Research Article

p53-Independent Apoptosis by Benzyl Isothiocyanate in Human Breast Cancer Cells Is Mediated by Suppression of XIAP Expression

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Abstract

We have shown previously that cruciferous vegetable constituent benzyl isothiocyanate (BITC) suppresses viability of cultured MCF-7 and MDA-MB-231 human breast cancer cells and retards mammary cancer development in MMTV-neu mice by causing apoptosis, but the mechanism of cell death is not fully understood. We now show that whereas p53 is dispensable for BITC-induced cell death, proapoptotic response to this promising chemopreventive agent is mediated by suppression of X-linked inhibitor of apoptosis (XIAP) protein expression. The BITC treatment increased levels of total and Ser15-phosphorylated p53 protein in MCF-7 cells, but the proapoptotic response to this agent was maintained even after knockdown of the p53 protein level. Exposure of MCF-7 and MDA-MB-231 cells to BITC resulted in a marked decrease in protein level of XIAP as early as 8 hours after treatment. Ectopic expression of XIAP conferred statistically significant protection against BITC-mediated cytoplasmic histone-associated apoptotic DNA fragmentation in both cell lines. Moreover, inhibition of MDA-MB-231 cell growth in vivo in female athymic mice by BITC administration correlated with a modest but statistically significant decrease in XIAP protein level in the tumor xenograft. The BITC treatment also resulted in induction as well as nuclear translocation of survivin only in the MCF-7 cells. The BITC-induced apoptosis was modestly but statistically significantly augmented by RNA interference of survivin in MCF-7 cells. In conclusion, the present study provides novel insight into the molecular circuitry of BITC-induced apoptosis to indicate suppression of XIAP expression as a critical mediator of this process.

Introduction

Despite remarkable progress toward screening efforts and targeted therapies (1, 2), breast cancer continues to be a leading cause of mortality among women around the world (3, 4). Risk factors implicated in mammary carcinogenesis include family history, Li-Fraumeni syndrome, atypical hyperplasia of the breast, late age at first full-term pregnancy, and early menarche and late menopause (5–7). Because some of these risk factors are not easily modifiable, other strategies to reduce mortality associated with this malignancy are attractive. This objective is partially fulfilled with the advent of selective estrogen receptor modulators such as tamoxifen and raloxifene, but this approach is ineffective against estrogen receptor-negative breast cancers (8–10). Moreover, long-term administration of selective estrogen receptor modulators inherits risk of severe side effects, including increased risk of uterine cancer, thromboembolism, and perimenopausal symptoms (8–10). Therefore, agents that are relatively safe but could offer protection against mammary carcinogenesis regardless of the estrogen receptor status are desirable. Dietary plants have attracted increasing momentum in recent years for the discovery of potential cancer chemopreventive agents (11, 12).

Benefit of a diet rich in cruciferous vegetables (e.g., broccoli, watercress, and garden cress) as a modifier of the breast cancer risk can be appreciated by the results of population-based case-control studies. A case-control study involving >300 breast cancer cases and matched controls showed an inverse association between urinary levels of isothiocyanates (ITC) as a biological indicator of cruciferous intake and the risk of breast cancer (13). Broccoli consumption was shown to be inversely correlated with the risk of mammary cancer in premenopausal women in another population-based case-control study (14). Antineoplastic effect of cruciferous vegetables is ascribed to organic ITCs that occur naturally as thioglucoside conjugates (15). Benzyl ITC (BITC) is one such compound that seems promising for prevention of mammary cancer (16). For example, our own work has revealed that dietary administration of 3 mmol BITC/kg
diet inhibits mammary hyperplasia and carcinoma incidence and/or burden in MMTV-neu mice without causing weight loss or any other side effects (16). The BITC-mediated prevention of mammary cancer in MMTV-neu mice correlated with suppression of cellular proliferation, increased apoptosis, and enhanced infiltration of T cells in the carcinoma (16).

