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Cell cycle arrest, apoptosis induction and inhibition of nuclear factor kappa B activation in anti-proliferative activity of benzyl isothiocyanate against human pancreatic cancer cells

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Benzyl isothiocyanate (BITC), a cruciferous vegetable-derived compound, has been shown to inhibit chemically induced cancer in animal models. Moreover, epidemiological studies have provided compelling evidence to suggest that cruciferous vegetables may be protective against cancer risk. Here, we report that BITC significantly inhibits growth of human pancreatic cancer BxPC-3 cells in a concentration-dependent manner with an IC_{50} of ~8 μM, a concentration that can be generated through dietary intake of cruciferous vegetables. Treatment of BxPC-3 cells with growth suppressive concentrations of BITC resulted in G_2/M phase cell cycle arrest that was associated with a marked decline in protein levels of G_2/M regulatory proteins including cyclin-dependent kinase 1 (Cdk1), cyclin B1 and cell division cycle 25B (Cdc25B). Further, BITC-mediated growth inhibition of BxPC-3 cells correlated with apoptosis induction that was characterized by an increase in Bax/Bcl-2 ratio, cleavage of procaspase-3 and poly(ADP-ribose)-polymerase (PARP), and an increase in cytoplasmic histone-associated DNA fragmentation. Interestingly, BITC treatment caused inhibition of nuclear factor κB (NF-κB) activation, which is constitutively activated in human pancreatic cancer. Western blotting revealed concentration-dependent decrease in NF-κB/Rel-p65 protein level in BxPC-3 cells upon exposure to BITC. An increase in protein level of inhibitory subunit κB (IκBa) in association with reduced serine-32 phosphorylation was also observed in BITC-treated BxPC-3 cells. Consistent with these findings, BITC treatment caused a decrease in nuclear translocation of NF-κB as reflected by reduced DNA-binding capacity of NF-κB. Furthermore, the protein level of cyclin D1, a transcriptional target of NF-κB, was reduced significantly in BITC-treated BxPC-3 cells. To the best of our knowledge, this study is the first published report to implicate suppression of NF-κB activation as a potential mechanism for anti-proliferative activity of BITC against human pancreatic cancer cells.

Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in men as well as in women in the United States (1). Currently available chemotherapeutic options for pancreatic cancer are not very effective mainly due to the emergence of drug resistance (2). The majority of patients with advanced pancreatic cancer survive <1 year following diagnosis (3,4). Novel preventive and therapeutic strategies are therefore, urgently needed to decrease the mortality associated with this malignancy (5). Molecular analysis of pancreatic adenocarcinoma has indicated activation of specific oncogenes and inactivation of tumor suppressor genes (6,7).

Aberrant expression of nuclear factor κB (NF-κB), belonging to the rel family of transcription factors, has been associated with pancreatic carcinogenesis (8–11). Several recent studies have demonstrated constitutive over-expression of NF-κB in human pancreatic cancer indicating its mitogenic and anti-apoptotic role (11,12). Activation of NF-κB, which is sequestered in the cytoplasm, requires phosphorylation of IκB at two specific serine residues at the N-terminus of IκB (13–15). This allows free NF-κB to translocate into the nucleus and bind to DNA to activate transcription of responsive genes such as cyclin D1 (16). Studies have suggested that activation of NF-κB/p65 promotes tumor cell survival by inhibiting apoptosis (17). A very recent study points out that blocking epidermal growth factor receptor (EGFR) with an anti-EGFR antibody inhibits NF-κB DNA-binding activity, thereby restoring apoptosis in pancreatic cancer cells and enhancing the effect of chemotherapy and radiation therapy (18). Therefore, inhibiting NF-κB activation pathways by chemopreventive agents could be one novel and logical approach to prevent and/or treat pancreatic cancer (18–20).

Epidemiological studies have indicated an inverse correlation between consumption of fruits and vegetables and the risk of various types of cancers (21). Several population-based, case-control studies from different geographical locations have concluded that increased consumption of cruciferous vegetables may also be protective against pancreatic cancer (22–24).

Laboratory studies indicate that anticancer effects of cruciferous vegetables are attributable to isothiocyanates (ITCs) (25,26). ITCs are widely distributed in cruciferous vegetables such as broccoli, gardencress, cabbage, Brussel sprouts, etc. (25,26). ITCs have a significant chemopreventive action against chemically induced cancer in experimental animal models (27–30). Modulation of phase I and/or phase II drug metabolizing enzymes is believed to be responsible for cancer protective effects of ITCs against chemically induced cancer (31). More recent studies including those from our laboratory have indicated that ITCs can inhibit proliferation of cancer cells in culture by causing cell cycle arrest and/or apoptosis (32–39).

Despite compelling epidemiological evidence for the protective effects of cruciferous vegetables against pancreatic cancer, activity of ITCs against pancreatic cancer has not been examined. The present studies were undertaken to study the effect of benzyl isothiocyanate (BITC), one of the most extensively studied ITC compounds, using BxPC-3 human

Abbreviations: BITC, benzyl isothiocyanate; Cdk1 cyclin-dependent kinase 1; ITC, isothiocyanate; NF-κB, nuclear factor kappa B; PARP, poly(ADP-ribose)polymerase.

pancreatic cancer cells as a cellular model. We demonstrate that BITC strongly suppresses growth of BxPC-3 cells by causing G_2/M cell cycle arrest and apoptosis. Our results also demonstrate that BITC-induced apoptosis is associated with inhibition of NF-κB activation.

