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Cancer Research

Bitter Melon (*Momordica charantia*) Extract Inhibits Breast Cancer Cell Proliferation by Modulating Cell Cycle Regulatory Genes and Promotes Apoptosis

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Abstract

Breast cancer is one of the most common cancers among women in the United States. Although there are effective drugs for treating advanced stages of breast cancers, women eventually develop resistance. One of the approaches to control breast cancer is prevention through diet, which inhibits one or more neoplastic events and reduces cancer risk. In this study, we have used human breast cancer cells, MCF-7 and MDA-MB-231, and primary human mammary epithelial cells as an *in vitro* model to assess the efficacy of bitter melon (*Momordica charantia*) extract (BME) as an anticancer agent. BME treatment of breast cancer cells resulted in a significant decrease in cell proliferation and induced apoptotic cell death. Apoptosis of breast cancer cells was accompanied by increased poly(ADP-ribose) polymerase cleavage and caspase activation. Subsequent studies showed that BME treatment of breast cancer cells inhibited survivin and claspin expression. Fluorescence-activated cell sorting analysis suggested that MCF-7 cells treated with BME accumulated during the G_2-M phase of the cell cycle. Further studies revealed that BME treatment enhanced p53, p21, and pChk1/2 and inhibited cyclin B1 and cyclin D1 expression, suggesting an additional mechanism involving cell cycle regulation. Together, these results show that BME modulates signal transduction pathways for inhibition of breast cancer cell growth and can be used as a dietary supplement for prevention of breast cancer. *Cancer Res; 70(5); 1925–31. ©2010 AACR.*

Introduction

Breast cancer is the most frequent neoplasm in women from Western countries. The etiology of breast cancer involves a complex interplay of genetic, hormonal, and probably dietary factors. There have been significant advances in breast cancer treatment that have improved patient survival and quality of life. However, women continue to die of the disease and new treatment strategies are essential. Cancer prevention by the use of naturally occurring dietary substances is considered as a practical approach to reduce the ever increasing incidence of cancer. The intervention of multistage carcinogenesis by modulating intracellular signaling pathways may provide a molecular basis for chemoprevention with a wide variety of dietary phytochemicals (1). Cancer cells acquire resistance to apoptosis by overexpression of antiapoptotic proteins and/or by downregulation or mutation of proapoptotic proteins. Generally, the growth rate of preneoplastic or neoplastic cells exceeds that of normal cells due to dysregulation of their cell growth and cell death machineries. Therefore, an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically deregulated, premalignant and malignant cells from the body is by inducing cell cycle arrest or apoptosis using dietary chemopreventive compounds.

Momordica charantia, also known as bitter melon, balsam pear, or karela, is widely cultivated in Asia, Africa, and South America and extensively used in folk medicines as a remedy for diabetes, specifically in India, China, and Central America (2). Freeze-dried bitter melon capsules are available and marketed in health food stores across North America and Western European countries (Eclectic Institute, Inc.). Animal studies have used either fresh bitter melon extract (BME) or crude organic fractions to evaluate its hypoglycemic and hypolipidemic effects (3–6). Compounds isolated from the fruit and seeds of bitter melon plant that are believed to contribute to its hypoglycemic activity include charantin (a steroid glycoside) and polypeptide "p" or plant insulin (a 166-residue insulin mimetic peptide; refs. 7, 8). Bitter melon is also known to contain additional glycosides such as mormordin, vitamin C, carotenoids, flavanoids, and polyphenols (9, 10). BME preparations from independent laboratories have shown comparable beneficial effects not only on glucose metabolism but also on plasma and hepatic lipids (11–13). In this study, we have used the crude BME to examine its efficacy against breast cancer cells as a model. Our data show that treatment of breast cancer cells with BME accumulates at the G_2-M phase of the cell cycle and induces cell cycle arrest and apoptosis.

