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Benzyl isothiocyanate sensitizes human pancreatic cancer cells to radiation therapy

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1. ABSTRACT

Increase in systemic toxicity and resistance are the major drawbacks of radiation therapy in the treatment of pancreatic cancer. We have shown previously that BITC inhibits the growth of human pancreatic cancer cells and induces apoptosis. Here we determined whether BITC could sensitize BxPC-3 cells and increase the therapeutic potential of γ-irradiation. Cells were pretreated with 2.5μM BITC for 24h followed by exposure to 5Gy of γ-irradiation and were allowed to grow for another 24 or 48h before being analyzed. Combination of BITC and γ-irradiation significantly reduced survival of cells and caused significantly enhanced arrest of cells in G2/M phase as compared to cells exposed to γ-irradiation alone. G2/M arrest was associated with DNA damage leading to the phosphorylation of ATR (Ser-428), Chk2 (Thr-68), Cdc25C (Ser-216), Cdk-1 (Tyr-15) and induction of $p21^{Waf1/Cip1}$. However, combination treatment after 48h caused 2.8-fold increase in apoptosis in BxPC-3 cells. Apoptosis at 48h was associated with NF-kB inhibition and p38 activation. Taken together, results of the present study suggest that the apoptosis-inducing effect of γ-irradiation can be increased by BITC.

2. INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States and one of the most difficult malignancies to manage (1). Clinical and molecular studies have revealed that the high mortality rate in pancreatic cancer is due to its poor response to chemotherapy and radiation therapy (2-3). In spite of recent advances in diagnostic imaging and improvements in radiation therapy techniques, the survival and mortality rate in pancreatic cancer has remained relatively constant (4-6). Therefore treatment with radiation therapy is a sub-optimal choice. Chemotherapy with 5-fluorouracil (5-FU) (7-9) or gemcitabine with radiation therapy has been found to produce better outcomes in various human malignancies including colorectal, lung, breast and pancreatic cancers (10-15). However, concurrent treatment with gemcitabine and radiotherapy for locally advanced pancreatic cancer was associated with severe toxicity resulting in incomplete delivery of the planned chemotherapy (13-14).

Intervention of cancer by dietary bioactive agents is becoming an alternative, safe and striking approach to

cancer treatment. Emerging evidence suggests that the anti-proliferative and anti-oxidant effects of some of these dietary agents might be utilized to both potentiate the response of cancer cells to radiotherapy and reduce radiation-induced toxicity to surrounding normal tissues (16-17). Epidemiological, pharmacological and case control studies support the fact that isothiocyanates present in cruciferous vegetable have substantial chemopreventive activity against human malignancies, including pancreatic cancer (17-20).

Benzyl isothiocyanate (BITC), an anticancer agent present in cruciferous vegetables such as watercress, broccoli etc., has been reported to inhibit chemically induced human cancers in experimental animals (21-24). In our previous studies, we demonstrated that BITC suppressess the growth of human pancreatic cancer cells by inducing DNA damage which causes G2/M cell cycle arrest and apoptosis (25) and is in part mediated by the inhibition of nuclear factor kappa B activation (26). Therefore, the goal of the present study was to determine whether BITC can sensitize human pancreatic cancer cells to γ-irradiation and increase the therapeutic potential of radiation at low doses. We demonstrated that BITC sensitizes human pancreatic cancer cells to low doses of γ-irradiation by arresting the proliferating cells in G2/M phase and inducing apoptosis.

3. MATERIAL AND METHODS

3.1. Chemicals

BITC, RPMI-1640 cell culture medium, RNase A, propidium iodide, and antibodies against actin were purchased from Sigma-Aldrich, St Louis MO. Electrophoresis reagents were from Bio-Rad Laboratories. Antibodies against Cdk-1 (#9112), p-Cdk-1 (Tyr-15) (#9111), checkpoint kinase 2 (Chk2) (#2662), p-Chk2 (Thr-68) (#2661), cell division cycle 25C (Cdc25C) (#4688), p-Cdc25C (Ser-216) (#9528), p-H2A.X (Ser-139) (#2577), p-ATR (Ser-428) (#2853), $P21^{Waf1/Cip1}$ (#2947), p-EGFR (Tyr-1068) (#2234), EGFR (#2232), p-ERK (Thr-202/Tyr-204) (#9101), ERK (#9102), p-P38 (Thr-180/Tyr-182) (#9216), P38 (#9212), NF-kB (#4764), Cyclin D1 (#2926), Caspase-3 (#9665), and poly (ADP-ribose) polymerase (PARP) (#9541) were from Cell Signaling Technology, Inc Danvers MA. Chemicals for cell culture such as penicillin/streptomycin (PSN) antibiotic mixture, sodium pyruvate, HEPES buffer, were purchased from GIBCO BRL Carlsbad CA. Heat inactivated fetal bovine serum and was purchased from Mediatech Cell Grow, Lawrence, Kansas. Western blotting enhanced chemiluminiscence (ECL) reagent was purchased from Perkin Elmer, Waltham MA. TransAM NF-kB p65 activation assay kit was from Active Motif, Carlsbad CA. NE-PER Nuclear and Cytoplasmic Extraction Kit was from Thermo Scientific, Rockford IL.