Materials and methods

Materials

BITC was purchased from Aldrich Chemical (Milwaukee, WI). Tissue culture media, fetal bovine serum and trypan blue were procured from GIBCO (Grand Island, NY). Sulforhodamine B and propidium iodide were from Sigma (St Louis, MO), and RNaseA was from Promega (Madison, WI). Cell Death Detection ELISA kit was from Roche Diagnostics GmbH (Mannheim, Germany), NE-PER Nuclear and Cytoplasmic Extraction kit was from Pierce (Rockford, IL), and TransAM ELISA kit was from Active Motif North America (Carlsbad, CA). Caspase-3 inhibitor z-DEVD-fmk was purchased from Enzyme Systems (Dublin, CA). The antibodies against cyclin B1, NF-κB and actin were from Oncogene Research Products (Boston, MA), antibodies against cleaved caspase-3, cleaved PARP, IκBα and phospho-IκBα were from Cell Signaling (Beverley, CA), antibodies against Bax, and cyclin-dependent kinase 1 (Cdk1), were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against Bcl-2 were from Dako Cytomation (Carpinteria, CA), and antibodies against Cdc25B were from BD PharMingen (San Diego, CA).

Cell culture

Human pancreatic cancer cell line BxPC-3 was obtained from the American Type Culture Collection (Rockville, MD). Monolayer cultures of BxPC-3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, PSN antibiotic mixture (Gibco BRL, Grand Island, NY) (10 ml/l), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 20% glucose. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell survival assays

The effect of BITC on survival/proliferation of BxPC-3 cells was determined by sulforhodamine B and trypan blue dye exclusion assay. Sulforhodamine B assay was performed as described by us previously (38). The IC_{50} value was determined from a plot of percentage of survival versus BITC concentrations. For trypan blue dye exclusion assay, 5×10^3 cells were plated in 6-well plates, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of BITC or DMSO (control), and the plates were incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Both floating and adherent cells were collected, and pelleted by centrifugation at 700 *g* for 5 min. The cells were re-suspended in 25 μl phosphate-buffered saline (PBS), mixed with 5 μl of 0.4% trypan blue solution and counted using a hemocytometer under an inverted microscope.

Cell cycle analysis

The effect of BITC on cell cycle distribution was determined by flow cytometric analysis of DNA content of nuclei of cells following staining with propidium iodide. BxPC-3 cells (10^6 cells) were seeded in T75 flasks, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of BITC or DMSO (control), and the flasks were incubated for 24 h at 37°C. The cells were washed with PBS and fixed in 70% ethanol overnight at 4°C. The cells were then treated with 80 μg/ml RNaseA and 50 μg/ml propidium iodide for 30 min, and analyzed using a Coulter Epics XL Flow Cytometer (Beckman Coulter, Miami, FL).

Determination of apoptosis

Apoptosis induction in control (DMSO-treated) or BITC-treated BxPC-3 cells was assessed by (i) quantification of cells with sub G_0/G_1 DNA content by flow cytometry following staining with propidium iodide, (ii) quantification of cytoplasmic histone associated DNA fragmentation using Cell Death Detection ELISA kit and (iii) DAPI staining. For analysis of cytoplasmic histone associated DNA fragments, 1×10^5 BxPC-3 cells were seeded in T25 flasks, allowed to attach, and treated with desired concentrations of BITC or DMSO for 24 h. Both floating and adherent cells were collected, and processed for quantification of cytoplasmic histone-associated DNA fragments according to the manufacturer's instructions. Apoptotic cells with condensed or fragmented nuclei were also visualized under a fluorescence microscope following staining with 10 ng/ml DAPI (40). Morphology of the cells was examined by using fluorescence microscope (Leica DM IRB).

Determination of NF-κB activity

NF-κB activity was determined using TransAM kit from Active Motif North America (Carlsbad, CA), according to the manufacturer's instructions. Nuclear and cytosolic fractions were prepared using NE-PER nuclear and cytoplasmic extraction kit from Pierce (Rockford, IL), according to manufacturer's instructions. Briefly, nuclear extract from control and BITC-treated BxPC-3 cells was added to 96-well plates pre-coated with the oligonucleotide containing NF-κB consensus sequence (5′-GGGACTTTCC-3′). Following incubation at room temperature for 1 h to facilitate the binding, a primary antibody, which recognizes only activated NF-κB/p65, was added to each well. The absorbance was read at 450 nm using a Lab System ELISA plate reader. This assay is specific for NF-κB/p65 activation and more sensitive than electrophoretic mobility shift assay.

Western blot analysis

Cells were exposed to varying concentrations of BITC for the indicated time periods as described above. The cells were washed twice with ice-cold PBS, lysed on ice with a solution containing 50 mM Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EGTA, 12 mM β-glycerol phosphate, 10 mM NaF, 16 μg/ml benzamidine hydrochloride, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 nM phenyl methyl sulfonyl fluoride. The cell lysate was cleared by centrifugation at 14 000 *g* for 15 min. Protein content in the supernatant fraction was determined by the method of Bradford (41). Lysate containing 20–60 μg protein was subjected to SDS–polyacrylamide gel electrophoresis according to the method of Laemmli (42), and the proteins were transferred onto polyvinylidene fluoride membrane (43). After blocking with 10% non-fat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1 h at the following dilutions: cyclin B1 (1:100 dilution), Cdc25B (1:1000 dilution), PARP (1:200 dilution), Cdk1 (1:100 dilution), Bcl-2 (1:100 dilution), Bax (1:500 dilution), caspase-3 (1:100 dilution), NF-κB (1:1000 dilution), IκBa (1:1000 dilution) and p-IκBa (1:1000 dilution). Subsequently, the membrane was incubated with appropriate secondary antibody, and the immunoreactive bands were visualized using enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. Each membrane was stripped and re-probed with antibody against actin (1:15 000 dilution) to ensure equal protein loading.

Densitometric scanning and statistical analysis

The intensity of immunoreactive bands was determined using a densitometer (Molecular Dynamics, Sunnyvale, CA) equipped with Image QuaNT software. Statistical significance of difference was determined by Student's *t*-test.

Results

BITC inhibits proliferation of cultured BxPC-3 cells

To determine the effect of BITC on cell growth, human pancreatic cancer cell line BxPC-3 was treated with increasing concentrations of BITC or DMSO for 24 h, and cell survival was assessed by sulforhodamine B assay. As shown in Figure 1A, BITC (1–50 μM) treatment resulted in a concentration-dependent inhibition of the proliferation of BxPC-3 cells with an IC_{50} of ~8 μM. Cell growth inhibition by BITC was confirmed by trypan blue dye exclusion method, and the results are shown in Figure 1B. Proliferation of BxPC-3 cells was significantly suppressed in the presence of BITC in a concentration-dependent manner. Concentration of DMSO was $<0.5\%$ in all the experiments conducted in this study.