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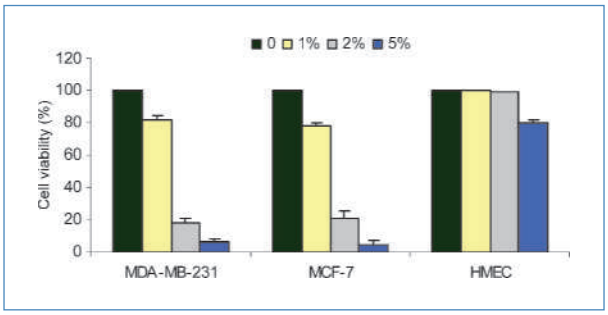

Figure 1. BME inhibits breast cancer cell proliferation. Breast cancer cells (MDA-MB-231 and MCF-7) and HMECs were treated with different concentrations of BME (1%, 2%, and 5%, v/v). Cell viability was measured after 48 h by trypan blue exclusion. Columns, mean of three separate experiments. The lowest level of significance was $P < 0.001$.

Materials and Methods

Preparation of BME. BME was prepared from the Chinese variety of young bitter melons (raw and green) as discussed previously (11). Briefly, BME was extracted using a household juicer and centrifuged at 560 × *g* at 4°C for 30 min. The supernatant BME was stored in aliquots at −80°C until further analysis.

Cells. MCF-7 and MDA-MB-231 breast cancer cells (obtained from American Type Culture Collection) were maintained in DMEM with 10% FCS. Human primary mammary epithelial cells (HMEC; obtained from Lonza) were maintained in mammary epithelial basal medium (Lonza).

Human apoptosis antibody array. The expression profile of apoptosis-related proteins was detected and analyzed using a human apoptosis array kit (ARY009, R&D Systems). This array contains duplicate spots of 35 apoptosis-related proteins. Briefly, the membrane containing immobilized apoptosis-related antibodies was blocked with bovine serum albumin for 1 h on a rocking platform at room temperature. The membrane was then incubated with lysates of MCF-7 or MDA-MB-231 cells treated with or without BME along with Detection Antibody Cocktail overnight at 2°C to 8°C on a rocking platform. The membrane was incubated with streptavidin-horseradish peroxidase conjugate followed by chemiluminescent detection reagent. The membrane was scanned and pixel density was presented by quantifying the mean spot densities from two experiments.

Fluorescence-activated cell sorting analysis. MCF-7 cells were treated with BME for 6 and 24 h. Cells were trypsinized and fixed in ice-cold 70% ethanol overnight at 4°C. Cells were washed, stained with propidium iodide for overnight, and subjected to fluorescence-activated cell sorting (FACS) analysis on a FACScan flow cytometer (BD PharMingen) as described previously (14). Data were analyzed using the CellQuest and ModFit softwares.

Western blot analysis. Breast cancer cells (MCF-7 and MDA-MB-231) were treated with or without BME, and cell lysates were prepared 48 h after treatment in 2× SDS sample buffer. Cell lysates were analyzed for Western blot analysis using poly(ADP-ribose) polymerase (PARP), caspase-3, caspase-7, claspin, survivin, cyclin D1, cyclin B1, p21, p53, and pRb antibodies (Santa Cruz Biotechnology or Cell Signaling), followed by enhanced chemiluminescence (Amersham Biosciences). Blots were reprobed with actin to compare protein load in each lane.

Results and Discussion

BME treatment induces human breast carcinoma cell death. MCF-7, MDA-MB-231 (breast cancer cells), and HMECs were treated with different concentrations (1%, 2%, and 5%, v/v) of BME. Cell viability was measured after 48 hours by trypan blue exclusion (Fig. 1). We have observed >80% cell death in MDA-MB-231 and MCF-7 cells treated with 2% (v/v) BME. On the other hand, primary epithelial cells did not display a significant cytotoxicity even after 5 d of incubation with BME.

