Benzyl isothiocyanate induces protective autophagy in human prostate cancer cells via inhibition of mTOR signaling

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Benzyl isothiocyanate (BITC) is a dietary chemopreventive agent that inhibits the growth of various human cancer cells by causing apoptotic cell death. In this study, we demonstrate that BITC not only induces apoptosis but also induces autophagy in human hormone-sensitive (Rv1) and -refractory (PC3) prostate cancer cells. In BITC-treated cells, the induction of autophagy was detected by monitoring the processing of an autophagy marker protein, microtubule-associated protein 1 light chain 3 (LC3), the aggregation of LC3 into granular structures and the formation of acidic organelles. Inhibition of autophagy using 3-methyladenine increased BITC-induced apoptosis, whereas the administration of caspase inhibitor suppressed BITC-induced cell death. Our data also showed that BITC inhibits mammalian target of rapamycin (mTOR) kinase activity in a dose-dependent manner. The expression of phospho-mTOR (Ser2481), an indicator of mTOR intrinsic catalytic activity, and phospho-UNC-51-like kinase 1 (Ser757), a direct substrate of mTOR, were decreased in BITC-treated cells. However, the increased expression of phospho-mTOR (Ser2448), phospho-AKT (Ser473) and antiapoptotic Bcl-2 were detected only in PC3 cells at later stages of BITC treatment. Collectively, our results show that BITC induces a protective autophagy response in Rv1 and PC3 cells through inhibition of the mTOR signaling pathway. Activation of the AKT survival pathway was only observed in PC3 cells, representing a resistance mechanism of advanced prostate cancer upon BITC treatment. These findings could potentially contribute to the beneficial effect of BITC in prostate cancer treatments.

Introduction

Prostate cancer is among the most prevalent cancers in men worldwide and the leading cause of cancer-related deaths in American men (1,2). Despite long-term efforts to develop targeted therapies, the current effective treatment for hormone-sensitive prostate cancer mainly relies on hormonal therapy with luteinizing hormone-releasing hormone agonists and antandrogens (3). In advanced prostate cancers that are hormone resistant and metastatic, the only option is chemotherapy and hormonone agonists and antiandrogens (3). In advanced prostate cancers that are hormone resistant and metastatic, the only option is chemotherapy. Our data also showed that BITC inhibits mammalian target of rapamycin (mTOR) kinase activity in a dose-dependent manner. The expression of phospho-mTOR (Ser2481), an indicator of mTOR intrinsic catalytic activity, and phospho-UNC-51-like kinase 1 (Ser757), a direct substrate of mTOR, were decreased in BITC-treated cells. However, the increased expression of phospho-mTOR (Ser2448), phospho-AKT (Ser473) and antiapoptotic Bcl-2 were detected only in PC3 cells at later stages of BITC treatment. Collectively, our results show that BITC induces a protective autophagy response in Rv1 and PC3 cells through inhibition of the mTOR signaling pathway. Activation of the AKT survival pathway was only observed in PC3 cells, representing a resistance mechanism of advanced prostate cancer upon BITC treatment. These findings could potentially contribute to the beneficial effect of BITC in prostate cancer treatments.

Materials and methods

Chemicals

BITC (purity ~98%) was purchased from Sigma (St Louis, MO). The stock solution of BITC was prepared at a concentration of 10 mM in dimethyl sulfoxide (DMSO), and aliquots were stored at −20°C. Reagents for cell culture, including RPMI 1640 medium, the penicillin and streptomycin antibiotic mixture, fetal bovine serum, non-essential amino acids, sodium pyruvate and glucose supplemented media were obtained from Invitrogen (Carlsbad, CA). All other chemicals, unless otherwise mentioned, were purchased from Sigma.

Cell culture

The human prostate cancer cell lines were obtained from the American Type Culture Collection and maintained as described previously (30). Each cell line was cultured at 37°C in an atmosphere of 5% CO2. Reagents for cell culture, including RPMI 1640 medium, the penicillin and streptomycin antibiotic mixture, fetal bovine serum, non-essential amino acids, sodium pyruvate and glucose supplements were obtained from Invitrogen (Carlsbad, CA). All other chemicals, unless otherwise mentioned, were purchased from Sigma.

