Pancreatic Tumor Suppression by Benzyl Isothiocyanate Is Associated with Inhibition of PI3K/AKT/FOXO Pathway

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Abstract

Purpose: Our previous studies have shown that benzyl isothiocyanate (BITC) suppress pancreatic cancer growth by inducing apoptosis but the molecular mechanism was unclear. In this study we hypothesized the involvement of PI3K/AKT/FOXO pathway in BITC-induced apoptosis.

Experimental Design: Mice were implanted BxPC-3 tumor xenografts and orally gavaged with 12 µmol BITC. Plasma and tumor BITC concentration was estimated by liquid chromatography/tandem mass spectrometry. BxPC-3 and Panc-1 cells were used to elucidate PI3K/AKT/FOXO pathway. Electrophoretic mobility shift assay (EMSA), DNA binding activity, immunofluorescence, and gene transfection were used to delineate the mechanism.

Results: BITC-treated mice showed 43% less tumor growth as compared with control mice and correlated well with the therapeutic concentrations of 6.5 µmol/L BITC achieved in plasma and 7.5 µmol/g BITC in tumor tissue. Western blot analyses and immunohistochemistry revealed that tumors from BITC-treated mice showed reduced phosphorylation of PI3K, AKT, PDK1, mTOR, FOXO1, and FOXO3a and increased apoptosis. Complementing our in vivo results, we made similar observations in a dose- and time-dependent manner in BITC-treated BxPC-3 and Panc-1 cells. Binding of FOXO1 with 14-3-3 proteins was also reduced drastically by BITC treatment indicating nuclear retention of FOXO1 and this observation was further confirmed with EMSA, immunofluorescence, DNA binding, and upregulation of FOXO-responsive proteins Bim, p27, and p21 in BxPC-3 cells. Overexpression of AKT by transient transfection significantly blocked the modulation of FOXO proteins and protected the cells from BITC-mediated apoptosis and growth suppression.

Conclusions: Our results provide convincing evidence on the involvement of PI3K/AKT/FOXO pathway in BITC-mediated pancreatic tumor growth suppression.

Introduction

The American Cancer Society reported that 42,470 Americans were diagnosed with pancreatic cancer in the year 2009, of which 35,420 died, making pancreatic cancer the fourth leading cause of cancer-related deaths (1). The high mortality rate is due to resistance to chemotherapy and radiotherapy, and the poor prognosis of the disease, and 40% to 50% patients rapidly progress to metastatic disease (2).

Phosphoinositide 3-kinase (PI3K)/AKT is a potent survival pathway that may mediate resistance to the apoptotic effects of chemotherapy drugs and radiation therapy in a variety of cancer types including pancreatic cancer (3). Although, majority of pancreatic cancer cell lines examined to date harbor constitutively activated AKT, the mechanism behind AKT activation in pancreatic cancer remains elusive (4). A recent study has shown that 59% of pancreatic adenocarcinomas showed hyperactivation of AKT (5). The mammalian FOXO family of transcription factors, consisting of FOXO1, FOXO3a, and FOXO4, which function downstream of PI3K signaling pathway, are important regulators of cell death and they promote cell survival and resistance (6). In fact, a number of anticancer drugs, such as doxorubicin and paclitaxel have been shown to induce apoptosis through oxidative stress, which enhances FOXO3a activity by stimulating its nuclear translocation (7) and thus causing overexpression of FOXO-responsive genes such as Bim, p27, and p21 (8). Regulation of FOXO factors by AKT pathway is receiving increasing attention in cancer research.

Currently, there is no effective treatment for pancreatic cancer because conventional chemotherapy and radiation treatment have shown very limited success in improving patient survival. Therefore, novel treatment strategies are urgently needed. Case-controlled epidemiologic studies
Translational Relevance

Despite much advancement in chemo- and radio-therapy, pancreatic adenocarcinoma remains a fatal disease, highly resistant to all treatment modalities. PI3K/AKT pathway is a potent survival signal that mediates resistance to the apoptotic effects of chemo drugs and radiation therapy. Recent studies have shown that approximately 59% of the pancreatic adenocarcinomas have hyperactivation of AKT. Many chemo-drugs are known to acquire resistance by activating AKT and/or FOXO proteins. In this study, we show that benzyl isothiocyanate inhibits the nuclear shuttling of FOXO proteins by suppressing the phosphorylation of AKT and subsequent FOXO proteins. These observations were further confirmed in an in vivo xenograft model. Thus, benzyl isothiocyanate could be used along with traditional chemotherapy, which causes activation of AKT or FOXO proteins.

continue to support the notion that consumption of cruciferous vegetables reduces the risk of pancreatic cancer (9–11). Benzyl isothiocyanate (BITC) is present in cruciferous vegetables such as garden cress (12–14). In our previous studies, we showed that BITC suppresses the proliferation of human pancreatic cancer cells by causing DNA damage resulting in G2/M cell cycle arrest and apoptosis (15). Our results also revealed that BITC was not toxic to normal pancreatic epithelial cells (16).