BITC-treated BxPC-3 cells are arrested in G_2/M phase of the cell cycle

Effect of BITC on cell cycle distribution was determined to gain insights into the mechanism of its anti-proliferative activity. As can be seen in Figure 2, a 24-h exposure of BxPC-3 cells to growth suppressive concentrations of BITC (5 and 10 μM) resulted in significant accumulation of cells in G_2/M phase that was accompanied by a decrease in cells with G_0/G_1 DNA content. For example, as compared with control, the percentage of cells in G_2/M phase was increased by ~2.9-fold upon treatment with 10 μM BITC for 24 h (Figure 2).

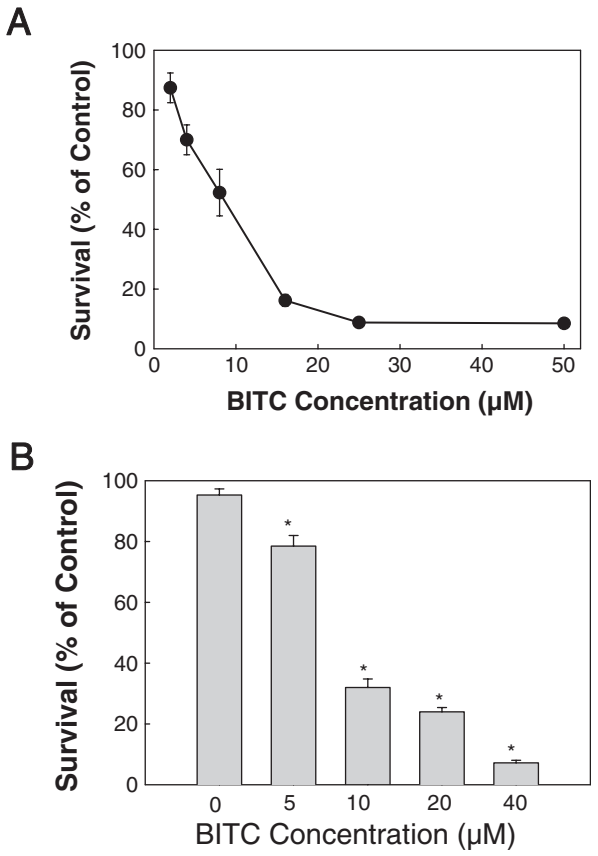


Fig. 1. Effect of BITC on proliferation of BxPC-3 cells determined by (**A**) sulforhodamine B and (**B**) trypan blue dye exclusion assay. Data are mean ± SD of two independent experiments in triplicate. *Significantly different compared with control ($P < 0.05$).

To understand the mechanism underlying G_2/M arrest in BITC-treated BxPC-3 cells, its effect on levels of proteins that regulate G_2/M transition was determined by immunoblotting, and representative blots are shown in Figure 3. Immunoblotting revealed that BITC treatment resulted in a significant reduction in the protein levels of cyclin B1, Cdk1 and Cdc25B in a concentration-dependent manner (Figure 3A–C). These results suggested that BITC treatment might cause a reduction in kinase activity of the Cdk1/cyclinB1 complex to elicit G_2/M phase cell cycle arrest mainly due to a decline in the protein levels of cyclinB1, Cdk1 and Cdc25B.

BITC treatment induces apoptosis in BxPC-3 cells

An increase in the fraction of cells with sub-G_0/G_1 DNA content upon treatment with BITC during analysis of cell cycle distribution (Figure 2, histograms for BITC-treated cells) suggested that BITC treatment might cause apoptosis to inhibit BxPC-3 cell survival. We therefore examined the apoptosis inducing effect of BITC in more detail by (i) quantifying cells with sub G_0/G_1 DNA content by flow cytometry, (ii) quantifying cytoplasmic histone-associated DNA fragmentation and (iii) analysis of cells with condensed nuclei. As can be seen in Figure 4A, exposure of BxPC-3 cells to 5 and 10 μM BITC resulted in a statistically significant accumulation of cells with sub-diploid DNA content. As shown in Figure 4B, BITC treatment caused ~2–3-fold increase in the levels of cytoplasmic histone associated DNA fragments as compared with control cells. Figure 4C shows nuclear fragmentation in BxPC-3 cells treated with BITC as determined by DAPI staining.

BITC modulates the protein levels of Bax and Bcl-2

Bax and Bcl-2 belong to a multi-gene family of proteins that play an important role in the regulation of apoptosis. Bcl-2 promotes cell survival, whereas Bax antagonizes this effect (44). Treatment of BxPC-3 cells for 24 h with increasing concentrations of BITC caused a dose-dependent increase in Bax protein level (Figure 5A). In contrast, the level of Bcl-2 protein was reduced significantly upon treatment with BITC in a dose-dependent manner (Figure 5B). As can be seen in Figure 5C, the ratio of Bax to Bcl-2 increased after BITC treatment in a dose-dependent manner, indicative of the apoptosis process.

Activation of caspase-3 by BITC treatment in BxPC-3 cells

Caspases are aspartate-specific cysteine proteases that play a key role in mediating apoptosis response. Caspases are sequentially activated due to cleavage of their inactive pro-caspase forms (45,46). Immunoblotting data shown in Figure 6A revealed that BITC treatment resulted in the activation of caspase-3 as evidenced by the appearance of 19 and 17 kDa cleavage intermediates. To determine whether activation of caspase-3 contributed to cell death by BITC, effect of z-DEVD-fmk, a specific inhibitor of caspase-3, on BITC-induced pro-caspase-3 cleavage and growth inhibition was determined. As shown in Figure 6B, BITC-induced cleavage of pro-caspase-3 was fully blocked in the presence of z-DEVD-fmk In agreement with these results, the growth inhibitory effect of BITC against BxPC-3 cells was also significantly attenuated upon treatment with z-DEVD-fmk (Figure 6C) confirming involvement of caspase-3 in BITC-mediated cell death.