Cell viability assays

Cell viability upon BITC treatment was assayed by the WST-1 reagent (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instructions. For the proliferation studies performed with 3-methyladenine (3-MA; Sigma), an autophagy inhibitor, and Z-VAD-FMK (Promega, Madison, WI), a broad-spectrum caspase inhibitor, cells were pretreated with 1 mM 3-MA or 20 μM Z-VAD-FMK for 2 h then administered with BITC in the presence of inhibitors for the indicated duration. Cell viability was determined as the percentage of the control. Each condition was assayed in eight replicate wells, and data were obtained from at least three separate experiments.

Abbreviations: AO, acridine orange; AVO, acidic vesicular organelle; Baf A1, bafilomycin A1; BITC, benzyl isothiocyanate; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinases; ITC, isothiocyanate; LC3, microtubule-associated protein 1 light chain 3; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; PI, propidium iodide; ULC1, UNC-51-like kinase 1; ZA, zolendronic acid.

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Detection of apoptosis

BITC-induced apoptosis was assessed by flow cytometry analysis of subdiploid (sub-G₀/G₁) cells and the activation of caspase 3/7 activity. After BITC treatment, one million cells were fixed in 70% ethanol at 4°C overnight. Cells were then washed with ice-cold phosphate-buffered saline twice, pelleted and resuspended in 0.04 mg/ml propidium iodide (PI) and 100 mg/ml ribonuclease in phosphate-buffered saline. The DNA content per cell was determined using a FACSCalibur flow cytometer, and the data were analyzed with CellQuest Pro software (Becton Dickinson, San Jose, CA). Activation of caspase 3/7 was assayed using (Z-DEVD)-R110 substrate (Bachem, Torrance, CA) as described (32). Briefly, cells were plated in 96-well plates and allowed to attach by overnight incubation. The cells were pretreated 2 h with the indicated inhibitors and then treated with DMSO (control) or the desired concentrations of BITC. Subsequently, the cells were directly lysed by adding caspase 3/7 assay buffer containing (Z-DEVD)-R110 substrate and incubated at 37°C for 1 h. The fluorescent intensity of proteolytically released fluorophore R110 was then measured using a plate reader (Victor X2, PerkinElmer, Waltham, MA) with an excitation of 485 nm and emission of 535 nm.

Detection of autophagy

Immunoblotting and immunofluorescence of LC3. To perform immunoblotting analysis of microtubule-associated protein 1 light chain 3 (LC3), cells subjected to the indicated treatments were harvested and lysed, and the protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). The lysates were separated by 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) before probing with LC3 antibodies (#180202a, MAP1LC3B; Abgent, San Diego, CA). Subsequent immunoblotting procedures were performed using a chemiluminescence procedure (Millipore) as per the manufacturer’s instructions. To perform immunofluorescence detection of LC3, cells were seeded in chamber slides (Becton Dickinson) and subjected to the indicated treatments. After removal of the medium, cells were washed twice with phosphate-buffered saline and fixed with ice-cold methanol at −20°C, followed by permeabilization with 0.25% Triton X-100 at 4°C. The primary antibody to LC3 was incubated at 4°C overnight before incubation with the fluorescein isothiocyanate secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Analysis was performed using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with the appropriate excitation and emission filters.

Acridine orange staining for autophagy detection. Cells were seeded in six-well plates and treated with BITC for 24 h before acridine orange (AO) staining as described previously (30). In the bafilomycin A1 (Baf A1; Santa Cruz Biotechnology, Santa Cruz, CA) cotreatment study, cells were treated with 200 nm Baf A1 for 1 h prior to the addition of AO.

Quantification of acidic vesicular organelles with AO staining using flow cytometry. In AO-stained cells, the acidic compartments fluoresce bright red, and the intensity of the red fluorescence is proportional to the degree of acidity. Therefore, the volume of the cellular acidic compartment can be quantified. Cells were stained with AO for 1 h, removed from the plate with trypsin-ethylenediaminetetraacetic acid and collected in phenol red-free medium. Green (510–530 nm) and red (650 nm) fluorescence emission from 1 × 10⁵ cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur using CellQuest software (Becton Dickinson).

mTOR kinase assay. The activity of mTOR was measured with colorimetric K-LISA mTOR activity assay kit (Calbiochem, San Diego, CA). In brief, cells treated with BITC for 24 h were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol, 1% Tween 20, 1 mM ethylenediaminetetraacetic acid, 20 mM microcystin-LR, 25 mM LaF) supplemented with complete protease cocktail inhibitors (Roche Diagnostics). After centrifugation at 10,000 g for 20 min to eliminate debris, protein concentration in the lysates was determined by the BCA protein assay (Pierce) and adjusted to 2 mg/ml. mTOR proteins were immunoprecipitated with mTOR antibody and protein A/G-agarose from 0.5 ml of the total protein lysates. A p70S6K-glutathione-S-transferase fusion protein, as a specific mTOR substrate, and adenosine triphosphate were added to the immunoprecipitated complex. After phosphorylation of S6K70 at T389 by the active mTOR in the sample, the supernatant was added to the glutathione-coated wells. The phosphorylated p70S6K was then detected with anti-p70S6K–pT389 antibody, followed by detection with horseradish peroxidase antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology). The mTOR activity was measured in terms of absorbance in microplate reader.

