Bowman–Birk inhibitor abates proteasome function and suppresses the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1

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The Bowman–Birk inhibitor (BBI), a soybean-derived protease inhibitor with well-characterized ability to inhibit trypsin and chymotrypsin activities, has been shown to be an effective suppressor of carcinogenesis and treated in human phase IIa clinical trial. However, the precise mechanisms by which BBI suppresses carcinogenesis are unknown. In this study, we demonstrated that BBI specifically and potently inhibits the proteasomal chymotrypsin-like activity in vitro and in vivo in MCF7 breast cancer cells. Proteasome inhibition by BBI is associated with accumulation of ubiquitinated proteins and the proteasome substrates, p21(WAF1) and p27(Kip1), accompanied with downregulation of ubiquitin and cyclin E which could arrest cell cycle at G(1)/S phase. Moreover, BBI suppressed MCF7 cell growth and had a novel effect on the decrease of phosphorylated extracellular signal-related kinases (ERK1/2). However, BBI was unable to inactivate ERK1/2 in the presence of a phosphatase inhibitor or a transcription inhibitor suggesting the involvement of a specific phosphatase. We found an induction of MAP kinase phosphatase-1 (MKP-1) in dose- and time-dependent manner correlated with dephosphorylation of ERK1/2 in BBI-treated MCF7 cells. In addition, BBI exhibited no inhibitory effects on EGF-stimulated activation of ERK1/2 and Akt. Together, we suggested that BBI abates proteasome function and results in upregulation of MKP-1, which in turn suppresses ERK1/2 activity. Our results support the notion that proteasome inhibition by BBI is a novel mechanism that contributes to prevention of cancer and further provides evidence that soybean products have the potential to advance as chemopreventive agents.

Introduction

A number of epidemiological data illustrate that large scale consumption of soybean products is associated with decreased cancer mortality rates in Asian population compared with that in the West (1–3). Particular compounds isolated from soybeans such as isoflavones and protease inhibitors are now being intensively studied in chemoprevention cancer. In recent times, protease inhibitors acting as important modulators in a wide variety of cellular physiological pathways (4) have been developed as a class of well-established cancer chemopreventive agents because of their strong anticarcinogenic activity in vivo and in vitro in cancer models (5,6). Soybeans are a particularly rich source of protease inhibitors and the most predominant protease inhibitor is the Bowman–Birk inhibitor (BBI). BBI, a serine protease inhibitor, has been shown to be a valid suppressor of carcinogenesis (7,8) in the human phase IIa clinical trial (9). BBI, identified and purified by Bowman (10) and Birk (11), is a 71-amino acid protein (~8 kDa) with seven disulfide bonds which stabilize its active configuration and has a double head structure with the well-characterized trypsin inhibitory domain on one head and the chymotrypsin inhibitory domain on the other (12). Interestingly, BBI is the only protease inhibitor derived from soybeans that possesses a chymotrypsin inhibitory activity (13) which might contribute to its anticarcinogenic activity, whereas trypsin inhibitory activity is not essential (14,15). As an anticarcinogenic agent, BBI has been studied both as purified BBI and as an extract of soybeans enriched in BBI termed BBI concentrate (BBIC). However, both BBI and BBIC have the same suppressive effects on liver and colon carcinogenesis induced by dimethylhydrazine in mice (16,17). BBI has anti-inflammatory (18) and radio-protective properties (15) and is able to inhibit free radical production (19) and carcinogen-induced transformation (20). In addition, BBI has the capacity to reverse the initiation of the carcinogenic process (21) and the radiation/carcinogen elevated levels of several oncogenes, such as c-myec (22) and c-fos (23). Although BBI has a broad spectrum of cancer-protective activities, but the exact mechanism(s) by which BBI exerts its anticarcinogenic effects remains limited.

The ubiquitin–proteasome system is the essential machinery in eukaryotic cells, playing a major role in the proteolysis of intracellular proteins (24). Within this system, the proteasome involved in the regulation of several physiological processes in cells is recently emerging as a target for cancer therapy (25). The 26S proteasome (2.5 MDa) is a multifunctional proteolytic complex that consists of a 20S proteolytic core and two 19S regulatory cap (26). Proteins conjugated with a poly-ubiquitin chain are recognized by the 19S proteasomal subunit and targeted to the 20S proteolytic core for degradation. There are three kinds of proteasomal activities, namely trypsin-like, chymotrypsin-like and peptidyl-glutamyl-peptide-hydrolytic (PGPH) activities (27). Inhibition of proteasome activity may influence many aspects of cellular functions and cause multiple complicated effects on living cells, further resulting in anti-inflammatory, anticarcinogenic, antiproliferative and apoptotic effects of cancer cells. The recent identification of

Abbreviations: ALLN, acetyl-Leu-Leu-norleucinal; AMC, 7-amido-4-methyl-coumarin; BBI, Bowman–Birk inhibitor; BBIC, BBI concentrate; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor receptor; ERK, extracellular signal-related kinase; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MKP-1, MAP kinase phosphatase-1; PGPH activity, peptidyl glutamyl peptide hydrolytic activity.
selective proteasome inhibitors has allowed a new definition of the role of proteasome in carcinogenesis and elicited appreciable interest because of their potential applications in cancer therapy and biotechnology. However, blockade of the chymotrypsin-like site of the proteasome causes a large reduction in the rates of protein breakdown (28) and this proteasomal chymotrypsin-like activity is associated with tumor cell survival and drug resistance (29). Most of the well-established valid proteasome inhibitors, which specifically inhibit the proteasomal chymotrypsin-like activity (30), have the abilities to induce apoptosis of cancer cells and suppress tumor cell growth in several experimental models and have been used as potential, novel anticancer agents in early clinical trials (31–33). This implies that proteasome inhibition may be a prerequisite for the development of chemopreventive agents.