In this study, our results show that orally feeding BITC significantly suppress the growth of BxPC-3 pancreatic tumor xenograft. The tumor growth inhibition by BITC was associated with inhibition of activation and expression levels of PI3K/AKT/FOXO proteins as analyzed by immunohistochemistry and Western blotting. We further confirmed our in vivo observations in BxPC-3 and Panc-1 cells.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines BxPC-3 and Panc-1 were procured from ATCC and normal human pancreatic duct epithelial cell line HPDE-6 was a generous gift from Dr. Ming-Sound Tsao (University of Toronto, Toronto, Canada). All cell lines were maintained as we have described previously (16).

Tumor therapy model

Tumor therapy experiment was performed as we have described previously (16) with minor modifications. The use of athymic nude mice and their treatment was approved by the Institutional Animal Care and Use Committee, Texas Tech University Health Sciences Center, and all the experiments were carried out in strict compliance with their regulations. Exponentially growing BxPC-3 (1 x 10^6) cells were injected subcutaneously into the left and right flanks of 10 mice. When the tumors reached a size of approximately 70 mm^3, mice were randomly segregated into 2 groups: test group of mice received 12 μmol BITC in PBS by oral gavage every day for 46 days, whereas control mice received vehicle alone. Tumor volume and animal weights were taken as we have described previously (16).

Western blot analysis

BxPC-3, Panc-1, and HPDE-6 cells were treated with varying concentrations of BITC (0, 5, 10, and 20 μmol/L) for 24 hours. For time-dependent experiment, cells were treated with 10 μmol/L BITC for 0, 2, 4, 8, 16, and 24 hours. The nuclear portion from control and treated cells or tumors was isolated by using a commercially available kit from Pierce, according to the manufacturer’s instruction. Protein (40 μg) was subjected to SDS-PAGE and Western blot was carried out as we have described previously (17).

Immunohistochemistry

Tumors excised from control and BITC-treated animals were fixed in formalin and 4-μm sections were used for immunohistochemistry of p-PI3K (Tyr458), p-AKT (Ser-473), and p-FOXO3a (Ser-253) as we have described previously (16).

Estimation of BITC in plasma and tumors

BITC was analyzed by liquid chromatography/tandem mass spectrometry (LC/MS–MS) as described previously (18). The LC/MS–MS system consisted of a 1200 LC/MS (Quad 42) mass spectrometer (Varian) equipped with a heated nebulizer interface, a Varian Prostar Model 210 pump, and an autosampler. HPLC separation was carried out on a C18 (particle size 5 μm; 150 x 2.0 mm) column (Varian) and the mobile phase consisted of acetonitrile/5 mmol/L formic acid (30:70, v/v). The flow rate was 0.2 mL/min and the injection volume was 10 μL. The mass spectrometer operated in a positive ionization mode, and the mass spectrometer setting was optimized for benzylthiourea and phenylthiourea (internal standard; IS) to give optimum ion yield. Multiple reaction monitoring of MS/MS was used for specific detection of the derivatives of phenethyl isothiocyanate (PEITC) as an IS and BITC by measuring the characteristic ion transitions for PEITC m/z 181 (parent ion) to m/z 105 (product ion) and BITC 167.0 (parent ion) to m/z 91 (product ion), respectively. BITC standards (0.001–50 μmol/L) were prepared in acetonitrile and 50 μL of each concentration were added to the plasma sample such that the final concentration of BITC in plasma ranged from 0.00008 to 4.13 μmol/L. Samples were extracted with n-hexane twice and ammonia (2 mol/L in 2-Methanol) was added for derivatization. The mixture was incubated for 6 hours at room temperature and dried under N2 stream and reconstituted with 200 μL of acetonitrile/H2O (60:40, v/v). The reconstituted sample was transferred into a 100-μL autosampler insert for analysis by LC/MS–MS.

AKT kinase assay

BxPC-3 cells were treated with various concentrations of BITC and cells were collected. Cell lysates were analyzed for
AKT kinase activity by using a kit (Assay Designs) according to the manufacturer’s instructions.

**Immunoprecipitation**

Immunoprecipitation was performed as we have described previously (19). Briefly, 1 x 10^6 BxPC-3 cells were plated in 100-mm dish and treated with different concentrations of BITC for 24 hours. Whole cell lysates were prepared by using radioimmunoprecipitation assay buffer and immunoprecipitated with FOXO1 antibody. Immune complexes were resolved on SDS-PAGE and immunoblotted for 14-3-3 binding motif. Same blot was stripped and reprobed for acetylated lysine.