3.2. Cell culture

BxPC-3 cells were obtained from ATCC. This is a well-differentiated epithelial pancreatic adenocarcinoma cell line obtained from a male Caucasian donor having wild type K-ras and mutated p53 and p16. A monolayer culture of BxPC-3 cells was maintained in RPMI-1640 medium supplemented with 2mM L-glutamine adjusted to contain 10% FBS, 1.5g/L sodium bicarbonate 4.5g/L glucose, 10mM HEPES, 1.0mM sodium pyruvate and antibiotics in a humidified incubator with 5% CO2 and 95% air. The stock solution of BITC was prepared in 100% dimethyl sulfoxide (DMSO) and subsequently diluted in medium so that the final concentration of DMSO was less than 0.2% in the medium. The cells were treated with BITC for 24 or 48 hours.

3.3. Cytotoxicity measurement by γ-irradiation

The cytotoxic effects of γ-irradiation on the proliferation of BxPC-3 cells was assessed by Sulforhodamine B (SRB) assay as mentioned previously (25). Briefly, 5000 cells were seeded in 96 well plate and treated with or without 2.5μM BITC for 24 hours followed by treatment with different doses (2.5, 5, 10 and 20Gy) of γ-irradiation using a Shepherd model 143–45A irradiator (J.L. Shepherd & Associates, San Fernando, CA) at a dose-rate of 4 Gy/minute (27). The cells were then allowed to grow for 24 or 48 hours before being analyzed for survival by SRB assay as described previously (25). The plates were read at 570 nm using a Bio Kinetics plate reader.

3.4. Cell cycle analysis

The effect of γ-irradiation and BITC individually and in combination on cell cycle distribution was assessed by flow cytometry after staining the cells with propidium iodide (PI). Briefly, 0.5×10^6 cells were plated and allowed to attach overnight. The medium was replaced with fresh complete medium containing the desired concentration of BITC and an equal volume of DMSO so that the final concentration of DMSO was less than 0.2%. After an incubation of cells at 37°C for a specified time, floating and adherent cells were collected by using 0.1% trypsin, washed twice with cold PBS, and fixed with ice-cold 70% ethanol overnight at 4°C. The cells were then treated with 80 μg/mL RNase A and 50 μg/mL propidium iodide for 30 min. The stained cells were analyzed using a Coulter Epics XL Flow Cytometer. Control cells were treated with DMSO and cultured in a similar manner. The cell cycle data were reanalyzed using MODFIT software.

3.5. Apoptosis determination

Apoptosis induction in control, BITC and γ-irradiated cells was determined by flow cytometry by quantitating: *1*) sub G_0/G_1 DNA contents of the PI stained cells by flow cytometry, or 2) by cleavage of caspase 3 and PARP by western blot analysis.

3.6. Western blot analysis.

BxPC-3 cells were pre-treated with 2.5μM BITC for 24 hours and then exposed to γ-irradiation (5Gy) and incubated for another 24 or 48 hours. Whole cell extracts were prepared as described by us previously (25, 28).