Western blot analysis. The protein levels of cells treated with 20 µM BITC for the indicated periods of time were examined using western blot analysis as described for the immunoblotting for LC3. Antibodies against mTOR, phospho-mTOR (Ser2448), phospho-p70S6K (Ser389), and phospho-AKT (Ser473) were from Cell Signaling Technology (Beverly, MA). Antibodies against extracellular signal-regulated kinase 1 (ERK1) and phospho-ERK were from Santa Cruz Biotechnology and those against Bcl-2 and β-actin were from Sigma. The intensity of immunoreactive bands was determined using Genetools software (Syngene, Frederick, MD) after scanning the developed films. Results are expressed as mean ± SD of three independent experiments.

Statistical analysis. All experiments were performed at least in triplicate, and data are expressed as the means ± SD. The statistical significance of the difference in the measured variables between the treatment and control groups was determined by Student’s t-test and was considered significant at P < 0.05.

Results

BITC treatment decreased the viability of Rv1 and PC3 cells by inducing apoptosis

The viability of Rv1 and PC3 cells, which represent hormone-sensitive and -refractory prostate cancer cells, respectively, was tested after BITC treatment. BITC significantly inhibited the growth of both Rv1 and PC3 cells in a time- and dose-dependent manner, as shown in Figure 1A and B. After 72 h of incubation, the viability of Rv1 and PC3 cells treated with 20 µM BITC was 10.03±4.12% and 53.09±7.72% relative to the DMSO controls, respectively. These results demonstrated the greater cytotoxicity of BITC in Rv1 cells compared with PC3 cells. The BITC-mediated inhibition of Rv1 and PC3 cell viability was accompanied by an induction of apoptosis, as judged by the increased sub-G₀/G₁ cell population (Figure 1C and D; also see Supplementary Figure S1A, available at Carcinogenesis Online, for representative flow cytometry histograms). After 24 h, Rv1 and PC3 cells treated with 20 µM BITC showed an increased level of sub-G₀/G₁ cells (Figure 1E and F; and see Supplementary Figure S1B, available at Carcinogenesis Online, for representative flow cytometry histograms) and caspase 3/7 activity (Figure 1G and H) compared with the control. The increase in sub-G₀/G₁ cells as well as the activated caspase 3/7 by BITC was reversed by cotreatment with Z-VAD-FMK, a broad-spectrum caspase inhibitor, further indicating that cell cytotoxicity upon BITC treatment occurred through apoptosis induction.

BITC treatment induced autophagy in prostate cancer cells

Elevated levels of the LC3-II protein in Rv1 and PC3 cells treated with BITC. Several ITCs are known to induce autophagy in several types of cancer cells (33). We, therefore, sought to determine whether BITC treatment causes induction of autophagy in prostate cancer cells. Processing of the LC3-II protein is characteristic of activation of the autophagy pathway (31). Total protein lysates from Rv1 and PC3 cells treated with vehicle (−) or 20 µM BITC (+) for 6, 16 and 24 h were assessed for LC3-II formation with an autophagy LC3 antibody that detects both LC3-I and LC3-II (APG8b, N-term; Abgent). As shown in Figure 2A, the LC3-II level was elevated in BITC-treated Rv1 and PC3 cells as early as 6 h and continued accumulating up to 24 h. We further extended the treatment duration from 24 h to 3 days with 10 and 20 µM BITC (Figure 2B); the amount of LC3-II in Rv1 cells increased with the dosage of BITC and incubation time, but its level was even greater in PC3 cells treated with 20 µM BITC.

Aggregation of the LC3 protein into vesicular organelles in cells treated with BITC. To further analyze autophagosome formation, we detected LC3 localization using cell-based immunofluorescence studies on Rv1 and PC3 cells. In cells treated with 20 µM BITC for 24 h, the LC3 protein increased in total abundance and aggregated in granular vesicular structures (Figure 2C). Different cell types have been shown to exhibit varying degrees of basal autophagy (28). Therefore, autophagosomes were quantified in control (DMSO) and BITC-treated cells to determine the fraction of cells exhibiting increased levels of LC3 aggregation, as shown in Figure 2D. The results demonstrated that BITC treatment significantly elevated the cellular abundance of autophagosomes (26.37% versus 1.3% in Rv1 cells and 43.52% versus 0.91% in PC3 cells).