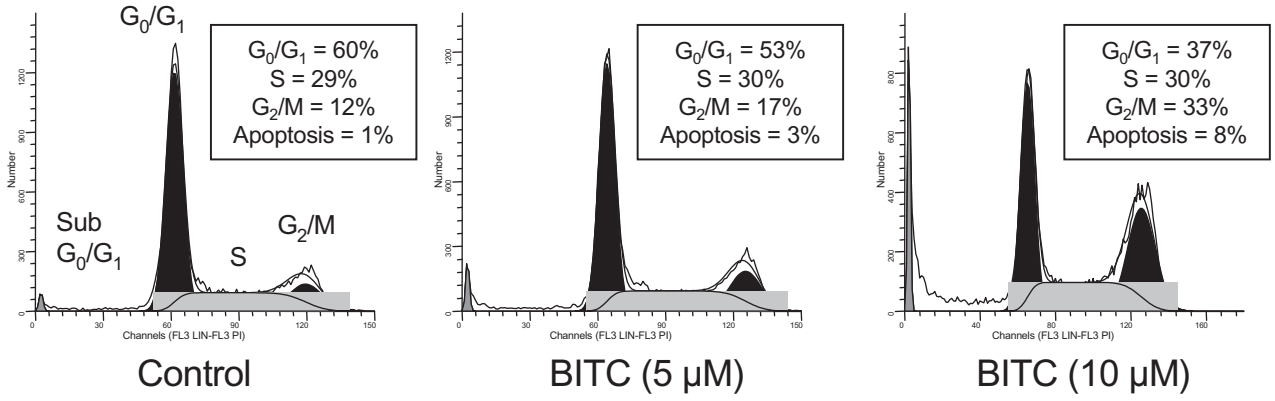
Treatment of BxPC-3 cells with BITC causes PARP cleavage

Caspase-3 is an executioner caspase whose activation leads to the cleavage of key cellular proteins including DNA repair enzyme poly(ADP-ribose)polymerase (PARP) (45,46). Treatment of BxPC-3 cells with increasing concentrations of BITC for 24 h resulted in cleavage of PARP, which was evidenced by appearance of the 89 kDa cleaved intermediate (Figure 7A). Cleaved PARP was not observed in cells treated with 40 μM BITC (Figure 7A, last lane), which may be due to necrotic death. As shown in Figure 7B, cleavage of PARP by BITC in BxPC-3 cells was nearly fully blocked when the cells were pre-treated with z-DEVD-fmk prior to BITC treatment.

BITC inhibits activation of NF-κB

Increased NF-κB activity has been demonstrated in pancreatic cancer, which is believed to enhance cancer cell survival by inhibiting apoptosis (11,17–20). NF-κB is retained in the cytoplasm in association with inhibitor protein IκBα (13,14). Upon phosphorylation on serine residues, IκBα is degraded allowing NF-κB to translocate to the nucleus and activate transcription of genes responsible for cell growth such as cyclin D1 (13–16).

Employing western blot analysis, we investigated the effect of BITC (5–40 μM, 24 h) on the levels of constitutively expressed NF-κB/p65 protein in BxPC-3 cells. As shown in Figure 8A, BITC treatment resulted in a concentration-dependent decrease in NF-κB/p65 protein levels in the cytosolic (Figure 8A) as well as nuclear (Figure 8B) fraction. Consistent with these observations, a decrease in NF-κB protein level correlated with a decrease in phospho-IκBα (serine 32) (Figure 8C) with a concomitant increase in the cytosolic levels of the IκBα protein



Treatment	G_0/G_1 phase	S phase	G_2/M phase
Control	61.52 ± 3.40	26.52 ± 2.31	11.95 ± 1.64
BITC 5 μM	58.77 ± 8.30	25.20 ± 6.12	16.02 ± 2.23*
BITC 10 μM	40.16 ± 4.68*	25.20 ± 2.69	34.63 ± 2.46*

*Statistically different when compared with control, $p<0.05$

Fig. 2. Effect of BITC on BxPC-3 cell cycle distribution. Representative histograms from DMSO-treated control and BITC-treated (5 or 10 μM for 24 h) BxPC-3 cells are shown. Data in the table are mean ± SD of two independent experiments in triplicate.

(Figure 8D). For example, exposure of BxPC-3 cells to 20 μM BITC for 24 h resulted in ~90% decrease in levels of NF-κB/p65 and phospho-IκBα, respectively. To determine if BITC-induced changes in the levels and/or phosphorylation of NF-κB/p65 and IκBα proteins, the effect of BITC on DNA-binding activity of NF-κB was determined, and the results are shown in Figure 8E. The NF-κB activity in nuclear fraction was inhibited upon BITC treatment for 24 h.

BITC treatment reduces level of cyclin D1 protein

Cyclin D1 is transcriptionally activated by NF-κB, and has been shown to be over-expressed in pancreatic cancers (47). BITC treatment in BxPC-3 cells resulted in a significant reduction in the protein level of cyclin D1 (Figure 8F), which is consistent with a decrease in NF-κB activity.

Discussion

The results of the present study demonstrate that BITC, a naturally occurring phytochemical present in cruciferous vegetables such as watercress, is a strong suppressor of human pancreatic cancer BxPC-3 cell proliferation. It is important to point out that growth inhibitory effects of BITC were observed at concentrations that may be generated through dietary intake of cruciferous vegetables. We also found that BITC exerts activity against proliferation of BxPC-3 cells by arresting cells in the G_2/M phase of the cell cycle and causing apoptosis. The BITC-induced apoptosis in BxPC-3 cells is mediated in part by inhibition of NF-κB activation. To the best of our knowledge, the present study is the first published report to document the novel activity of BITC as an inhibitor of the NF-κB signaling pathway in human pancreatic cancer cells.