BME treatment of MCF-7 cells induces PARP cleavage and caspase activation. Cleavage of the DNA repair enzyme PARP from a 116-kDa protein to a signature peptide of 86 kDa is associated with a variety of apoptotic responses. PARP is a nuclear protein and a downstream substrate of activated caspase-3/7. To examine the effect of BME on the apoptotic signaling pathway, PARP cleavage was investigated

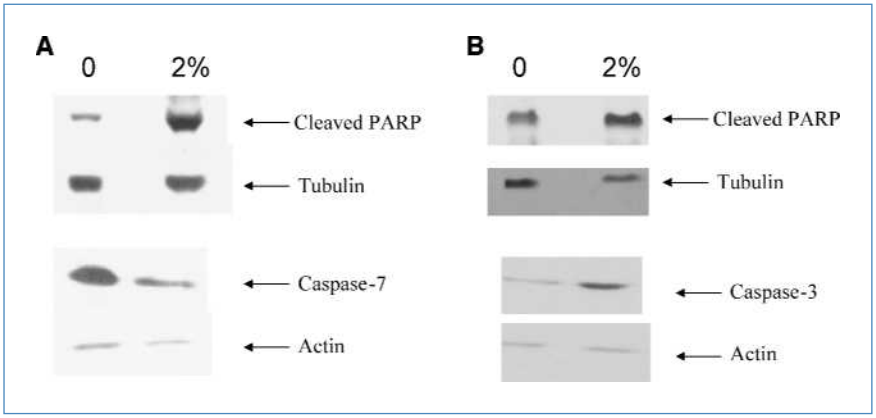

Figure 2. BME-mediated cell death involves PARP cleavage and caspase activation. Lysates were prepared from MCF-7 cells (A) or MDA-MB-231 cells (B) treated with BME (2%) for 48 h and subjected to Western blot analysis using a specific antibody to PARP or caspases. After treatment of BME, PARP was cleaved to an 86-kDa signature peptide (top). Treatment of MCF-7 cells with BME induces caspase-7 (A). The antibody used in this experiment only recognized the procaspase-7 form. On the other hand, BME treatment in MDA-MB-231 cells induces caspase-3 (B; cleaved caspase-3). The blot was reprobed with an antibody to actin for comparison of equal protein load.

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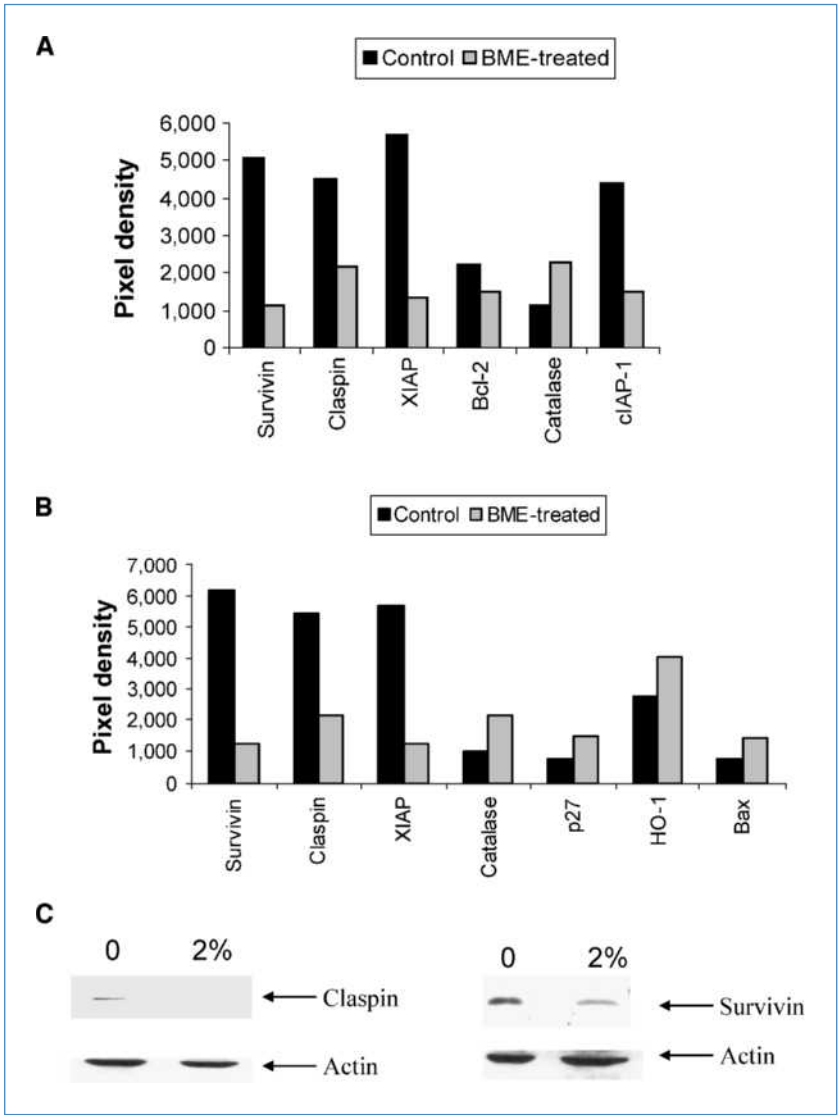