BITC treatment caused the formation of acidic vesicular organelles. Another characteristic of autophagy is the formation of acidic...
vesicular organelles (AVOs), which can be detected by vital staining using AO (30,31,34). As depicted in Figure 2E and F, vital staining of Rv1 and PC3 cells with AO showed the accumulation of AVOs in the cytoplasm of cells treated with 20 µM BITC (Figure 2, E2 and F2). This result further confirmed that the autophagic process was activated by BITC treatment. Baf A1 is a H+ adenosine triphosphatase inhibitor that prevents the transition of autophagosomes to autophagolysosomes by disrupting lysosome fusion (34). The accumulation of AVOs induced by BITC treatment was markedly reduced with 2 h pretreatment of Baf A1 in BITC-treated cells (Figure 2, E4 and F4). Moreover, the Baf A1 treatment also caused the accumulation of LC3-II in BITC-treated cells (Figure 2G). Taken together, these results indicate that BITC treatment resulted in the formation of autophagolysosomes via autophagosomes fusion with lysosomes. In order to further confirm that BITC causes autophagy induction, the cells treated with BITC were analyzed for AVO-positive populations in a concentration- and time-dependent manner. As shown in Figure 2H and I (also see Supplementary Figure S2A, available at Carcinogenesis Online, in the supplemental material for representative flow cytometry histograms), AVO-positive cells were increased in cells treated with BITC from 2.5 to 20 µM; however, at the dose of 40 µM, the AVO-positive cells were decreased. When treated with 20 µM BITC, the AVO-positive cells were increased with treatment duration (Figure 2I and K; Supplementary Figure S2B, available at Carcinogenesis Online, for the representative flow cytometry histograms).

**Autophagy inhibited BITC-induced apoptosis**

We next raised the question whether induction of autophagy affects BITC-induced apoptosis. 3-MA, a known inhibitor of class III PI3-kinase (31), was employed to address this question. The decreased level of LC3-II was detected in BITC-treated cells with 2 h pretreatment of 1 mM 3-MA (Figure 3A and B), indicating that pretreatment of 3-MA inhibited BITC-induced autophagy. The effects of 3-MA on BITC-induced apoptosis were determined by the flow cytometry analysis of sub-G0/G1 cells stained with PI and the activation of caspase 3/7 activity. The results for Rv1 and PC3 cultures treated for 24 h with either DMSO or 20 µM BITC in the presence or absence of 3-MA are shown in Figure 3. BITC treatment caused a nearly 3-fold increase in the percentage of apoptotic cells with sub-G0/G1 content within both the Rv1 and PC3 (Figure 3C and D, respectively; also see Supplementary Figure S3, available at Carcinogenesis Online, for the representative flow cytometry histograms) cell populations compared with the DMSO-treated control. BITC-induced apoptosis was exacerbated by the presence of 3-MA, whereas 3-MA treatment alone did not cause an accumulation of subdiploid cells. The percentage of sub-G0/G1 cells increased in the Rv1 and PC3 cell populations by 1.34- and 1.39-fold, respectively, upon cotreatment of cells with BITC and 3-MA compared with BITC treatment alone. The caspase 3/7 activities were also higher in the presence of 3-MA compared with BITC treatment alone (Figure 3E and F). The data presented above indicate that BITC treatment induces both apoptosis and autophagy, and BITC-induced autophagy inhibits apoptosis in prostate cancer cells. We next evaluated whether the induction of autophagy in BITC-treated cells actually contributes to cell death by cotreating with 3-MA or Z-VAD-FMK to inhibit autophagy or apoptosis, respectively. As shown in Figure 4, the viability of Rv1 (Figure 4A) and PC3 (Figure 4B) cells treated with 20 µM BITC for 24 h decreased to 42.86±5.71% and 63.68±2.03% compared with the DMSO-treated controls. Treatment with 1 mM 3-MA or 20 µM Z-VAD-FMK alone slightly decreased and increased, respectively, the viability of both Rv1 and PC3 cells. Cotreatment of BITC with 3-MA decreased cell viability from 42.86±5.71% to 32.05±3.92% and 63.68±2.03% to 50.05±2.02% in Rv1 and PC3 cells, respectively. Cotreatment of BITC with Z-VAD-FMK nearly restored cell viability.

