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Research Article

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# Benzyl Isothiocyanate–Mediated Inhibition of Histone Deacetylase Leads to NF-κB Turnoff in Human Pancreatic Carcinoma Cells

Sanjay Batra, Ravi P. Sahu, Prabodh K. Kandala, and Sanjay K. Srivastava

## Abstract

NF-κB/p65 is constitutively activated in pancreatic cancers, where it plays a critical role in the transcriptional activation of multiple cell survival genes. We have previously shown the apoptosis-inducing effects of benzyl isothiocyanate (BITC) in pancreatic cancer cells. We hypothesized that inhibition of NF-κB/p65 could be the mechanism of BITC-induced apoptosis. Therefore, the effect of BITC on NF-κB/p65 was evaluated in BxPC-3, Capan-2, and normal HPDE-6 cells by Western blotting, transcriptional and DNA-binding activity, and immunohistochemistry in the xenografted tumors. Our results reveal a remarkable decrease in the phosphorylation of NF-κB/p65 at $Ser^{536}$ in both BxPC-3 and Capan-2 cells by BITC treatment. The expression of NF-κB/p65 was downregulated significantly in BxPC-3 cells, whereas it remained unchanged in Capan-2 cells. BITC treatment caused a significant decrease in NF-κB transcriptional and DNA-binding activity in both BxPC-3 and Capan-2 cells. A drastic decrease was observed in the expression and reporter activity of cyclin D1 in both the cell lines. Moreover, BITC also caused a significant decrease in the expression and activity of histone deacetylase (HDAC) 1 and HDAC3 in BxPC-3 and HDAC3 in Capan-2 cells. Overexpression of HDAC1 or HDAC3 abrogated the effects of BITC. BITC treatment did not cause any change in HDAC expression in normal HPDE-6 cells. Immunohistochemical analysis of tumors from BITC-treated mice showed significantly reduced staining for NF-κB, cyclin D1, HDAC1, and HDAC3 compared with control. Our results suggest inhibition of HDAC1/HDAC3 by BITC as a plausible mechanism of NF-κB inactivation, resulting in the *in vitro* and *in vivo* growth suppression of pancreatic cancer cells. *Mol Cancer Ther; 9(6); OF1–13. ©2010 AACR.*

## Introduction

A number of studies support the fact that food phytochemicals, including isothiocyanates (ITC) belonging to organosulfur group of compounds, protect against cancer (1, 2). ITCs occur as glucosinolates in cruciferous vegetables of *Brassica* species. The effectiveness of ITCs as chemoprotective agents against chemical carcinogenesis in experimental animals has been well documented (3). Besides inhibiting phase I enzymes, which required for the bioactivation of carcinogens and increase carcinogen excretion or inducing detoxification by phase II enzyme including glutathione *S*-transferase in various model systems (4, 5), ITCs are also known to induce caspase-mediated apoptosis through multiple signaling pathways (6, 7).

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In cancer cells, including pancreatic, breast, colon, and prostate, NF-κB is constitutively active, which protects the cells from apoptosis and, in some cases, stimulates their growth (8, 9). NF-κB is involved in the maintenance of normal cellular functions, including cell cycle progression, cellular motility, cell to cell communication, and cell lineage development (8–12). However, aberrant regulation of NF-κB has been observed in tumorigenesis, malignant transformation, metastasis, and angiogenesis (8, 9). Modulation of NF-κB activity is therefore extremely critical in regulating the fate of cancer cells (9). Thus, many anticancer drugs seek to inhibit NF-κB activation for inhibiting tumor growth or sensitizing tumor cells to chemotherapy (13, 14). NF-κB typically resides in cytoplasm bound to its inhibitory protein IκBα (15). On cellular stimulation, IκBα proteins are phosphorylated by IκB kinase (IKK), liberating NF-κB, which translocates to the nucleus and gets involved in the transcription of various genes (10–12). There are reports that suggest that NF-κB may shuttle between cytoplasm and nucleus in nonstimulated cells as well (16, 17). IKKα and IKKβ upstream kinases modulate the antiapoptotic response and also cell growth by regulating the activation of NF-κB (18). IKK activation in turn is regulated by its upstream NF-κB–activating kinase, which activates IKKβ through direct phosphorylation (19). Besides phosphorylation,

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several other modifications, including ubiquitination, acetylation, sumoylation, and nitrosylation, also regulate NF-κB (20). Furthermore, NF-κB activity is also regulated by transcriptional coactivators or corepressors, which include histone acetyltransferases (HAT) and histone deacetylases (HDAC; refs. 21–23). HDAC1, HDAC2, and HDAC3 have been reported to interact with NF-κB; however, their roles in the regulation of NF-κB activity are controversial (21–23). Acetylation of p65 either suppresses or activates NF-κB transcription depending on the biological context of the cell (24–26). Five lysine residues in the DNA-binding domain of NF-κB/p65 ($Lys^{122}$, $Lys^{123}$, $Lys^{218}$, $Lys^{221}$, and $Lys^{310}$) play vital role in regulating its transcriptional activity and intranuclear mobility (27). Acetylation of $Lys^{218}$, $Lys^{221}$, and $Lys^{310}$ causes activation of NF-κB/p65 by inhibiting its IκBα binding and thereby decreasing nuclear export of this complex, whereas acetylation at $Lys^{122}$ and $Lys^{123}$ suppresses transcriptional activity of NF-κB by reducing its DNA binding (27). HDAC inhibitors also lead to growth arrest by inducing $p21^{WAF1}$ and downregulate transcription of cyclin D1 by inhibiting NF-κB DNA binding (28, 29).

Previous studies from our laboratory showed that benzyl ITC (BITC)–mediated cell cycle arrest and apoptosis in pancreatic cancer cells were associated with the modulation of cell cycle regulatory proteins, including induction of $p21^{WAF1}$ and downregulation of cyclin D1 (30, 31). Because cyclin D1 is an important transcriptional target of NF-κB, we hypothesized that apoptosis in human pancreatic cancer cells by BITC could be due to inactivation of NF-κB.

In the present study, we provide evidence that BITC specifically inhibits the expression and activity of HDAC1 and HDAC3 in BxPC-3 and HDAC3 in Capan-2 pancreatic cancer cells without causing any effect on HPDE-6 normal pancreatic ductal epithelial cells. We found that BITC inhibits NF-κB transcriptional activity and p65 DNA-binding capacity in both BxPC-3 and Capan-2 cell lines, which exhibit differential p65 phosphorylation. Overexpression of HDAC1 and HDAC3 in BxPC-3 and HDAC3 in Capan-2 cells significantly blocked the effects of BITC with respect to p65 DNA binding, NF-κB transcriptional activity, and cyclin D1 reporter activity, leading to the attenuation of BITC-mediated apoptosis. Further, BxPC-3 tumor xenografts from BITC-treated mice revealed reduced expression of NF-κB, cyclin D1, HDAC1, and HDAC3. Taken together, our findings suggest that BITC could be a novel HDAC inhibitor that regulates pancreatic cancer cell survival by inhibiting HDAC1 and HDAC3.

## Materials and Methods

### Chemicals and plasmids

Antibodies against HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), IKKα, IκBα, IκBα $Ser^{32/36}$, phospho–NF-κB/p65 $Ser^{536}$/$Ser^{276}$, p300/CBP, $p21^{WAF1}$, and cyclin D1 were purchased from Cell Signaling Technology; HDAC8, NF-κB/p65, and IKKγ were procured from Santa Cruz Biotechnology; and BITC and β-actin were from Sigma-Aldrich. Chemicals for cell culture, such as penicillin/streptomycin antibiotic mixture (PSN) and Opti-MEM I reduced serum medium, were purchased from Life Technologies. Vorinostat [suberoylanilide hydroxamic acid (SAHA)] was obtained from Cayman Chemicals, and pure NF-κB protein was from Panomics. Heat-inactivated fetal bovine serum and RPMI 1640 and McCoy's medium were purchased from Mediatech Cellgro. Electrophoresis reagents were procured from Amresco.

The Flag-HDAC expression constructs were kindly provided by Dr. Edward Seto (H. Lee Moffitt Cancer Center, Tampa, FL; ref. 32), whereas luciferase reporter plasmids encoding the full-length human cyclin D1 promoter (−1745CD1LUC) were a kind gift from Dr. Richard Pestell (Thomas Jefferson University, Philadelphia, PA; ref. 33). pRL-*Renilla* luciferase vector (control) construct was generously provided by Dr. Erguang Lee (The Scripps Research Institute, La Jolla, CA), whereas NF-κB luciferase reporter gene construct was a kind gift from Dr. Glen D. Rosen (Stanford University, Stanford, CA; ref. 34).