3.7. NF-kB determination

The effect of γ-irradiation and BITC individually and in combination on NF-kB DNA binding was determined by TransAM NF-kB p65 Activation assay according to the manual's instruction. Briefly, BxPC-3

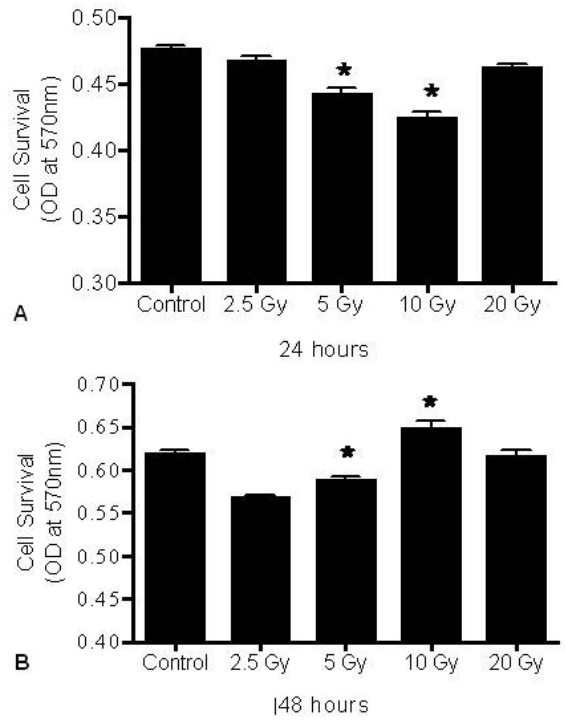

Figure 1. Effect of γ-irradiation on the survival of BxPC-3 cells. A) BxPC-3 human pancreatic cancer cells were irradiated at different doses (0, 2.5, 5, 10 and 20Gy) as described in methods and incubated for 24 hours. The effect of irradiation on the survival of cells was measured by SRB assay. B) The effects of irradiation at these different doses were measured after 48 hours. Values are means ± SEM of 3 independent experiments (each conducted in triplicate). Data were analyzed by non-parametric ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. Differences between tested groups were analyzed and considered significant at $P<0.05$ from control. * denotes statistical significant difference between control and γ-irradiation treated groups.

cells were treated either with 2.5μM BITC or 5Gy γ-irradiation alone or in combination followed by extraction of nuclear lysates using NE-PER Nuclear and Cytoplasmic Extraction Kit. 10μg nuclear lysates with 30μl complete binding buffer were added to the 96 well ELISA plate followed by incubation for 1 hour at room temperature (RT). After incubation, the plate was washed with washing buffer and incubated with NFkB primary antibody (1:1000) for 1 hour at RT followed by incubation with HRP-conjugated secondary antibody (1:1000) for 1 hour at RT. The plate was then washed and developed with developing solution by incubating 5 minutes at RT and read at 450 nm using a Bio Kinetics plate reader.

3.8. Densitometric scanning and statistical analysis

The intensity of immunoreactive bands was determined using a densitometer (Molecular Dynamics, Sunnyvale, CA) equipped with Image QuaNTsoftware. Unless otherwise stated, each experiment was repeated independently three times and expressed as mean values with 95% confidence intervals. All statistical calculations were performed using InStat software and GraphPad Prizm 4.0. Nonparametric analysis of variance followed by Bonferroni post hoc multiple comparison tests were used to test the statistical significance of difference between control and treated groups. Differences were considered significant at $P<0.05$.

4. RESULTS

4.1. Effect of γ-irradiation on the survival of BxPC-3 cells

Various doses of γ-irradiation have been used to reduce the growth of pancreatic tumors (13-15). However, we wanted to determine the optimum dose of γ-irradiation required to inhibit the growth of BxPC-3 cells. We exposed the cells to varying doses of γ-irradiation (0, 2.5, 5, 10 and 20 Gy) and after 24 or 48 hours evaluated the survival of the cells by SRB assay. Our results show that a γ-irradiation dose of 5 Gy significantly reduced the survival of BxPC-3 cells after 24 and 48 hours of treatment as compared to control cells (Figure 1A, B). Surprisingly, at higher doses of γ-irradiation, the survival of the cells was not affected (Figure 1A, B), suggesting the development of resistance to irradiation. Therefore, in our subsequent experiments we used 5Gy to γ-irradiate the cells.

4.2. Synergistic effect of BITC with γ-irradiation

Our next step was to determine whether pretreatment of BxPC-3 cells with BITC could sensitize the cells to the growth inhibitory effects of γ-irradiation and if yes, at what optimum dose of BITC. In our previous studies, we demonstrated that 10μM BITC significantly induced apoptosis in BxPC-3 cells (28). We therefore tried low doses of (2.5 and 5μM) BITC in our experiments to treat the cells for 24 hours followed by γ-irradiation at 5Gy. The survival of these cells was evaluated 24 or 48 hours later. As shown in Figure 2A and B, our results clearly show that cells that were treated with 2.5μM BITC for 24 hours and then treated with 5Gy γ-irradiation exhibited significantly lower survival as compared to cells which were treated with BITC or γ-irradiation alone. However the cells which were treated with 5μM BITC for 24 hours and then γ-irradiated showed massive cell death. In order to determine the mechanism of enhanced growth inhibitory effects of the combination treatment, we used 2.5μM BITC and 5Gy γ-irradiation in our subsequent experiments.

