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Inhibition of mitochondrial fusion is an early and critical event in breast cancer cell apoptosis by dietary chemopreventative benzyl isothiocyanate

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Abstract

Benzyl isothiocyanate (BITC) is a highly promising phytochemical abundant in cruciferous vegetables with preclinical evidence of *in vivo* efficacy against breast cancer in xenograft and transgenic mouse models. Mammary cancer chemoprevention by BITC is associated with apoptotic cell death but the underlying mechanism is not fully understood. Herein, we demonstrate for the first time that altered mitochondrial dynamics is an early and critical event in BITC-induced apoptosis in breast cancer cells. Exposure of MCF-7 and MDA-MB-231 cells to plasma achievable doses of BITC resulted in rapid collapse of mitochondrial filamentous network. BITC treatment also inhibited polyethyleneglycol-induced mitochondrial fusion. In contrast, a normal human mammary epithelial cell line (MCF-10A) that was derived from fibrocystic breast disease, was resistant to BITC-mediated alterations in mitochondrial dynamics as well as apoptosis. Transient or sustained decrease in levels of proteins engaged in regulation of mitochondrial fission and fusion was clearly evident after BITC treatment in both cancer cell lines. A trend for a decrease in the levels of mitochondrial fission- and fusion-related proteins was also observed *in vivo* in tumors of BITC-treated mice compared with control. Immortalized mouse embryonic

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fibroblasts from Drp1 knockout mice were resistant to BITC-induced apoptosis when compared with those from wild-type mice. Upon treatment with BITC, Bak dissociated from mitofusin 2 in both MCF-7 and MDA-MB-231 cells suggesting a crucial role for interaction of Bak and mitofusins in BITC-mediated inhibition of fusion and morphological dynamics. In conclusion, the present study provides novel insights into the molecular complexity of BITC-induced cell death.

Keywords

benzyl isothiocyanate; mitochondrial dynamics; apoptosis; breast cancer; chemoprevention

1. Introduction

Epidemiological studies have consistently argued for the possibility of breast cancer risk reduction with increasing dietary intake of cruciferous vegetables (Suzuki et al., 2013; Liu and Lv, 2013). For example, a recent population-based prospective cohort study of 47,289 Japanese women suggested a statistically significant inverse association between intake of cruciferous vegetables and the risk of breast cancer in premenopausal subjects (95% confidence interval = 0.38-1.10; p -trend = 0.046) (Suzuki et al., 2013). Similar association was also suggested in a meta-analysis of prior epidemiological data (Liu and Lv, 2013). Organic isothiocyanates (ITCs) generated after cutting or chewing of the cruciferous vegetables are believed to be responsible for the anticancer effects of this class of dietary plants (Fahey et al., 2001). Even though >100 glucosinolate precursors of ITCs have been identified in different plants, benzyl isothiocyanate (BITC) is one of the best studied member of this class of cancer chemopreventive phytochemicals (Wattenberg, 1977). BITC and its close structural analogue phenethyl isothiocyanate as well as a synthetic compound (phenyl isothiocyanate) all inhibited 7,12-dimethylbenz[*a*]anthracene-induced mammary tumor development in female Sprague-Dawley rats when administered 4 hours before carcinogen treatment providing evidence for inhibition of cancer initiation (Wattenberg, 1977). BITC administration after 1 week of carcinogen challenge also exerted an inhibitory effect on chemically-induced breast cancer development in rats providing evidence for post-initiation cancer chemoprevention efficacy (Wattenberg, 1981). Our laboratory is the first to demonstrate BITC-mediated chemoprevention of breast cancer in a transgenic mouse model as well as *in vivo* suppression of MDA-MB-231 xenograft growth in athymic mice (Warin et al., 2009; Warin et al., 2010). Using mouse mammary tumor virus-*neu* (MMTV-*neu*) transgenic mouse model, we demonstrated a significant decrease in cumulative incidence of mammary hyperplasia and carcinoma upon feeding of BITC-supplemented diet (Warin et al., 2009). Consistent with our findings, oral administration of BITC resulted in a significant decrease in growth of 4T1 mouse breast cancer cells implanted in mammary fat pad of syngeneic Balb/c mice (Kim et al., 2011a). A reduction in multiplicity and burden of pulmonary metastasis upon BITC treatment was also observed in this study (Kim et al., 2011a). Other noteworthy anticancer properties of BITC in breast cancer cells include repression of estrogen receptor- α expression, inhibition of oncogenic actions of leptin, and suppression of epithelial to mesenchymal transition (Kang et al., 2009; Kim et al., 2011b; Sehrawat and Singh, 2011; Sehrawat et al., 2013). Inhibition of epithelial to mesenchymal transition may partly explain anti-metastatic effect of BITC (Kim et al., 2011a; Sehrawat and

Singh, 2011; Sehrawat et al., 2013). More recent studies from our laboratory have also demonstrated BITC-mediated inhibition of breast cancer stem cells *in vitro* and *in vivo* (Kim et al., 2013). Together, these observations provide compelling *in vitro* and *in vivo* evidence for anti-neoplastic effect of BITC in cellular and animal models of breast cancer.