Over the last decade, there have been increasing studies about the role of signaling pathways in tumor progression and scientists have focused on members of the mitogen-activated protein kinase (MAPK) pathways. The MAPK pathways are critical for the conversion of diverse extracellular signals to biological responses, which modulate many cellular processes. The dynamic modulation among three MAPK subfamilies, specifically p42/44 extracellular signal-related kinases (ERK 1/2), c-Jun N-terminal kinase and p38 kinase (34), is responsible for the cell fate, whether the cells undergo apoptosis, differentiation or proliferation. The balance MAPKs and the specific protein phosphatases play an important role in controlling MAPKs activation. So far, the most studied signal transduction cascade involved in the control of cell proliferation is the ERK pathway and its activation may be closely associated with the increase of cell death threshold (35). Among MAPK phosphatases (MKPs), MKP-1 is the first identified mammalian dual specificity phosphatase and displays marked substrate selectivity for ERK in vitro and in vivo by dephosphorylating phospho-Thr/Thr residues of ERK1/2 (36). However, the role for ERK signaling in the proteasome inhibition by potent proteasome inhibitors has not been described, and we propose to investigate this issue further.

To elucidate the precise mechanisms by which BBI suppresses carcinogenesis, we speculate that the chymotrypsin inhibitory activity of BBI might also inhibit the chymotrypsin-like activity of 26S proteasome. In the present study, we demonstrate that BBI specifically inhibits the proteasomal chymotrypsin-like activity in vitro and in vivo in MCF7 breast cancer cells. This is associated with the accumulation of the known proteasome substrates followed by the downregulation of cell cycle regulatory cyclins. Furthermore, BBI suppressed MCF7 cell growth and led to the inactivation of ERK1/2 which were correlated with an induction of dual specific MKP-1. On the other hand, BBI specifically displayed inhibitory influence on ERK signaling in comparison with distinct proteasome inhibitors and soy-derived isoflavones. This study supports the notion that the inhibition of proteasome activity by BBI may contribute to its cancer preventative effects.

Materials and methods

**Materials**

BBI, genistein, sodium orthovanadate, EGF, purified calpain (human erythrocytes), acetyl-Leu-Leu-norleucinal (ALLN), fluorogenic peptide substrates, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), benzoyloxycarbonyl-Leu-Leu-GLu-AMC (for the proteasomal PGP activity), Suc-Leu-Tyr-AMC (for the calpain activity) and the specific calpain inhibitor, calpeptin, were obtained from Calbiochem (Darmstadt, Germany). Z-Gly-Gly-Arg-AMC (for the proteasomal trypsin-like activity) was purchased from Bachem (King of Prussia, PA). Anticyclin D2 was purchased from Tocris Cookson (Bristol, UK).

**Cell culture**

Human breast cancer MCF7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan, UT) containing 10% (v/v) fetal calf serum (FCS) (Biological Industries), 100 U/ml of penicillin and 100 μg/ml streptomycin. All cells were maintained in a humidified atmosphere of 5% CO2 at 37°C. Cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and homogenized in proteasome lysis buffer for proteasome activity assay (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) or gold lysis buffer for western blot assay (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 100 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% SDS, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) for 30 min at 4°C. Afterward, the lysates were centrifuged at 12 000 g for 30 min and the supernatants were collected as whole cell extracts.

**Assay for proteasome activity of whole MCF7 cell extract**

To measure the proteasome activities of cancer cells in vitro and in vivo, we performed cell-free proteasome activity assay as described previously (37). A whole cell extract (10 μg) of MCF-7 cells was incubated at 37°C for 90 min with 20 μM of fluorogenic peptide substrate, Suc-Leu-Leu-Tyr-AMC for chymotrypsin-like or Z-Leu-Leu-GLu-AMC for PGP or Z-Gly-Gly-Arg-AMC for trypsin-like activities, in 200 μl of the assay buffer (20 mM Tris–HCl, pH 8.0) with or without a series of concentrations of BBI. After incubation, the reaction mixture was diluted with 200 μl of assay buffer followed by a measurement of the hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a VersaFluorTM Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad, Hercules, CA).

**Assay for proteasome activity of intact MCF7 cells**

MCF7 cells were seeded in a 12-well plate (5 × 104 cells/well) overnight. These cells then were treated with an indicated concentration of BBI for 24 h, followed by an addition of 20 μM of fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like activity) at 37°C for 2 h. Afterward, 100 μl of the cell medium was collected and diluted with 1 × PBS to 400 μl. Measurement of free AMC groups was described as above.