**FOXO1 DNA binding assay**

BxPC-3 cells were treated with different concentrations of BITC for 24 hours and nuclear fraction was isolated. Equal protein (15 μg) from control and BITC-treated cells were subjected to DNA binding assay by using a kit (Universal EZ-TFA Kit; Millipore) according to the manufacturer’s instructions.

**Immunofluorescence**

BxPC-3 cells were treated with 10 μmol/L BITC for 24 hours. Treated and untreated cells were immunostained with anti-FOXO1 antibody as described previously (20).

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**Figure 1.** BITC inhibits the growth of in vivo xenograft by inhibiting the PI3K/AKT/FOXO pathway. Athymic nude mice were kept on antioxidant-free diet for 1 week before starting the experiment. Exponentially growing 1 x 10^6 BxPC-3 cells were injected into both right and left flanks of each animal in PBS/Matrigel suspension. When tumors reached 70 mm^3 in size, mice were randomly divided into 2 groups with 10 mice in each group. Treated group received 12 μmol BITC by oral gavage everyday whereas control group received vehicle alone. Tumors were measured 3 times a week. Effect of BITC on tumor volume (A), tumor weight (B), and mice body weight (C) was evaluated. D, the mechanism of tumor growth inhibition by BITC was determined in the tumor lysates from control and BITC-treated mice. Tumors were homogenized, lysed, and 40 μg protein was resolved on SDS-PAGE and probed for p-PI3K (Tyr-458), PI3K, p-AKT (Ser-473), AKT, p-mTOR (Ser-2448), mTOR, p-FOXO1 (Ser-256), FOXO1, p-FOXO3a (Ser-253), FOXO3a, Bim, PCNA, cleaved (Cl.) Caspase-3, and cleaved PARP. The blots were stripped and reprobed for actin to ensure equal protein loading. Bottom, left, the quantitation of tumor Western blots. A and B, values are mean ± SEM of 20 samples; C and D, values are mean ± SD of 10 samples.* P < 0.05 statistically significant when compared with control.
Electrophoretic mobility shift assay

Cells treated with 10 µmol/L BITC for 24 hours were collected and nuclear fraction was isolated. Nuclear FOXO1 protein was captured by using biotin labeled FOXO1 binding site oligomer 5’-CAAAACAACAAAACAACAAAACAA-3’ and subjected to DNA binding activity by using the commercially available kit from Panomics.

AKT transient transfection

BxPC-3 cells were transiently transfected with plasmid containing wild-type AKT (a generous gift from Dr. Daniel Altschuler, University of Pittsburgh, Pittsburgh, Pennsylvania) by using lipofectamin LTX (Invitrogen). Briefly, 3 × 10^5 cells were transfected with 0.5 µg of the AKT plasmid diluted in Opti-MEM serum-free medium to which lipofectamine reagent was added before the mixture was added to cells. Cells were incubated with plasmid-lipofectamine mixture for 5 hours and then media was replaced with fresh growth medium and incubated for another 24 hours. Transfected cells were treated with 10 µmol/L BITC for 24 hours and analyzed for cell toxicity or apoptosis. Whole cell lysates (40 µg) were subjected to Western blot analysis and actin was used as loading control.

Annexin V–FITC apoptosis assay

Cells were treated with various concentrations of BITC for 24 hours and apoptosis was evaluated by using Annexin V–FITC kit (Calbiochem) by flow-cytometer (Accuri C6) according to the manufacturer’s instructions.

Statistical analysis

All the statistical analyses were performed by Prism 5.0 (GraphPad Software Inc.). Results were expressed as mean ± SD or SEM of at least 3 independent experiments. Data were analyzed by Student’s t test or 1-way ANOVA followed by Bonferroni’s post hoc analysis for multiple comparisons. Differences were considered statistically significant at P < 0.05.

Results

BITC suppress tumor growth in nude mice

To test the possibility that BITC treatment would suppress pancreatic tumor growth, BxPC-3 tumor-bearing mice were fed 12 µmol BITC every day and tumor growth was periodically recorded. Our results show that oral gavage of 12 µmol BITC significantly reduced the growth of the tumors starting day 10 of treatment and continued till the end of the experiment (Fig. 1A). At day 46 of the treatment, tumor volume in the treated group was reduced by 43% as compared with control groups (465.8 ± 30.8 mm^3 versus 266.7 ± 35.4 mm^3; n = 20; Fig. 1A). Similarly, weight of the tumors dissected from treated mice was approximately 45% less than the weight of the tumors from control mice (Fig. 1B). The weight of the mice did not change significantly, indicating no apparent systemic toxicity in BITC-treated mice (Fig. 1C).