BITC is an interesting small molecule that induces both apoptotic and autophagic cell death in human breast cancer cells (Xiao et al., 2006; Xiao et al., 2008; Kim and Singh, 2010; Antony et al., 2012; Xiao et al., 2012). Breast cancer cells are also sensitive to apoptosis induction *in vivo* after BITC administration (Warin et al., 2009; Kim et al., 2011a). For example, tumors from MMTV-*neu* transgenic mice fed BITC-supplemented diet exhibited up to 2.5-fold increase in apoptotic bodies when compared with tumors of mice on basal diet (Warin et al., 2009). While autophagy induction by BITC is regulated by Forkhead BoxO1 (Xiao et al., 2012), molecular complexity of BITC-induced apoptotic cell death is still not fully resolved. Nevertheless, prior cellular studies have revealed a critical role for multidomain proapoptotic proteins Bax and Bak in apoptosis induction by BITC (Xiao et al., 2006). Bax and Bak deficiency confers near complete protection against BITC-induced apoptosis in immortalized mouse embryonic fibroblasts (Xiao et al., 2006). Because the Bcl-2 family proteins play a critical role in regulation of mitochondrial morphology and dynamics (Brooks and Dong, 2007), the present study was designed to answer the question of whether apoptotic cell death induction by BITC was associated with altered mitochondrial dynamics (fission and/or fusion). Well-characterized human breast cancer cell lines (MCF-7 and MDA-MB-231), a normal human mammary epithelial cell line (MCF-10A), and tumor tissues from control and BITC-fed MMTV-*neu* mice were utilized to address this question.

2. Materials and methods

2.1. Reagents

BITC (purity >98%) was purchased from LKT laboratories (St. Paul, MN). Reagents for cell culture such as fetal bovine serum, culture media, and antibiotic mixture were purchased from Invitrogen-Life Technologies (Carlsbad, CA). Sources of the antibodies were as follows: anti-phospho (S616) dynamin-1-like protein (Drp1), anti-total Drp1, anti-mitofusin (Mfn) 2, and anti-cleaved caspase 3 antibodies were from Cell Signaling Technology (Beverly, MA); anti-Mfn1, anti-mitochondrial fission 1 protein (Fis1), anti-Bak and anti-Bax antibody was from Santa Cruz Biotechnology (Dallas, TX); anti-optic atrophy 1 (Opa1) antibody was from Abcam (Cambridge, MA); anti-active Bax (for immunoprecipitation) antibodies were from BD Biosciences (San Jose, CA); anti-actin antibody was from Sigma Aldrich (St. Louis, MO); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from GeneTex (Irvine, CA); and anti-active Bak (for immunoprecipitation) antibody was from Calbiochem (Billerica, MA). MitoTracker green and Hoechst 33342 were purchased from Invitrogen-Life Technologies whereas 4',6-diamidino-2-phenylindole (DAPI) was from Sigma-Aldrich. Annexin V-FITC Apoptosis Detection kit was purchased from BD Biosciences. Polyethylene glycol (PEG) 1500 was purchased from Roche Life Sciences (Indianapolis, IN). The pAc-green fluorescence protein (GFP)1-Mito and

pDsRed2-Mito plasmids were kindly provided by Prof. Bennett van Houten (University of Pittsburgh, Pittsburgh, PA).

2.2. Cell lines

MDA-MB-231, MCF-7 and MCF-10A cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured according to the supplier's recommendations. MDA-MB-231 and MCF-7 cells stably transfected with mitochondria targeting pAc-GFP or pDsRed2 plasmids were cultured in medium supplemented with 100 µg/mL G418. Mouse embryonic fibroblasts (MEF) from wild-type (Drp1^{+/+}) and Drp1-deficient (Drp1^{-/-}) mice were a generous gift from Dr. K. Mihara (Ishihara et al., 2009). Immortalized mouse embryonic fibroblasts derived from wild-type (WT), Bak knockout (Bak^{-/-}) and Bax and Bak double knockout (DKO) mice were generously provided by the late Dr. Stanley J. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA) and maintained, as described by us previously (Xiao et al., 2006).

2.3. Apoptosis assay

Apoptosis was quantified by flow cytometry using Annexin V-FITC kit. Manufacturer's protocol was followed for this assay. Briefly, after treatment with dimethyl sulfoxide (DMSO) or BITC, the cells were harvested by trypsin treatment and washed with phosphate-buffered saline (PBS). Equal number of cells from each group (1×10^5) were suspended in 100 µL of binding buffer and stained in dark with 4 µL of Annexin V-FITC and 2 µL of propidium iodide solution for 30 minutes at room temperature. Samples were then diluted with 200 µL of binding buffer, and stained cells were analyzed using a BD AccuriTM C6 Flow Cytometer.