**BITC treatment inhibited mTOR signaling in prostate cancer cells**

It is now well known that mTOR is a regulator of autophagy in cells under starvation or various other forms of cellular stress (35). Inhibition of mTOR increases the expression of autophagy-related...
BITC induces protective autophagy by mTOR inhibition

Genes (Atgs), such as Atg8 (LC3), and the kinase activity of Atg1 (36). Both are key components in autophagy. We, therefore, detected mTOR kinase activity in cells treated with different doses of BITC. The mTOR kinase activity was decreased at 24 h post-BITC treatment in a dose-dependent manner. Moreover, the inhibition of kinase activity was more profound in PC3 compared with Rv1 cells (Figure 5A and B). We next detected the expression of mTOR, phospho-mTOR (Ser2448 and Ser2481), ULK1, phospho-ULK1 (Ser757) in cells treated with 20 µM of BITC. As shown in Figure 5C and D, a time course study using cell lysates from BITC-treated Rv1 and
PC3 cells for 6–72 h showed inhibition of mTOR signaling. BITC treatment slightly increased level of phospho-mTOR (Ser2481), an indicator of mTOR intrinsic catalytic activity, in Rv1 cells at 6 and 12 h, and then the expression started to decline at 24–72 h. In PC3 cells, the expression level of phospho-mTOR (Ser2481) was decreased at all time points tested, suggesting the inhibition of mTOR activity upon BITC treatment (Figure 5E and F). The expression level ULK1 was decreased in both cells treated with BITC, and the expression
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In previous studies, BITC showed cytotoxic effects in several cultured cell lines (16–23). Our data demonstrated that treatment with BITC affected cell viability and induced apoptosis in Rv1 and PC3 cells (Figure 1). BITC-induced autophagy resulted in the formation of LC3-II in Rv1 and PC3 cells (Figure 1). BITC-induced autophagy formed autophagic vacuoles (AVOs) and aggregated LC3-II, suggesting that BITC triggers protective autophagy in human prostate cancer cells.

Autophagy is an important cellular defense mechanism in normal development (40). In prostate cancer cells, several ITCs, such as sulforaphane (31) and phenethyl isothiocyanate (41), were reported to induce not only apoptosis but also autophagy. Recent studies also demonstrated that BITC induces mitochondria-dependent apoptosis in DU145 prostate cancer cells (42), but whether BITC induces autophagy in DU145 was not investigated. Because the induction of autophagy is considered a defense mechanism against sulforaphane-induced apoptosis in prostate cancer cells (31), elucidation of BITC-induced autophagy in prostate cancer cells may provide useful information on this dietary chemopreventive agent and benefit its future application.

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**Discussion**

This study provides the first experimental evidence of autophagy induction in human prostate cancer cells treated with BITC. BITC-mediated autophagy in Rv1 and PC3 cells was characterized by the induction of LC3-II followed by the recruitment of processed LC3-II to autophagosomes and the formation of AVOs. Inhibition of autophagy using specific inhibitors, 3-MA, increased apoptosis and failed to rescue the cell death caused by BITC treatment, suggesting that BITC triggers protective autophagy in human prostate cancer cells.

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**Fig. 5. Inhibition of mTOR signaling in BITC-treated Rv1 and PC3 cells.** The mTOR kinase activity was detected in Rv1 (A) and PC3 (B) cells treated with 0, 10, 20 and 40 μM BITC for 24 h. Immunoblotting for mTOR (mTOR-Total), phospho-mTOR (p-mTOR, Ser 2448), phospho-mTOR (p-mTOR, Ser 2481), ULK1, phospho-ULK1 (p-ULK1, Ser 757), ERK 1/2 (ERK), phospho-ERK 1/2 (p-ERK) and β-actin (as a loading control) was performed using lysates from Rv1 (C) and PC3 (D) cells cultured in the presence of 20 μM BITC for the indicated times. The relative expression level of phospho-mTOR (Ser2448), phospho-mTOR (Ser2481) and phospho-ULK was quantitated by densitometric scanning and is presented as the percentages of 0 h control in (C) and (D) for Rv1 and PC3 cells, respectively. The statistical calculation from blots of three independent experiments is shown. The results are presented as the means ± SD; *P < 0.05.