ITCs have been shown previously to exert anticancer activity against a variety of chemically induced cancer in animal model by inhibiting phase I enzymes (Cytochrome P-450) responsible for the activation of carcinogens and/or by inducing detoxification enzymes such as glutathione *S*-transferase (31). Recently, a few studies including those from our laboratory have indicated that ITCs are anti-proliferative to cancer cells and cause cell cycle arrest and/or induce apoptosis through different signal transduction mechanisms (32–39). These studies support the notion that ITCs may not only prevent initiation but also inhibit the promotion phase of the carcinogenesis process.

Cancer progression has been suggested to involve the loss of cell cycle checkpoint controls that regulate the passage through cell cycle. Checkpoints are control mechanisms that ensure the proper timing of cell cycle events and monitor the integrity of the DNA (48,49). Entry into mitosis is blocked by G_2/M checkpoint mechanisms when DNA is damaged. Blocking damaged cells in G_2/M provides time to repair DNA damage or an opportunity to permanently arrest cells if

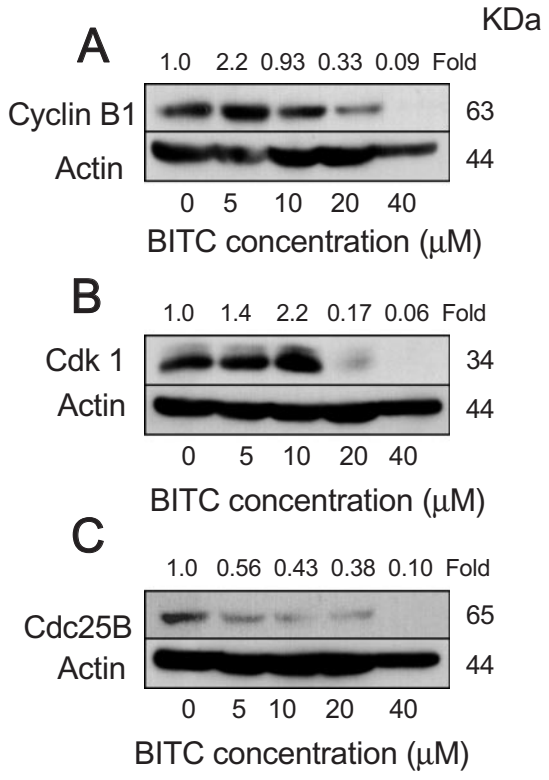


Fig. 3. Representative immunoblots showing effect of BITC treatment on levels of (**A**) cyclin B1, (**B**) Cdk1 and (**C**) Cdc25B. BxPC-3 cells were treated with DMSO (control) or indicated concentrations of BITC for 24 h prior to preparation of lysates. Each blot was stripped and re-probed with anti-actin antibody to ensure equal protein loading. Intensities of the immunoreactive bands were quantified by densitometric scanning. A change in the protein level is indicated at the top of the band following normalization to actin control.

the damage is severe. Both of these responses are important to protect organisms from tumor formation driven by the accumulation of mutations (48,49). Regulation of G_2/M transition is dependent on activation of Cdk1/cyclinB1 (48,49). The Cdk1/cyclinB1 kinase complex is maintained in an inactive state by reversible phosphorylations on tyrosine 15 and threonine 14 of Cdk1 (49,50,52). At the onset of mitosis both of these residues are dephosphorylated by the Cdc25 family of phosphatases (48,49). By supplementing existing cell cycle machinery with extrinsic cell cycle regulators, it may be possible to block initiation or progression of cancer. There is growing evidence that ablation of G_2/M checkpoint in human tumor cell lines increases sensitivity to anticancer agents. For example, several flavanoids, such as quercetin and genistein that have been shown to inhibit tumor growth in animal models are able to interrupt cell cycle progression in either G_1 or G_2 phase (50,51). In the present study, we observed that treatment of BxPC-3 cells with BITC results in arrest of cells in G_2/M phase and that BITC-mediated G_2/M arrest is associated with a decrease in the protein levels of Cdk1, cyclinB1 and Cdc25B. While the precise mechanism by which BITC reduces levels of the above proteins remains to be elucidated, the results of the present study are in agreement with our previous observations with other ITC analogs including allyl-ITC (38).

Apoptosis is an important mechanism to eliminate unwanted cells in a wide variety of physiological processes, and deregulation of this process is implicated in pathogenesis of many chronic diseases, including cancer, autoimmunity