**Assay for calpain activity**

An aliquot of 1 μg of purified calpain was incubated with 40 μM of fluorogenic peptide calpain substrate, Suc-Leu-Leu-Val-Tyr-AMC, at 37°C for 90 min in 200 μl of calpain assay buffer (50 mM Tris–HCl, pH 7.5, 50 mM f-EDTA, 1 mM Z-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like activity) at 37°C for 2 h. Afterward, 100 μl of the cell medium was collected and diluted with 1 × PBS to 400 μl. Measurement of free AMC groups was described as above.

**Western blot analysis**

The MCF7 cells (5 × 107) were seeded onto a 60-mm tissue culture dish in 10% (v/v) FCS/DMEM and cultured for 24 h. The cells were then incubated in DMEM treated with various doses of BBI for the indicated hours (see figure legends). Protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories). A total of 40 μg of protein was separated by SDS–PAGE and transferred onto PVDF membranes (Schleicher & Schuell, Keene, NH). Non-specific binding on the PVDF membranes was minimized with a blocking buffer containing 5% of bovine serum albumin (v/v) in PBS at room temperature for 1 h. Then the membranes were incubated with primary antibodies (1:1000 dilution) at room temperature for 2 h and washed with 1× PBST (1× PBS,0.2% Tween 20, v/v, Pharmacia Biotech, Buckinghamshire, UK). The following primary antibodies: phospho-ERK1/2 (Thr202/Tyr204), phospho-MEK1/2 (Ser218/Ser222), MEK1, ERK1 and MKP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-Akt (Ser473) and Akt antibodies were purchased from Cell Signaling (Beverly, MA); cyclin D1, cyclin E, CDK2, CDK4, actin, p27 and Hsp90 antibodies were from BD Transduction Laboratory; p21 was from Upstate Biotechnology (Lake Placid, NY). Anti-ubiquitin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, MD). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, which were from BD Transduction Laboratory (1:2000 dilution) at 37°C, for 1 h. Immunoreactivity was detected with an enhanced chemiluminescence system (PerkinElmer Life Sciences, Boston, MA). The intensity of the bands was quantified using Phospho-Image system.
Flow cytometric cell analysis

MCF7 cells were cultured in 10 cm Petri dishes and incubated for 24 h. The cells that had been treated with BBI were harvested by trypsinization at a different time course, washed with ice-cold PBS resuspended in 200 ml PBS, and fixed in 800 ml of ice-cold 100% ethanol at −20°C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 mg/ml RNase) and incubated at 37°C for 30 min. Then 1 ml of propidium iodide solution (50 mg/ml) was added and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

MTT assay

Cells were seeded in a 24-well plate (2 × 10^4 cells/well) overnight and then treated with varying concentrations of BBI for an additional 24 h. The effect of BBI on cell growth was examined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. In brief, 40 µl of MTT solution (2 mg/ml in 1 × PBS, Sigma Chemical Co, St Louis, MO) was added to each well and incubated for 2–4 h at 37°C. The supernatant was aspirated and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 500 µl of dimethyl sulfoxide. Finally, the absorbance was monitored by a microplate reader at a wavelength of 550 nm.

Cell proliferation assay

The inhibitory effects of BBI on proliferation in MCF7 cells were evaluated by the trypan blue exclusion assay. In brief, MCF7 cells were seeded on 6-well plates at a density of 5 × 10^5 cells in DMEM containing 10% FCS (v/v) overnight. The medium was then changed and incubated with or without indicated concentrations of BBI every 24 h. At the end of the incubation period, the cells were harvested by trypsinization. The cells were mixed well with trypan blue solution. Both the living cells (without dye staining inside), and the dead cells (with dye staining inside), were counted by a hemocytometer under a microscope. The survival fraction was calculated based on the number of living cells normalized to all counted cells, including the living cells and the dead cells.

Statistical analysis

All values were expressed as mean ± SD. Each value is the mean of at least three separate experiments in each group. The differences in the effects of compound treatment when compared with vehicle-treated control values were analyzed by t-test as appropriate. Asterisk indicates that the values are significantly different from the control (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results

BBI specifically inhibited the proteasomal chymotrypsin-like activity in MCF7 cells

To examine whether BBI, which has inhibitory effects on chymotrypsin and trypsin activities, also specifically inhibits the proteasomal chymotrypsin-like activity, protein extracts were prepared from MCF7 cells and in vitro proteasome activity assay was performed. The result showed that 10 µM of BBI potently and specifically inhibited ~50% of the proteasomal chymotrypsin-like activity in MCF7 cell extracts rather than proteasomal trypsin-like and PGPH activities which showed ~25 and 10% inhibition, respectively (Figure 1A); whereas, the well-characterized proteasome inhibitor, ALLN (38), displayed more potent inhibitory effects on the three proteasomal activities than BBI. In addition, BBI significantly inhibited the proteasomal chymotrypsin-like activity in MCF7 cell extracts in a dose-dependent manner with an IC_{50} value of 20 µM (Figure 1B).