2.4. Confocal microscopy

Desired cells (mtGFP-MDA-MB-231 or mtGFP-MCF-7) were cultured on Lab-Tek II chamber slides (Sigma-Aldrich), allowed to attach, and then exposed to DMSO (control) or 2.5 or 5 µM BITC for 1-, 2- or 4 hours. In MCF-10A cells, direct labelling of mitochondria was achieved by staining live cells with the fluorescent probe MitoTracker green (200 nM for 30 minutes). After washing with PBS, cells were fixed with 4% paraformaldehyde for 20 minutes, and nuclear DNA was stained with DAPI. Confocal images were acquired at randomly selected fields using a Nikon A1 confocal microscope at 60× or 100× objective magnification. Percentage of mitochondrial network area per cell was quantified in a total of 50-100 cells in 6-9 fields per slide using Nikon NIS-Elements software.

2.5. Live cell confocal microscopy

Live cell imaging was performed in an environmentally controlled FCS2 live cell chamber (37°C temp and 5% CO₂) (Bioprotech, Butler, PA) system using a confocal microscope (Nikon A1, Nikon) controlled by NIS-Elements software. Prior to imaging, 2×10^5 cells were plated on 35 mm glass bottom microwell dish (Mat-Tek, Ashland, MA) and incubated for 24 hours. Cells were then incubated for 2-3 minutes with Hoechst 33342 in complete media to label nuclei. The dishes were then immediately assembled into a heated chamber and time-lapse imaging was initiated immediately. The Z stacks images of selected cells were

acquired every 5 minutes using excitation at 488 nm (for GFP) and 403.8 nm (for Hoechst 33342). Fluorescence was captured through an Plan Apo VC 60× oil immersion objective, NA = 1.40 (Nikon) by using perfect focus system to correct possible focus drift during time lapse imaging. Culture medium containing BITC was used, and the temperature of the chamber was maintained at 37°C during the imaging. GFP excitation was kept as low as possible to avoid photo-destruction of the cell. For all studies, 3-6 fields per dish and duplicate dishes per condition were evaluated in three independent experiments. Images were processed using MetaMorph (Molecular Devices, Sunnyvale, CA), and Adobe Photoshop.

2.6. Quantitative analysis of PEG-induced mitochondrial fusion

For PEG-induced mitochondrial fusion assay, 1×10^5 mtGFP-MDA-MB-231 or mtGFP-MCF-7 cells were co-plated with the same number of mito-DsRed2 expressing MDA-MB-231 or MCF-7 cells onto glass coverslips in 12 well plates. Cycloheximide (20 µg/mL) was added 30 minutes before fusion and kept in all solutions used subsequently to inhibit *de novo* synthesis of proteins. Cells were then washed with serum free medium and incubated with pre-warmed solution of PEG 1500 (50% wt/vol) for 1 minute at room temperature. After extensive washing with medium containing 10% serum, cells were treated with DMSO or BITC (2.5 or 5 µM) for 6 hours. Next, the cells were fixed for 30 minutes with ice-cold 4% paraformaldehyde in PBS, stained with DAPI and mounted onto glass slides. Randomly selected heterokaryons were acquired using confocal microscope (Nikon A1) from which the percentage of co-localized fluorescence was calculated using MetaMorph software and expressed as mitochondrial fusion percentage.

2.7. Western blotting

Control and BITC-treated cells were processed for immunoblotting as described by us previously (Xiao et al., 2003). Tumor tissues from MMTV-*neu* mice archived from our previous study (Warin et al., 2009) were also used for western blotting. Supernatants from MMTV-*neu* tumors for immunoblotting were prepared as described previously (Powolny et al., 2011). Probing with anti-actin or anti-GAPDH antibody was performed to correct for protein loading differences. Some actin or GAPDH bands may be common in different blots due to multiplexing. Densitometric quantitation was done using UN-SCAN-IT software version 5.1 (Silk Scientific Corporation, Orem, Utah).

2.8. Immunoprecipitation-immunoblotting

MDA-MB-231 or MCF-7 cells were treated with 2.5 or 5 µM BITC for 6 hours and lysed using a solution containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 1% CHAPS, and protease inhibitor cocktail. Aliquots containing 500 µg of whole cells lysate protein in 0.5 mL of lysis buffer were incubated overnight at 4°C with 2 µg anti-active Bax monoclonal antibody (clone 6A7) or 1 µg anti-active Bak monoclonal antibody (clone TC100). A 40 µL aliquot of protein G-agarose beads (Santa Cruz Biotechnology) was then added to each sample and the incubation was continued for 2 hours at 4°C. The immunoprecipitated complexes were washed thrice with lysis buffer and subjected to gel electrophoresis followed by immunoblotting using Mfn1, Mfn2 and polyclonal anti-Bax or anti-Bak antibody.