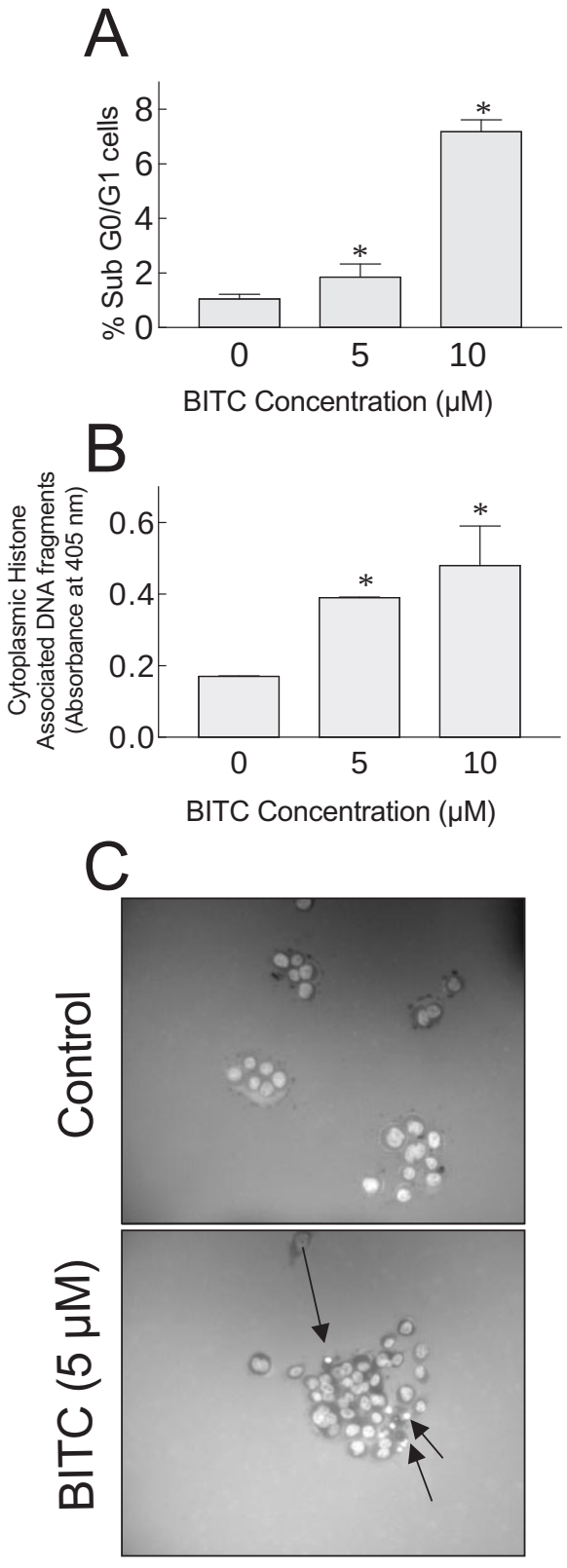


Fig. 4. Effect of BITC treatment on apoptosis induction assessed by (**A**) flow cytometric analysis of cells with sub-G_0/G_1 DNA content following exposure of BxPC-3 cells to DMSO (control) or 5 or 10 μM BITC for 24 h, (**B**) analysis of histone-associated DNA fragments following exposure of BxPC-3 cells to DMSO (control) or 5 or 10 μM BITC for 24 h and (**C**) DAPI staining of BxPC-3 cells following treatment with DMSO (control) or 5 μM BITC for 24 h. Data are mean ± SD of at least two independent determinations. *Significantly different compared with control ($P < 0.05$ by Student's *t*-test).

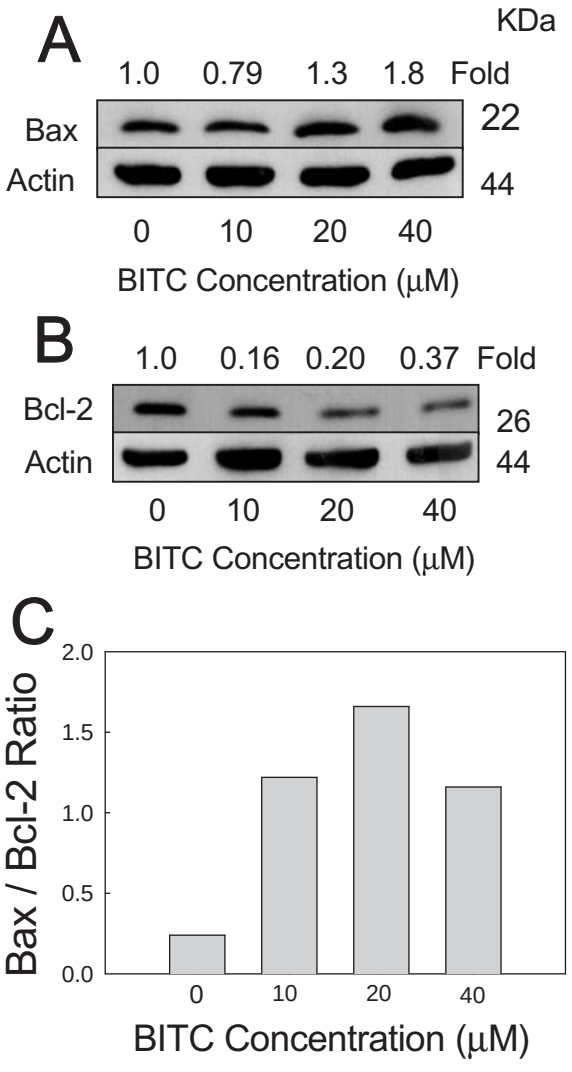


Fig. 5. Effect of BITC treatment on protein levels of (**A**) Bax, and (**B**) Bcl-2 determined by immunoblotting. (**C**) Bar diagram summarizing the effect of BITC on Bax/Bcl-2 ratio. Equal amount of protein (40 μg) was subjected to western blotting. Each blot was stripped and re-probed with anti-actin antibody to ensure equal protein loading. Intensities of the immunoreactive bands were quantified by densitometric scanning. Protein level relative to control is indicated at the top of each band following normalization to actin control. Data are representative of at least two independent experiments with similar results.

