

Cytotoxic Effects of 7-*O*-Butyl Naringenin on Human Breast Cancer MCF-7 Cells

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Abstract The effect of 7-*O*-butyl naringenin (BN), a chemically synthesized derivative of naringenin, was tested on the proliferation of human breast cancer MCF-7 cells. BN inhibited the proliferation of MCF-7 cells in dose-dependent manner (IC_{50} : $67.5 \pm 2.1 \mu\text{M}$), resulting in an increase in the sub-G1 phase cell population. BN induced the generation of intracellular reactive oxygen species (ROS), which were reduced by pretreatment with *N*-acetylcysteine (NAC). BN also increased the phosphorylation of stress-activated protein kinase/c-Jun NH₄-terminal kinase 1/2 (SAPK/JNK1/2), c-Jun, and p38. However, the phosphorylation of extracellular-regulated kinase 1/2 (Erk1/2) was decreased in BN-treated cells. Pretreatment of cells with the specific inhibitors SP600125 and SB203580 diminished the BN-induced activation of SAPK/JNK1/2 and p38, respectively. These results indicate that the BN-induced cytotoxicity of MCF-7 cells is mediated by the generation of ROS as well as through the p38, SAPK/JNK1/2, and c-Jun activation signaling pathways. BN may therefore possess chemotherapeutic

potential as an anti-proliferative agent.

Keywords: 7-*O*-butyl naringenin, reactive oxygen species (ROS), stress-activated protein kinase/c-Jun NH₄-terminal kinase 1/2 (SAPK/JNK1/2), p38, c-Jun

Introduction

Flavonoids, which are natural compounds widely distributed in plants such as fruits and vegetables, are known to possess anti-tumor activity against various human cancer cell lines and human tumor xenograft systems, suggesting that they hold potential as tumor suppressive agents (1-4). Flavonoids also have anti-oxidative, tissue-protective, cell cycle progressive, and suppressive effects. Therefore many studies have attempted to investigate their role in human disease and health (5-8).

Naringenin (Ng), a flavanone compound found in citrus fruits such as grapefruits, oranges, and tomato skin (9,10), has been reported to possess anti-oxidant, free radical scavenger, anti-inflammatory, and immunomodulator activities (11-13). Ng is also known to cause cytotoxic and apoptotic effects in several cancer cell lines in a dose-dependent manner as well as inhibits tumor growth in sarcoma S-180 implanted mice, suggesting that Ng can potentially be used to inhibit tumor growth (14-16). Cytotoxic effects were also induced in human cancer cell lines when high concentrations of Ng were administered (50% effective concentration: $150\text{-}560 \mu\text{M}$). However, the use of flavonoids as cancer chemopreventive or chemotherapeutic agents requires the development of novel flavonoids or Ng derivatives that can induce cytotoxicity at low concentrations in a cell type-dependent manner. Previously, we evaluated the effects of Ng along with 3 Ng derivatives [7-*O*-*tert*-butoxycarbonylmethyl

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naringenin (TN), 7-*O*-butyl naringenin (BN), and 7-*O*-(α -methoxycarbonyl)benzyl naringenin (MN)] on the growth of human breast cancer MCF-7 cells. Maximum cell growth inhibition was observed in BN-treated cells (data not shown). Recently, Kim *et al.* (17) reported that BN had anti-microbial effects on *Helicobacter pylori* through the inhibition of urease activity. BN has been also suggested as a potential anti-inflammatory agent for the inhibition of hyaluronidase (18), which catalyzes the hydrolysis of hyaluronic acid and is involved in allergic reactions, cancer metastasis, and inflammation (19,20). However, the effect of BN on human cancer cell lines has not been previously reported. Here, we report the molecular mechanisms involved in the BN-induced cell growth inhibition of MCF-7 cells.

Materials and Methods

Materials Cell culture media and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. TN, BN, and MN were kindly supplied by Prof. Yong-Sun Park of the Chemistry Department, Konkuk University, Seoul, Korea. Briefly, BN mainly used in this work was synthesized from 12 hr stirring reaction of Ng (500 mg, 1.84 nmol), butyl bromide (1.5 equiv), and K_2CO_3 (1.0 equiv) in dimethyl formamide (DMF, 10 mL) (18).

