis and inhibit NFκB activation in BxPC3 pancreatic cancer cells via inhibition of Notch-1 and NFκB signaling pathways, and to sensitize pancreatic cancer cells to docetaxel and cisplatin (29). Furthermore, in another study, genistein potentiated the effect of erlotinib in BxPC3, ASPC1, and CAPAN2 pancreatic cancer cells through inhibition of AKT and NFκB (30). Resveratrol, a polyphenol found in grape skins, has been shown to inhibit the growth of pancreatic cancer cells and enhance the antitumor activity of gemcitabine in an orthotopic mouse model (10).

The precise molecular mechanism(s) underlying the anti-cancer properties of I3C on neoplastic cells are not yet completely clear; however, substantial evidence indicates that the antitumor effect of I3C is attributable to its ability to target a plethora of signaling pathways governing apoptosis, cell-cycle progression, hormonal homeostasis, DNA repair, and angiogenesis (31). Multiple aspects of cancer cell-cycle regulation and survival are targeted, including Akt-NFκB signaling, caspase activation, cyclin-dependent kinase activities, estrogen metabolism, estrogen receptor signaling, endoplasmic reticulum stress, and *BRCA* gene expression. This broad spectrum of antitumor activities in conjunction with low toxicity emphasizes the translational value of I3C in cancer therapy. Previous studies have shown that I3C modulated the constitutively activated STAT3 transcription factor, which activates a number of cancer genes and its ability to induced apoptosis in pancreatic cancer cells was further demonstrated (32).

Considering the pleiotropic effects on multiple signaling targets relevant to cell survival, I3C has also shown an ability to sensitize cancer cells to apoptosis induced by various anti-cancer agents and radiation. I3C and various therapeutic agents work through different signal transduction pathways in a synergistic manner to suppress cell viability of various types of cancer cells. For example, I3C has been shown to be an effective sensitizer of TRAIL treatment against TRAIL-resistant LNCaP prostate cancer cells by up-regulating the expression of two TRAIL death receptors, DR4and DR5 (33). In addition, treatment of MCF-7 cells with I3C and tamoxifen synergistically ablated expression of the phosphorylated retinoblastoma protein (Rb), an endogenous substrate for the G1 cyclin-dependent kinases (CDKs), through specific down-regulation of the expression of CDK6 (34).

Results from the current study demonstrate that I3C can also effect epigenetic changes. I3C reactivated the $p16^{INK4a}$ tumor suppressor gene through hypomethylation of the promoter. As a tumor suppressor gene, $p16^{INK4a}$ is the universal and key regulator of the G1-S phase transition of the cell cycle (35). $p16^{INK4a}$ was selected for the present study because it is a key tumor suppressive gene and a strong candidate for mediating cellular senescence (36). As an essential cell cycle regulation factor, $p16^{INK4a}$ inhibits the cyclin D-dependent protein kinases

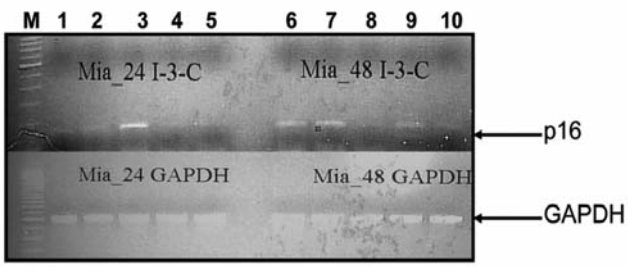

Figure 4. *Gene expression analysis of MiaPaCa2 cells using RT-PCR. MiaPaCa2 cells (male-derived) were treated with 0.1, 1, 10, or 100 μM of indole-3-carbinol. Lanes 1-5 are 24 h treatment and lanes 6-10 are 48 h treatment. Lanes 1 and 6, controls (untreated).*

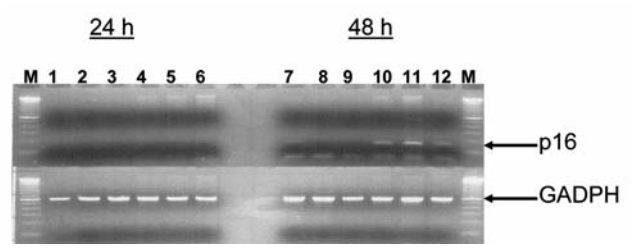

Figure 6. *Gene expression analysis of MiaPaCa2 cells using RT-PCR. MiaPaCa2 cells (male) were treated with 0.1, 1, 10, 100, 1000 μM 5-azacytidine. Lanes 1-5 are 24 h treatment and lanes 6-10 are 48 h treatment. Lane 1 and 7, controls (untreated).*