We further investigated to see whether BBI specifically inhibits the proteasomal activities. The effects of BBI on other protease activities such as calpain were then examined. Calpain is a cysteine protease that is activated by a number of processes, including increased intracellular Ca^{2+}, phosphorylation and proteolysis and appears to regulate cell adhesion and migration (39). The activity of purified calpain was significantly inhibited by the specific calpain inhibitor, calpeptin (40), whereas BBI displayed slight inhibitory effects on the calpain activity (Figure 1C). Furthermore, we tested if BBI could inhibit the activity of 26S proteasome in intact MCF7 cells. We compared the inhibitory effects of BBI with those of ALLN on the 26S proteasome of MCF7 cells. Figure 1D showed that BBI had more potent inhibitory effects on the proteasomal chymotrypsin-like activity of 26S proteasome in intact MCF7 cells in a concentration-dependent manner (with an IC_{50} value of ~8 µM) than that of ALLN, indicating that BBI is ~3–4 times stronger than ALLN as an inhibitor of proteasome. These results suggest that BBI is a potent and efficient proteasome inhibitor that specifically inhibits the proteasomal chymotrypsin-like activity of 26S proteasome in MCF7 cell extracts and living MCF7 cells over other protease activities.

Uppregulation of proteasome target proteins as a marker of proteasome inhibition mediated by BBI

To further illustrate that BBI could inhibit tumor cellular proteasome activity in vivo, we studied the levels of proteasome target proteins such as cyclin-dependent kinase inhibitors (CDKIs), p27kip1 and p21cip1/WAF1 (41,42) in MCF7 cells which were treated with various concentrations of BBI at indicated times. A 24-h treatment of MCF7 cells with a series of concentrations of BBI increased the levels of p27kip1 and p21cip1/WAF1 (Figure 2A). A concentration of 5 µM of BBI apparently upregulated the levels of p27kip1 and p21cip1/WAF1 (27 and 13%, respectively) when compared with that of control. Under normal conditions, cellular proteins targeted to proteolysis are conjugated with a polyubiquitin chain prior to degradation by the proteasome (43). Using anti-ubiquitin antibodies, the level of ubiquitinated proteins is visualized by western blot analysis as a broad range of molecular weight proteins. As shown in Figure 2A, polyubiquitin-conjugated proteins were also upregulated via the blockade of the proteasome function in BBI-treated MCF7 cells. A 24-h of BBI treatment resulted in a 43%-increase of ubiquitinated proteins and achieved maximum induction (~2-fold) at a higher dose. Furthermore, we tested the effects of BBI on the temporal expression of p27kip1 and p21cip1/WAF1 proteins. The levels of p27kip1 and p21cip1/WAF1 were upregulated in a time-dependent manner, whereas BBI exhibited more potent induction on the levels of p27kip1 than that of p21cip1/WAF1 (3-fold versus 2-fold) (Figure 2B). However, the expression of both CDK inhibitors slightly decreased after a 24 h treatment. A similar finding was observed in the accumulation of polyubiquitinated proteins. The levels of p27kip1 and p21cip1/WAF1 proteins, and ubiquitinated proteins accumulated in MCF7 cells as early as 3 h and remained stable until 24 h followed by treatment with 20 µM of BBI. These results suggest that the increased levels of proteasome target proteins, p27kip1 and p21cip1/WAF1, and upregulation of polyubiquitinated proteins with regard to both dose and time resulted from the inhibition of the proteasome function mediated by BBI in vivo and these data could further confirm the previous results (Figure 1).

Modulation of G_{1}/S cell cycle regulators in BBI-treated MCF7 cells

It is well known that CKIs, cyclin D, cyclin E, CDK2 and CDK4 are regulators involved in G_{1}/S phase progression (44). To investigate whether the accumulated p27kip1 and p21cip1/WAF1, which bind to cyclin–CDK complexes to inhibit their catalytic activity and induce cell cycle arrest (45), are
functional, we examined the levels of the downstream targets of p27Kip1 and p21Cip1/WAF1, CDKs and cyclins in BBI-treated MCF7 cells. As expected, the expression levels of cyclin D1 and cyclin E were obviously decreased by 60–80% after a 12-h treatment of BBI and reached the nadir after a 24-h treatment when compared with vehicle-treated control (Figure 2C). Conversely, there were no significant decreases of CDK2 and CDK4 proteins (data not shown). Previous studies indicated that both the upregulation of CKIs and the downregulation of G1/S-related cyclins simultaneously stabilize the formation and reduce the activity of cyclin–CDK complexes, following induction of cell cycle arrest at G1 phase (46,47). To further evaluate the DNA content of MCF7, nuclei were measured by flow cytometry after the addition of BBI. As shown in Figure 2D, a 12 h treatment with 20 μM BBI resulted in a higher proportion of cells in the G1 phase (73.84%) compared with the untreated cell (58.42%). Thus, we demonstrated that BBI-induced upregulation of p27Kip1 and p21Cip1/WAF1 accompanied by downregulation of cyclin D1 and cyclin E could result in G1/S phase arrest of MCF7 cells.