### Cell culture

Human pancreatic cancer cell lines BxPC-3 and Capan-2 were obtained from the American Type Culture Collection. Monolayer cultures of BxPC-3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, PSN antibiotic mixture (1%, v/v; Life Technologies), 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 20% glucose, whereas monolayer cultures of Capan-2 cells were maintained in McCoy's medium supplemented with 10% fetal bovine serum and PSN antibiotic mixture (1%, v/v) at 37°C in a humidified chamber of 95% air and 5% $CO_2$. Normal human pancreatic duct epithelial cells (HPDE-6) were a generous gift from Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada). HPDE-6 cells were cultured in keratinocyte serum-free medium supplemented with 4 mmol/L L-glutamine and adjusted to contain 0.2 ng/mL epidermal growth factor, 30 μg/mL bovine pituitary extract, and 1% (v/v) PSN as described by us previously (7, 35).

### Cell extract preparation and immunoblotting

Briefly, $1.0 \times 10^6$ human pancreatic cells BxPC-3, Capan-2, or HPDE-6 were treated with increasing concentrations of BITC for 24 hours or with 10 μmol/L BITC for varying time intervals. In another experiment, BxPC-3 cells were treated with 0 to 80 μmol/L SAHA for 24 hours. Cell pellets were collected after treatment and washed with ice-cold PBS. For Western blot analysis, whole-cell extracts were prepared using lysis buffer (8 mol/L urea, 4% CHAPS, 40 mmol/L Tris-HCl containing 10 mmol/L sodium glycerophosphate, 5 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L

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orthovanadate, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL of protease inhibitors: chymostatin, pepstatin, antipain, and leupeptin). Lysates were sonicated for five short pulses of 5 seconds each (Fisher Scientific Sonic Dismembrator Model 100 at 2 W output power). Extracts were centrifuged for 15 minutes at 14,000 × *g* at 4°C, and supernatants were aliquoted and stored at −70°C for future analysis. Protein content was determined by Bradford's reagent. Equal amounts of protein were loaded and separated by SDS-PAGE and electroblotted onto nitrocellulose membrane (Hybond ECL, Amersham Life Science). Membranes were blocked for 1 hour at 4°C in TBS containing 0.1% Tween 20 and 5% nonfat dry milk at room temperature for 1 hour, followed by overnight incubation with primary antibody. The blots were then incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (1:3,000; Santa Cruz Biotechnology) for 45 minutes in blocking buffer. Immunoblots were developed by enhanced chemiluminescence reagent according to the manufacturer's instructions (Amersham). Nuclear extracts were prepared using Fraction-PREP Cell Fractionation kit (Biovision) as per the manufacturer's protocol.

### DNA binding

NF-κB/p65 binding assays were done as per the instructions of manufacturer's protocol (TransAM ELISA kit). Basically, the DNA-binding motif of NF-κB (5′-GGGACTTTCC-3′) was coated to a 96-well plate. Transcriptionally active nuclear NF-κB binds to DNA and is recognized by antibody against p65. Briefly, nuclear lysates of treated/nontreated and/or transfected BxPC-3 and Capan-2 cells were prepared using nuclear extract kit from Active Motif for DNA-binding assays. Five micrograms of nuclear extract mixed with binding buffer were added to the precoated plate. After 1 hour of incubation, wells were washed and plates were incubated with NF-κB/p65 antibody for additional 1 hour. Following incubation, plates were washed thrice with wash buffer and HRP-conjugated anti-rabbit IgG (Molecular Probes) was added to each well. Plates were read at 450 nm after adding the developing reagent.

In another experiment, pure 50 ng NF-κB protein in 10 mmol/L Tris-HCl (pH 7.4) was incubated with or without 10 to 20 μmol/L BITC for 4 hours at 37°C, and the DNA-binding activity was done using TransAM ELISA kit as described above.

Electrophoretic mobility shift assay (EMSA) was done using commercially available kit from Panomics with slight modification. Ten micrograms of nuclear protein extracted from control or BITC-treated BxPC-3 cells were incubated in the presence of poly(deoxyinosinic-deoxycytidylic acid) at room temperature. Biotin-labeled NF-κB–binding site oligomer 5′-AGTTGAGGGGACTTTCCCAGGC was then added and protein-DNA complexes were separated using 5% nondenaturing polyacrylamide gels in Tris-borate/EDTA buffer (0.1 mol/L Tris, 0.09 mol/L boric acid containing 1 mmol/L EDTA) at 4°C. Complexes were transferred to nylon membranes, and transferred oligos were immobilized by UV cross-linking for 3 minutes. For detection of bound oligos, membranes were blocked using blocking buffer (Panomics EMSA Gel-Shift kit) followed by the addition of streptavidin-horseradish peroxidase, and blots were developed by enhanced chemiluminescence according to the manufacturer's instructions.

### Transient transfection

About $0.3 \times 10^6$ or $5 \times 10^3$ BxPC-3 or Capan-2 cells were plated in 6- or 96-well plates and transiently transfected with 0.75 μg of Flag, Flag-HDAC1, and Flag-HDAC3 plasmid DNA or cotransfected with NF-κB or cyclin D1 luciferase reporter plasmid DNA using Fugene 6 transfection reagent for 24 hours. pRL-*Renilla* luciferase reporter plasmid was used as control. Transfected or cotransfected cells were treated with DMSO or 10 μmol/L BITC for 24 hours followed by sulforhodamine B (SRB) cell survival assay, dual-luciferase assay (Promega), DNA-binding assay, or Western blotting.

### Dual-luciferase activity

Cotransfected cells were lysed using passive lysis buffer from the dual-luciferase reporter assay system kit (Promega). Cells were ruptured by passive lysis buffer as per the manufacturer's instructions, and 20 μL of cell lysate were added to a tube containing 100 μL luciferase assay buffer. Luciferase activity was determined using $20/20^n$ luminometer from Turner Biosystems. The reaction was then stopped by adding 100 μL Stop and Glo buffer, and the samples were read to determine *Renilla* luciferase activity.

### Cell survival assays

The effect of BITC on the survival of BxPC-3 and Capan-2 cells transfected with empty vector, Flag-HDAC1, or Flag-HDAC3 was determined by SRB assay as described by us previously (31, 36). Briefly, transfected cells were treated with BITC, SAHA, or DMSO (control) for 24 hours followed by SRB assay. Plates were read at 570 nm using Bio Kinetics plate reader EL-800 from BioTek Instrument, Inc.

### Immunohistochemistry of control and BITC-treated tumors

In our previous studies, we have shown that oral feeding of 12 μmol BITC for 6 weeks significantly suppressed the growth of BxPC-3 tumor xenografts in athymic nude mice (7). The tumor sections from control and BITC-treated mice were taken, and immunohistochemistry for NF-κB, cyclin D1, HDAC1, and HDAC3 was done as described previously (7). Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated by washing the sections in xylene, 100% ethanol, 95% ethanol, and finally distilled water. Antigens were unmasked by boiling the sections in 10 mmol/L sodium citrate buffer (pH 6.0) and incubating in 3% hydrogen peroxide for

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10 minutes. Tumor sections were washed twice in PBS containing 0.1% Tween 20, blocked in 5% horse serum diluted in TBS-Tween 20 for 1 hour at room temperature, and then incubated with anti–NF-κB, cyclin D1, HDAC3, and HDAC1 antibody (1:300 in TBS-Tween 20) overnight at 4°C. After removal of the primary antibody, sections were washed thrice in wash buffer for 5 minutes each followed by incubation with 200 μL of HRP-conjugated secondary antibody diluted 1:5,000 in blocking solution for 30 minutes. Subsequently, sections were washed with wash buffer and incubated with 200 μL of avidin-biotin conjugate reagent containing avidin and biotinylated HRP for 30 minutes at room temperature using avidin-biotin conjugate staining kit according to the manufacturer's instructions (Santa Cruz Biotechnology). Three drops of peroxidase substrate were added to each section and incubated until the desired color developed. The sections were counterstained with hematoxylin and mounted and analyzed under a phase-contrast Olympus microscope (Olympus America, Inc.).