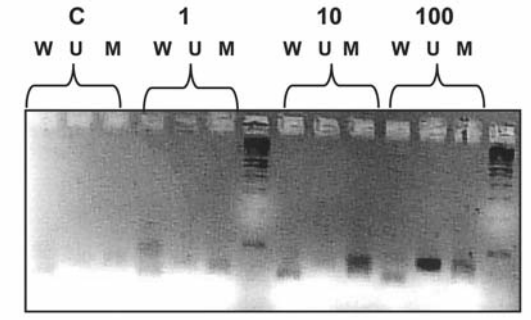

Figure 5. *Methylation-specific PCR (MSP) analysis of MiaPaCa2 cells (male) treated with 1, 10 or 100 μM indole-3-carbinol. W=wild type, U=unmethylation and M=methylated.*

CDK4 and CDK6, the main function of which appears to be the phosphorylation of RB. $p16^{INK4a}$ is functionally inactivated by promoter hypermethylation in a wide variety of cancers, including breast, colon, prostate, esophageal, pancreatic carcinomas, head and neck cancers, and other cell lines (25). Results from a recent study suggest that accumulation of DNA methylation of multiple tumor-related genes, including $p16^{INK4a}$, is involved in multistage carcinogenesis of the pancreas, from early precancerous stages to malignant progression, and that DNA methyltransferase 1 protein overexpression may by responsible for this aberrant DNA methylation (37). Restoration of $p16^{INK4a}$ expression through novel demethylating agents in combination with cytotoxic drugs may represent an important therapeutic intervention for human cancer.

Other mechanisms involving epigenetic alterations may be involved. Gemcitabine is a prodrug requiring intracellular phosphorylation to 2',2'-difluoro-2'-deoxycytidine monophosphate (dFdCMP) by deoxycytidine kinase (dCK) then converted to 2',2'-difluoro-2'-deoxycytidine di- and triphosphate (dFdCDP and dFdCTP, respectively) (38). Cytidine deaminase (CDA) catalyzes gemcitabine degradation. dCK is important in gemcitabine sensitivity, since induction of this enzyme resulted in re-sensitization of cells to gemcitabine (39). Factors influencing dCK transcriptional regulation and function have been explored. Treatment with 5-azacytidine in human lymphoid cell lines resulted in an increase in dCK activity up to 37%, which suggests that epigenetic control may be responsible for dCK gene silencing during development of gemcitabine resistance (40). Thus, opportunities for therapeutic intervention may include modulating dCK activity at the transcriptional level with epigenetic agents in combination with cytotoxic nucleosides. Because CDA catalyzes deamination of cytidine, deoxycytidine, and their analogs (41), it might be expected that its up-regulation may induce gemcitabine resistance. There is an indication that 5-aza-2'-deoxycytidine, a potent inhibitor of DNA methylation, induces CDA activity in human HL-60 myeloid leukemic cells, which correlates with induction of their differentiation. Whether this increased CDA activity by 5-aza-2'-deoxycytidine is due to CDA promoter demethylation or gene expression remains to be demonstrated. Hence, it is important to investigate whether gemcitabine-resistant cells have differential expression of this gene that is modulated by epigenetic mechanisms (42).

In conclusion, this study showed the ability of I3C to enhance the cytotoxicity of gemcitabine, the cancer drug used as a standard regimen for pancreatic cancer. This effect was shown in both male- and female-derived pancreatic cancer cells, although cells from the female cell line were more highly resistance to gemcitabine alone. Given the need to develop improved treatments for this disease, these results suggest a potential for combinational therapy. The use of non-toxic dietary agents is particularly attractive. I3C was also shown to reactivate the $p16^{INK4a}$ tumor suppressor gene through hypomethylation of the promoter. The renewed expression of epigenetically silenced genes following treatment with the demethylating agent may provide an important approach to increase the sensitivity of anti-cancer drugs. Additional studies are needed to clearly delineate the usefulness of dietary agents in chemotherapy.

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