4.3. Cell cycle analysis of BxPC-3 cells treated with BITC and γ-irradiation

In our previous studies, we demonstrated that BITC strongly suppressess the growth of human pancreatic cancer cells by causing cell cycle arrest and apoptosis (25). To gain further insight into the mechanism of the growth inhibitory effects of BITC with γ-irradiation, we assessed the cell cycle distribution of BxPC-3 cells by flow cytometry (Table 1 and 2). A significantly increased number of cells were observed in G2/M phase in response to combination treatment as compared to γ-irradiation alone after 24 hours of treatment (Table 1). We did not observe G2/M cell cycle arrest after 48 hours of treatment, however about a 2.8 fold increased number of cells were observed in sub G0/G1 phase in the combination treatment as compared to γ-irradiation alone (Table 2).

Table 1. Cell cycle distribution of BxPC-3 cells treated with BITC, γ-irradiation or combination of both and analyzed after 24 hours of treatment

	G1	S	G2/M	Sub G0/G1
Control	56.0±2.82	8.5±0.70	23.5±0.70	2.4±1.90
γ-Irradiation	31.3±1.52	14.3±1.50	40.7±3.00	3.7±0.57
2.5μM BITC	50.0±1.41	12.5±0.70	26.5±2.10	5.5±0.70
2.5μM BITC + γ-Irradiation	22.7±0.57	10.0±1.00	51.0±1.00[1]	5.6±1.50

[1] Statistically significant when compared with γ-irradiation, P<0.05.

Table 2. Cell cycle distribution of BxPC-3 cells treated with BITC, γ-irradiation or combination of both and analyzed after 24 hours of treatment

	G1	S	G2/M	Sub G0/G1
Control	49.7±0.57	10.0±1.00	23.3±1.50	6.0±0.00
γ-Irradiation	47.3±2.00	7.6±1.15	24.0±1.00	7.6±0.57
2.5μM BITC	51.3±2.51	9.0±1.72	28.0±1.00	4.0±0.00
2.5μM BITC +γ-Irradiation	51.6±4.50	3.0±0.00	14.0±0.00[1]	21.3±0.57[1]

[1] Statiscally significant when compared with γ-irradiation, P<0.05

4.4. Modulation of cell cycle regulatory proteins in BxPC-3 cells treated with BITC and γ-irradiation

Since we observed significant G2/M cell cycle arrest after combination treatment, we next sought to determine whether these treatments caused DNA damage. We examined the phosphorylation of H2A.X at Ser-139, which is considered to be an important marker for the presence of DNA double strand breaks. We observed increased phosphorylation of H2.A.X in the cells treated with BITC and γ-irradiation combination as compared to BITC or γ-irradiation alone after 24 hours of treatment (Figure 3A), whereas after 48 hours, not much change was observed (data not shown).

The next logical step was to determine whether combination treatment mediated DNA damage activates check point kinase 2 (Chk2). Our results show an increased phosphorylation of Chk2 at Thr-68 in the cells treated with the combination as compared to individual treatments at the 24 hour time point (Figure 3A). The protein level of Chk2 remained unchanged during these treatments. However, after 48 hours there was no change in the expression or phosphorylation of Chk2 (Thr-68) (data not shown).

We further observed a modest increase in the phosphorylation of ATR at Ser-428 in the cells treated with the combination, indicating that the phosphorylation of Chk2 at Thr-68 is perhaps mediated by activated ATR. We did not observe any change in the levels of ATM at Ser-1981 (data not shown). Our results also show a subtle increase in the phosphorylation of Cdc25C at Ser-216 and Cdk-1 at Tyr-15 in the cells treated with the combination of BITC and γ-irradiation as compared to BITC or irradiation alone (Figure 3A), indicating that G2/M cell cycle arrest in these cells is mediated by the activation of Chk2 and other G2/M regulatory molecules. Several recent studies suggest that $p21^{Waf1/Cip1}$ regulates the entry of cells by activating G2/M checkpoints and apoptosis (29-32). Our western blot results demonstrated the induction of $p21^{Waf1/Cip1}$ in the cells treated with the combination as compared to individual treatment. Interestingly, none of the above-mentioned changes were observed in the cells after 48 hours of treatment with or without combination treatment (data not shown); the reason being that the cells after 48 hours of combination treatment shifted from G2/M to sub G0/G1 phase.