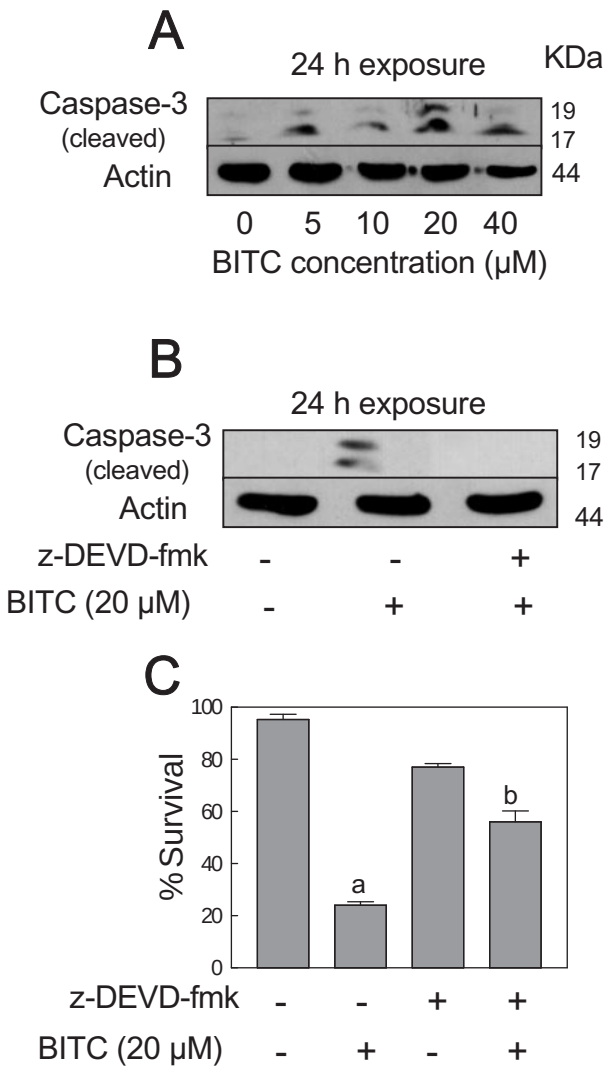


Fig. 6. (**A**) Western blot analysis for the effect of BITC treatment on cleavage of pro-caspase-3. (**B**) Effect of caspase-3-specific inhibitor z-DEVD-fmk on BITC-induced cleavage of pro-caspase-3. BxPC-3 cells were pre-treated with 40 μM of caspase-3 inhibitor for 2 h prior to treatment with 20 μM BITC for 24 h. For data in panels (A) and (B), blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. (**C**) Effect of caspase-3-specific inhibitor on survival of BxPC-3 cells in the presence or absence of BITC as assessed by trypan blue dye exclusion assay. Data are mean ± SD of at least two independent determinations. [a]Significantly different compared with DMSO control ($P < 0.05$ by Student's *t*-test), and [b]significantly different compared with BITC alone treatment group ($P < 0.05$ by Student's *t*-test).

and neurodegenerative disorders (52). Among the growing number of genes that regulate apoptosis induced by a wide variety of stimuli, the Bcl-2 family of proteins have acquired center stage in the regulation of this complex yet tightly regulated process (44). Some proteins within this family, including Bcl-2 and Bcl-X_L, inhibit apoptosis, while others such as Bax and Bak promote apoptosis (44). Bcl-2 and related anti-apoptotic proteins seem to dimerize with a proapoptotic molecule, e.g. Bax and modulate the sensitivity of cell to apoptosis (44). Hence, an alteration in the levels of anti- and pro-apoptotic Bcl-2 family proteins is likely to influence apoptosis. The results of the present study indicate that BITC treatment reduces Bcl-2 protein level and increases the level of pro-apoptotic protein Bax thereby increasing the Bax/Bcl-2 ratio. Thus, it seems logical to postulate that BITC-induced apoptosis in BxPC-3 cells may be caused by a reduction in heterodimerization of Bcl-2 with Bax. However, further studies are needed to systematically explore this possibility.

Caspases are cysteine proteases that a play critical role in the execution of apoptosis (45,46). Several chemotherapeutic and chemopreventive agents have been shown to cause apoptotic cell death through mediation of caspases (45,46). For example, Yu *et al*. (33) were the first to observe induction of caspase-3-like activity in HeLa cells treated with phenethyl-ITC, a close structural analog of BITC (33). Caspase-3 is an executioner caspase, which upon activation can systematically dismantle cells by cleaving key proteins such as PARP (45,46). Cleavage of pro-caspase-3 and PARP following treatment with BITC was observed in BxPC-3 cells (present study). In addition,

BITC-induced cleavage of caspase-3 as well as PARP was significantly attenuated in the presence of z-DEVD-fmk (present study), suggesting a caspase-dependent mechanism in cell death by this agent. Activation of caspase-3 can be mediated by a mitochondrial pathway involving release of apoptosis promoting factors, e.g. cytochrome c, from mitochondria to the cytosol leading to recruitment and activation of caspase-9 (45,46). Alternatively, activation of caspase-3 in some systems is mediated by a death-receptor pathway involving caspase-8 (45,46). Even though further studies are needed to ascertain whether BITC-induced caspase-3 activation in BxPC-3 cells is caused by mitochondrial or death-receptor pathway, a very recent study has suggested involvement of mitochondria in BITC-induced apoptosis in RL-34 cells (53).

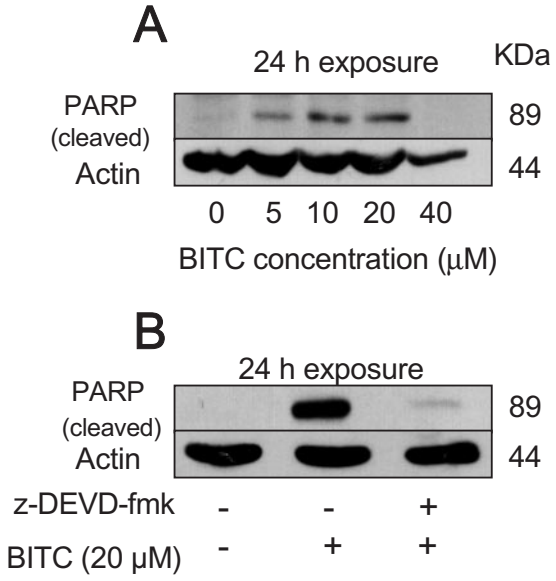


Fig. 7. (**A**) Immunoblotting for PARP cleavage using lysates from control (DMSO-treated) and BITC-treated BxPC-3 cells. (**B**) Effect of caspase-3-specific inhibitor on BITC-induced cleavage of PARP. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. Similar results were observed in at least two independent experiments.