### Statistical analysis

All the statistical calculations were done using GraphPad Prism 4.0 software. For comparisons that involved multiple variables and observations, ANOVA was used followed by Bonferroni or Newman-Keuls post hoc multiple comparison tests. The Student's *t* test was used to compare variations in control and treated groups only. All data are expressed as means ± SD. Differences were considered statistically significant when *P* value was <0.05.

## Results

### Inactivation of NF-κB/p65 by BITC in pancreatic cancer cells

Previous studies from our laboratory showed that BITC induces caspase-3–dependent apoptosis in BxPC-3 and Capan-2 cells (7, 30, 31, 36). However, the exact mechanism of BITC-mediated apoptosis was not clear. Because NF-κB is involved in apoptosis and cell survival, we wanted to see whether BITC-induced apoptosis in our model is mediated by modulation of NF-κB pathway. Therefore, to unveil the molecular mechanism of BITC, we first determined the concentration- dependent effect on the phosphorylation of NF-κB/p65 at $Ser^{536}$ in BxPC-3 cells. BxPC-3 cells treated with 10 and 20 μmol/L BITC for 24 hours resulted in about 90% to 99% decrease in the phosphorylation of NF-κB/p65 at $Ser^{536}$ and $Ser^{276}$ (Fig. 1A). Similarly, we observed a significant decrease in the basal level of NF-κB/p65 after BITC treatment in BxPC-3 cells (Fig. 1A). On cellular stimulation, IKKα activates IκBα by phosphorylation at $Ser^{32/36}$. Activated IκBα is ubiquitinated and degraded proteasomally, liberating NF-κB to translocate to nucleus. Phosphorylation of NF-κB at $Ser^{536}$ by IKK is also required for the transcriptional activity of NF-κB. The effect of BITC was thus evaluated on the expression and phosphorylation of IKK and IκBα. As shown in Fig. 1A, BITC treatment reduced the expression level of IKKα without affecting the phosphorylation or protein expression of IκBα. Consistent with these observations, we observed a drastic decrease in the phosphorylation and protein expression of NF-κB in the nuclear fraction of the cells treated with BITC for 24 hours compared with control (Fig. 1A). Time-dependent experiment in BxPC-3 cells revealed a decrease in the phosphorylation or protein level of NF-κB/p65 starting from 8 hours of treatment with 10 μmol/L BITC and persisted until the duration of the experiment (Fig. 1A).

### BITC drastically decreases transcriptional activity of NF-κB

Phosphorylation of NF-κB/p65 at $Ser^{536}$ is required for the transcriptional activity of p65. Because our results showed decrease in p65 phosphorylation and protein level in BxPC-3 cells, we next determined the effect of BITC on the transcriptional activity of NF-κB. BxPC-3 cells were transiently transfected for 24 hours with NF-κB luciferase reporter construct and pRL-*Renilla* luciferase reporter plasmid as internal control, followed by treatment with 10 μmol/L BITC for additional 24 hours. Our results show that BITC was able to drastically reduce the transcriptional activity of NF-κB compared with control group in BxPC-3 cells (Fig. 1B). For example, 10 μmol/L BITC treatment for 24 hours reduced NF-κB transcriptional activity by 90% in BxPC-3 cells compared with control (Fig. 1B).

### BITC suppresses NF-κB DNA-binding capacity

Because we observed significant decrease in NF-κB transcriptional activity by BITC treatment, we subsequently investigated the effect of BITC on κB DNA-binding capacity of p65 in BxPC-3 cells. Significantly low p65 DNA binding was observed in BITC-treated BxPC-3 cells as assessed by EMSA (Fig. 1C, top). These results were further confirmed by ELISA-based p65 DNA-binding assay. In agreement with EMSA results, decrease in DNA-binding capacity of p65 was observed in response to BITC treatment (Fig. 1C, middle). To further determine whether the decrease in DNA-binding activity of NF-κB is due to direct modification of NF-κB by BITC, we did *in vitro* assay where pure NF-κB protein was exposed to 10 and 20 μmol/L BITC at 37°C for 4 hours and DNA-binding assay was done. BITC treatment up to 20 μmol/L did not affect the DNA-binding activity of NF-κB, ruling out the physical interaction of BITC with NF-κB (data not shown). However, further structural studies are required to prove whether BITC at higher concentrations modifies NF-κB by physically binding.

### BITC decreases cyclin D1 protein expression and cyclin D1 promoter reporter activity

Because our results showed significant decrease in NF-κB DNA binding, we next raised a question

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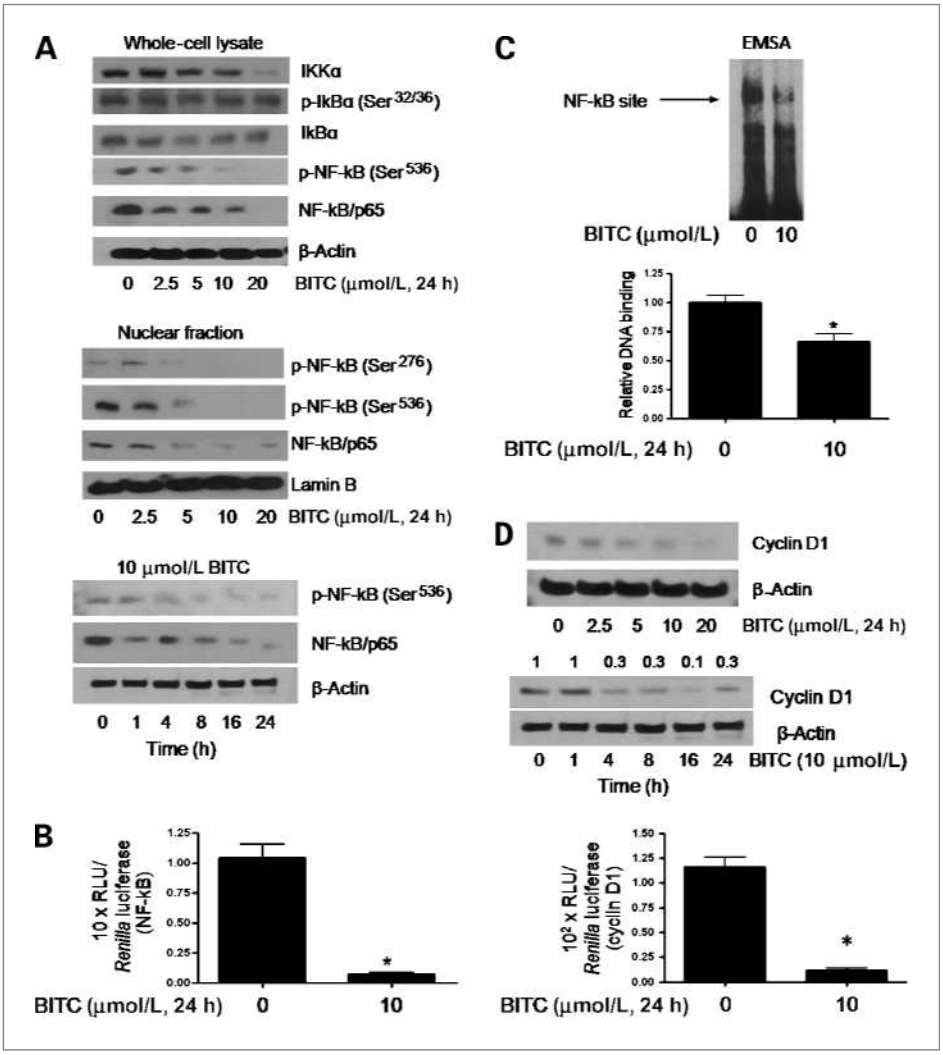


Figure 1. BITC treatment causes inhibition of NF-κB and cyclin D1 in BxPC-3 pancreatic cancer cells. A, BxPC-3 cells were treated with 0 to 20 μmol/L BITC for 24 h or with 10 μmol/L BITC for different time intervals. Cells were lysed and 40 μg protein (whole cell or nuclear) was resolved by SDS-PAGE. Each blot was stripped and reprobed with β-actin for cytosolic and lamin B for nuclear proteins to ensure equal protein loading. Each experiment was repeated thrice with similar results. B, the NF-κB luciferase assay was determined in control and BITC-treated BxPC-3 cells using luminometer with the dual-luciferase substrate system and luciferase activity was normalized with *Renilla* luciferase as internal control. C, EMSA was done using nuclear protein obtained from BITC-treated BxPC-3. In addition, NF-κB/p65 DNA binding by ELISA method was determined using 5 μg of nuclear protein from control and BITC-treated cells and assayed for the presence of activated p65 using antibody specific for p65 following binding to NF-κB consensus sequence using TransAM NF-κB/p65 ELISA kit. D, BxPC-3 cells were treated with 0 to 20 μmol/L BITC for 24 h or treated with 10 μmol/L BITC for varying time intervals and the whole-cell lysates were subjected to SDS-PAGE. The membranes were probed with cyclin D1 antibody. Each blot was stripped and reprobed with β-actin to ensure equal protein loading. Each experiment was repeated thrice with similar results. BxPC-3 cells were also transiently transfected with cyclin D1 promoter luciferase construct and *Renilla* luciferase plasmid as a control, followed by treatment with 10 μmol/L BITC or DMSO for 24 h. Cell lysates were subjected to luciferase activity and normalized with control *Renilla* luciferase. Columns, mean of three independent experiments each done in triplicates; bars, SD. *, $P < 0.05$, compared with control.