Previous studies, including those from our laboratory, have shown that BITC treatment effectively inhibits growth of cultured human breast cancer cells by causing apoptotic cell death (17–20). Interestingly, a spontaneously immortalized nontumorigenic human mammary epithelial cell line (MCF-10A) is highly resistant to growth inhibition and apoptosis induction by BITC in comparison with breast cancer cells (17). We also showed that the molecular circuitry of BITC-induced apoptosis in human breast cancer cells entails production of reactive oxygen species due to inhibition of complex III of the mitochondrial respiratory chain (20). Interestingly, a spontaneous suppression of BITC treatment is dispensable for BITC-induced cell death.

Western blotting and detection of apoptosis

After treatment with DMSO (control) or the desired concentrations of BITC for specified time intervals, floating and attached cells were collected and lysed as described by us previously (21). Cytosolic and nuclear fractions from control and BITC-treated cells were prepared using a kit from Pierce. Immunoblotting was done as previously described by us (17, 20, 21). Apoptosis induction was measured by analysis of cytoplasmic histone-associated DNA fragmentation (22).

RNA interference

The MDA-MB-231 (5 × 10⁴) and MCF-7 (1.5 × 10⁵) cells were seeded in six-well plates and transfected at ~50% confluency with 100 nmol/L of p53- or survivin-targeted siRNA using Oligofectamine. Twenty-four hours after transfection, the cells were treated for 24 hours with either DMSO (control) or 5 μmol/L BITC. Subsequently, the cells were collected and processed for immunoblotting and measurement for cytoplasmic histone-associated DNA fragmentation.

Ectopic expression of XIAP by transient transfection

The MDA-MB-231 (5 × 10⁴) and MCF-7 (1.5 × 10⁵) cells were transiently transfected at ~50% confluency with the empty pcDNA3.1 vector or pcDNA3.1 encoding for XIAP (Addgene) using FuGENE6 transfection reagent. Twenty-four hours after transfection, the cells were treated with DMSO (control) or BITC for specified time period. Cells were collected and processed for immunoblotting and measurement for apoptosis.

Immunohistochemical analysis of XIAP expression in MDA-MB-231 xenografts

We have shown previously that BITC administration retards growth of MDA-MB-231 cells implanted in female athymic mice (23). We used tumor sections from the same study to determine the effect of BITC administration on expression of XIAP protein by immunohistochemistry. Briefly, mice were randomized into two groups of five mice per group. The mice were injected i.p. with either vehicle or vehicle containing 7.5 μmol BITC per mouse on Monday to Friday for 2 weeks before tumor cell injection (23). Exponentially growing MDA-MB-231 cells were suspended in PBS and mixed in a 1:1 ratio with Matrigel. A 0.1-mL suspension containing 2.5 × 10⁸ cells was injected s.c. on flank of each mouse above the hind limb. A portion of the tumor tissue removed from the control and BITC-treated mice was fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 4- to 5-μm thickness. Representative tumor sections from control and BITC-treated mice were processed for H&E staining and immunohistochemical analysis of XIAP expression as described previously by us for other proteins (16, 24, 25). At least four nonoverlapping representative images of each tumor section from five mice of each group were captured and analyzed using Image-Pro Plus 5.0 software for quantitation of XIAP expression.
Reverse transcription-PCR
Total RNA from MCF-7 and MDA-MB-231 cells treated for 16 hours with DMSO (control) or BITC (2.5 and 5.0 μmol/L) was extracted with the use of the RNeasy kit (Invitrogen). The cDNA was synthesized with the use of 1 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen) with oligo(dT) primer. PCR was done with specific primers (survivin forward primer, 5′-AGGACGGCCCTCTTGGAGG-3′; survivin reverse primer, 5′-CTTTTTATGTCTTCTATGGGGTC-3′) with the use of the following amplification conditions: 94°C for 2 minutes, 40 cycles at 94°C for 15 seconds, at 60°C for 20 seconds, and at 68°C for 15 seconds. Human GAPDH was used as a control as in our previous studies (26).