NF-κB exists as a heterodimer of p50 and p65 subunits, and is sequestered in the cytoplasm as an inactive complex bound to an endogenous inhibitor IκB (13–15). Following cellular stimulation, IκB proteins are phosphorylated at two specific serine residues at the N-terminus IκBα (serine 32/36) or IκBβ (Ser19/23) by IκB kinase. The phosphorylation of IκB promotes its ubiquitination and degradation through the 26S proteasome (13–15). Degradation of IκB protein liberates NF-κB allowing it to translocate to the nucleus (13–15). NF-κB activation in cancer cells correlates with resistance to apoptosis and increased levels of anti-apoptotic Bcl-2 family proteins (13–15). NF-κB also activates genes involved in cell proliferation (cyclin D1), angiogenesis (vascular endothelial growth factor) and metastasis thereby promoting tumor growth and metastasis (13–16). It is becoming increasingly clear that compounds that block NF-κB activation could be highly useful for the treatment of cancers including pancreatic cancer (19, 20). Data presented herein indicate that BITC treatment significantly inhibits NF-κB activation by reducing the level of phospho-IκBα (serine 32). Suppression of pancreatic tumor-

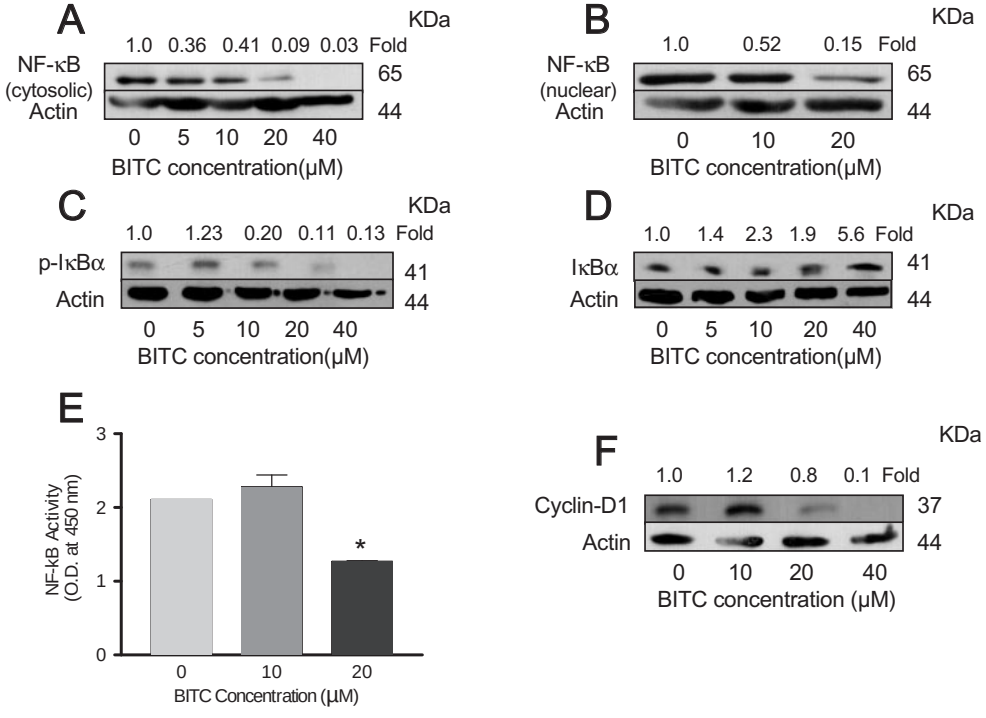


Fig. 8. Immunoblotting for the effect of BITC on (**A**) NF-κB/p65 protein level in the cytosolic extract, and (**B**) NF-κB/p65 protein level in the nuclear extract. Immunoblotting for (**C**) p-IκBa and (**D**) IκBa using lysates from BxPC-3 cells treated with varying doses of BITC for 24 h. (**E**) Effect of BITC on NF-κB/p65 activity determined by an ELISA-based assay as described in Materials and Methods. *Significantly different compared with control ($P < 0.05$ by Student's *t*-test). (**F**) Western blot analysis for level of cyclin D1 protein using lysates from BxPC-3 cells treated with DMSO (control) and varying concentrations of BITC for 24 h. The blots in each experiment were stripped and re-probed with anti-actin antibody to ensure equal protein loading. Intensities of the immunoreactive bands were quantified by densitometric scanning. Protein level relative to control is indicated at the top of each band following normalization to actin control. Data are representative of at least two independent experiments with similar results.

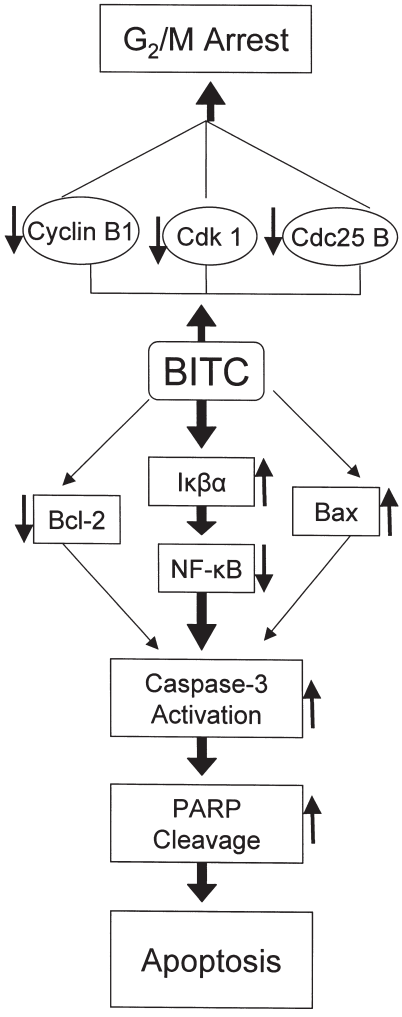


Fig. 9. Proposed mechanisms of BITC-induced apoptosis and G_2/M phase cell cycle arrest in BxPC-3 cells.

igenesis by inhibiting constitutive NF-κB activity by using mutated IκBα has been shown recently (54). We also found that BITC treatment reduces the level of cyclin D1 protein, which confirms that BITC inhibits NF-κB activation since cyclin D1 is a downstream target of NF-κB. Interestingly, a recent study indicated an inverse correlation between survival of patients with pancreatic cancer and over-expression of cyclin D1 (47). Based on the results of the present study, the mechanisms by which BITC causes cell cycle arrest and apoptosis in BxPC-3 cells are summarized in Figure 9. Taken together, the results of the present study suggest that inhibition of NF-κB activation may be an important mechanism for growth suppressive activity of BITC against human pancreatic cancer cells. However, the precise mechanism of BITC-mediated inhibition of NF-κB activation in BxPC-3 cells is not clear, which is of considerable interest to us in our future investigations.

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