whether this change could affect its downstream transcriptional target cyclin D1 (30, 37). As shown in Fig. 1D, BITC treatment caused a substantial decrease in the expression of cyclin D1 in a concentration- and time-dependent manner in BxPC-3 cells. We further evaluated the effect of BITC on cyclin D1 promoter reporter activity and observed about 70% to 90% decrease in relative luciferase activity (Fig. 1D, bottom).

## BITC is a HAT and HDAC inhibitor

HATs and HDACs are enzymes involved in acetyl modification of histone and nonhistone proteins such as NF-κB and act as critical gene silencer or activator (21, 22). The effect of BITC was evaluated in pancreatic cancer cells on the expression and/or activity of HDACs and p300/CBP. Our results show a concentration- and time-dependent decrease in HDAC1 and HDAC3

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expression in BITC-treated BxPC-3 cells (Fig. 2A). For example, treatment with 10 to 20 μmol/L BITC resulted in about 55% to 60% decrease in HDAC1 and 65% to 70% decrease in HDAC3 expression in BxPC-3 cells (Fig. 2A, top). In a time-dependent experiment in BxPC-3 cells, a decrease in HDAC1 expression was observed after 16 and 24 hours of BITC treatment (Fig. 2A, bottom). The expression of HDAC2, HDAC5, HDAC6, HDAC7, and HDAC8 nevertheless did not change in response to BITC treatment in BxPC-3 cells (Fig. 2A, top). We further confirmed our observations on HDAC inhibition by evaluating the effect of BITC on the deacetylase activity of HDACs. As shown in Fig. 2B, we observed 30% to 35% decrease in deacetylase activity of both HDAC1 and HDAC3 in 10 μmol/L BITC–treated BxPC-3 cells. Further, we compared BITC-mediated inhibition of HDAC1/3 with a known HDAC inhibitor, SAHA (vorinostat). Although 5 μmol/L SAHA treatment for 24 hours decreased the expression of HDAC1, HDAC3, NF-κB, and cyclin D1, it failed to significantly suppress the survival of BxPC-3 cells compared with BITC (Fig. 2C). Our results also show that 10 μmol/L BITC was able to downregulate ~35% of p300/CBP expression in BxPC-3 cells (Fig. 2D).

## BITC induces p21$^{WAF1}$ expression

As HDAC inhibitors such as trichostatin A and depsipeptide are known to induce p21$^{WAF1}$ expression (38, 39), we wanted to see whether HDAC inhibition by BITC in

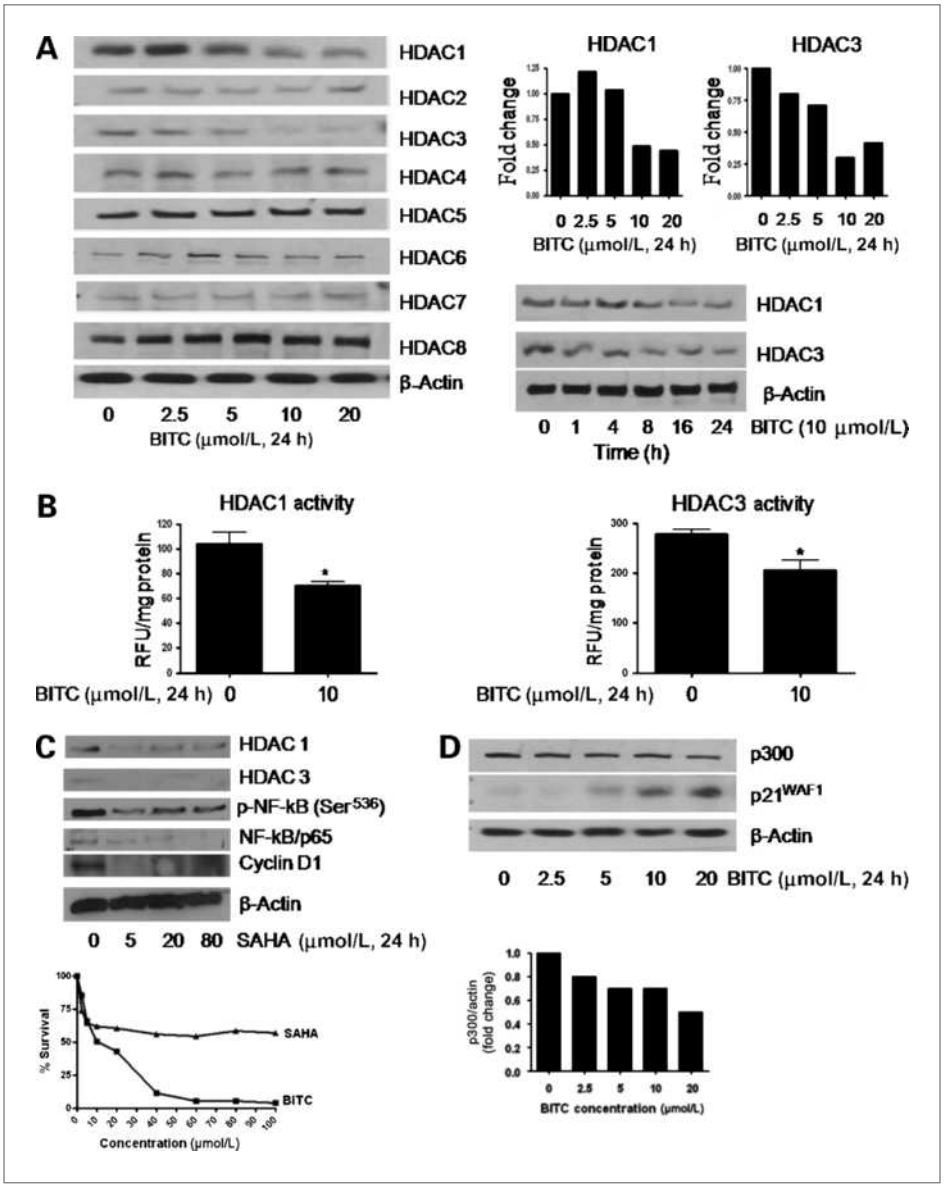


Figure 2. BITC causes decrease in HDAC1 and HDAC3 expression and activity level. A, BxPC-3 cells were treated with different concentrations of BITC for 24 h or treated with 10 μmol/L BITC for varying time intervals and the whole-cell lysates were subjected to SDS-PAGE. Representative immunoblots show the effect of BITC treatment on the expression of HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, and HDAC8. Each blot was stripped and reprobed with anti–β-actin to ensure equal protein loading. Each experiment was repeated thrice with similar results. B, HDAC1 and HDAC3 activity was determined in the nuclear lysates of DMSO and BITC-treated BxPC-3 cells using HDAC Fluorescent Activity Assay kit. *Statistically different when compared with control, $P < 0.05$. C, effect of SAHA was determined in BxPC-3 cells and compared with the effects of BITC. Cells were exposed to 0-100 μmol/L SAHA or BITC for 24 h and evaluated for NF-κB, cyclin D1, and HDAC1/3 expression by Western blotting and cell survival by SRB assay. D, BxPC-3 cells were treated with 0-20 μmol/L BITC for 24 h. Whole-cell lysates were subjected to SDS-PAGE and the membranes were probed with p300/CBP, p21$^{WAF1}$, and β-actin antibody. Each experiment was repeated thrice with similar results.

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our model also causes an increase in $p21^{WAF1}$ expression. As shown in Fig. 2D, treatment of BxPC-3 cells with 0 to 20 μmol/L BITC for 24 hours led to a substantial increase in $p21^{WAF1}$ expression.