Cell and cell culture Human breast cancer MCF-7 cells were obtained from KCLB[®] (Korean Cell Line Bank, Korea) and were cultured in Dulbecco's modified Eagle's minimum media (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin solution at 37°C in a 5% CO_2 incubator. All experiments were performed on 60 cm^2 petri dishes unless stated otherwise. Cells were seeded onto 60 cm^2 petri dishes at a density of 1×10^6 cells/dish and incubated for 24 hr prior to the experiment. Cells were washed with phosphate buffered saline (PBS, pH 7.4) and treated with fresh media containing different concentrations of Ng or BN dissolved in dimethyl sulfoxide (DMSO). The vehicle control received only DMSO (0.05%, v/v).

Cell growth inhibition study using the MTT assay

Cell growth inhibition was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells cultured overnight on 96-well plates were washed twice with PBS, administered media containing different concentrations of BN and incubated for 24 hr. Ten μ L of MTT stock solution (5 mg/mL) were added to each well, followed by incubation for an additional 4 hr. Blue formazans were eluted from cells by the addition of 100 μ L of DMSO with gentle shaking for 10 min at room temperature. Absorbances were measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (SpectraMAX; Molecular Devices, Sunnyvale, CA, USA).

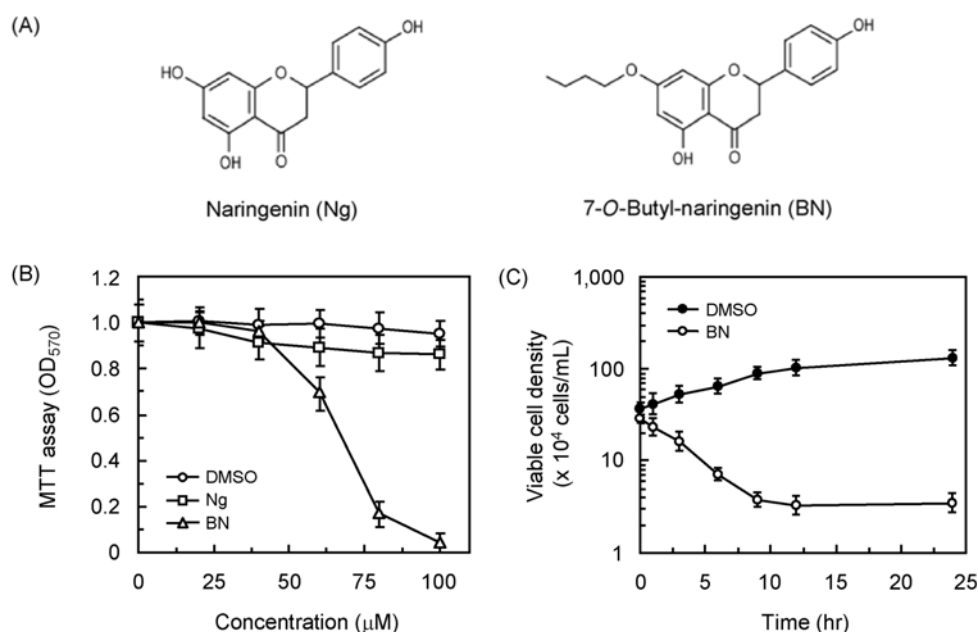


Fig. 1. Effect of BN on MCF-7 cell proliferation. (A) Chemical structures of naringenin (Ng) and 7-*O*-butyl naringenin (BN), (B) MCF-7 cells were treated with different concentrations of BN for 24 hr, followed by MTT assay, (C) MCF-7 cells were treated with 100 μ M BN for the indicated times and cell viability was determined. Data are mean \pm SD from 3 independent experiments and each experiment was conducted in triplicate.

Cell viability Cell viability was determined by trypan blue dye exclusion assay. A cell suspension was prepared using brief trypsinization. One-hundred μL of cell suspension were mixed with 100 μL of 1% trypan blue solution, which was left for 5 min at room temperature. Stained (dead) cells and total cells per square of cell chamber (4 squares/suspension) were counted under a microscope (Olympus, Tokyo, Japan).