Immunocytochemical localization of survivin
The MCF-7 cells were cultured on coverslips and treated with DMSO (control) or 5 μmol/L BITC for 16 hours. The cells were treated with 200 nmol/L MitoTracker Red at 37°C for 30 minutes to stain mitochondria. After washing with PBS, the cells were fixed with 4% parafomaldehyde and permeabilized using 0.1% Triton X-100. The cells were incubated with anti-survivin antibody overnight at 4°C. The cells were then washed with PBS and incubated with Alexa Fluor 488–conjugated secondary antibody (1:2,000 dilution; Molecular Probes) for 1 hour at room temperature. After washing, cells were stained with 4′,6-diamidino-2-phenylindole (10 ng/mL) for 5 minutes at room temperature. The cells were visualized using a Leica DC300F fluorescence microscope.

Statistical analysis
Each experiment was repeated at least twice with triplicate measurements for quantitative comparisons. Statistical significance of difference in measured variables between control and treated groups was determined by t test or one-way ANOVA. Difference was considered significant at P < 0.05.

Results

p53 tumor suppressor was dispensable in BITC-induced apoptosis
We have shown previously that BITC treatment causes apoptotic cell death in MCF-7 cell line, which expresses wild-type p53 (17). In the present study, we used the same cell line to test whether BITC-induced apoptosis was influenced by p53, which is a well-accepted facilitator of apoptotic cell death by different stimuli (27). As can be seen in Fig. 1A, BITC exposure caused a concentration- and time-dependent increase in the levels of total as well as Ser15-phosphorylated p53. The Ser15 phosphorylation of p53 has been implicated in apoptosis (27). Next, we used siRNA technology to directly test involvement of p53 in regulation of BITC-induced apoptosis. Transient transfection of MCF-7 cells with a p53-targeted siRNA resulted in complete silencing of the p53 protein expression (Fig. 1B). Moreover, the BITC-mediated induction of p53 protein was abolished in MCF-7 cells transfected with the p53-specific siRNA (Fig. 1B). However, enrichment of cytoplasmic histone-associated apoptotic DNA fragmentation resulting from BITC exposure (5.0 μmol/L for 24 h) over DMSO-treated control was comparable in MCF-7 cells transfected with the control nonspecific siRNA and p53-targeted siRNA (Fig. 1C). Collectively, these results
indicated that the BITC-induced apoptosis was not influenced by the \( p53 \) status at least in the MCF-7 cell line.

**BITC treatment downregulated expression of XIAP protein**

IAP proteins, including cIAP1, XIAP, and survivin, have emerged as critical regulators of apoptotic cell death \((28–30)\). We raised the question of whether the BITC-induced apoptosis in breast cancer cells was accompanied by alterations in expression of IAP family proteins. The level of cIAP1 protein was increased on BITC treatment in both MCF-7 and MDA-MB-231 cell lines, especially at the 5.0 \( \mu \)mol/L concentration, but this effect was not sustainable (Fig. 2). For example, BITC-mediated induction of cIAP1 protein was nearly completely abolished at the 24-hour time point in the MCF-7 cell line. The XIAP protein expression was markedly decreased on treatment of MCF-7 and MDA-MB-231 cells with BITC at both 2.5 and 5.0 \( \mu \)mol/L concentrations. The BITC-mediated decline in XIAP protein level was evident as early as 8 hours after treatment, and this effect was maintained for the duration of the experiment (24 h after treatment) in both cell lines (Fig. 2). The BITC treatment elicited a cell line–specific response on survivin protein expression (Fig. 2). The BITC treatment resulted in upregulation of survivin protein in the MCF-7 cells especially at the 16- and 24-hour time points (Fig. 2). The BITC-mediated induction of survivin in MCF-7 cells was preceded by \( \sim 40\% \) decrease in its level at the 8-hour time point. On the other hand, the BITC treatment did not have an appreciable effect on survivin protein level in the MDA-MB-231 cell line. Collectively, these results indicated that BITC treatment caused a sustained decrease in the level of XIAP protein in both cell lines and caused induction of survivin selectively in the MCF-7 cells.