Tumor growth inhibition was associated with inhibition of PI3K/AKT/FOXO pathway

PI3K/AKT is constitutively activated in majority of pancreatic tumors (5). We hypothesized that the pancreatic tumor growth inhibition by BITC in our model was due to inhibition of PI3K/AKT pathway. To test our hypothesis, constitutive levels of PI3K and AKT were examined in the tumor lysates by Western blotting. As shown in Figure 1D, phosphorylation of PI3K at Tyr-458 and AKT at Ser-473 was drastically suppressed by BITC treatment. The expression level of AKT, but not PI3K, was also reduced in the tumors of BITC-treated mice (Fig. 1D). Next, we investigated the downstream molecules of AKT pathway such as mTOR, FOXO1, and FOXO3a, which are known to play critical role in tumorigenesis. Our results show that phosphorylated and protein levels of mTOR and phosphorylated levels, but not protein levels, of FOXO1 and FOXO3a were decreased in the tumors of BITC-treated mice (Fig. 1D). On the contrary, the levels of FOXO-regulated proapoptotic protein Bim were substantially increased in the tumors of BITC-treated mice as compared with control animals (Fig. 1D) indicating that PI3K/AKT/FOXO pathway plays a critical role in
BITC-mediated pancreatic tumor suppression. In addition, we observed cleaved products of caspase-3 and PARP in the tumors from BITC-treated mice indicating apoptosis (Fig. 1D). PCNA levels were decreased in the tumors from BITC-treated mice indicating reduced mitosis (Fig. 1D). To further confirm the mechanism by which BITC feeding reduced tumor growth, tumors from control and BITC-treated mice were evaluated by immunohistochemistry (Supplementary Fig. 1). As analyzed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay, substantially increased numbers of apoptotic bodies were observed in the tumor sections.
BxPC-3, 24 h treatment

A

p-mTOR (Ser-2448)
mTOR
IKK α
p-FOXO1 (Ser-256)
FOXO1
p-FOXO3a (Ser-253)
FOXO3a
Actin
0 5 10 20 BITC μmol/L

Quantification of BxPC-3 Western blots

Cytosolic fraction

FOXO1
FOXO3a
Actin
0 5 10 20 BITC μmol/L

Nuclear fraction

FOXO1
FOXO3a
Lamin B
0 5 10 20 BITC μmol/L

14-3-3 binding motif
IP: FOXO1
IB: 14-3-3 binding motif

BITC (10 μmol/L, 24 h) - +

PanC-1, 24 h treatment

B

p-mTOR (Ser-2448)
mTOR
IKK α
p-FOXO1 (Ser-256)
FOXO1
p-FOXO3a (Ser-253)
FOXO3a
Actin
0 5 10 20 BITC μmol/L

Quatntiation of PanC-1 Western blots

D

BxPC, FOXO1 Immunofluorescence

BITC (10 μmol/L, 24 h) - +

EMSA

FOXO1

0.0 0.1 0.2 0.3 0.4
FOXO1 nuclear DNA binding activity (OD 450 nm)

P < 0.05
P < 0.05

10 μmol/L BITC - +
obtained from BITC-treated mice as compared with control mice, whereas reduced staining for PCNA was noticed in the similar sections, suggesting that the suppression of tumor growth in BITC-treated mice was due to reduced mitosis and increased apoptosis. Furthermore, as compared with controls, BITC treatment substantially reduced the staining of p-PI3K (Tyr-458), p-AKT (Ser-473), and p-FOXO3a (Ser-253) in the tumor sections (Supplementary Fig. 1). To determine the nuclear localization, FOXO proteins were determined in the nuclear and cytosolic fractions of the tumors from control and BITC-treated mice. As shown in Figure 2A, in control tumors, FOXO1 expression was reduced in the nucleus as compared with cytosol, whereas in the tumors from BITC-treated mice, FOXO1 was retained in the nucleus. Similar observations were made for FOXO3a (Fig. 2A). These observations further confirm that BITC suppress tumor growth by inducing apoptosis which is associated with the inhibition of PI3K/AKT/FOXO pathway.

**BITC concentration in plasma and tumor**

To see whether therapeutic concentration of BITC in the plasma and tumor was achieved by oral gavage, we evaluated BITC in the plasma and tumors of BITC-fed mice by LC/MS–MS. The mean BITC concentration in plasma after 1 hour of BITC (12 μmol) oral gavage was 6.5 ± 0.1 μmol/L (n = 10), whereas accumulated BITC concentration in the tumors after 46 days was 7.5 ± 0.3 μmol/g (n = 10; Fig. 2B). These results indicate that the therapeutic concentration of BITC could be achieved in vivo by oral feeding and that the tumor growth suppression correlated with the concentration of BITC in plasma and tumor. It is important to mention that we only evaluated BITC and not the metabolites of BITC. The detailed study of the bioavailability and pharmacokinetics of BITC in mice is currently underway in our laboratory.