Flow cytometric analysis Cells grown for 12 hr in the absence or presence of different concentrations of BN in 60 cm^2 petri dishes were collected by trypsinization, washed twice with PBS, and fixed in 1 mL of ice-cold 70% ethanol for 1 hr at 4°C. Cell pellets were collected by centrifugation (800 \times g for 3 min), washed with PBS and resuspended in 1 mL of propidium iodide (PI) solution (50 $\mu\text{g}/\text{mL}$ in PBS) containing 10 $\mu\text{g}/\text{mL}$ of RNase A. After incubation in the dark for 30 min, cells were analyzed by a flow cytometer

(FACSCalibur, BD Biosciences, San Jose, CA, USA). Fluorescences emitted from PI-DNA complexes were estimated using a minimum of 10,000 cells/sample and analyzed using Cell Quest Alias software (BD Biosciences).

Determination of ROS Intracellular reactive oxygen species (ROS) generated by BN or hydrogen peroxide (H_2O_2) were measured based on the intracellular peroxide-dependent oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) to the fluorescent compound 2',7'-dichlorofluorescein (DCF), as previously described (14,21). Cells were seeded onto 24-well plates at a density of 5×10^4 cells/well and cultured for 24 hr. After washing twice with PBS, fresh medium containing 80 μM BN or 1 mM H_2O_2 was added and the cells were incubated for 6 hr. Cells were then administered 20 μM of DCFH-DA and incubated for 30 min at 37°C. After rising with PBS 2 times, an additional