4.5. Induction of apoptosis by combination treatment

Twenty four hour treatment showed modest cleavage of PARP (Figure 3A). However, after 48 hours of combination treatment, we observed a substantial increase in the population of cells with sub-diploid DNA content as compared to γ-irradiation treatment alone (Figure 3B). We therefore further explored the mechanism behind the induction of apoptosis after 48 hours of combination treatment. Our results show that γ-irradiation alone activated EGFR by phosphorylation at Tyr-1068 (Figure 4A). However, BITC treatment together with the γ-irradiation blocked this activation (Figure 4A). We examined the involvement of MAPK signaling molecules such as ERK, JNK and P38 which are known to be regulated by EGFR. Although we did not observe any change in the phosphorylation of ERK or JNK, a significant increase in the phosphorylation of P38 at Thr-180/Tyr182 was observed in the cells 48 hours after treatment with BITC and γ-irradiation combination as compared to γ-irradiation alone (Figure 4A). Activation of P38 is suggestive of the induction of apoptosis (33). Accordingly, we observed increased cleavage of PARP in response to combination treatment (Figure 4A). Our results also show that γ-irradiation substantially induced the expression of NF-kB in BxPC-3 cells and this increase in NF-kB was attenuated by combination treatment (Figure 4A). The expression of cyclin D1, which is the downstream transcriptional target of NF-kB was also consistently decreased by combination treatment. In addition, our results show that the DNA binding activity of NF-kB was significantly decreased by the combination treatment in BxPC-3 cells (Figure 4B) suggesting the involvement of NF-kB pathway in the induction of apoptosis in BxPC-3 cells. However, further studies are required to dissect out the exact mechanism.

5. DISCUSSION

Although the prevalence of pancreatic carcinoma among all human malignancies diagnosed in USA is only 2%, it is one of the most difficult cancers to manage and remains the fourth leading cause of cancer related deaths (1). Due to its poor prognosis and late onset of diagnosis, only 10-15% of patients present with resectable disease (34). The remaining 85%-90% present with locally advanced unresectable or metastatic disease with a media

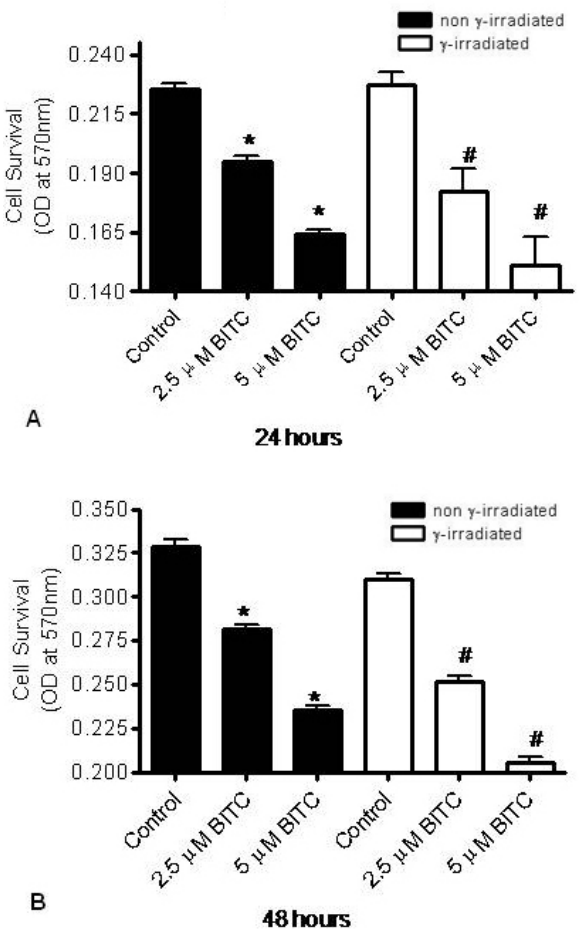

Figure 2. Effect of combination treatment on cell survival. A) Cells were treated with two different concentrations of BITC (2.5 and 5μM) alone for 24 hours or with BITC (2.5 and 5μM for 24 hours) and irradiation (5Gy) and survival of cells were evaluated after 24 hours by SRB assay. B) Cells were treated with BITC alone or with combination treatment and survival of cells was measured after 48 hours by SRB assay. Values are means ± SEM of 2 independent experiments (each conducted in triplicate). Data were analyzed by non-parametric ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. Differences between tested groups were analyzed and considered significant at $P<0.05$ from control. *denotes statistical significant difference between control and BITC alone treated groups, whereas # denotes differences between γ-irradiated control and combination treatment groups.