3. Results

3.1. BITC-induced apoptosis in breast cancer cells was associated with disruption of mitochondrial integrity

Initially, we determined the kinetics of BITC-induced apoptosis at plasma achievable doses of 2.5 and 5 μ M. Dose-dependent apoptosis after treatment with BITC was evident at 4- and 6-hour time points in both MDA-MB-231 (Fig. 1A) and MCF-7 cells (Fig. 1B). Confocal microscopy revealed normal filamentous mitochondrial network in DMSO-treated control cells that was severely disrupted (mitochondrial fragmentation) after BITC treatment in both cell lines (Fig. 1C).

Next, we determined the impact of BITC treatment on mitochondrial integrity by quantitation of mitochondrial surface area using cells stably transfected with mitochondria-targeted GFP (mtGFP-MDA-MB-231 and mtGFP-MCF-7). Representative confocal images of mitochondrial network in fixed mtGFP-MDA-MB-231 and mtGFP-MCF-7 cells without or with BITC treatment are shown in Fig. 2A and 2B, respectively. The mitochondrial network surface area was decreased significantly after 1-, 2- or 4-hour treatment with 2.5 or 5 μ M BITC compared with vehicle-treated control cells (Fig. 2C,D). In a follow-up experiment, the effect of BITC treatment on mitochondrial morphology was studied by time-lapse live cell confocal microscopy at 5 minute intervals. Data from time-lapse analysis of live mtGFP-MDA-MB-231 (Fig. 2E) and mtGFP-MCF-7 cells (data not shown) in the presence of BITC from different experiments revealed that mitochondrial fragmentation typically occurred by ~10 minutes post-BITC treatment. Collectively, these results indicated that BITC-induced apoptosis in breast cancer cells was preceded by disruption of mitochondrial network.

3.2. BITC treatment inhibited PEG-induced mitochondrial fusion

Mitochondrial fragmentation during apoptosis may be the result of increased fission and/or decreased fusion (Suen et al., 2008; Westermann, 2010; van der Bliek et al., 2013). Mitochondrial fusion allows the mixing of matrix contents of different mitochondria (van der Bliek et al., 2013). We proceeded to quantify the effect of BITC treatment on mitochondrial fusion activity in breast cancer cells. Representative images exemplifying PEG-induced fusion in MDA-MB-231 and MCF-7 cells are shown in Fig. 3A and Fig. 3B, respectively. Merging of green and red fluorescence is indicative of mitochondrial fusion (enlarged images of selected mitochondria are shown in the right hand panel of Fig. 3A and 3B). There was a dose dependent and significant decrease in the fusion activity after treatment with BITC in both MDA-MB-231 (up to 50% decrease compared with DMSO-treated control) and MCF-7 cells (up to 37% decrease compared with DMSO-treated control) (Fig. 3C). Based on these observations, we conclude that BITC treatment inhibits mitochondrial fusion in breast cancer cells.

3.3. A normal mammary epithelial cell line was resistant to BITC-mediated disruption of mitochondrial network and apoptosis

The MCF-10A cell line was derived from fibrocystic breast pathology and spontaneously immortalized. This cell line is non-tumorigenic in athymic mice and a well-characterized

representative of normal mammary epithelial cells. Unlike breast cancer cells (Fig. 2A,B), BITC failed to alter mitochondrial network surface area in MCF-10A cells (Fig. 4A,B). Consistent with our prior observations (Xiao et al., 2006), the MCF-10A cell line was also resistant to apoptosis induction by BITC (Fig. 4C). These results indicated that disruptive effect of BITC on mitochondrial integrity was cancer cell-selective at least for breast cancer.

3.4. Effect of BITC treatment on expression of proteins involved in mitochondrial fission and fusion

We determined the effect of BITC treatment on levels of proteins involved in regulation of mitochondrial dynamics (Hoppins et al., 2007; van der Blik et al., 2013; Lennon and Salgia, 2014). Levels of p-S616 Drp1 and Fis1 were decreased markedly after treatment with 2.5 and 5 μ M BITC in both cell lines (Fig. 5). Transient (MDA-MB-231) or sustained (MCF-7) decrease in total Drp1 protein level was also observed (Fig. 5). Levels of Mfn1 and Mfn2 proteins were decreased markedly upon 2-, 4- and 6-hour treatment with BITC in both MDA-MB-231 and MCF-7 cells (Fig. 5). We also found accumulation of short isoform of Opa1 that was accompanied by a loss in levels of long isoform at all time points (Fig. 5). These results indicated that BITC negatively targeted both fission and fusion machinery of mitochondrial dynamics in breast cancer cells.