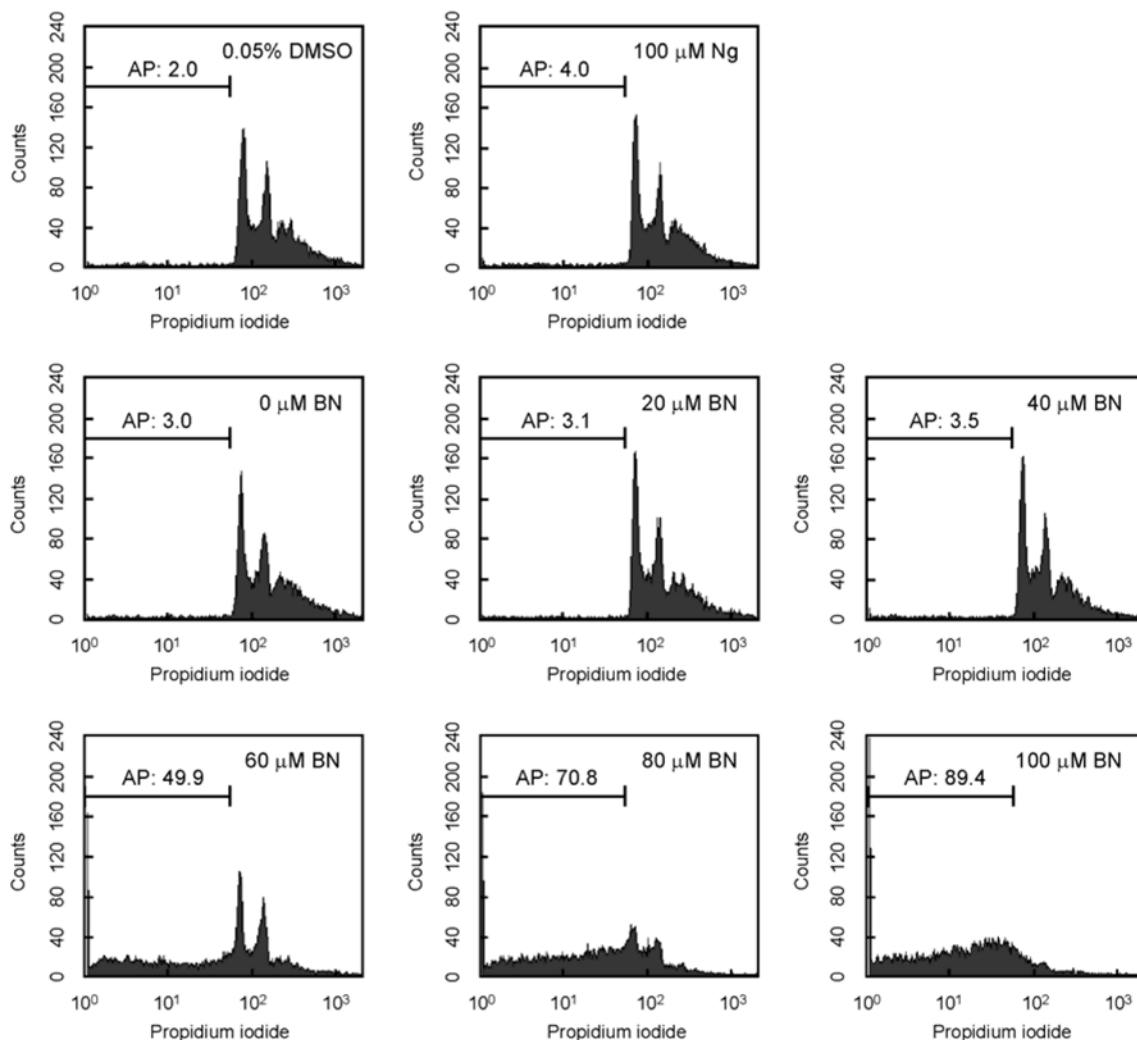


Fig. 2. Flow cytometric analysis of BN-treated MCF-7 cells. Cells were treated with different concentrations of BN for 12 hr. AP values mean the percentage of sub-G1 cells. Data represent the results of 3 independent experiments.

1 mL of PBS was added to each well and the fluorescent intensity was determined using a fluorescence microscope (Carl Zeiss, Gaottingen, Germany) with excitation at 485 and emission at 530 nm. Fluorescence emitted from DCF was also analyzed by flow cytometry as described above.

Cells grown in 24-well plates for 24 hr were administered the anti-oxidant *N*-acetylcysteine (NAC, 5 mM) for 1 hr prior to treatment with 80 μ M BN or 1 mM H₂O₂ for 12 hr. This was followed by the addition of 20 μ M DCFH-DA and incubation for 30 min at 37°C. Cells were treated as described above to determine changes in DCF fluorescence.

Protein extraction and Western blot analysis Cells grown in the absence or presence of different concentrations of BN were washed with ice-cold PBS and lysed with 100 μ L of lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM NaF, 1% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail] for 30 min at 4°C. Protein extracts were collected after centrifugation at 16,000 \times g for 10 min. Protein concentrations were determined using a DC protein

assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein extracts were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked by incubation in 5% non-fat dry milk in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05%(v/v) Tween-20] for 2 hr and probed with primary antibodies. After washing with TBST 3 times with gentle shaking, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 hr. Membranes were again washed 3 times with gentle shaking and chemiluminescent signals were visualized using an enhanced chemiluminescence reagent (ECL; Pierce, Rockford, IL, USA) as described by the manufacturer. All antibodies used in this experiment were purchased from Cell signaling (Beverly, MA, USA; phospho-p38 and phospho-SAPK/JNK1/2 antibodies) and Santa Cruz Biotechnology (Santa Cruz, CA, USA; phospho-Erk1/2, Erk1/2, phospho-c-Jun, c-Jun, β -actin, and secondary anti-mouse or rabbit antibodies). For Western blot and