**BITC downregulates PI3K/AKT pathway in BxPC-3 and PanC-1 but not in HPDE-6 cells**

To model and elucidate the molecular observations made in vivo, we treated BxPC-3 and PanC-1 cells with varying concentrations of BITC for 24 hours. As shown in Figure 3A, BITC treatment significantly suppressed the phosphorylation of PI3K (Tyr-458) in BxPC-3 cells in a concentration-dependent manner. In a time-dependent study, 10 μmol/L BITC suppressed the phosphorylation of PI3K as early as 4 hours after treatment and continued till 24 hours (Fig. 3A). The protein levels of PI3K remained unchanged even after 24 hours of treatment, indicating that BITC specifically targets the activation of PI3K. Furthermore, BITC treatment significantly reduced the protein levels and phosphorylation of AKT at Ser-473 and Ser-308 in both BxPC-3 and PanC-1 cells (Fig. 3A and B). We then investigated the effect of BITC on PDK1, the upstream regulator of AKT. Our results indicate that BITC treatment significantly suppressed the phosphorylation of PDK1 at Ser-241 (Fig. 3B). Interestingly, phosphorylation of PI3K or AKT was not detected in normal human pancreatic ductal epithelial (HPDE-6) cells (Fig. 3C), which is consistent with the general understanding that PI3K or AKT are activated in transformed cells (21). Moreover, BITC treatment did not alter the protein levels of PI3K or AKT in HPDE-6 cells indicating the specificity of BITC toward cancer cells.

**AKT kinase activity**

Because we observed a decrease in AKT phosphorylation and expression by BITC treatment, we wanted to see whether this corresponds to a decrease in the functionality of AKT, by measuring the kinase activity. As shown in Figure 3D, a significant reduction in the kinase activity of AKT was observed in BxPC-3 cells. For example, treatment of cells with 10 to 20 μmol/L BITC for 24 hours resulted in the inhibition of approximately 45% to 75% of AKT kinase activity as compared with control cells.

**BITC regulates downstream molecules of AKT**

AKT regulates various cellular processes by directly acting on the downstream molecules such as mTOR, FOXO, and IKK. First, we examined the effect of BITC on mTOR and IKK-α. Our results reveal that BITC treatment substantially suppressed the protein levels and phosphorylation of mTOR (Ser-2448) in both BxPC-3 and PanC-1 cells (Fig. 4A and B). The protein level of IKK-α was also reduced by BITC treatment in both the cell lines (Fig. 4A and B). Next, we evaluated the effect of BITC on FOXO transcriptional factors, which are downstream to AKT. Our results show that BITC treatment for 24 hours significantly reduced the phosphorylation of FOXO1 (Ser-256) and FOXO3a (Ser-253) in BxPC-3 and PanC-1 cells (Fig. 4A and B). The reduced phosphorylated levels of FOXOs were not due to decreased protein levels because protein levels of FOXO1 and FOXO3a did not change by BITC treatment (Fig. 4A and B).

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**Figure 4.** BITC downregulates cytosolic mTOR, IKK-α, FOXO and sequester FOXO1, and FOXO3a in nucleus. The effect of BITC on mTOR, IKK-α, FOXO1, and FOXO3a was determined in BxPC-3 (A) and PanC-1 (B) cells. Cells were treated with different concentrations of BITC for 24 hours, lysed and 40 μg cell lysate was resolved by SDS-PAGE. Membranes were stripped and reprobed for β-actin to ensure equal protein loading. The experiment was repeated 3 times with similar observations. Bar diagram shows the quantitation of respective Western blots. C, effect of BITC on nuclear retention of FOXO. To track the subcellular localization of FOXO proteins, nuclear proteins, and cytosolic proteins from control and BITC-treated BxPC-3 cells was probed for FOXO1 and FOXO3a. Same membrane was stripped and reprobed for actin or lamin B to ensure equal protein loading. In another experiment, whole cell lysate from control and BITC-treated BxPC-3 cells was incubated with FOXO1 antibody overnight, resolved on SDS-PAGE and immunoblotted for 14-3-3 binding motif. To further confirm nuclear retention of FOXO1, BxPC-3 cells were immunostained with FOXO1 (red) and actin (green) antibodies and visualized under microscope (Olympus Inc.). D, FOXO1 DNA binding activity was evaluated by EMSA and Universal DNA binding assay. Values are mean ± SD of 3 individual experiments.
BITC regulates nuclear shuttling of FOXO proteins