Figure 3. BME treatment in breast cancer cells inhibits survivin and claspin expression. Modulation of apoptosis signaling molecules in BME-treated MCF-7 (A) or MDA-MB-231 (B) cells. Breast cancer cells were treated with BME for 48 h and cell lysates were used for antibody array. The blot was scanned for presentation, and the data presented as pixel density. Western blot analysis was done for claspin (left) and survivin (right) expression using specific antibodies (C). The blot was reprobed with an antibody to actin for comparison of equal protein load.

in MCF-7 cells treated with different concentrations of BME by Western blot analysis using a specific antibody. Cells treated with BME displayed a cleaved 86-kDa signature peptide as shown in Fig. 2A. MCF-7 cells treated with BME displayed activation of caspase-7 (Fig. 2A). BME treatment in MDA-MB-231 cells also induced PARP cleavage and activated caspase-3 (Fig. 2B).

Breast cancer cells treated with BME inhibits survivin and claspin expression. To investigate the involvement of apoptotic signaling molecules following BME treatment, we performed an apoptosis antibody array using the Human Apoptosis Array from R&D Systems. For this, MCF-7 or MDA-MB-231 cells were treated with BME (2%, v/v) for 48 h. Cell lysates were prepared and apoptosis antibody array was done following the manufacturer's protocol. Our results showed that a number of apoptotic signaling proteins were modulated following treatment of BME. Survivin, XIAP, and claspin were significantly inhibited following treatment of BME in both MCF-7 and MDA-MB-231 cells (Fig. 3A and B, respectively). On the other hand, the proapoptotic gene *catalase* was upregulated in both cell lines after BME treatment. Antiapoptotic proteins Bcl-2 and cIAP-1 were inhibited only in BME-treated MCF-7 cells, whereas p27, HO-1, and Bax protein expressions were increased in BME-treated MDA-MB-231 cells. We further verified the expression of survivin and claspin, proteins involved in inhibition of cell growth and induction of apoptosis, by Western blot analysis using specific antibodies. Our results verified an inhibition of both proteins following BME treatment in MCF-7 cells (Fig. 3C).

Modulation of proteins involved in the cell cycle process following BME treatment. Because both claspin and survivin are involved in cell cycle regulation (15, 16), we next examined whether BME treatment modulates cell cycle progression in MCF-7 cells by FACS analysis. Cells were treated with BME for 6 and 24 h. Control (untreated) and treated

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cells were collected and stained with propidium iodide followed by FACS analysis. A significant increase in G_2-M peak (19% at 6 hours, 45% at 24 hours) was observed in BME-treated MCF-7 cells, compared with only a 10% increase in the control cells (Fig. 4), suggesting an accumulation at the G_2-M phase of the cell cycle. We have also observed a trend of inhibition of cyclins and enhancement of p21 at the 6-hour time point (data not shown). Therefore, these results suggest that BME treatment in MCF-7 cells affects their normal regulation of cell cycle progression.

We also examined key cell cycle regulatory molecules following BME treatment. MCF-7 or MDA-MB-231 cells were exposed to BME (2%, v/v) for 24 h. Cell lysates were prepared and Western blot was done using specific antibodies. We chose to examine the molecules that are downstream of survivin and/or claspin. BME-treated MCF-7 cell lysates displayed inhibition of cyclin B1 and cyclin D1 expression and upregulation of p53 and p21 (Fig. 5A). We further examined the status of phospho-Chk1/2 because these molecules are activated in DNA stress (15). Our results showed an increase in phospho-Chk1/2 (S345Chk1 and T68Chk2) following BME treatment (Fig. 5B). Similar results were observed from MDA-MB-231 cells (data not shown). These results suggest that BME uses several signaling pathways that induce breast cancer cell death.