3.5. BITC-induced apoptosis was significantly attenuated by Drp1 deficiency

A role for Drp1 in apoptosis regulation has been established previously (Inoue-Yamauchi and Oda, 2012). Therefore, we further evaluated the role of this protein in proapoptotic effect of BITC by using MEF from wild-type (Drp1^{+/+}) and Drp1 knockout (Drp1^{-/-}) mice (Fig. 6A). BITC treatment for 2 hours resulted in dose-dependent cleavage of caspase 3, a marker of apoptosis, in Drp1^{+/+} MEF that was not observed in Drp1^{-/-} MEF (Fig. 6A). Fig. 6B shows representative flow histograms for Annexin V-FITC-positive and propidium iodide-negative (early apoptotic cells) and Annexin V-FITC-positive and propidium iodide-positive (late apoptotic cells) fractions in Drp1^{+/+} and Drp1^{-/-} MEF cultures after 2-hour treatment with DMSO or BITC. Consistent with caspase 3 activation data (Fig. 6A), BITC treatment (1, 2.5 or 5 μ M for 2 hours) caused a dose-dependent and significant increase in the apoptotic fraction in Drp1^{+/+} MEF (Fig. 6C). Moreover, in agreement with published data (Inoue-Yamauchi and Oda, 2012), Drp1 deficiency modestly increased apoptosis in the absence of BITC. However, the BITC-induced apoptosis in Drp1^{-/-} MEF was significantly lower when compared with Drp1^{+/+} MEF at least at the 5 μ M dose (Fig. 6C). Taken together, these data indicate that Drp1 plays a critical role in BITC-induced apoptosis.

3.6. Bak and/or Bax deficiency conferred protection against BITC-induced alterations in mitochondrial dynamics regulating proteins

The Bcl-2 family proteins are critical regulators of mitochondrial integrity during apoptosis (Brooks and Dong, 2007). Because our prior work revealed a critical role for Bax and Bak in BITC-induced apoptosis (Xiao et al., 2006), it was of interest to determine if the changes in mitochondrial dynamics regulating proteins were affected by Bax and/or Bak status. To address this question, we utilized SV-40 immortalized MEF from wild-type (WT) mice, Bak knockout (Bak^{-/-}) mice, and from Bax and Bak double knockout (DKO) mice. BITC-mediated decrease in levels of p-S616 phosphorylated Drp1 and total Drp1 was abolished in

Bak^{-/-} and DKO cells (Fig. 7). Both Bak^{-/-} and DKO MEF exhibited a marked increase in Fis1 protein expression compared with WT MEF in the absence of BITC treatment. However, BITC-mediated downregulation of Fis1 protein was maintained in Bak^{-/-} and DKO MEF (Fig. 7). On the other hand, BITC-mediated downregulation of Mfn1 or Mfn2 was partially reversed in DKO. Consistent with breast cancer cells, BITC treatment caused accumulation of Opa1 short isoform and loss of long isoform in WT MEF. These effects were partly reversible in Bak^{-/-} and nearly fully abrogated in DKO MEF. These observations provide experimental evidence for involvement of Bak and Bax in BITC-mediated changes in proteins involved in regulation of mitochondrial dynamics.

3.7. BITC treatment affected interaction of Bak/Bax with Mfn1/2

Bak and Bax are known to regulate mitochondrial dynamics (Karbowksi et al., 2006; Brooks and Dong, 2007; Hoppins et al., 2011). For example, during apoptosis Mfn2 dissociates from Bak but its interaction with Mfn1 is increased. To explore the possibility of whether BITC treatment affects Bak/Bax interaction with Mfn isoforms, we immunoprecipitated active-Bak or active-Bax using monoclonal antibodies and then performed immunoblotting using anti-Mfn1, anti-Mfn2, anti-Bak or anti-Bax antibody. BITC treatment for 6 hours resulted in increased interaction of Bak with Mfn1 in MDA-MB-231 and MCF-7 cells, which was accompanied by a decrease in Bak-Mfn2 interaction (Fig. 8A). Increased interaction between Bak and Mfn1 can't be attributed to increased expression or difference in immunoprecipitation of the Bak protein. Increased interaction of Bak with Mfn1 was observed at 2.5 μ M without any increase in Bak protein expression or immunoprecipitation (Fig. 8A). Effect of BITC on Bax and Mfn interaction was ambiguous and inconsistent in different experiments (Fig. 8B). These results demonstrated alterations in Bak interaction with Mfn1/2 upon BITC treatment in breast cancer cells.