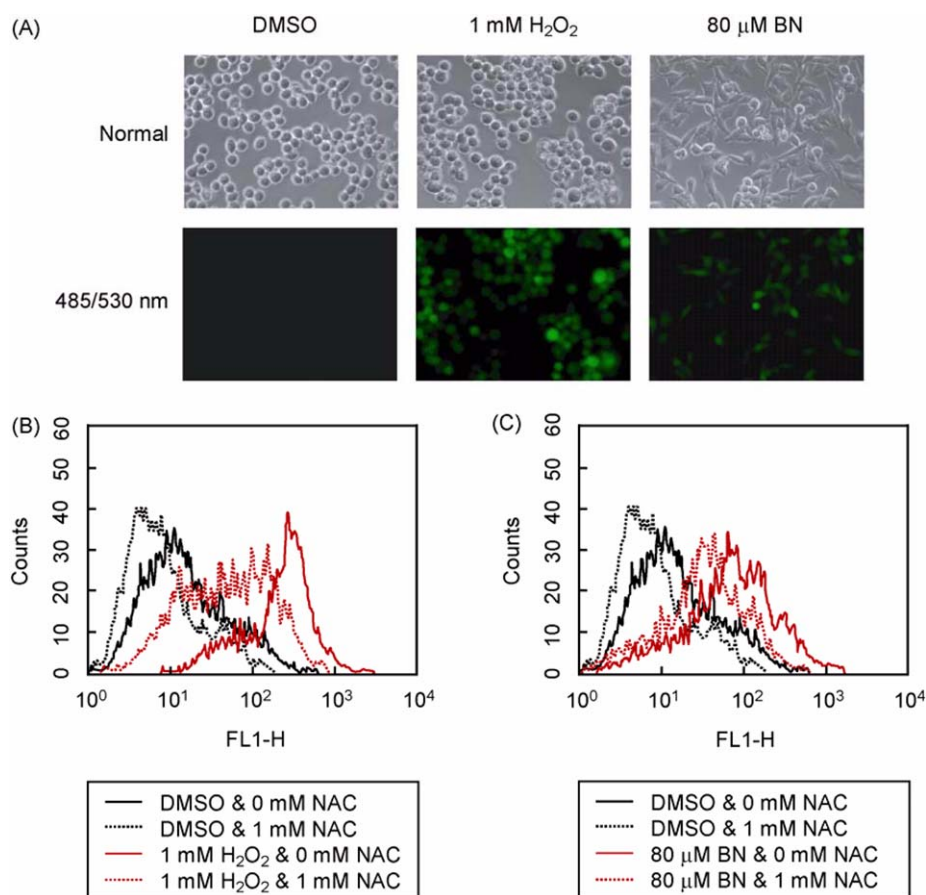


Fig. 3. ROS generation in BN-treated MCF-7 cells. (A) MCF-7 cells were treated with 1 mM H₂O₂ and 80 μ M BN for 6 hr, followed by incubation with 20 μ M DCFH-DA for 30 min. DCF fluorescence was determined by fluorescence microscopy, (B & C) NAC-pretreated or untreated MCF-7 cells for 1 hr were incubated with 1 mM H₂O₂ (B) and 80 μ M BN (C) for 6 hr. After fixation, DCF fluorescence was determined by flow cytometry.

autoradiographic images, the density of each band was measured using Image acquisition and analysis software ver 4.5 (UVP, Upland, CA, USA).

Results and Discussion

BN-induced cell growth inhibition of MCF-7 cells To determine the effect of Ng or BN on MCF-7 cell proliferation, cells cultured overnight were treated with different concentrations of Ng or BN for 24 hr and cell growth was measured by MTT assay (Fig. 1B). BN inhibited cell growth with an ID_{50} of $67.5 \pm 2.1 \mu\text{M}$. However, Ng showed only about 8% cell growth inhibition. Cell growth inhibition by BN was also confirmed by measuring viable cell densities by trypan blue dye exclusion assay (Fig. 1C). Cells cultured overnight were treated with $100 \mu\text{M}$ BN for 2, 4, 6, 8, 12, and 24 hr, followed by determination of viable cell densities. BN inhibited the growth of MCF-7 cells, as most cells lost viability after 12 hr of BN treatment. To investigate the sub-G1 cell population induced by apoptotic DNA fragmentation, BN-treated MCF-7 cells were cultured for 12 hr, stained with PI, and their DNA contents were

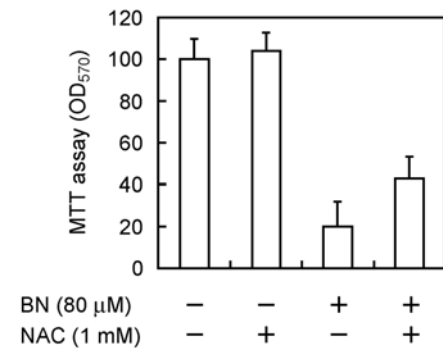


Fig. 4. Effect of NAC on BN-induced MCF-7 cell growth inhibition. NAC-pretreated MCF-7 cells were incubated with $80 \mu\text{M}$ BN for 12 hr and cell proliferation was determined by MTT assay. Data are mean \pm SD from 3 independent experiments and each experiment was conducted in triplicate. The value obtained from BN- and NAC-untreated cells was obtained as 100.