### Overexpression of HDAC1 and HDAC3 increases NF-κB transcriptional activity and DNA binding

To establish the involvement of HDACs in BITC-mediated regulation of NF-κB, cells were transfected with HDAC1 and HDAC3 expression plasmids and treated with 10 μmol/L BITC for 24 hours. As shown in Fig. 3A, BxPC-3 cells transfected with HDAC1 or HDAC3 plasmid resulted in the increased expression of HDAC1 and HDAC3 protein by ~6-fold compared with vector (Flag)–transfected cells. Because BITC efficiently inhibits NF-κB transcriptional activity and its κB DNA binding, we next determined the effect of HDAC1 and HDAC3 overexpression on BITC-mediated regulation of transcriptional activity and κB DNA binding of p65. BxPC-3 cells were transfected with Flag, Flag-HDAC1, Flag-HDAC3 with/without NF-κB luciferase reporter plasmid, and *Renilla* luciferase as control followed by treatment with 10 μmol/L BITC for 24 hours. A 7- to 10-fold increase in NF-κB luciferase activity (Fig. 3A, middle) and a 20% to 30% increase in relative DNA binding of p65 (Fig. 3A, bottom) were observed in HDAC1- and HDAC3-overexpressing BxPC-3 cells, altogether neutralizing the effect of BITC.

### HDAC overexpression increases cyclin D1 expression and promoter reporter activity in BITC-treated cells

As BITC treatment led to drastically reduced cyclin D1 expression and promoter activity in BxPC-3 cells after inhibiting NF-κB/p65 DNA binding, we next wanted to see if HDAC overexpression can prevent BITC-mediated degradation of cyclin D1. Interestingly, overexpression of HDAC1 led to 3.5-fold increase in cyclin D1 and HDAC3 overexpression caused ~2.4-fold increase in cyclin D1 expression in BxPC-3 cells following BITC treatment compared with control cells (Fig. 3B). We next cotransfected BxPC-3 cells with Flag, Flag-HDAC1, or Flag-HDAC3 along with cyclin D1 promoter reporter and *Renilla* luciferase reporter and subsequently treated the cells with 10 μmol/L BITC for 24 hours. Our results reveal about 3- to 5-fold increase in cyclin D1 promoter reporter activity in HDAC1- and HDAC3-overexpressing BxPC-3 cells compared with the cells transfected with empty vector (Fig. 3B, bottom). Although these results suggest a connection between HDAC1/3 and cyclin D1, we further confirmed this by immunoprecipitating cyclin D1 from control and BITC-treated BxPC-3 cells and immunoblotted with HDAC1 and HDAC3 antibody. Compared with control, expression of both HDAC1 and HDAC3 was reduced in response to BITC treatment in cyclin D1 immunoprecipitated samples, indicating an association of cyclin D1 with HDAC1 and HDAC3. Overall, our results establish HDAC1/3 as molecular target of BITC in BxPC-3 cells.

### Overexpression of HDACs protects BxPC-3 cells from BITC-mediated apoptosis

Thus far, our results suggested that HDAC overexpression abrogated the effect of BITC-mediated inhibition of NF-κB and HDACs. We therefore next wanted to see whether HDAC inhibition is directly linked to BITC-induced apoptosis. To do that, BxPC-3 cells were transfected with Flag, Flag-HDAC1, and Flag-HDAC3 expression plasmids and then treated with 10 μmol/L BITC for 24 hours. It is interesting to point out that the concentrations at which BITC significantly inhibit HDAC expression and activity also induced apoptosis in both the cell lines (36). Overexpression of HDAC1 and HDAC3 decreased the cleavage of caspase-3 and PARP in BITC-treated BxPC-3 cells compared with control cells, indicating protection from BITC (Fig. 3C).

### HDAC overexpression confers resistance to BITC in pancreatic cancer cells

Overexpression of HDAC1 and HDAC3 in BxPC-3 cells offered about 35% to 40% protection against BITC treatment in terms of cell survival compared with vector-transfected cells (Fig. 3D). These results suggest that overexpression of HDAC1 and HDAC3 confers resistance to apoptosis and growth-suppressive effects of BITC, and establish a critical role of HDACs in the survival of pancreatic cancer cells.

### Effect of BITC on NF-κB and HDACs in Capan-2 cells

To rule out the cell-specific effects of BITC, we evaluated the effect of BITC in Capan-2 pancreatic cancer cells and normal HPDE-6 cells. Compared with BxPC-3 cells, treatment of Capan-2 cells with BITC also resulted in the decrease in the phosphorylation of NF-κB at $Ser^{536}$; however, the protein level was modestly reduced at higher BITC concentration (Fig. 4A). In a time-dependent experiment, BITC treatment caused changes in the phosphorylation and protein level only at 24 hours (Fig. 4A, bottom) as opposed to BxPC-3 cells where the effect of BITC was as early as 4 hours (Fig. 1A). The transcriptional activity of NF-κB was determined in Capan-2 cells using NF-κB luciferase reporter construct and pRL-*Renilla* luciferase reporter plasmid as internal control, as described in BxPC-3 cells. After 24 hours, treatment of Capan-2 cells with 10 μmol/L BITC resulted in ~45% reduction in NF-κB transcriptional activity (Fig. 4B). Similarly, we observed ~50% inhibition of the DNA-binding capacity of NF-κB in Capan-2 cells in response to BITC treatment (Fig. 4B, bottom). The next step was to determine the effect of BITC on the expression and transcriptional level of cyclin D1, which is the downstream transcriptional target of NF-κB. As shown in Fig. 4C, BITC treatment of Capan-2 cells resulted in the substantial reduction in the expression of cyclin D1 in a concentration- and time-dependent manner. The effect of BITC on the expression of cyclin D1 was evident just after 1 hour of treatment (Fig. 4C, middle). The transcriptional activity

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of cyclin D1 was also reduced drastically by BITC treatment in Capan-2 cells (Fig. 4C, bottom). Further, the effect of BITC was evaluated on the expression of various HDACs in Capan-2 cells (Fig. 4D). Unlike BxPC-3 cells, BITC treatment was able to decrease only HDAC3 expression in Capan-2 cells and this effect was evident at 24 hours (Fig. 4D). Nevertheless, BITC treatment significantly downregulated the expression of p300/CBP in Capan-2 cells (Fig. 5A). On the contrary, expression of p21 was significantly increased in this cell line by BITC treatment (Fig. 5A).

To confirm the role of HDAC3 in BITC-mediated NF-κB and cyclin D1 inhibition leading to decreased