Once phosphorylated by AKT, FOXO1 binds to 14-3-3 chaperone proteins, which serve as escort proteins for FOXO1 to move out of the nucleus (22) leading to transcriptional downregulation of Bim, p21, and p27 (8). Hence, we determined the interaction of FOXO proteins with 14-3-3 chaperones. As expected, BITC treatment drastically decreased 14-3-3 binding sites on FOXO1 proteins indicating its retention in the nucleus (Fig. 4C). To confirm nuclear localization of FOXO proteins, we immunoblotted nuclear and cytosolic proteins and our results show that both FOXO1 and FOXO3a protein levels steadily increased in nuclear fraction and decreased in cytosolic fraction of BITC-treated BxPC-3 cells (Fig. 4C). Further, our immunofluorescence studies show intensified staining of FOXO1 in the nucleus of BITC-treated cells as compared with control cells, confirming the nuclear accumulation of FOXO1 proteins (Fig. 4C). Next, we sought to determine the DNA binding activity of FOXO1 by electrophoretic mobility shift assay (EMSA) to see whether the retention of FOXO in the nucleus causes increased DNA binding resulting in the transcription of responsive genes Bim and p27. Indeed, EMSA results shows that BITC treatment significantly increased the DNA binding ability of FOXO1 protein (Fig. 4D). The DNA binding activity of FOXO1 was further confirmed by Universal-DNA Binding Assay. Our results shows that BITC treatment increased the DNA binding activity of FOXO1 in the nuclear fraction of the cells in a concentration-dependent manner (Fig. 4D).

Nuclear translocation of FOXO proteins are expected to upregulate the transcription of FOXO-responsive genes such as Bim, p21, and p27 (23). Our results clearly show

Figure 5. BITC induces the expression of Bim and p27. Effect of BITC on Bim and p27 was evaluated in BxPC-3 (A) or PanC-1 (B) cells. Cells were treated with BITC for 24 hours and 40 μg protein was resolved by SDS-PAGE. The membranes were probed for Bim, p27, and p21. Same membrane was stripped and reprobed for β-actin to ensure equal protein loading. The experiment was repeated and similar results were obtained. Bar diagrams shows the quantitation of respective Western blots. C, deacetylation of lysine by BITC was determined in BxPC-3 cells. FOXO1 was immunoprecipitated from control and BITC-treated cells and immunoblotted against acetylated lysine. D, BITC-treated cells were examined for the expression of CBP/p300, SirT1, SirT2, SirT3, and SirT6. The experiments were repeated 3 times and similar results were obtained.
that BITC treatment substantially enhanced the expression of Bim and p27 in BxPC-3 and PanC-1 cells (Fig. 5A and B), indicating that BITC induces nuclear localization of FOXO transcription factor.

**BITC regulates FOXO transcriptional activity by acetylation**

Another level of regulation of FOXO transcription is by acetylation or deacetylation of critical lysine residues on FOXO by CBP (CREB binding protein) and SirTs, respectively (24). Hence, we evaluated the acetylated levels of lysine. FOXO1 proteins from control and BITC-treated BxPC-3 cells were immunoprecipitated with FOXO1 antibody and immunoblotted for acetylated lysine. Our results show that acetylated lysine levels on FOXO1 were significantly decreased by BITC treatment (Fig. 5C). We also observed that CBP/p300 protein levels were decreased by BITC treatment (Fig. 5D). Interestingly, none of the SirT levels were affected by BITC treatment (Fig. 5D).