**Inhibition of MCF7 cell growth by BBI without showing cytotoxicity**

We examined the cytotoxic effects of BBI on MCF7 cells. The cytotoxicity of BBI in MCF7 cells was assessed by the MTT assay, based on the reduction of MTT to formazan precipitates by metabolically active cells. After a 24 h treatment with varying concentrations of BBI ranging from 5 to 100 μM,
BBI had little effect (<30% reduction) on MCF7 cell viability even at 80 μM (Figure 3A). However, a 50%-lethal effect on MCF7 cells was detected until treating with 88 μM of BBI. Meanwhile, the survival fraction, based on the number of living cells, which were normalized to all counted cells (including living cells and dead cells), showed a slight decrease when cells were continuously treated with BBI (Figure 3B).

Fig. 2. Accumulation of proteasome target proteins and down-regulation of G1/S phase regulatory proteins mediated by BBI. MCF7 cells were treated with varying concentrations of BBI for 24 h (A) or 20 μM of BBI for indicated times (B). After treatment, western blot analysis was performed to detect the levels of p27Kip1, p21Cip1/WAF1 and polyubiquitinated proteins using specific antibodies as described under Materials and methods section. Immunoblotting of Hsp90 was used as an internal control for equivalent protein loading. (C) MCF7 cells were starved for 24 h before incubation with 20 μM of BBI for indicated times in complete medium (10% FCS). The levels of cyclin D1, cyclin E proteins were determined by western blot analysis. The expression of actin was used as the internal control for equivalent loading. The western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent change in protein expression of the bands normalized to the levels of house-keeping proteins which represent internal control such as Hsp90 or actin. (D) Determination of cell-cycle distribution in control and BBI treated MCF7 cells by flow cytometry. MCF7 cells were treated without or with 20 μM of BBI for 3, 6, 9 and 12 h. The method of flow cytometry used is described in the Materials and methods section.
We speculated that BBI, acting as a proteasome inhibitor with the ability to induce apoptosis and tumor growth arrest in human malignancies (48), might inhibit MCF7 cell proliferation. The magnitude of growth inhibition by BBI was determined by trypan blue exclusion assay that examined the live cells without dye staining inside, which were counted by a hemocytometer under a microscope. As shown in Figure 3C, the significantly inhibitory effects on MCF7 cell growth mediated by BBI were observed 2 days after a 24-h treatment with 10 \( \mu \text{M} \) of BBI. Furthermore, 20 \( \mu \text{M} \) of BBI displayed more potent inhibition of cell growth (~40%) with another 24 h treatment. This reveals that BBI could inhibit MCF7 cell growth in a sustained manner. The results agreed with our previous data that BBI induced the upregulation of CDK inhibitors and downregulation of \( G_{1}/S \) cell cycle regulators (Figure 2), demonstrating that BBI suppressed proliferation of MCF7 cells through the inhibition of 26S proteasome.

Dephosphorylation of p42/p44 MAPK induced by BBI in MCF7 cells

It is presumed that BBI, with ability to suppress cell proliferation of MCF7, may play a specific role in mediated activation of the ERK1/2 pathway whose activation generally leads to an increase in the threshold for cell death (49). To study the relation between antiproliferation of MCF7 cells induced by BBI and the impact of BBI on ERK1/2 signaling, we monitored the dose- and time-course responses of BBI-treated MCF7 cells. Analysis of cell extracts revealed the levels of dually phosphorylated, active ERK1/2 in MCF7 cells with the specific antibody recognizing phosphorylated ERK1/2 (Thr202/Tyr204). Treatment with 5 \( \mu \text{M} \) of BBI for 24 h resulted in a significantly decreased level of phospho-ERK1/2 and reached maximum inhibition with 20 \( \mu \text{M} \) of BBI incubation without any loss of total ERK1/2 protein levels under these conditions (Figure 4A). In addition, time-course experiments exhibited that phospho-ERK1/2 were potently attenuated as early as 3 h and further maintained until 24 h after exposure to BBI in the presence of serum stimulation (Figure 4B). However, inactivation of ERK1/2 signaling by BBI was transient in MCF7 cells because of the levels of phospho-ERK1/2 slowly returning to normal at 24 h. It is possible that the decline in ERK inactivation, consistent with that in proteasome target proteins (Figure 2A and B), was because of the disappearance of the active proteasome inhibitor. These results showed that BBI induced downregulation of dually phosphorylated ERK1/2 in a concentration- and time-related manner in comparison with ALLN and genistein, one of the predominant isoflavones found in soybean with multiple chemopreventive effects (50–52), on the suppression of ERK1/2 signaling. In contrast to the inhibitory effect of BBI on ERK1/2 activity, genistein and ALLN induced the phosphorylation of ERK1/2 after a 6 h treatment (data not shown). This result indicated that the suppression of ERK1/2 activity is the novel effect of BBI.