**Ectopic expression of XIAP conferred protection against BITC-induced apoptosis**

Because BITC treatment exhibited the most striking effect on XIAP protein expression in both cell lines (Fig. 2), we designed experiments to determine functional significance of these observations. Transient transfection of MCF-7 cells with a vector encoding for XIAP resulted in \( \sim 2\)-fold increase in its protein level compared with empty vector–transfected cells (Fig. 3A). The BITC treatment (5.0 \( \mu \)mol/L for 24 h) caused a decrease in the level of XIAP protein both in empty vector–transfected MCF-7 cells and in XIAP-overexpressing cells (Fig. 3A). In addition, overexpression of XIAP conferred significant protection against BITC-induced cytoplasmic histone-associated DNA fragmentation in MCF-7 cells (Fig. 3B). Statistically significant inhibition of BITC-induced cytoplasmic histone-associated DNA fragmentation by forced overexpression of XIAP was also observed in the MDA-MB-231 cell line (Fig. 3C and D). These results indicated that XIAP was a target of BITC-induced apoptosis in breast cancer cells.

**BITC-mediated inhibition of MDA-MB-231 cell growth in vivo correlated with suppression of XIAP expression in the tumor xenograft**

We have shown previously that BITC administration significantly retards growth of MDA-MB-231 cells implanted in female athymic mice without causing weight loss or any other side effects \((23)\). For example, 50 days after tumor cell injection, the average tumor volume in vehicle-treated
control mice (1581 ± 240 mm³) was approximately 2.5- to 3-fold higher compared with mice i.p. administered with 2.5 and 7.5 μmol BITC, five times per week (23). In the present study, we used tumor sections from the same experiment to determine if BITC-mediated growth inhibition of MDA-MB-231 cells in vivo was accompanied by suppression of XIAP expression. Figure 4 depicts H&E staining and immunohistochemical analysis for XIAP expression in representative MDA-MB-231 tumor section of a control mouse and a 7.5 μmol BITC–treated mouse. Tumors from BITC-treated mice exhibited a modest but statistically significant decrease in XIAP protein expression compared with control tumors (Fig. 4). These observations indicated that, similar to cultured cells, BITC administration caused suppression of XIAP protein level in the MDA-MB-231 xenografts in vivo.

**BITC treatment caused nuclear translocation of survivin in MCF-7 cells**

Reverse transcription-PCR was done to determine if the BITC-mediated induction of survivin in MCF-7 cells (Fig. 2) was due to transcriptional upregulation. As can be seen in Fig. 5A, exposure of MCF-7 cells to 2.5 and 5.0 μmol/L BITC for 16 hours resulted in a marked increase in the levels of survivin mRNA. Consistent with the results of immunoblotting, BITC-mediated increase in survivin mRNA was not observed in the MDA-MB-231 cells (Fig. 5A).