survival time of 4-11 months (35-36). Current treatment modalities to treat cancer include surgery, radiotherapy and chemotherapy. Radiation therapy works by damaging the DNA of the tumor cells thus stopping their growth. Radiation is not specifically targeted to cancer cells and therefore causes injury to normal cells surrounding the tumor (37). The adverse effects of radiotherapy are the major limiting factor for its successful outcome. The effectiveness of radiation therapy can be enhanced by combination with radiosensitizers. Recent studies have revealed that several anticancer agents such as 5-fluorouracil, gemcitabine and curcumin have potent radiosensitizing properties and could enhance the effect of radiation induced apoptosis against a variety of human malignancies (7-13, 38-39).

Therefore the mission to successfully treat human malignancies would be to find and evaluate the effectiveness of non-toxic agents with known mechanisms of action which could increase the efficacy of γ-irradiation at low doses and thus reduce overall systemic toxicity. BITC is a dietary agent abundantly present in many cruciferous vegetables and consumed by humans on a daily basis. Epidemiologic studies continue to support the notion that dietary intake of cruciferous vegetables may reduce the risk of different types of malignancies, including pancreatic cancer (17-20). BITC was observed to be relatively safe to normal pancreatic cells as demonstrated by us previously (25, 28). We have shown previously that BITC inhibits the growth of human pancreatic cancer cells by inducing DNA damage leading to G2/M cell cycle arrest and apoptosis (25). The aim of the present study was to determine whether BITC could sensitize pancreatic cancer cells to γ-irradiation therapy.

In this present study, we demonstrated that BITC sensitizes pancreatic cancer cells to γ-irradiation at low doses by arresting cells in the G2/M phase of the cell cycle. Higher doses of γ-irradiation failed to reduce the survival of cells in our model. The possible reason for this paradox could be that these cells became resistant at the higher doses as suggested in previous studies (40-41). It is well known that cells with severe DNA damage enter into apoptotic cell death (42). Our results show that a combination of BITC and γ-irradiation treatment resulted in increased phosphorylation of H2A.X at Ser-139 compared to γ-irradiation or BITC alone treatment, suggesting the presence of DNA double strand breaks. The cell cycle checkpoints are activated in response to DNA damage leading to cell cycle arrest in G2/M phase (30-31). Chk1 and Chk2 are two check point kinases and although being structurally different from each other share overlapping functions (42-43). We observed significant activation of Chk2 by phosphorylation at Thr-68 in the cells treated with a combination of BITC and γ-irradiation as compared to individual treatment. This is in line with the previously published study where sulforaphane induced G2/M arrest was mediated through the activation of Chk2 and Cdc25C in PC3 prostate cancer cells (44). DNA damage is predominantly associated with the activation of ATM whereas ATR is activated by stalling of replication fork induced by UV, nucleotide imbalance and DNA cross linking (45). Our data show modest activation of ATR at Ser-428 in the combination treatment. These results are in agreement with previous a study where cisplatin treatment caused DNA damage induced apoptosis through the activation of ATR-Chk2 in immortalized rat kidney proximal tubular cells (46). A Chk2 act as a signal distributor and upon activation disperses checkpoint signals to downstream targets and phosphorylates Cdc25C at Ser-216 which in turn activates Cdk1 by phosphorylation at Thr-15 leading to G2/M arrest (45, 47). We further