Decreased ERK1/2 signaling is caused by a phosphatase, MKP-1

We examined the effects of BBI on MEK activity to find out whether the dephosphorylation of ERK1/2 could be because of decreased MEK activity, which may in turn phosphorylate both threonine and tyrosine residues of ERK1/2. When cells were exposed to BBI in time-course experiments, a decrease of ~44% in phospho-MEK1/2 levels was noted as compared
with control after a 12-h BBI treatment (Figure 5A). However, there was no further decrease in the phospho-MEK1/2 levels when cells were treated with BBI for further periods such as 18 or 24 h. To further elucidate, whether any phosphatase might be involved in the inactivation of BBI-mediated ERK1/2, cells were pretreated with BBI for 4 h and then after adding sodium orthovanadate, a general inhibitor for protein tyrosine phosphatases, for another 2 h. As Figure 5B shows, sodium orthovanadate not only increased baseline levels of phospho-ERK1/2, but also significantly abrogated the ability of BBI to induce dephosphorylation of ERK1/2. This result suggested that an upregulation of vanadate-sensitive tyrosine phosphatase may be responsible for the decreased level of phospho-ERK1/2 mediated by BBI. We next investigated whether BBI led to the induction of a phosphatase or the activation of an inactive phosphatase. Pretreatment of actinomycin D, the de novo transcription inhibitor, completely eliminated the inhibitory effects of BBI on the activation of ERK1/2 (Figure 5C). These findings showed that dephosphorylation of ERK1/2 mediated by BBI resulted from the induction of a tyrosine phosphatase. Because of the inducible MAP kinase phosphatase-1 (MKP-1) has the ability to dephosphorylate phospho-Ser-Thr or phospho-Tyr residues and shows selectivity for ERK1/2 in vitro (53). Thus, to further probe the possible link between MKP-1 and BBI-induced dephosphorylation of ERK1/2, we evaluated the concentration and time dependence of MKP-1 in MCF7 cells exposed to BBI by western blotting. A significant induction of MKP-1 was noted in a manner that is dependent on the various concentrations of BBI, when compared with low levels of MKP-1 in the absence of BBI (Figure 6A). Moreover, the levels of MKP-1 proteins accumulated as early as 3 h, with subsequent increase in a time dependent manner after the initial treatment with 20 μM of BBI (Figure 6B). Importantly, MKP-1 induction appeared to correlate with ERK1/2 dephosphorylation in BBI-treated MCF7 cells and both showed increased levels in a dose- and time-dependent manner, although a more continuous induction of MKP-1 was seen. Interestingly, MKP-1 is one of the proteasome target proteins which degrade via the ubiquitin–proteasome pathway (54). Taken together, these findings suggested that the molecular mechanism responsible for decreased ERK1/2 signaling resulted from the accumulation of MKP-1 via proteasome inhibition mediated by BBI. The notion that the proteasome inhibition by BBI may contribute to its cancer preventive effects was consistent with our results.
**Effects of BBI treatment on EGF-induced activation of ERK1/2 and Akt signaling**

With the emerging role of the activated epidermal growth factor receptor (EGFR) in breast cancer, it would be important to verify the chemopreventive effects of BBI on two EGFR-stimulated downstream survival pathways, ERK1/2 and Akt. We next investigated the possibility that BBI might compete with EGF for binding to EGFR and block EGF-stimulated proliferation of MCF7 cells. When MCF7 cells were treated with 40 ng/ml EGF in the absence of serum, the maximum activation of ERK1/2 detected by phospho-ERK1/2 (Thr202/Tyr204) antibody was seen at 5 min and then declined to beneath basal activity by 15–30 min without affecting total protein levels of ERK1/2 (Figure 7A). A similar time frame was shown for Akt activation. The results showed that EGF is capable of specifically activating ERK1/2 and Akt. Simultaneously, treatment with BBI and EGF did not prevent EGF-stimulated ERK1/2 activation as well as Akt activation, indicating that BBI exerted its inhibitory effects on ERK1/2 activity without binding to EGFR. Moreover, pretreatment of BBI for 6 h before the addition of EGF exhibited more potent attenuation on EGF-stimulated ERK1/2 activation than vehicle-treated controls (Figure 7B) whereas no significant change in EGF-stimulated Akt activation was detected (data not shown), suggesting that BBI specifically executes its inhibitory effects on ERK1/2 signaling via accumulation of MKP-1 protein which required a period of time for blockade of proteasome-mediated degradation induced by BBI. Therefore, we infer that BBI-mediated abrogation of the proteasome function may be a precedent to the attenuation of ERK1/2 activity which transduces proliferative signals to cells.

**Discussion**

Despite significant preclinical advances that BBI shows promise of becoming an effective nontoxic chemopreventive agent in phase IIa clinical trial, only a few of the underlying mechanisms by which BBI exerts its anticarcinogenetic effects are definitely understood. In this study, interest was sparked on the effects of BBI on proteasome activities in solid breast cancer MCF7 cells. We demonstrated that BBI, which acts as a chymotrypsin and trypsin inhibitor, potently and specifically inhibited the proteasomal chymotrypsin-like activity in intact MCF7 cells. We also found that BBI more potently suppressed proteasomal chymotrypsin-like activity than ALLN in intact MCF7 cells (Figure 1D). Previous studies indicated that BBI is an extraordinary protein with the ability to survive the digestion process and widely distribute to all organs in a fully active form (55). This implies that BBI may be more resistant to cellular enzymes and efficiently penetrate through cellular membranes to perform its inhibitory effects on the proteasome function. The activity of BBI is dependent on the interactions between the reactive site of BBI and the active site of the proteasome. Therefore, BBI with two separate inhibitory domains, may typically allow simultaneous inhibition of proteasomal chymotrypsin-like and trypsin-like activities. It is possible that the exposed bulky conformation of BBI forms a stereohindrance for proteasome substrate processing and

**Fig. 6.** Upregulation of MKP-1 mediated by BBI in MCF7 cells. Cells after treatment with varying concentrations of BBI for 24 h (A) or 20 μM of BBI for indicated times (B) were harvested as described in the Materials and methods section. The levels of MKP-1 protein were analyzed by western blot with anti-MKP-1 antibody. Results are from a single experiment that is representative of three separate experiments. The values below the figures represent the changes in protein expression of the bands normalized to the levels of Hsp90.