**AKT overexpression abrogates BITC-mediated nuclear localization of FOXO and apoptosis**

To confirm the role of AKT signaling in BITC-mediated tumor growth suppression, BxPC-3 cells were transfected with 0.5 μg of AKT plasmid for 24 hours by using Lipofectamine-LTX and treated with different concentrations of BITC for another 24 hours and analyzed by cell survival assay. Results were confirmed by 3 independent experiments. B, control and BITC-treated AKT-transfected cells were analyzed for p-AKT (Ser-473), AKT, p-FOXO1 (Ser-256), FOXO1, p-FOXO3a (Ser-253), FOXO3a, Bim, cleaved (Cl.) caspase-3, and cleaved PARP. Same blots were stripped and reprobed for β-actin. C, cells from above-mentioned treatment were also analyzed for apoptosis by using Annexin V by Accuri flow-cytometer. Each experiment was performed in triplicate. Results are expressed as mean ± SD. D, effect of BITC with LY-294002. BxPC-3 cells were treated with LY-294002 and then with BITC (10 μmol/L, 24 hours). Cell lysates were analyzed by Western blot.
with AKT plasmid and the effect of BITC on FOXO/Bim pathway was evaluated. We were able to achieve approximately 4.8-fold enhanced expression of AKT over constitutive level in nontransfected cells. Our results show that AKT overexpression significantly increased the viability of BITC-treated BxPC-3 cells as compared with BITC-treated nontransfected cells (Fig. 6A). The percent survival of wild-type BxPC-3 cells by 10 μmol/L BITC treatment was 53.2 ± 2.6% whereas in AKT-transfected BxPC-3 cells survival was 85.6 ± 8.6% indicating a 32.4% survival advantage (Fig. 6A). We next examined the effect of BITC on FOXO and Bim levels in AKT-transfected cells. Our results show that the decline in FOXO1 and FOXO3a phosphorylation by BITC treatment was blocked by AKT overexpression (Fig. 6B). We also observed that BITC-induced Bim protein expression was significantly reduced in cells overexpressing AKT (Fig. 6B). We then determined apoptosis in control and AKT-transfected BxPC-3 cells after BITC treatment. Western blot analysis shows significantly reduced cleavage of caspase-3 and PARP in AKT-transfected BxPC-3 cells as compared with BITC-treated nontransfected cells (Fig. 6B). We confirmed apoptosis by Annexin V–FITC (fluorescein isothiocyanate) by flow cytometry. Our results show that 10 μmol/L BITC induced approximately 57.4% apoptosis whereas, in AKT-transfected cells, apoptosis was reduced to 25% indicating 32.4% decrease in apoptosis (Fig. 6C). To further confirm AKT as a target of BITC, we pretreated BxPC-3 cells with LY-294002 (10 μmol/L, 1 hour) followed by BITC (10 μmol/L, 24 hours). Our results show that LY-294002, which is a known inhibitor of PI3K, together with BITC substantially decreased the phosphorylation of AKT and FOXO and increased apoptosis as compared with BITC alone treated BxPC-3 cells (Fig. 6D). Taken together, these results establish the critical role of AKT inhibition in BITC-induced apoptosis through FOXO-Bim in our model.

Discussion

We have shown previously that BITC suppress the growth of pancreatic cancer by inducing apoptosis; however, the exact mechanism of BITC-mediated induction of apoptosis was not clear. Our current results show that orally feeding 12 μmol BITC to athymic nude mice significantly suppressed the growth of pancreatic tumor xenograft. Interestingly, 6.4 μmol/L BITC was observed in plasma after 24 hours of oral gavage of 12 μmol BITC and a cumulative concentration of 7.5 μmol BITC/g tumor tissue was observed indicating that the therapeutic concentration of BITC can be achieved in vivo and is directly linked to the tumor growth suppression in this study. Furthermore, tumor growth suppression by BITC treatment was associated with increased apoptosis in the tumor cells, which in turn was linked with the inhibition of PI3K, AKT, and FOXO activation. Proapoptotic protein Bim, which is negatively regulated by AKT through FOXO proteins (25) was upregulated in the tumors of the BITC-treated mice. Inhibition of activated levels of PI3K, AKT, and FOXO were confirmed in BxPC-3 and PanC-1 pancreatic cancer cells by Western blotting, EMSA, kinase activity and immunofluorescence. Overexpression of AKT by transient transfection substantially blocked the growth-suppressive and apoptosis-inducing effects of BITC. To the best of our knowledge, our results for the first time show the involvement of PI3K/AKT/FOXO in BITC-mediated pancreatic tumor suppression.

 Constitutive activation of AKT has been reported in various cancer types including breast, colon, ovarian, prostate, and pancreatic cancers (26). A study conducted by West and colleagues has shown that more than 55% of the cancers have hyperactivation of AKT, making it as an attractive molecular target (27). Furthermore, most of the cancers acquire drug resistance due to the activation of PI3K/AKT pathway (28). For instance, paclitaxel (29), doxorubicin (30) in breast cancer, and gemcitabine in pancreatic cancer (31) acquire drug resistance due to hyperactivation of PI3K/AKT survival pathway. Our results showed that BITC treatment substantially suppressed the phosphorylation of AKT at both Ser-473 and Ser-308 in a dose- and time-dependent manner in BxPC-3 and PanC-1 cells. In agreement with these results, tumors from BITC-treated mice also showed drastic suppression of AKT phosphorylation at Ser-473. Under normal physiologic condition, AKT is under the control of PI3K catalyzed products IP3 and PIP3 and these products bind to the PH domain of AKT causing translocation of AKT to plasma membrane (32). The full activation of AKT requires phosphorylation of AKT at Ser-308 and Ser-473 (33). Phosphorylation of AKT at Ser-308 is catalyzed by PDK1 (34), which is again regulated PI3K, but the second kinase PDK2 that is responsible for phosphorylation at Ser-473 is not yet fully understood (35). BITC treatment drastically reduced the phosphorylated level, but not protein level, of PI3K in both BxPC-3 and PanC-1, and BITC-treated tumors. On the contrary, phosphorylated PI3K levels were undetectable in normal pancreatic HPDE-6 cells, which is in agreement with previous investigations showing that PI3K is constitutively activated mostly in cancer cells (36). Our results indicate that BITC suppress the growth of pancreatic cancer cells by targeting constitutively activated PI3K without affecting the protein levels. However, a recent study has shown that BITC reduces protein levels of PI3K in HT-29 cells, indicating that BITC acts differentially in different cancer cells (37). Previous investigations have indicated that pancreatic cancer growth can be modulated by PTEN-regulating agents such as TGF-β (38) and PPAR-γ PTEN (39). Our results show that BITC did not alter the protein or phosphorylated levels of PTEN in either BxPC-3 or PanC-1 cells (data not shown). However, activated levels but not protein levels of PDK1 were downregulated by BITC treatment. In conclusion, BITC modulates AKT in pancreatic cancer cells by inhibiting the upstream molecules PI3K and PDK1.