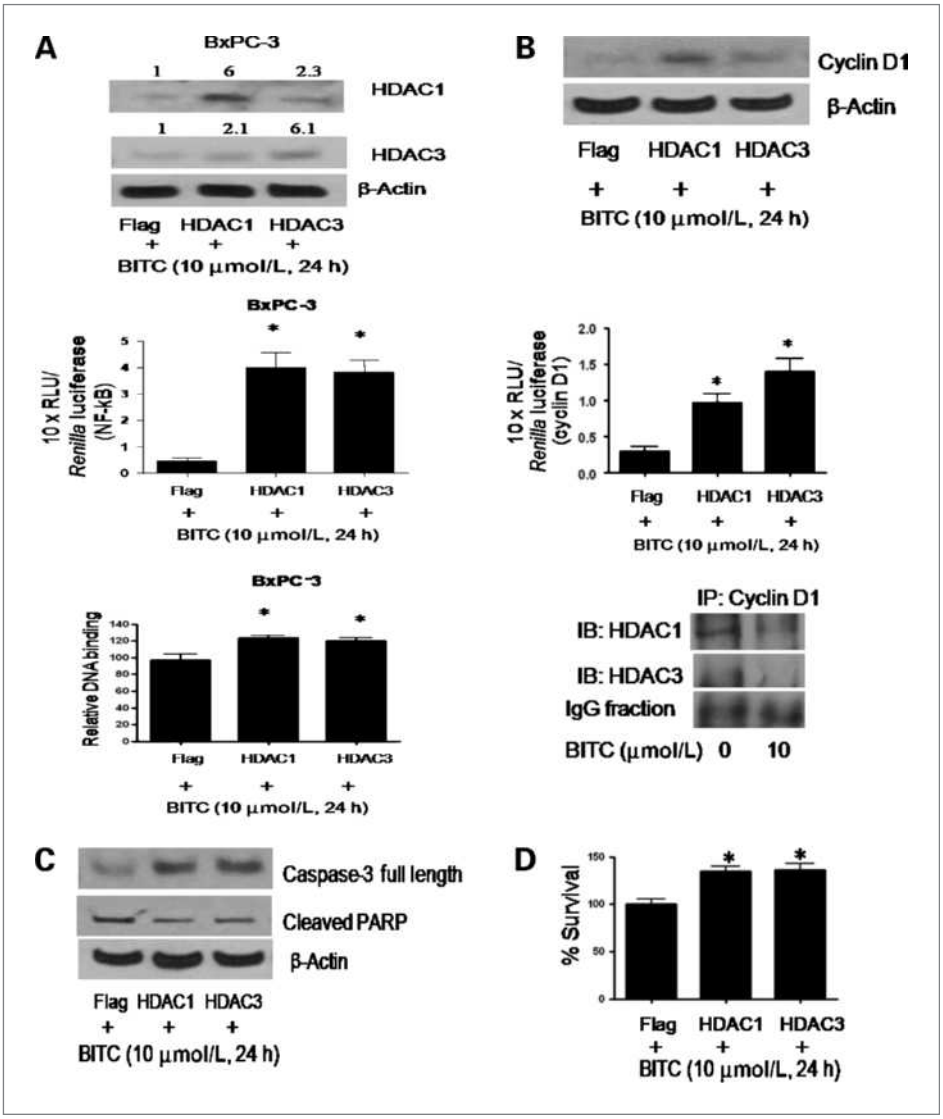


Figure 3. HDAC1 and HDAC3 overexpression blocks BITC-mediated inactivation of NF-κB and cyclin D1 and induction of apoptosis. A, BxPC-3 cells were transfected with Flag, Flag-HDAC1, or Flag-HDAC3 plasmid with/without NF-κB luciferase plasmid and *Renilla* luciferase as control for 24 h followed by treatment with 10 μmol/L BITC for 24 h. Whole-cell lysate from transfected and/or treated cells was subjected to SDS-PAGE, and the immunoblots were probed with HDAC1 and HDAC3 antibody. Equal loading was determined with β-actin antibody. The relative NF-κB luciferase activity was done in BxPC-3 cells as described in Fig. 1 and normalized with *Renilla* luciferase as a control. Nuclear lysates from transfected and BITC-treated BxPC-3 cells were used to determine relative DNA binding of p65 by ELISA kit as described above. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, compared with control. B, BxPC-3 cells were transiently transfected with Flag, Flag-HDAC1, and Flag-HDAC3 plasmid DNA with or without cyclin D1 luciferase reporter plasmid and *Renilla* luciferase as internal control followed by treatment with 10 μmol/L BITC for 24 h. Cell lysates were resolved using SDS-PAGE, and the membrane was probed with cyclin D1 antibody. For equal loading, the membrane was blotted with β-actin antibody. Relative cyclin D1 reporter luciferase activities were done in transfected cells as described above and normalized against *Renilla* luciferase as a control. Cyclin D1 was further immunoprecipitated from control and BITC-treated BxPC-3 cells, and the lysate was immunoblotted for HDAC1 and HDAC3. Columns, mean of three independent experiments; bars, SD. C, cells were transiently transfected with Flag, Flag-HDAC1, and Flag-HDAC3 plasmids as described above and treated with 10 μmol/L BITC or DMSO for 24 h and analyzed for full-length caspase-3 and cleaved PARP. Each experiment was repeated thrice with similar results. D, HDAC-overexpressing cells after BITC treatment were subjected to SRB cell survival assay. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, compared with control.

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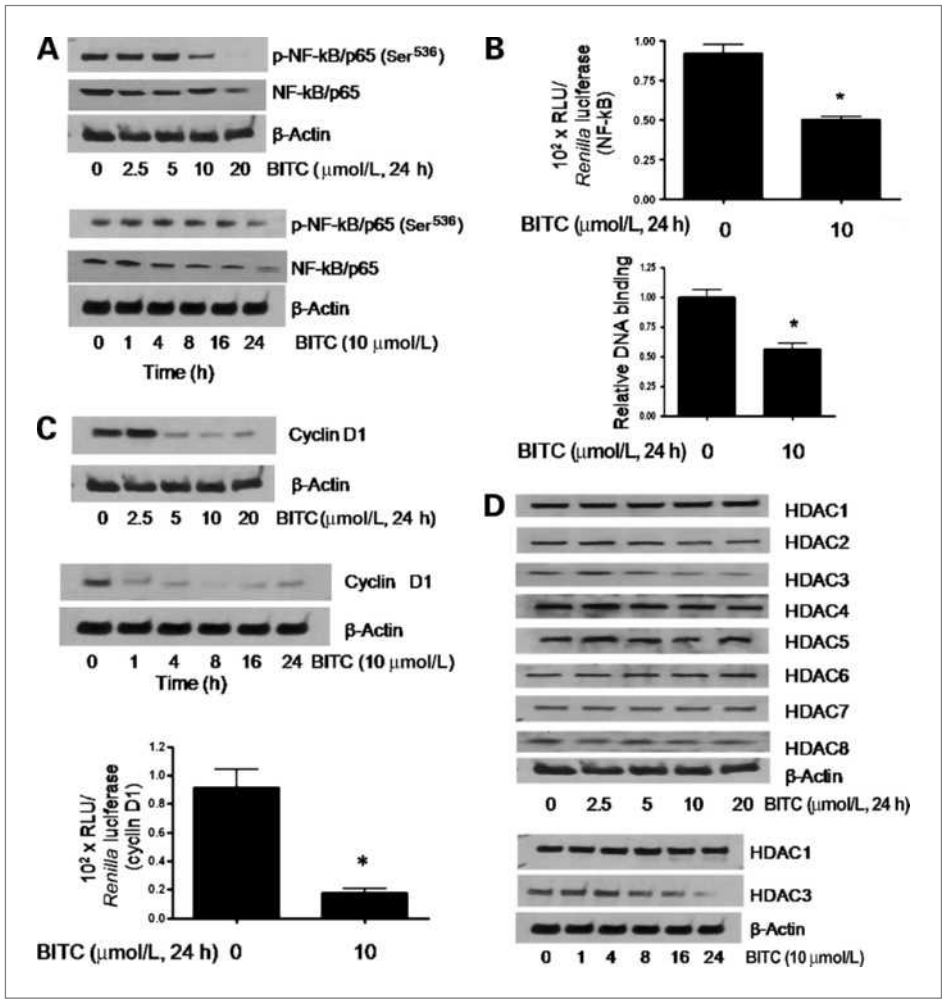


Figure 4. Effect of BITC on NF-κB, cyclin D1, and HDACs in Capan-2 cells. A, Capan-2 cells were treated with 0 to 20 μmol/L BITC for 24 h or with 10 μmol/L BITC for different time intervals. Proteins were resolved by SDS-PAGE and immunoblotted with phospho–NF-κB/p65 (Ser$^{536}$) and NF-κB/p65 antibodies. Each blot was stripped and reprobed with β-actin antibody to ensure equal protein loading. Each experiment was repeated thrice with similar results. B, control and BITC-treated cells were transfected with NF-κB luciferase reporter plasmid as described in Fig. 1. The luciferase assay was done with the dual-luciferase substrate system, and luciferase activity was normalized with *Renilla* luciferase as internal control. In addition, NF-κB/p65 DNA binding was determined using TransAM NF-κB/p65 ELISA kit. Columns, mean of three independent experiments each done in triplicates; bars, SD. *, $P < 0.05$, compared with control. C, Capan-2 cells were treated with 0 to 20 μmol/L BITC for 24 h or treated with 10 μmol/L BITC for varying time intervals, and the whole-cell lysates were subjected to SDS-PAGE. The membranes were probed with cyclin D1 antibodies. Control and BITC-treated cells were also transiently transfected with cyclin D1 promoter luciferase construct and subjected to luciferase activity as described in Fig. 1. Columns, mean of three independent experiments each done in triplicates; bars, SD. *, $P < 0.05$, compared with control. D, Capan-2 cells were treated with different concentrations of BITC for 24 h or treated with 10 μmol/L BITC for varying time intervals, and whole-cell lysates were subjected to SDS-PAGE. Representative immunoblots show the effect of BITC on the expression of HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, and HDAC8. Each blot was stripped and reprobed with anti–β-actin to ensure equal protein loading. Each experiment was repeated thrice with similar results.

cell survival, Capan-2 cells were transfected with HDAC plasmids as explained in detail in Materials and Methods, followed by treatment with BITC. A significant increase in NF-κB luciferase activity and DNA binding of p65 was observed in HDAC3-overexpressing BITC-treated Capan-2 cells, but not much change was observed by HDAC1 overexpression (Fig. 5B). Similarly, a 3-fold increase in cyclin D1 promoter reporter activity in HDAC3-overexpressing Capan-2 cells was observed (Fig. 5B, bottom). Further, HDAC3 overexpression offered significant cell survival advantage in BITC-treated Capan-2 cells (Fig. 5C). Taken together, these results clearly indicate the role of HDAC3 but not HDAC1 in the survival of Capan-2 cells.