analyzed by flow cytometry (Fig. 2). The amount of cells in the sub-G1 population was increased in a dose-dependent manner, resulting in increases of 3, 3.1, 49.9, 70.8, and 89.4% after incubation with 0, 20, 40, 60, 80, and $100 \mu\text{M}$ BN, respectively. Ng has been reported to inhibit the proliferation of MCF-7 cells by down-regulating

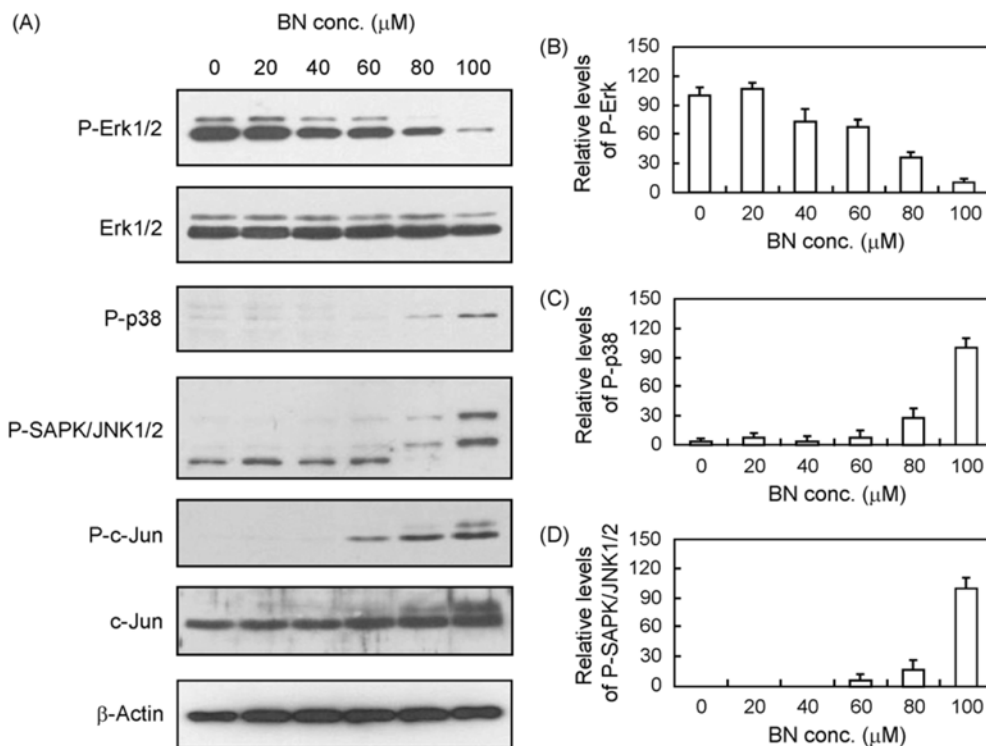


Fig. 5. Effect of BN on MAPK activation. (A) MCF-7 cells were treated with different concentrations of BN for 12 hr. Identical amounts of whole cell extracts were separated by SDS-PAGE. P-Erk1/2, Erk1/2, P-p38, P-SAPK/JNK1/2, P-c-Jun, and c-Jun were detected by Western blot analysis. β -Actin was used as a control. (B-D) The contents of P-Erk1/2 (B), P-p38 (C), and P-SAPK/JNK1/2 (D) obtained from 3 independent experiments of (A) were quantified and relative levels were plotted as bar diagrams. The protein content obtained from BN-untreated cells was estimated as 100 (B). The contents of P-p38 and P-SAPK/JNK1/2 obtained from $100 \mu\text{M}$ BN-treated cells were estimated as 100 (C & D).