Uncontrolled cell growth and resistance to apoptosis are major defects in neoplasia. Development of approaches that induce apoptotic machinery within cancer cells could be effective against their proliferation and invasive potential (17). A number of agents such as γ-irradiation, immunotherapy, and chemotherapy induce apoptosis in tumor cells as the primary mode of action for most anticancer therapies.

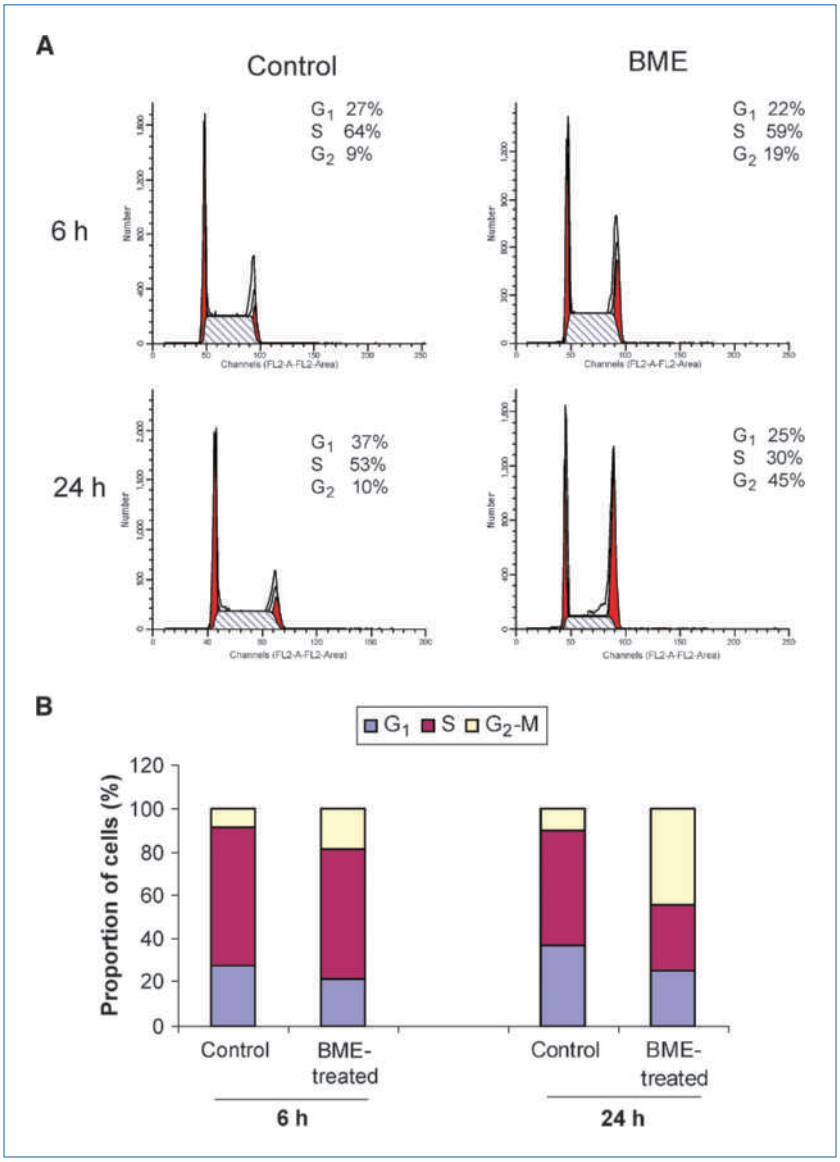

Figure 4. Treatment of BME in MCF-7 cells resulted in accumulation of cells at the G_2-M phase. A, cells were treated with BME for 6 and 24 h and stained with propidium iodide. DNA content was analyzed by flow cytometry. Results are represented as percent of cell population in G_1, S, and G_2-M phases of the cell cycle. $P < 0.01$. B, the population of cells at different cell cycle phases is shown by a bar diagram.