Checkpoint kinase 2 (Chk2)–dependent release of survivin from mitochondria has been shown to counteract cell death in tumor cells induced by chemotherapeutic agents (31). We next asked whether BITC treatment affected survivin localization by activating Chk2. This was a strong possibility in light of our previous findings showing activation of Chk2 by sulforaphane, a structural analogue of BITC (32). Initially, we explored this possibility by determining the effect of BITC treatment (2.5 and 5.0 μmol/L for 8, 16, and 24 h) on activating phosphorylation of Chk2 (Thr68). Treatment of MCF-7 cells with 2.5 and 5.0 μmol/L BITC resulted in increased Thr68 phosphorylation of Chk2 at 16- and 24-hour time points (results not shown). We used immunocytochemistry to determine localization of survivin in control (DMSO, 16 h) and BITC-treated MCF-7 cells (5.0 μmol/L, 16 h). As shown in Fig. 5B, a small fraction of cells exhibited mitochondrial localization of survivin in DMSO-treated control MCF-7 cells (identified by an arrow), as judged by merging of the mitochondria-associated red fluorescence and survivin-associated green fluorescence around 4′,6-diamidino-2-phenylindole–stained nuclei (blue color). Nuclear localization of survivin was also observed in some DMSO-treated control MCF-7 cells. Interestingly, BITC treatment resulted in a robust increase in nuclear as well as cytosolic levels of survivin in comparison with DMSO-treated control (Fig. 5B). We confirmed these observations by immunoblotting of survivin using isolated cytosolic and nuclear fractions prepared from control (DMSO, 16 h) and BITC-treated MCF-7 cells (5.0 μmol/L, 16 h). In agreement with the results of...
immunocytochemistry (Fig. 5B), the level of survivin protein was increased in both cytosolic and nuclear fractions on treatment of MCF-7 cells with BITC (Fig. 5C). The blots were stripped and reprobed with anti-poly(ADP-ribose) polymerase and anti-α-tubulin antibodies to normalize for differences in protein loading as well as to rule out cross-contamination of cytosolic and nuclear fractions (Fig. 5C). These results indicated that BITC treatment caused an increase in cytosolic and nuclear levels of survivin in MCF-7 cells.

**Survivin knockdown modestly augmented BITC-induced apoptosis in MCF-7 cells**

Next, we proceeded to experimentally test functional significance of survivin induction in proapoptotic response
to BITC using MCF-7 cells. The MDA-MB-231 cell line was included as a negative control. Similar to untransfected cells (Fig. 2), exposure of nonspecific siRNA-transfected MCF-7 cells to 5.0 μmol/L BITC for 24 hours resulted in ∼3-fold increase in survivin protein level compared with DMSO-treated control (Fig. 6A). The level of survivin protein was decreased by >90% in MCF-7 cells transfected with a survivin-targeted siRNA (Fig. 6A). Moreover, the BITC-mediated induction of survivin protein expression was nearly completely abolished in survivin siRNA-transfected cells. As shown in Fig. 6B, the cytoplasmic histone-associated DNA fragmentation enrichment resulting from BITC exposure over DMSO-treated control was modestly but statistically significantly greater in survivin-silenced cells in comparison with nonspecific siRNA-transfected MCF-7 cells (Fig. 6B). On the other hand, knockdown of survivin protein level (Fig. 6C) did not have an appreciable effect on BITC-induced apoptosis in the MDA-MB-231 cell line (Fig. 6D). We conclude that induction of survivin is marginally cytoprotective against BITC-mediated cell death in the MCF-7 cells.

Discussion

BITC is a highly promising cancer chemopreventive constituent of edible cruciferous vegetables (e.g., garden cress) with inhibitory effect in various chemically induced rodent cancer models and transgenic mice prone to spontaneous cancer development (15, 16, 33–35). Recent studies have provided convincing evidence to implicate apoptosis induction as the main mechanism in cancer chemoprevention by BITC (16, 35). For example, prevention of mammary cancer development in MMTV-neu mice by dietary administration of BITC correlates with increased apoptosis in carcinoma lesions (16). The main objective of the present study was to gain insight into the mechanism of BITC-induced apoptosis, which is not fully understood. Intrinsic value of defining the mechanism of proapoptotic response to BITC can eventually be appreciated during rational design of novel BITC-based preventive interventional regimens. Our initial inquiry focused on the possible role of p53 in execution of BITC-induced apoptotic cell death for two main reasons. First, the p53 tumor suppressor was shown to be essential for apoptosis induction by phenethyl ITC, which is a close structural analogue of BITC (36). Conversely, p53 has been shown to be a negative regulator of apoptosis induction by BITC in human colon CCD-18Co cells (37). The present study reveals that p53 tumor suppressor is dispensable for apoptosis induction by BITC because its knockdown has no influence on proapoptotic response to BITC in MCF-7 cells. Lack of p53 dependence should be viewed as a therapeutic advantage
for BITC, as loss-of-function mutation of this tumor suppressor is quite frequent in human cancers (27).