**Fig. 7.** Effects of BBI treatment on EGF-induced activation of ERK1/2 and Akt. MMCF7 cells were synchronized by serum starvation for 24 h and then stimulated with EGF (40 ng/ml) in the presence or absence of 20 μM of BBI (A) or pretreated with 20 μM of BBI for 6 h before EGF was added (B). After incubation for the indicated time periods, cells were harvested and western blot analysis performed to detect the levels of proteins as described in the Materials and methods section. The membrane was probed with phospho-ERK1/2, ERK1/2, phospho-Akt and Akt antibodies. Results are from a single experiment that is representative of three separate experiments. The values below the figures represent the changes in protein expression of the bands normalized to the levels of ERK1/2 or Akt.
lodges in the proteolytic region of the 26S proteasome, resulting in the blockade of the proteasome function.

Several studies have recently established that cyclin/CDK complexes, whose activity is constrained by CKIs, regulate cell progression through the G1 phase and the initiation of the mitosis phase or the entry into the S phase. In normal cells, CDK2 forms a complex with cyclin E, whereas CDK4 associates with cyclin D, both required to catalyze the G1/S transition. The collaboration between cyclins and CKIs such as p27Kip1 and p21Cip1/WAF1, governs the cell cycle control during G1 phase (56–58). The previous report illustrated that upregulation of CKIs attenuated the activity of CDKs to phosphorylate their target proteins and stabilized the association of cyclins and CDKs (44). In this study, our results showed that BBI treatment resulted in the accumulation of p27Kip1 and p21Cip1/WAF1, and the consequent downregulation of cyclin D1 and cyclin E that modulate the G1/S phase progression (Figure 2), suggesting that BBI might induce cell-cycle arrest at G1/S phase. However, no significant changes of the CDK2 and CDK4 proteins were detected. It is possible that BBI-induced accumulation of p27Kip1 and p21Cip1/WAF1 might lead to an alteration in the CDK–cyclin complexes by a decrease in the protein levels of cyclins or kinase activity of CDKs rather than the downregulation of CDK proteins. Furthermore, BBI potentely arrested the growth of MCF7 cells after a 24-h incubation (Figure 3C). On the other hand, BBI also suppressed ERK1/2 signaling, which is the well-characterized signal transduction cascade involved in the control of cell proliferation (34), in a dose- and time-dependent manner (Figure 4). Several groups averred that the possible role of MAPK activation in cell-cycle progression may be linked to increased expression of cyclin molecules, e.g. cyclin D1 (59,60). Inhibition of the MAPK pathway may increase the susceptibility of carcinoma cells to cytotoxic drugs by modulating cell-cycle progression and/or by interfering with downstream events. In agreement with these studies, BBI certainly attenuated ERK1/2 activity, which was accompanied by downregulation of cyclin D1 and possibly followed by dysregulation of cell cycle progression. Together, these results supported the notion that proteasome inhibition induced by BBI may contribute to the growth inhibitory effects of BBI on MCF7 cells.

Given the crucial role of MAPK activity in breast cancer (61,62), we highlighted the chemopreventive effects of BBI on ERK signaling. We found the dephosphorylation of ERK1/2 at as early as 3 h of exposure to BBI in the continued presence of FCS (Figure 4). However, phospho-ERK1/2 levels were decreased after an 18-h BBI treatment without affecting the activity of MEK1/2, the upstream event of ERK1/2 (Figure 5A), but the exclusion of BBI-attenuated ERK1/2 activity was detected in the presence of the phosphatase inhibitor or actinomycin D (Figure 5B and C). It suggested that dephosphorylation of ERK1/2 was on account of a phosphatase. Several pieces of evidence implied that MKP-1, the major phosphatase involved in the inactivation of ERK1/2 signaling (36,53), is degraded via the ubiquitin–proteasome pathway (54). Based on these studies, we found that MKP-1 was strongly induced by BBI in a dose- and time-dependent manner (Figure 6), indicating that proteasome inhibition mediated by BBI is sufficient to increase the protein levels of MKP-1.