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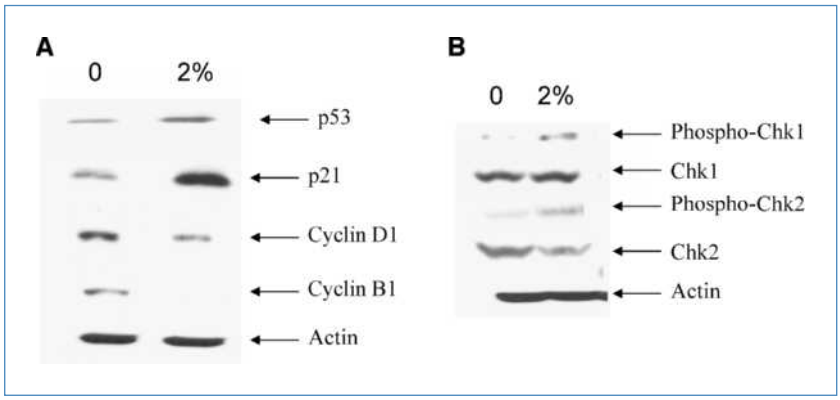

Figure 5. Modulation cell cycle regulatory proteins following BME treatment in breast cancer cells. A, MCF-7 cells were treated with BME for 24 h. Cell lysates were analyzed for the expression of cyclin D1, p53, cyclin B1, and p21 by Western blot using specific antibodies. The blots were reprobed with an antibody to actin for comparison of protein load. B, Western blot analysis was done for phosphorylated and total Chk1 and Chk2 expression of MCF-7 cells treated with BME for 48 h using specific antibodies. The blots were reprobed with an antibody to actin for comparison of equal protein load.

Impairment of this pathway is implicated in treatment resistance (18). In fact, many chemopreventive agents of natural origin have shown promising anticancer properties by induction of the apoptotic pathway in transformed or tumor cells (1, 13). In this present study, we have shown that BME exerts a significant effect on inhibition of cell growth and induction of apoptosis in breast cancer cells mediated by cell cycle and apoptosis regulatory proteins. The expression of critical cell cycle regulatory proteins cyclin B1 and cyclin D1 was significantly decreased and the G_2-M phase was blocked in MCF-7 cells following BME treatment. This is the first report, to our knowledge, describing the mechanism of BME-mediated inhibition of breast cancer cell growth. Future *in vivo* study will reveal the anticancer efficacy of crude BME in breast cancer animal models.

Over the years, there has been a worldwide interest in BME as a dietary supplement because of its various health beneficial effects including lowering diabetes and lipidemia (6, 12). A recent study by us reveals that BME is well tolerated, and has been termed as relatively safe in acute, subchronic, and chronic doses in animal studies (6). Recently, α-eleostearic acid and the dihydroxy derivative from bitter melon were suggested to be the major inducers of apoptosis in HL60 cells (19). MCP30, isolated from bitter melon seeds, selectively induces prostate cancer apoptosis and inhibits histone deacetylase-1 activity (20). While our article was under review, Grossmann and colleagues (21) reported that α-eleostearic acid, which makes up ~60% of *M. charantia* seed oil, blocks MDA-MB-231-ERα cell proliferation and induces apoptosis.

We have examined the effect of BME in both estrogen receptor (ER)–positive and ER-negative breast cancer cells and observed inhibition of survivin expression. Survivin, a member of the BIR-containing inhibitor of apoptosis protein (IAP) family, involved in inhibition of apoptosis, exerts multiple effects throughout the cell cycle (reviewed in ref. 16). The importance of survivin in inhibiting cell death and promoting cell proliferation is emphasized by its overexpression in many human tumors including breast cancer. BME downregulates survivin expression in breast cancer cells along with other antiapoptotic molecules. We have observed that BME-mediated inhibition of survivin also reduced cyclin B1 and cyclin D1 expression. Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis and also is regulated by survivin (22, 23). We have observed downregulation of Bcl-2 following BME treatment in MCF-7 cells (data not shown). Therefore, it is conceivable that the downregulation of survivin is mechanistically linked with BME-mediated cell growth inhibition and apoptosis. Our results also indicated that cyclin B1 was downregulated in BME-treated breast cancer cells, whereas the cyclin-dependent kinase (CDK) inhibitor p21 was upregulated, suggesting that BME inhibited breast cancer cell growth through the arrest of cell cycle and inhibition of proliferation. The CDK inhibitors (p21, p27, and p57) have been shown to arrest the cell cycle and inhibit the growth of cancer cells (24–26). Inhibition of cell growth by BME could be due to the induction of apoptosis in addition to cell cycle arrest. Overexpression of survivin is also involved in resistance to chemotherapy (27). Therefore, the downregulation of survivin by BME could also be a useful strategy for chemosensitization