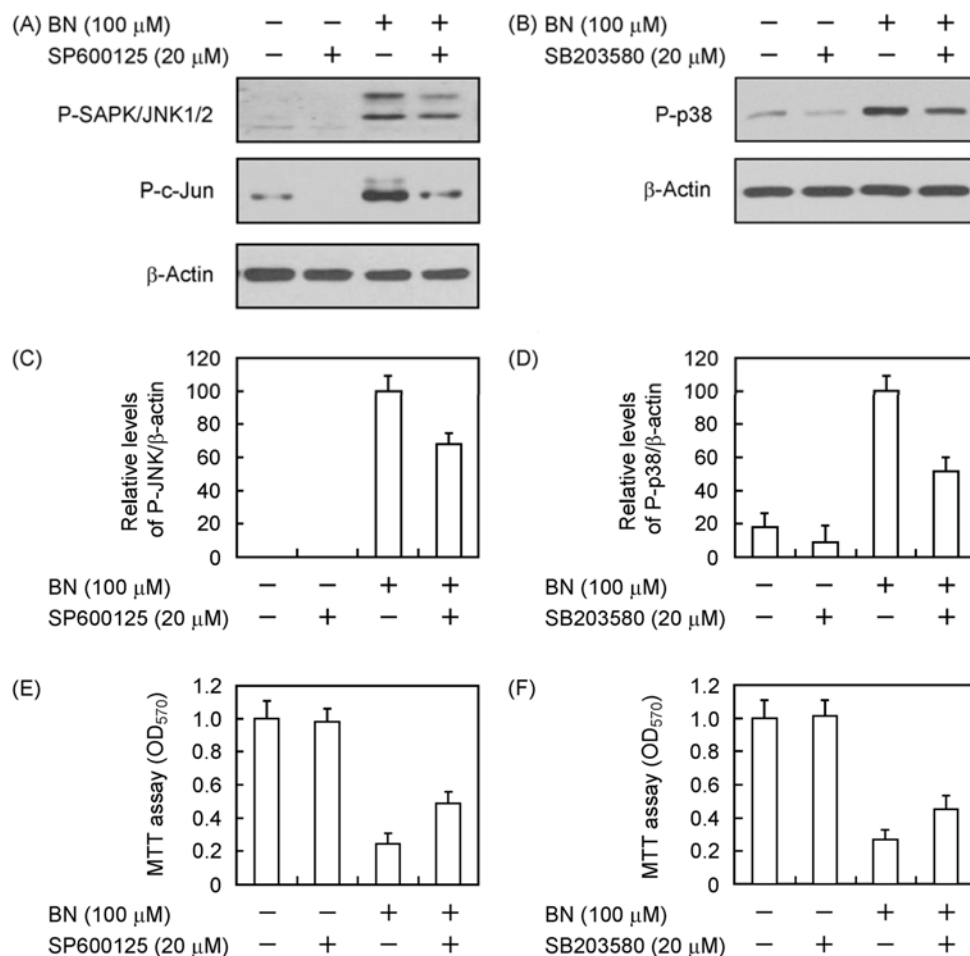


Fig. 6. Effect of SP600125 and SB203580 on BN-treated MCF-7 cells. (A & B) SP600125 (A)- or SB203580 (B)-pretreated MCF-7 cells for 1 hr were incubated with 100 μ M BN for 12 hr. Identical amounts of whole cell extracts were separated by SDS-PAGE. P-SAPK/JNK1/2, P-c-Jun, and P-p38 were detected by Western blot analysis. β -Actin was used as a control. (C & D) The contents of P-SAPK/JNK1/2 (C) and P-p38 (D) obtained from 3 independent experiments of (A & B) were quantified and relative levels were plotted as bar diagrams. The protein content obtained from BN- and SP200125/SB203580-untreated cells was estimated as 100. (E & F) SP600125 (E) or SB203580 (F)-pretreated MCF-7 cells were incubated with 100 μ M BN for 12 hr and cell proliferation was determined by MTT assay. Data are mean \pm SD from 3 independent experiments and each experiment was conducted in triplicate.

insulin-stimulated glucose uptake, suggesting that Ng may possess therapeutic potential as an anti-proliferative agent (22). Furthermore, Kano *et al.* (15) reported that Ng treatment induced 50% growth inhibition of MCF-7 cells at 240 ± 23 μ M after 48 hr. We also determined MCF-7 cell proliferation at high concentrations (maximum 200 μ M) of Ng or the Ng derivatives, TN or MN. Interestingly, 50% growth inhibition was not observed even at a concentration of 200 μ M (data not shown). However, BN induced 50% growth inhibition of MCF-7 cells at 67.5 ± 2.1 μ M over 24 hr (Fig. 1B). This indicates that BN inhibits the proliferation of MCF-7 cells more effectively than Ng or the other Ng derivatives, TN and MN.