In addition to the correlation of ERK inactivation and MKP-1 activity, there are other regulators involved in the MAPK pathway such as MKP-3, which dephosphorylates cytosolic phospho-ERK1/2 (63) and blocks its nuclear translocation (64), and protein serine/threonine phosphatase (PP) 2A, which is a ubiquitous enzyme with pleiotropic functions and has the ability to inactivate phospho-MEK1/2 (65,66). This might be one possible reason that phospho-MEK1/2 was slightly inhibited, owing to the actions of PP2A or MKP-3, when cells were exposed to BBI for 12 h (Figure 5A). The cellular effects of proteasome inhibitors are similar to that of oxidative stressors on mitochondrial membrane potential and cell viability (67,68). However, multiple pathways are involved in the expression of MKP-1, which was identified as an immediate early gene responsive to serum stimulation as well as oxidative stress (69). Accordingly, the induction of MKP-1 is coincident with ERK inactivation and partly dependent on ERK activity which is a consequence of the complicated modulations among the cellular signal pathways. Consequently, these findings supported the hypothesis that the induction of MKP-1 by BBI might in part have resulted in decreased proteolytic rate and oxidative stress caused by the attenuation of the proteasome function in this study.

It is intriguing that the MKP-1 expression more steadily increased over the time course of the BBI treatment when compared with the recovery of phospho-ERK1/2 at 24 h (Figure 4B versus 6B). Catalytic activation of MKPs is achieved though a substrate-induced activation mechanism (70). The report by Brunet et al., who noted that expression of the inactive form of MKP-3 sequestered phospho-ERK1/2 in the cytoplasm (64), suggested that MKP-3 may play an important role in nuclear translocation and the activity of ERK1/2, even though upregulation of MKP-1 was observed in BBI-treated MCF7 cells. However, the means by which MKP-1 executes such regulatory effects on ERK1/2 after BBI treatment remain elusive. Our current observation is consistent with the suppressive effects of BBI on phospho-ERK1/2 levels, demonstrating the correlation between upregulation of MKP-1 levels and ERK1/2 inactivation in BBI-treated MCF7 cells. Taken together, these data illustrated that decreased levels of phospho-ERK1/2 are primarily due to the accumulation of MKP-1, which dephosphorylates phospho-ERK1/2 through the blockade of the proteasome function by BBI.

Elevated Ras–Raf–MKK1/2 signaling or maintenance of ERK1/2 activity is strongly associated with many types of cancers and triggers the degradation of MKP-1 via the ubiquitin–proteasome pathway (71). In this study, we demonstrated that BBI has the ability to abate the proteasome function followed by cell growth arrest which agrees with the suppression of ERK1/2 activity through upregulation of MKP-1. Therefore, BBI potently exerts its effects on proteasome inhibition that plays an important role in applications to chemotherapy for drug-resistant cancer cells bearing elevated ERK activity. However, mitogen-activated ERK is able to induce MKP-1 expression which in turn decreases ERK1/2 activity, indicating that MKP-1 acts as a negative feedback regulator to limit sustained ERK1/2 signaling (72). The quantitative blockade of MKP-1 induction using actinomycin D attenuated the inhibitory effect of MKP-1 on the feedback loop and completely preserved the activity of ERK1/2 (Figure 6C). Although the BBI-mediated increase in the protein levels of MKP-1 is because of protein stabilization by proteasome inhibition, the possibility that BBI may transcriptionally induce MKP-1 expression cannot be excluded. Additionally, a combination of other phosphatase activities such as the serine/threonine protein phosphatase 5, which is induced by proteasome inhibition in MCF7 cells (73), may also be
needed to fully dephosphorylate ERK1/2. This possibility requires further study.

The structure of BBI consisting of 71 residues with seven disulfide bridges is similar to that of EGF, which is a 53-residue protein with three disulfide bonds. It would be interesting to see whether BBI competes with EGF for binding to EGFR and blocks the proliferation of tumor cells. To examine the possibility that BBI-induced antiproliferation might be due to the blockade of EGF-stimulated EGFR signaling, no significant difference in the levels of phospho-ERK1/2 and phospho-Akt in BBI-treated cells was detected when compared with those of vehicle-treated controls (Figure 7A). It suggests that BBI did not compete with EGF for binding to EGFR. Pretreatment of BBI for 6 h displayed more inhibition on EGF-stimulated ERK1/2 activity (Figure 7B), whereas BBI has no inhibitory effects on Akt activity in dose- or time-dependent frame in the presence of serum or EGF (data not shown). Thus, these results further confirm that BBI certainly increases MKP-1 proteins via proteasome inhibition and leads to decreased ERK1/2 activity.

To date, there have been more investigations into the anti-cancer effects of genistein than that of BBI. In fact, soybean contains a greater proportion of protease inhibitors than genistein (2.4 and 0.05%, respectively). Animal carcinogenesis studies have demonstrated that dietary levels containing as little as 0.01% BBI can suppress liver carcinoma in mice (74). It is assumed that 50 mg of BBI activity from dietary 2 g soybean would be necessary in the 500 g human dietary intake for the prevention of some kinds of cancer. Therefore, we infer that BBI is a more potent and effective cancer preventive agent than genistein if there is a daily soyfood intake. Since protease inhibitors regulate effects on cell proliferation and apoptosis by controlling protein levels of cellular key elements, dysfunction of proteasome by proteasome inhibitors appears to have cytotoxic effects on cancer cells. In conclusion, our results first report that BBI which specifically inhibits 26S proteasome dysfunction of proteasome by proteasome inhibitors appears to decreased ERK1/2 activity.

Conflict of Interest Statement: None declared.

References


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