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**Table 1.** Inhibition of mTOR signaling in BITC-treated Rv1 and PC3 cells. The mTOR kinase activity was detected in Rv1 (A) and PC3 (B) cells treated with 0, 10, 20 and 40 μM BITC for 24 h. Immunoblotting for mTOR (mTOR-Total), phospho-mTOR (p-mTOR, Ser 2448), phospho-mTOR (p-mTOR, Ser 2481), ULK1, phospho-ULK1 (p-ULK1, Ser 757), ERK 1/2 (ERK), phospho-ERK 1/2 (p-ERK) and β-actin (as a loading control) was performed using lysates from Rv1 (C) and PC3 (D) cells cultured in the presence of 20 μM BITC for the indicated times. The relative expression level of phospho-mTOR (Ser2448), phospho-mTOR (Ser2481) and phospho-ULK was quantitated by densitometric scanning and is presented as the percentages of 0 h control in (C) and (D) for Rv1 and PC3 cells, respectively. The statistical calculation from blots of three independent experiments is shown. The results are presented as the means ± SD; *P < 0.05.
We started with the same concentration of 3-MA to inhibit autophagy was administered to prostate cancer cells treated with 100 µM BITC (Figure 3A and B). The results showed an increase in the percentage of sub-G₀/G₁ cells and caspase 3/7 activity (30), inhibition of LC3-II formation was achieved when 1 mM 3-MA was used in Rv1 and PC3 cells, suggesting that autophagy is the main pathway in BITC-induced cell death.

mTOR is considered a key regulator in autophagy induction and serves as a defensive mechanism in cells under starvation or stress (35). Therefore, we examined the mTOR activity in BITC-treated cells. The mTOR activity was decreased in both Rv1 and PC3 cells in a concentration-dependent manner (Figure 5A and B). The expression of phospho-mTOR (Ser2481) was decreased in BITC-treated Rv1 and PC3 cells at 24–72 h. The expression of phospho-ULK1 (Ser757) was decreased in BITC-treated Rv1 cells. However, the expression of phospho-mTOR (2448) and phospho-ULK1 (Ser757) was increased in PC3 cells at later stages of BITC treatment (Figure 5C–F). Phosphorylation of ULK1 at Ser757 by mTOR under nutrient sufficiency has been reported (37); however, we did not observe a correlated expression of phospho-mTOR (Ser2481) with phospho-ULK1 (Ser757) in BITC-treated cells. It is possible that other unknown pathways affected by BITC may interfere with the activation of ULK1 and need further investigation. Activation of ERK 1/2 has been shown to stimulate autophagy (44,45), we did not observe ERK 1/2 activation in BITC-treated cells (Figure 5A and B). These data suggest that BITC-induced autophagy occurs through mTOR inhibition.

We noticed that BITC exhibited superior cytotoxicity in Rv1 cells compared with PC3 cells, and the inhibition of mTOR in PC3 cells was reduced at later stages of BITC treatment. PI3K-Akt-mTOR is well known as a cancer survival pathway, and a recent study demonstrated that inhibition of mTOR in DU145 cells causes activation of AKT kinase (39). We, therefore, investigated the status of AKT phosphorylation in a time course study. The results indicated decreased and increased p-AKT in Rv1 and PC3 cells, respectively, upon exposure to BITC (Figure 6). The expression level of the Bcl-2 and active caspase 3 proteins showed an increasing and decreasing trend, respectively, in Rv1 cells, which was consistent with apoptosis that detected by the increment of sub-G₀/G₁ cells.
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In PC3 cells, the level of Bcl-2 increased at 24–72 h of BITC treatment, which also correlated with the decreased level of active caspase 3. Inhibition of mTOR was demonstrated to increase upstream receptor tyrosine kinase signaling and activate AKT in metastatic prostate cancer cells (39). The activation of AKT in later stages of BITC-treated PC3 cells may, therefore, represent a defensive mechanism of advanced prostate cancer cells upon anticancer drug treatment. However, further investigation using a specific inhibitor of AKT activation is needed to elucidate the relationship between AKT activation and BITC resistance in PC3 cells.

In conclusion, this study showed that BITC has anticancer activity in human prostate cancer cells through the induction of apoptotic cell death. BITC also induces early protective autophagy pathways by inhibiting mTOR signaling in these cells regardless of their androgen sensitivity. However, the hormone-refractory PC3 cells showed greater resistance to BITC treatment, which was related to the on-set activation of AKT. These data provide basic mechanisms for the chemotherapeutic properties of BITC in prostate cancer cells and show that advanced prostate cancer may induce prosurvival signals to evade BITC treatment.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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