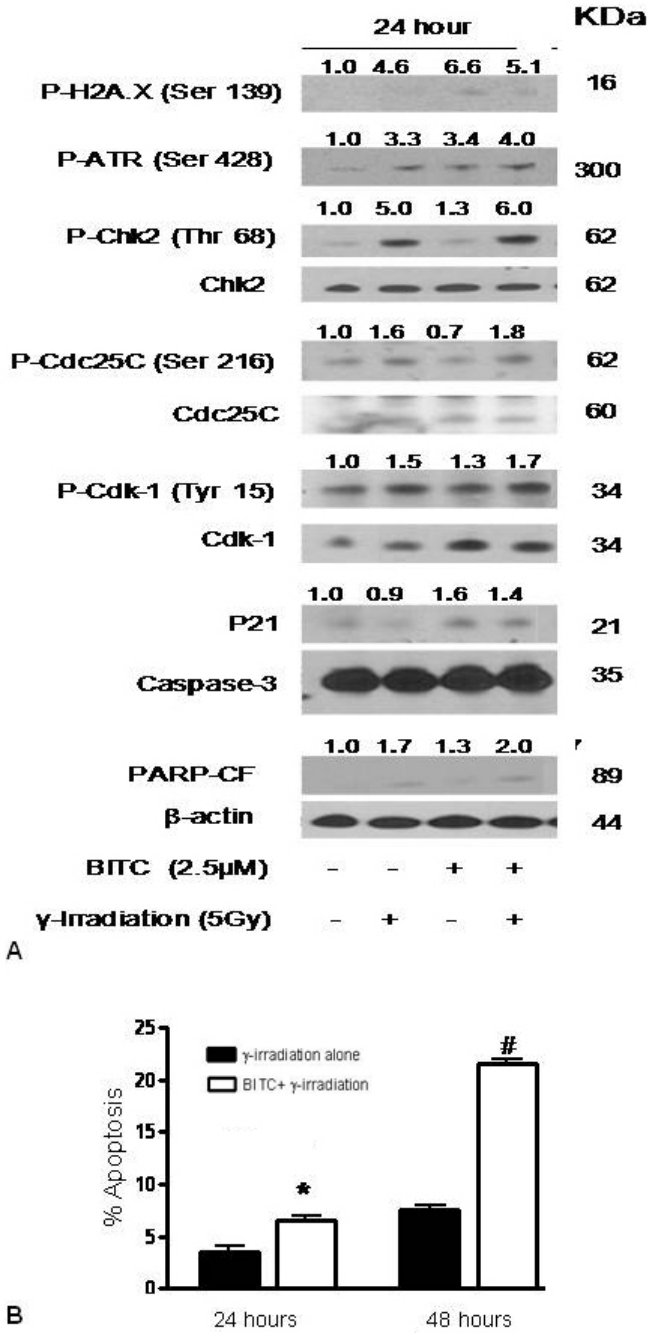

Figure 3. Effect of BITC, γ-irradiation or combination treatment on cell cycle regulatory proteins. A) BxPC-3 cells were treated with 2.5μM BITC for 24 hours followed by irradiation at 5Gy or treated with BITC and γ-irradiation alone. The cells were collected 24 hours later and lysed as described in the method section. Representative immunoblots show the effect of these treatments on the phosphorylation of H2A.X (Ser 139), ATR (Ser 428), Chk2 (Thr 68), Cdc25C (ser 216), Cdk1 (Tyr 15) and protein expressions of Chk2, Cdc25C, Cdk1, $P21^{Waf1/Cip1}$, caspase-3 and cleaved fragment of PARP. Each blot was stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Intensities of immunoreactive bands were quantified by densitometric scanning. B) The effects of γ-irradiation alone or combination (BITC and γ-irradiation) on apoptosis was measured after 24 or 48 hour treatment by quantitating cells in sub G_0/G_1 phase by flow cytometry. Values are means ± SEM of 2 independent experiments (each conducted in triplicate). Data were analyzed by non-parametric ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. Differences between tested groups were analyzed and considered significant at $P<0.05$ from control.