3.8. Mammary cancer chemoprevention by BITC administration was associated with downregulation of fission and fusion protein expression *in vivo*

We have shown previously that dietary BITC (3 μ mol BITC/kg diet) administration prevents mammary cancer development *in vivo* in MMTV-*neu* mouse model without causing weight loss or any other side effects (Warin et al., 2009). We used remaining archived tumor tissues to test whether expression of fission and fusion-regulating proteins was affected by BITC administration. Immunoblots for fission and fusion-regulating proteins using tumor supernatants from control and BITC-fed mice (n=3 for both) are shown in Fig. 8C. Protein levels of Drp1, Fis1, and Mfn1 were clearly decreased in tumors of BITC-treated mice compared with control although the difference was not significant due to small sample size (Fig. 8D). The results were visually less clear for Mfn2 and Opa1. Nevertheless, these results provided *in vivo* evidence for a trend for BITC-mediated downregulation of proteins involved in regulation of mitochondrial dynamics.

4. Discussion

The present study reveals that BITC-induced apoptosis in breast cancer cells is evident as early as 4 hour post-treatment. The primary route of metabolism of ITCs, including BITC is their urinary excretion *via* mercapturic acid pathway (Conaway et al., 2002). A

pharmacokinetic study in humans showed that BITC metabolites, including BITC-*N*-acetylcysteine, BITC-cysteine, BITC-cysteinylglycine and/or BITC-glutathione could be detected in plasma for up to 6 hours after ingestion of glucosinolate precursor of BITC as 10 g of freeze-dried nasturtium plant (*Tropaeolum majus*, commonly known as Indian cress) (Platz et al., 2013). Levels of these metabolites ranged between 0.19 and 2.61 μM for BITC-cysteinylglycine and between 0.14 and 1.25 μM for BITC-*N*-acetyl-L-cysteine (Platz et al., 2013). Interestingly, BITC-*N*-acetylcysteine was shown to retain anticancer response in cancer cells *in vitro* (Tang et al., 2006). The mean concentration of BITC in plasma of mice after 1 hour of oral treatment with 12 μmol BITC approached 6.5 μM (Boreddy et al., 2011). While metabolite levels were not measured in this study, BITC was also detectable in the tumor xenografts (7.5 $\mu\text{mol/g}$) after 46 days of treatment (Boreddy et al., 2011). Even though pharmacokinetics of BITC in humans is yet to be determined, it is reasonable to conclude that BITC can trigger apoptosis within the pharmacokinetic window at least in breast cancer cells *in vitro*.

Continual fission and fusion of mitochondria, which are the cellular powerhouse, is essential for their integrity and consequently normal physiology (Detmer and Chan, 2007). Mitochondrial dynamics is also implicated in apoptosis regulation (Brooks and Dong, 2007; Suen et al., 2008). For example, Opa1 oligomerization has been shown to inhibit mitochondrial cristae remodeling required for cytochrome *c* release (Arnoult et al., 2005; Frezza et al., 2006; Yamaguchi et al., 2008). In general, mitochondrial fusion is suggested to inhibit apoptosis whereas mitochondrial fission serves to promote release of apoptogenic molecules and hence apoptosis (Brooks et al., 2007; Boland et al., 2013). In cells after apoptosis triggered by external stimuli, the normal mitochondrial filamentous network changes to fragmented (punctate and spherical) shape either due to increased fission or inhibition of fusion. The present study reveals that BITC treatment not only disrupts mitochondrial filamentous network but also inhibits their fusion. We propose that these changes in mitochondrial dynamics represent a critical event in BITC-induced apoptosis in breast cancer cells as mitochondrial integrity is not affected in BITC-treated MCF-10A cells, which are also resistant to apoptosis induction by this agent. Moreover, changes in mitochondrial integrity are evident very early after BITC exposure.

The role of regulatory components of mitochondrial dynamics in cancer is still unresolved but Drp1 is upregulated in invasive breast carcinoma and lymph node metastasis (Zhao et al., 2013a). Drp1, which is the primary regulator of mitochondria fission, is a GTPase residing in the cytosol but recruited to the fission sites in mitochondrial membrane (Lennon and Salgia, 2014). Drp1 is phosphorylated at multiple sites including S616 (Lennon and Salgia, 2014). Phosphorylation of Drp1 at S616 is mediated by cyclinB/cyclin dependent kinase 1 complex (Taguchi et al., 2007). Our prior work coupled with this study suggests that Drp1 may be a critical target in anticancer effects of BITC in breast cancer cells, including apoptosis induction, G₂/M phase cell cycle arrest, and inhibition of cell migration. This conclusion is based on the following findings: (a) BITC decreases protein level and S616 phosphorylation of Drp1 (present study); (b) BITC-mediated G₂/M phase cell cycle arrest in MDA-MB-231 and MCF-7 cells is accompanied by downregulation of cyclinB/cyclin-dependent kinase complex (Xiao et al., 2006); (c) BITC-induced apoptosis is significantly attenuated in Drp1 deficient mouse embryonic fibroblasts (present study); and (d) BITC

inhibits cell migration in MDA-MB-231 and MCF-7 cells (Kim et al., 2012) and Drp1 silencing in breast cancer cells reduces their capacity to migrate *in vitro* (Zhao et al., 2013a).