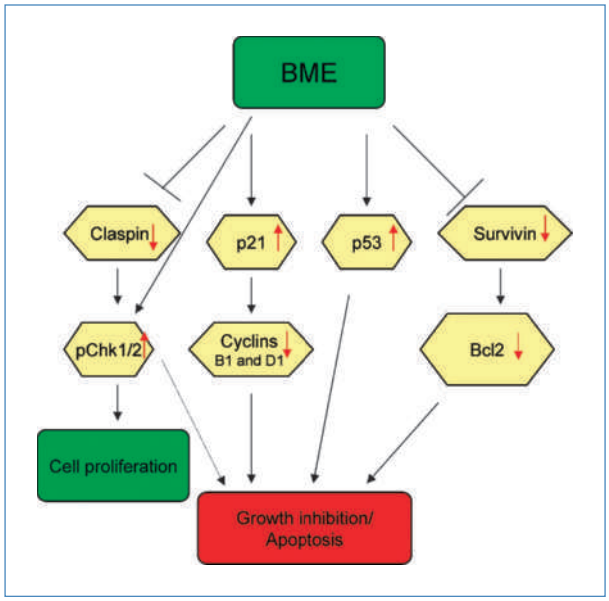

Figure 6. Proposed mechanisms showing BME-mediated cell growth inhibition and apoptosis in breast cancer cells.

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of metastatic breast cancer cells. Further in-depth investigations are needed to establish the cause-and-effect relationship of the *survivin* gene regulation and BME-mediated cell growth inhibition and apoptosis in breast cancer in animal models.

Claspin expression is inhibited in MCF-7 and MDA-MB-231 cells following BME treatment. In normal cells and tissues, claspin expression was weak, whereas increased levels were observed in cancer cell lines and tumor specimens (15). Claspin is required for the phosphorylation and activation of the Chk1 protein kinase by ATR during DNA replication and in response to DNA damage. Chk1 and Chk2 are key mediators of intra-S-phase checkpoint signaling. How this pathway couples to cell survival is controversial, especially with respect to p53, and is associated with activation or inhibition of apoptosis after stress or DNA damage (27). We have observed an increase of phospho-Chk1 (S345) and phospho-Chk2 (T68) expression following BME treatment in MCF-7 and MDA-MB-231 cells. These results are in agreement with previous studies where curcumin, diallyl trisulfide, resveratrol, and lithium caused activation of Chk1 by phosphorylation at Ser-345 residue in human pancreatic (28), prostate (29), ovarian (30), and hepatocellular (31, 32) carcinoma cells. Cortez (33) also reported that phosphorylation of Chk1/2 was increased in a dose-dependent manner by caffeine treatment along with hydroxyurea, irradiation, or aphidicolin. However, the inhibition of claspin and activation of Chk1 by BME in breast cancer cells suggests another function of claspin besides activation of Chk1 and warrants further investigation.

In response to DNA damage, p53 is unregulated and phosphorylates various kinases such ATM, ATR, and DNA-dependent protein kinase. Activated (phosphorylated) ATM also phosphorylates Chk2. We have observed upregulation of p53 and phospho-Chk. Further study is needed to examine the regulation of upstream signaling molecules of Chk and p53 in BME-treated breast cancer cells.

In summary, we have shown that BME exerts a significant effect on inhibition of cell growth and induction of apoptosis in breast cancer cells mediated by cell cycle and apoptosis regulatory proteins, as illustrated in Fig. 6. Together, these results suggest that BME modulates several signal transduction pathways that additively or synergistically induce breast cancer cell death and can be used as a dietary supplement for prevention of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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