### BITC failed to alter NF-κB or HDAC expression in normal HPDE cells

We next wanted to see if BITC treatment can modulate the expression of NF-κB and HDAC expression in normal HPDE cells. Consistent with the notion that NF-κB is activated in transformed or malignant cells but not in normal cells, we did not observe any constitutive

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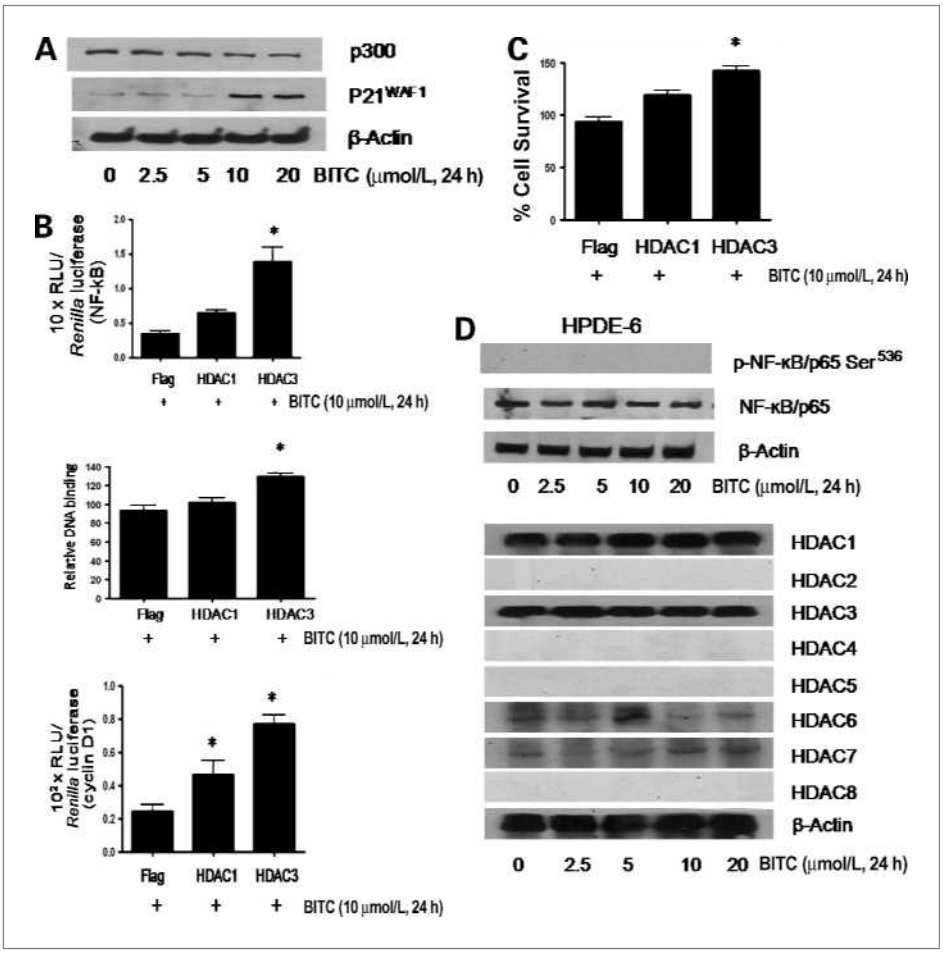


Figure 5. HDAC3 overexpression protects the cells from BITC-mediated changes in Capan-2 cells, and HPDE-6 cells are unaffected by BITC treatment. A, Capan-2 cells were treated with 0 to 20 μmol/L BITC for 24 h and whole-cell lysate was subjected to SDS-PAGE and the membranes were probed with p300/CBP, $p21^{WAF1}$, and β-actin antibody. B, NF-κB luciferase activity, DNA binding, and cyclin D1 luciferase activity were done in control and BITC-treated Capan-2 cells transfected with Flag, Flag-HDAC1, or Flag-HDAC3 plasmid DNA with/without NF-κB luciferase plasmid and *Renilla* luciferase as described in Fig. 3. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, compared with control. C, HDAC-overexpressing cells treated with BITC were subjected to SRB B-cell survival assay. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, compared with control. D, HPDE-6 cells were treated with different concentrations of BITC for 24 h and whole-cell lysates were subjected to SDS-PAGE. Representative immunoblots show the effect of BITC treatment on the expression of phospho–NF-κB/p65 $Ser^{536}$, NF-κB/p65, HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, and HDAC8. Each blot was stripped and reprobed with anti–β-actin to ensure equal protein loading. Each experiment was repeated thrice with similar results.

phosphorylation of NF-κB in HPDE cells (Fig. 5D, top). Further BITC treatment did not change the constitutive p65 expression of NF-κB in HPDE cells (Fig. 5D). We did not observe any constitutive expression of HDAC2, HDAC4, HDAC5, or HDAC6 in HPDE cells, and BITC treatment failed to cause any change in the expression of HDAC1, HDAC3, HDAC6, or HDAC7 (Fig. 5D). We have already shown in our previous publication that HPDE cells were resistant to the deleterious effects of BITC (36).

### Oral BITC administration suppresses the growth of pancreatic tumor xenograft by downregulating NF-κB, cyclin D1, HDAC1, and HDAC3 *in vivo*

In our previously published study, we have shown that oral administration of 12 μmol (90 mg/kg body weight) BITC in PBS, 5 days per week for 6 weeks, significantly suppressed the growth of BxPC-3 pancreatic tumor xenografts in athymic nude mice (7). To further establish whether NF-κB and HDAC1/3 play any role in the growth of pancreatic tumor, the tumors from control and BITC-treated mice were analyzed by immunohistochemistry for the expression of NF-κB, cyclin D1, HDAC1, and HDAC3. As shown in Fig. 6, reduced staining of NF-κB, cyclin D1, HDAC1, and HDAC3 was observed in the tumor sections from BITC-treated mice compared with tumor sections from control mice. Based on these observations, it can be concluded that the reduced tumor growth in BITC-fed mice was due to reduced expression of NF-κB, cyclin D1, HDAC1, and HDAC3.

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## Discussion

In this study, we explored the mechanism of BITC-mediated regulation of NF-κB in pancreatic cancer cells. We used BxPC-3 and Capan-2 human pancreatic cancer cell lines and compared their sensitivity toward BITC in normal pancreatic cell line HPDE-6. Interestingly, we observed that BITC treatment led to significant inhibition in the activation of NF-κB/p65 by suppressing the phosphorylation of p65 at $Ser^{536}$ in BxPC-3 and Capan-2 cells. The expression of NF-κB/p65 was significantly reduced in BxPC-3 but not in Capan-2 cells by BITC treatment. Phosphorylation of NF-κB at $Ser^{536}$ by IKKα is required for the transcriptional activity of NF-κB. IKKα also phosphorylates IκBα at $Ser^{32/36}$, resulting in liberating NF-κB, which then translocates to nucleus. Our results show reduced levels of IKK after BITC treatment without affecting IκBα, indicating that the reduced phosphorylation of NF-κB is due to reduced IKKα. On the contrary, BITC treatment did not show any effect on NF-κB activation or expression in normal HPDE-6 cells. In fact, we did not observe any basal level of NF-κB phosphorylation in HPDE-6 cells. These results agree with previous studies, which suggest that NF-κB is activated in tumor cells but not in normal cells (40, 41). Following activation, NF-κB translocates to the nucleus where it binds to specific elements (κB sites) within the promoters of responsive genes to activate their transcription (10–12). We observed a decrease in nuclear translocation of NF-κB/p65 in BxPC-3 cells. Further, BITC treatment caused a drastic decrease in NF-κB/p65 transcriptional activity and κB DNA binding in both BxPC-3 and Capan-2 cells treated with BITC. We also observed that BITC treatment significantly reduced expression and promoter reporter activity of cyclin D1, a NF-κB–responsive gene, in both BxPC-3 and Capan-2 cells. NF-κB–mediated cyclin D1 regulation has been reported earlier (29, 37). It is important to point out that expression of cyclin D1 has been inversely correlated with the decreased median survival of patients with pancreatic cancer (42).