Mitochondrial fusion, which is clearly inhibited after BITC treatment in MDA-MB-231 and MCF-7 cells, is regulated by dynamin-related GTPase Mfn1, Mfn2, and Opa1 (Hoppins et al., 2007; van der Bliek et al., 2013; Lennon and Salgia, 2014;). While Mfn1 and Mfn2 are required for fusion of outer mitochondrial membrane, Opa1 regulates the fusion of inner membrane (Hoppins et al., 2007; van der Bliek et al., 2013; Lennon and Salgia, 2014). Expression of all the protein is decreased upon BITC treatment in both MDA-MB-231 and MCF-7 cells. Consistent with these observations, PEG-induced fusion is also decreased in BITC-treated breast cancer cells. Activity of Opa1 itself is controlled by splice variants and proteolytic cleavage resulting in short and long forms of the protein (Lee et al., 2004; Song et al., 2007; Lennon and Salgia, 2014). The current mechanistic model suggests that mitochondrial fusion depends upon correct ratio of long and short isoforms of Opa1. The GTPase activity of Opa1 is regulated by interaction of long and short isoforms with each other (Escobar-Henriques and Anton, 2013). The short Opa1 isoform is associated with apoptosis induction (Yamaguchi et al., 2008; Lennon and Salgia, 2014). An imbalance in long and short isoforms of Opa1 is clearly evident in BITC-treated cells. The faster migrating short form is increased while long Opa1 level is decreased in BITC-treated breast cancer cells. Opa1 is also involved in regulation of apoptosis by clinically-used anticancer agents. For example, Opa1 downregulation is associated with sorafenib-induced apoptosis in hepatocellular carcinoma cells (Zhao et al., 2013b). In lung adenocarcinoma cells, Opa1 overexpression confers cisplatin resistance through inactivation of caspase-dependent apoptosis (Fang et al., 2012). It is interesting to note that BITC sensitizes different types of cancer cells to cisplatin (Di Pasqua et al., 2010; Lee et al., 2012; Wolf and Claudio, 2014), which may be partly attributable to change in Opa1 short and long isoform ratio, and possibly inhibition of mitochondrial fusion. Further work is needed to explore this possibility.

During apoptosis, changes in mitochondrial fusion-fission are reported to occur downstream of Bak and Bax, resulting in the loss of mitochondrial membrane potential and release of cytochrome *c* and activation of the intrinsic pathway of apoptosis (Brooks et al., 2007). In our experimental settings, BITC treatment also changes the interactions of Bak with Mfn1 and Mfn2. We saw that in the presence of BITC, Bak dissociates from Mfn2 and increase its association with Mfn1 in both MDA-MB-231 and MCF-7 cells. In healthy cells, Bak interacts with both Mfn1 and Mfn2.

In conclusion, the present study is the first to implicate changes in mitochondrial dynamics in BITC-induced apoptosis. Furthermore, we provide evidence to indicate that the BITC mediated inhibition of mitochondrial fusion activity is regulated by multidomain proapoptotic proteins Bax and Bak in breast cancer cells.

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Abbreviations

ANOVA	analysis of variance
BITC	benzyl isothiocyanate
DAPI	4',6-diamidino-2-phenylindole
DKO	mouse embryonic fibroblasts isolated from Bak and Bax double knockout mice
DMSO	dimethyl sulfoxide
Drp1	dynamamin related protein 1
Fis1	mitochondrial fission 1 protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescence protein
ITCs	isothiocyanates
Mfn	mitofusin
MEF	mouse embryonic fibroblasts
MMTV-<i>neu</i>	mouse mammary tumor virus- <i>neu</i>
PEG	polyethylene glycol
Opa1	optic atrophy 1
PBS	phosphate-buffered saline
WT	wild-type

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Fig. 1.
BITC-induced apoptosis in human breast cancer cells is associated with collapse of mitochondrial integrity. Quantitation of apoptotic fraction (early + late apoptotic cells) in MDA-MB-231 (A) and MCF-7 cells (B) after 4- or 6-hour treatment with DMSO (control) or BITC (2.5 or 5 μM). Percentage of apoptosis is shown as mean ± SD (n=3). *Significant ($p < 0.05$) compared with control by one-way analysis of variance (ANOVA) followed by Dunnett's adjustment. C, Representative images showing mitochondrial morphology in mtGFP-MDA-MB-231 and mtGFP-MCF-7 cells without or with 4 hour treatment with BITC. The letter "n" signifies nucleus.

Fig. 2.