ROS generation in BN-treated MCF-7 cells The generation of intracellular ROS by BN in MCF-7 cells was evaluated by measuring the intracellular peroxide-

dependent oxidation of DCFH-DA. MCF-7 cells were treated with 80 μ M BN for 6 hr followed by the determination of DCF fluorescence by fluorescence microscopy (Fig. 3A). H_2O_2 (1 mM)-treated cells were used as a positive control and were compared with BN-treated cells. BN-treated cells displayed DCF fluorescence values lower than those produced by H_2O_2 -treated cells. To further investigate ROS generation by BN, cells pretreated with NAC, a ROS trapping and inhibiting agent, were administered BN or H_2O_2 for 6 hr in the presence of NAC. DCF fluorescence was analyzed by flow cytometry (Fig. 3B, 3C). H_2O_2 and BN treatment increased the cell populations containing high levels of DCF fluorescence (Fig. 3B, 3C, red solid lines). The presence of NAC decreased H_2O_2 or BN-induced DCF fluorescence (Fig. 3B, 3C, black and red dotted lines). Furthermore, NAC pretreatment decreased BN-induced cell growth inhibition

(Fig. 4). These results demonstrate that BN-induced inhibition of MCF-7 cell growth involves intracellular ROS generation.

Mitogen-activated protein kinase (MAPK) activity is changed in BN-treated MCF-7 cells MAPKs, including extracellular-regulated kinase 1/2 (Erk1/2), p38, and stress-activated protein kinase/c-Jun NH₂-terminal kinase 1/2 (SAPK/JNK1/2), have been shown to play roles in signaling pathways of cell growth stimulation, inhibition, survival, and apoptosis (23,24). To determine the involvement of MAPKs in BN-induced MCF-7 cell growth inhibition, MCF-7 cells were treated with different concentrations of BN for 12 hr and the phosphorylation of Erk1/2, p38, SAPK/JNK1/2, and c-Jun was determined by Western blot analysis. As shown in Fig. 5, BN increased the levels of Phospho-p38 (P-p38) and Phospho-SAPK/JNK1/2 (P-SAPK/JNK1/2), which are activated by pro-inflammatory cytokines and oxidative stress, resulting in cell growth inhibition and apoptosis (25,26). BN also increased the level of Phospho-c-Jun (P-c-Jun) generated by SAPK/JNK1/2 activation. However, BN decreased the level of Phospho-Erk1/2 (P-Erk1/2), which induces cell growth and survival after activation by mitogens and growth factors through a Ras/Raf/MEK signaling cascade. To further explore the activation of SAPK/JNK1/2 and p38 by BN treatment, MCF-7 cells were pretreated with the specific SAPK/JNK1/2 inhibitor, SP600125 (EMD Chemicals, Gibbstown, NJ, USA), or the p38 inhibitor, SB203580 (EMS Chemicals), for 1 hr and administered with 100 μ M BN for 12 hr (Fig. 6A-6D). Pretreatment with SP600125 reduced the BN-induced phosphorylation of SAPK/JNK1/2 by 33% (Fig. 6A, 6C). Pretreatment with SB203580 also reduced the BN-induced phosphorylation of p38 by 49% (Fig. 6B, 6D). As a result, the growth of SP600125 and SB203580-pretreated MCF-7 cells was increased by 102 and 70%, respectively, compared to BN-treated cells (Fig. 6E, 6F). This implies that BN-induced cell growth inhibition is mediated by the activation of SAPK/JNK1/2 and p-38 and inactivation of Erk1/2.

In summary, we investigated the effect of BN on MCF-7 cell proliferation as well as the molecular mechanisms involved in BN-induced cell growth inhibition. BN effectively inhibited the proliferation of MCF-7 cells, compared with natural Ng and other Ng derivatives, suggesting that BN may possess chemotherapeutic potential as an anti-proliferative agent. BN also induced intracellular ROS generation. We further determined that BN achieves MCF-7 cell growth inhibition through the activation of SAPK/JNK1/2 and p38 and inactivation of Erk1/2.

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