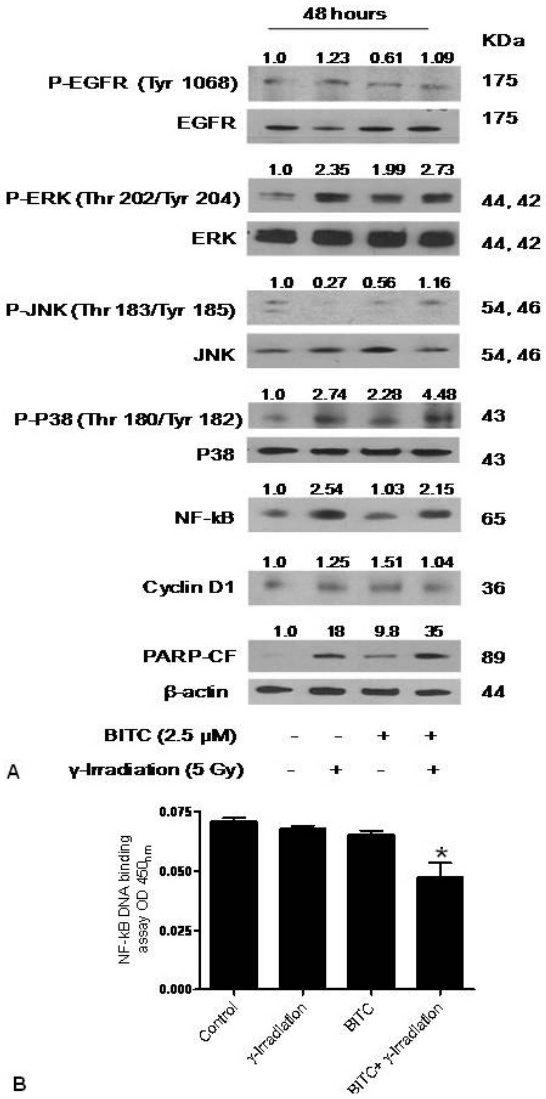

Figure 4. Effect of BITC, γ-irradiation or combination treatment on cell survival signaling proteins. A) Cells were treated with BITC, γ-irradiation or with the combination and lysates were prepared as described above. Representative immunoblots show the effects of these treatments on the phosphorylation of EGFR (Tyr 1068), ERK (Thr 202/Tyr 204), JNK (Thr 183/Tyr 185), P38 (Thr 180/Tyr 182) and protein expressions of EGFR, ERK, JNK, P38, NF-kB, cyclin D1 and cleavage of PARP. Each blot was stripped and reprobed with anti-β-actin antibody to ensure equal protein loading. Intensities of immunoreactive bands were quantified by densitometric scanning. B) Cells were treated as described above followed by measurement of NF-kB DNA binding activity using a commercially available kit as described in the methods. Values are means ± SEM of 2 independent experiments (each conducted in triplicate). Data were analyzed by non-parametric ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. Differences between tested groups were analyzed and considered significant at $P<0.05$ from control. *denotes statistical significant difference between γ-irradiated and combination treatment groups.

observed increased phosphorylation of Cdc25C at Ser-216 and Cdk1 at Tyr-15 in the combination treatment at the 24 hour time point. Recent reports suggest that $p21^{Waf1/Cip1}$ negatively regulates the entry of cells in G2/M phase and induces apoptosis (30, 32). Our data also demonstrate an increase in the expression of p21Waf1/Cip1 in response to combination treatment indicating its role in G2/M arrest as suggested by other reports (29-32). However no change in the activation of these G2/M cell cycle regulatory proteins was observed after 48 hours of treatment. During DNA damage, signals generated by different genotoxic stress block key cell cycle transitions until DNA is repaired (30, 47), or lead to apoptotic cell death in case of severe DNA damage (45). We observed a modest cleavage of PARP at 24 hours; however, an increase in the cleavage of PARP was demonstrated in the combination treatment after 48 hours as compared with individual treatment, indicating that at later time points apoptosis is the main mechanism for cell death. Studies have shown the involvement of multiple intracellular signaling pathways including EGFR/ MAPK and NF-kB in radiation induced apoptosis (48-50). Our results show that γ-irradiation alone substantially activates EGFR, NF-kB and its downstream target cyclin D1, which was attenuated by combination treatment. Ultraviolet irradiation has been shown to activate AP-1 and NF-kB, which is inhibited by the chemopreventive agent aspirin (49). We further observed significant activation of P38 by combination treatment compared to individual treatment after 48 hours. Our results are in agreement with previous studies where irradiation induced apoptosis was mediated through the modulation of EGFR, P38 and NF-kB pathways (48-50).

Taken together, the results of our study reveal that the combination of BITC with γ-irradiation at low doses induces apoptosis in human pancreatic cancer cells by causing cell cycle arrest and inhibition of the EGFR/NF-kB pathway. Thus the systemic toxicity caused by γ-irradiation can be decreased in the clinical setting without compromising its therapeutic effects.

6. ACKNOWLEDGEMENT

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Abbreviation: BITC; Benzyl isothiocyanate

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