BITC treatment decreases mitochondrial surface area in breast cancer cells. Representative images showing mitochondrial network in mitoGFP-MDA-MB-231 (A) and mitoGFP-MCF-7 (B) cells after 1-, 2- and 4-hour treatment with DMSO (control) or BITC (2.5 or 5 μ M). Quantitation of percentage of mitochondrial surface/cell in mitoGFP-MDA-MB-231 (C; n=9) and mitoGFP-MCF-7 cells (D; n=8). Results shown are mean $\pm$ SD. *Significant ($p < 0.05$) compared with control by one-way ANOVA with Dunnett's adjustment. E, Live cell images of mitochondria in mitoGFP-MDA-MB-231 cells after addition of 2.5 μ M BITC.

In this experiment, time-lapse images were acquired at 5-minute intervals over a 2-hour period. Data shown in Fig. 2E are at 30 minute interval (for first image after initial captured at 15 seconds) followed by images at 15 minute intervals.

Fig. 3. BITC treatment inhibits PEG-induced mitochondrial fusion. MDA-MB-231 and MCF-7 cells stably expressing mitochondria-targeted DsRed2 or GFP were used for PEG fusion assay. Representative images from one of the two independent experiments are shown for, MDA-MB-231 (A) or MCF-7 (B) cells. Enlarged images are shown in the right panel for each cell line. C, Quantitation of BITC-mediated inhibition of mitochondrial fusion relative to DMSO-treated control. Percentage of co-localized fluorescent signal was determined in randomly selected heterokaryons in multiple fields with the use of the MetaMorph software.

Results shown are mean $\pm$ SD. *Significant ($p < 0.05$) compared with control by one-way ANOVA with Dunnett's adjustment.

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Fig. 4. Mitochondrial integrity is not disrupted by BITC treatment in the MCF-10A cell line. A, Representative confocal images depicting mitochondria (green color) in MCF-10A cells without or with BITC treatment (2.5 μ M for 6 hours). The nuclei were visualized by DAPI staining (blue color). B, Percentage of mitochondrial network surface/cell after 6-hour treatment of MCF-10A cells with DMSO or BITC. At least 100 cells from three different slides were analyzed. C, Quantitation of apoptotic fraction (early + late apoptotic cells) in MCF-10A cells after 4- and 6-hour treatment with DMSO (control) or BITC (2.5 or 5 μ M).

Percentage of apoptosis is shown as mean $\pm$ SD (n=3). Similar results were obtained from replicate experiments.

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Fig. 5.

Effect of BITC treatment on levels of proteins involved in regulation of mitochondrial fission and fusion. Western blotting for p-Drp1 (S616), Drp1, Fis1, Mfn1, Mfn2, and Opa1 proteins using lysates from MDA-MB-231 and MCF-7 cells after 2-, 4- or 6-hour treatment with DMSO or BITC (2.5 or 5 μM). Western blotting for each protein was performed at least twice using independently prepared lysates and the results were comparable.

Fig. 6. MEF from Drp1 knockout mice are resistant to BITC-induced apoptosis. A, Immunoblotting for Drp1 and cleaved caspase 3 using lysates from Drp1^{+/+} and Drp1^{-/-} MEF treated for 2 hours with DMSO or BITC. B, Representative flow histograms depicting apoptotic fraction in Drp1^{+/+} and Drp1^{-/-} MEF after 2-hour treatment with DMSO or BITC (5 μM). C, Percentage of apoptotic fraction (early + late apoptotic cells) from the experiments shown in panels A and B (mean ± SD; n=3). Significant ($p < 0.05$) compared with (*) respective

DMSO-treated control and (\$) between Drp1^{+/+} and Drp1^{-/-} MEF by one-way ANOVA followed by Bonferroni's correction.

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Fig. 7. Critical role of Bak and Bax in BITC mediated changes in mitochondrial dynamics related proteins. Immunoblotting for p-Drp1(S616), Drp1, Fis1, Mfn1, Mfn2, and Opa1 proteins using lysates from SV40 immortalized MEF derived from wild-type (WT) mice, Bak knockout (BAK^{-/-}) mice and Bak and Bax double knockout (DKO) mice after 24 hour treatment with DMSO or the indicated concentrations of BITC.

Fig. 8.

BITC treatment affects interaction of Bak with Mfn proteins. Immunoprecipitation/immunoblotting for interaction of Bak with Mfn1 and Mfn2 (A) and interaction of Bax with Mfn1 and Mfn2 (B) in MDA-MB-231 or MCF-7 cells after 6-hour treatment with DMSO or BITC (2.5 or 5 μ M). Whole cell lysates were subjected to immunoprecipitation using anti-activeBak or anti-active Bax antibody. The resultant immunoprecipitates were subjected to immunoblotting with Mfn1, Mfn2, and anti-polyclonal Bak or Bax antibody. C, Western blotting for Drp1, Fis1, Mfn1, Mfn2, and Opa1 proteins using tumor lysates from control

and BITC-treated MMTV-*neu* mice. D, Quantitation of the band intensity of proteins in panel C (n=3).

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