AKT indirectly regulates apoptosis by inhibiting the transcription of Bim through phosphorylation of FOXO transcription factors and directly inhibits the apoptosis by regulating CREB or IKK (40). In addition, AKT also
regulates protein synthesis through mTOR pathway during oxidative stress (41). Our previous results have shown that BITC causes oxidative stress in pancreatic cancer cells by ROS generation (17). Our current results reveal that both activated and protein levels of mTOR were decreased by BITC treatment. These results are in agreement with other investigators who reported that PEITC, a close analogue of BITC, suppresses mTOR pathway in prostate cancer (42). Our results also reveal that BITC treatment decrease the expression of IKK-α in BxPC-3 and PanC-1 cells. These results provide the evidence that BITC induces apoptosis by inhibiting AKT-regulated proteins.

The major mechanism by which AKT regulates FOXO transcription factors is by subcellular localization and phosphorylation of FOXO (43). On phosphorylation by AKT, FOXO proteins specifically binds to 14-3-3 chaperones leading to conformational change that causes masking of nuclear localization signal and exposing the nuclear export signal. This results in moving FOXO from the nucleus into cytosol and prevents the transactivation of responsive genes such as Bim and p27 (44). We hypothesized that inhibition of AKT by BITC treatment would lead to nuclear sequestration of FOXO proteins and increased transcription of responsive genes such as Bim and p27. In agreement with our hypothesis, our results showed that phosphorylated levels of FOXO1 and FOXO3a were decreased with BITC treatment resulting in the nuclear retention of these proteins. These observations were further supported by our results showing that binding of FOXO1 with 14-3-3 binding sites were decreased by BITC treatment. Furthermore, FOXO-responsive proteins p27 and Bim were upregulated providing evidence that BITC treatment causes localization of FOXO proteins in the nucleus thus leading the cells to apoptosis.

Another plausible mechanism by which FOXO proteins are regulated is through acetylation and deacetylation by CBP and SirT protein, respectively, at conserved lysine residues in the DNA binding site of FOXO proteins, resulting in the reduced DNA binding ability of FOXO proteins (45). CBP proteins such as p300 levels were reduced with BITC treatment suggesting reduced acetylation of FOXO proteins. Inhibition of histone deacetylases such as HDAC1/3 by BITC was recently observed by us in pancreatic cancer cells (19). These observations were further confirmed with reduced acetylation of lysine in BITC-treated cells in this study. Increased DNA binding capacity of FOXO1 was observed in response to BITC treatment as compared with control cells in the nucleus resulting in the transcription of FOXO1-responsive proteins p27 and Bim. Overexpression of AKT severely abrogated the apoptosis-inducing and growth-suppressive effects of BITC.

The pharmacokinetics of BITC in humans is not yet reported. However, consumption of 100 g watercress by human volunteers resulted in approximately 928 ± 250 nmol/L PEITC in the plasma (19). In another study, consumption of a single dose of hydrolyzed extract of 3-day-old broccoli sprouts (containing approximately 200 μmol total isothiocyanates) resulted in a peak concentration of 0.94 to 2.27 μmol/L isothiocyanates in the plasma, serum, and erythrocytes within 1 hour of broccoli consumption in humans (46). In a recent study, oral administration of 10 μmol/kg PEITC in rats resulted in approximately 9.2 ± 0.6 μmol/L PEITC in the plasma after 0.44 hour of treatment (47). In agreement with these observations, our current studies reveal that oral administration of 12 μmol BITC to athymic nude mice after 1 hour resulted in approximately 6.5 ± 0.1 μmol/L BITC in the plasma and approximately 7.5 ± 0.3 μmol/g cumulative concentration of BITC in the tumors after 46 days of treatment. These results indicate that the therapeutic concentration of BITC can be achieved. Nonetheless, detailed pharmacokinetic studies on BITC are required and the focus of our future studies.

In conclusion, our in vitro and in vivo results show that BITC suppress the growth of the pancreatic tumor in strong association with an inhibition and targeting of PI3K/AKT, and probably in major part, their downstream FOXO pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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