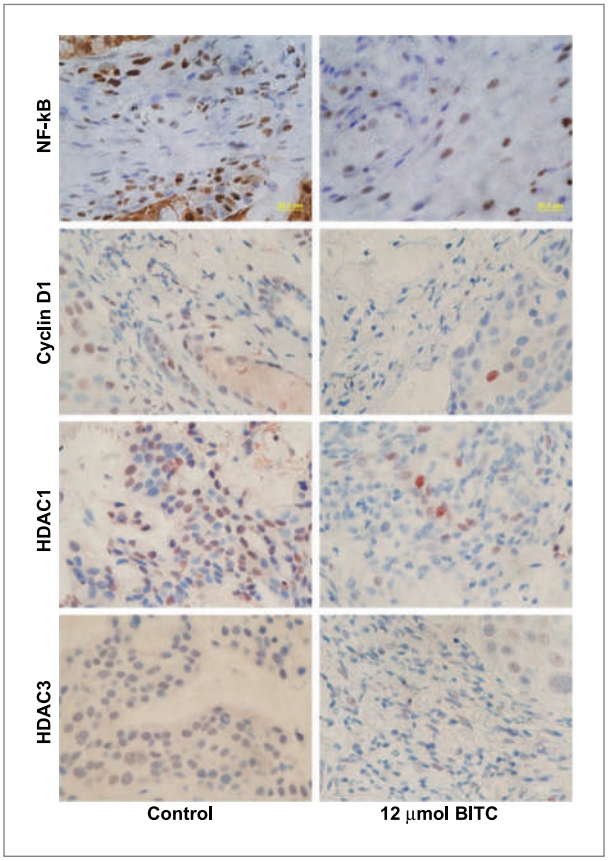


Figure 6. BITC treatment suppresses the expression of NF-κB, cyclin D1, HDAC1, and HDAC3 *in vivo*. Tumor sections from control and 12 μmol BITC-treated mice were analyzed for the expression of NF-κB, cyclin D1, HDAC1, and HDAC3 by immunohistochemistry. Each section was analyzed under the microscope at ×200 magnification.

Many signaling pathways and transcriptional factors are regulated by HDACs (22, 25, 29). Several studies have reported the inhibition of NF-κB transcriptional activity by HDAC inhibitors, although the mechanism seemed different in various cell lineages (43). On the other hand, activation of NF-κB by HDAC inhibitors has also been described in the literature (44, 45). Thus, HDACs are not only transcriptional activators but also transcriptional repressors. Previous studies suggested that NF-κB/p65 not only associates with but its transcriptional activity is also regulated by HDAC1, HDAC2, and HDAC3 (21, 22). Based on our results, we suggest that BITC inactivates NF-κB in BxPC-3 and Capan-2 cells by dephosphorylation, leading to its turnoff in BxPC-3 cells by modulating the action of coactivators and/or corepressors such as HATs and HDACs. p300/CBP plays a major role in acetylation of NF-κB/p65 at $Lys^{122}$ and $Lys^{123}$ residues by its intrinsic HAT activity (20). Our results show that BITC significantly repress the expression of p300/CBP in Capan-2 cells; however, its effect in BxPC-3 cells was of lower magnitude. We further observed that BITC treatment decreases the expression and activity of HDAC1 and HDAC3 in BxPC-3 cells and HDAC3 but not HDAC1 in Capan-2 cells. Differential inhibition of HDAC1/3 by BITC could be cell line specific, as both the cell lines differ from each other significantly. BxPC-3 cells have wild-type K-Ras and mutated p53, whereas Capan-2 cells harbor wild-type p53 and mutated K-Ras. HDAC1/3 inhibition by BITC in our model provides some indirect evidence that increased acetylation of lysine residues terminates NF-κB/p65 transcriptional activity. Acetylation of $Lys^{122}$ and $Lys^{123}$ is known to suppress the transcriptional activity of NF-κB by reducing its DNA binding (46, 47). Nonetheless, more studies are required to link the involvement of lysine residues and their acetylation in BITC-mediated inhibition of NF-κB activity. Regulation of NF-κB activity by HDAC inhibition has been shown by other anticancer agents (29, 43). Vorinostat (SAHA) is a well-established HDAC inhibitor and is

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currently used for the treatment of cutaneous T-cell lymphoma. Although SAHA reduced HDAC1/3, NF-κB, and cyclin D1 expression and reduced the viability of BxPC-3 cells, BITC treatment at similar concentrations had much pronounced effect and reduced the survival of BxPC-3 cells by ~95%, indicating the involvement of other pathways. Because HDAC inhibition is also associated with the induction of the cell cycle inhibitor $p21^{WAF1}$, its effect on normal cell proliferation and differentiation could be related to the antineoplastic effects of HDAC inhibitors (38, 39). In agreement, our results show the induction of $p21^{WAF1}$ by BITC treatment. Our results thus indicate that BITC-mediated differential posttranslational modification of NF-κB by HDACs adversely affects the growth of pancreatic cancer cells.

Overexpression of either HDAC1 or HDAC3 nevertheless increased NF-κB transcriptional activity, its κB DNA-binding capacity, cyclin D1 expression, and promoter reporter activity in BITC-treated pancreatic cancer cells. Studies have shown direct correlation between cyclin D1 expression and HDAC1 activity (29). Cyclin $D1^{-/-}$ mouse embryonic fibroblasts showed reduced HDAC1 activity, whereas its reconstitution resulted in increased HDAC1 activity. Furthermore, coimmunoprecipitation studies in cyclin $D1^{+/+}$ mouse embryonic fibroblasts revealed a direct association of both HDAC1 and HDAC3 with cyclin D1 (48). A recent study also revealed NF-κB–mediated transcriptional regulation of cyclin D1 and control of cell growth and differentiation in 10T1/2 fibroblasts (37). Studies are in progress to study the association of cyclin D1 with HDACs and NF-κB in pancreatic cancer cells using cyclin $D1^{-/-}$ mouse embryonic fibroblasts. Furthermore, overexpression of HDAC1 and HDAC3 led to a significant decrease in BITC-mediated caspase-3 and PARP cleavage. Our *in vivo* data also clearly indicate that oral administration of 12 μmol BITC significantly suppress the growth of BxPC-3 tumor xenografts (7) and that the tumor growth suppression was associated with the reduced expression of NF-κB, cyclin D1, HDAC1, and HDAC3, complementing our *in vitro* observations.

As the pharmacokinetics of BITC in humans has not been documented, it is difficult to predict how much cruciferous vegetable would need to be consumed to achieve serum concentration of 10 μmol/L BITC. However, a very recent study suggested that orally feeding male Sprague-Dawley rats with 10 or 100 μmol PEITC/kg (an analogue of BITC) resulted in the rapid absorption and reached peak concentration of 9.2 ± 0.6 and 42.1 ± 11.4 μmol/L PEITC, respectively, in the plasma after 0.44 ± 0.1 and 2.0 ± 1 hour of PEITC feeding, respectively, suggesting that micromolar concentrations may be achieved *in vivo* (49). In another pharmacokinetic study, four human volunteers were fed with a single dose of myrosinase hydrolyzed extract of 3-day-old broccoli sprouts (containing ~200 μmol total ITCs), and peak concentration of 0.94 to 2.27 μmol/L isothiocyanates was reached in the plasma, serum, and erythrocytes at 1 hour after broccoli extract ingestion (50). Nevertheless, a detailed pharmacokinetic study on BITC is required and is the focus of our laboratory.

Present *in vitro* and *in vivo* findings indicate that (*a*) BITC regulates NF-κB at multiple levels in pancreatic cancer cells, (*b*) the importance of HDAC1 and HDAC3 in regulating pancreatic cancer cell growth and differentiation, and (*c*) HDACs as the molecular target of BITC in human pancreatic cancer cells. Taken together, our results suggest BITC-mediated inhibition of HDAC1 and HDAC3 as a probable mechanism of NF-κB inactivation, leading to decreased expression of cyclin D1, thus resulting in the *in vitro* and *in vivo* growth suppression of pancreatic cancer cells.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Benzyl Isothiocyanate–Mediated Inhibition of Histone Deacetylase Leads to NF- κB Turnoff in Human Pancreatic Carcinoma Cells

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