The IAP family of proteins has emerged as a critical regulator of apoptosis in response to different stimuli, including death receptor activation, growth factor withdrawal, radiation, and genotoxic insults (30, 38, 39). The IAPs play important roles in adaptive response to cellular stress, differentiation, motility, and immune response (39). This family of proteins is characterized by the presence of baculovirus IAP repeat (BIR) domains (40). Of the eight IAP members identified to date, XIAP is the best-characterized and most potent inhibitor of caspase-3 and caspase-7 (38). Anti-caspase activity of XIAP is attributed to its BIR domains; BIR3 domain inhibits caspase-9, whereas the BIR2 linker region is implicated in inhibition of caspase-3 and caspase-7 (38). Moreover, XIAP overexpression correlates with poor prognosis in some (e.g., childhood acute myelogenous leukemia and bladder cancer) but not all cancers (38, 41, 42). The present study reveals that BITC exposure decreases protein level of XIAP in breast cancer cells. In addition, the BITC-mediated inhibition of MDA-MB-231 cell growth in vivo is accompanied by suppression of XIAP protein expression in the tumor xenograft (Fig. 4). The BITC-mediated suppression of XIAP expression is not a cell line-specific response because BITC treatment decreases protein level of XIAP in both MCF-7 and MDA-MB-231 cells (Fig. 2). Moreover, RNA interference of XIAP confirms statistically significant protection against BITC-mediated DNA fragmentation in MCF-7 as well as in MDA-MB-231 cells (Fig. 3). We have shown previously that the BITC treatment causes proteolytic cleavage (activation) of procaspase-3 and procaspase-9 in MDA-MB-231 cells and pharmacologic inhibition of caspase-9 attenuates BITC-mediated apoptosis (17). It is possible that caspase activation by BITC is facilitated by suppression of XIAP protein level.

The precise mechanism by which BITC treatment suppresses expression of XIAP protein is yet to be determined, but several possibilities exist. One such possibility relates to the BITC-mediated proteosomal degradation of XIAP, which is capable of autoubiquitination and can be stabilized by inhibition of the proteasome (43, 44). It is also plausible that BITC treatment inhibits transcription of XIAP. Finally, BITC-mediated inhibition of XIAP translation is another possibility deserving attention. Previous studies have shown that MDM2 physically interacts with the internal ribosome entry segment (IRES) of the 5′-untranslated region of XIAP and positively regulates XIAP IRES activity (45). The XIAP IRES-dependent translation is increased in cells transfected with MDM2 (45). However, further studies are needed to systematically explore these possibilities.

Survivin is another IAP family member that contains a single BIR domain and an extended COOH-terminal helical coiled-coil domain. However, unlike other IAPs, survivin is devoid of the RING finger domain (28, 30, 39). Recent studies have pointed toward important roles of survivin in both cell cycle regulation and apoptosis control (46). Subcellular localization of survivin in mitochondria seems important for its antiapoptotic function (31, 46, 47). Survivin expression is absent or low in most terminally differentiated normal tissues, but this protein is overexpressed in different tumor types (46, 47). Furthermore, survivin overexpression in tumors correlates with clinical pathologic variables of the aggressive disease (46, 48) and confers treatment resistance in cancer cells (48). We found that BITC treatment results in transcriptional upregulation of survivin in MCF-7 cells but not in the MDA-MB-231 cell line. Although the mechanism behind cell-specific transcriptional upregulation of survivin by BITC treatment is not yet clear, silencing of survivin renders MCF-7 cells modestly more sensitive to BITC-induced apoptosis. We conclude that survivin induction is marginally cytoprotective against BITC-induced apoptosis, and this correlation is cell specific.

In conclusion, we provide experimental evidence to show that the BITC-induced apoptosis in human breast cancer cells is independent of p53 but mediated by suppression of XIAP protein level irrespective of the estrogen receptor status. The BITC-mediated suppression of XIAP is also observed in MDA-MB-231 xenograft in vivo. Accordingly, XIAP expression may be a viable biomarker of BITC response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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