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A novel anticancer agent Broussoflavonol B downregulates estrogen receptor (ER)-α36 expression and inhibits growth of ER-negative breast cancer MDA-MB-231 cells

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ABSTRACT

Estrogen receptor (ER)-negative breast cancers are aggressive and unresponsive to antiestrogens, and current therapeutic modalities for ER-negative breast cancer patients are usually associated with strong toxicity and side effects. Less toxic and more effective targeted therapies are urgently needed to treat this type of breast cancer. Here, we report that Broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree (*Broussonetia papyrifera*) exhibited potent growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells at sub-micromolar concentrations. Broussoflavonol B induced cell cycle arrest at both the G_0/G_1 and G_2/M phases accompanied by a downregulation of c-Myc protein, a upregulation of the cell cycle inhibitory proteins $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ and a down-regulation of the expression levels of the G_2/M regulatory proteins such as cyclin B1, cdc2 and cdc25C. Broussoflavonol B also induced apoptotic cell death characterized by accumulation of the annexin V- and propidium iodide-positive cells, and cleavage of caspases 8, 9 and 3. In addition, Broussoflavonol B treatment also decreased the steady state levels of the epidermal growth factor receptor (EGFR) and ER-α36, a variant of estrogen receptor-α, and restricted growth of the stem-like cells in ER-negative breast cancer MDA-MB-231 cells. Our results thus indicate that Broussoflavonol B is a potent growth inhibitor for ER-negative breast cancer cells and provide a rational for preclinical and clinical evaluation of Broussoflavonol B for ER-negative breast cancer therapy.

1. Introduction

Depending on the existence of one of the estrogen receptors, ER-α, human breast cancers are divided into ER-positive or ER-negative. Approximately 70% of breast cancer patients are positive for ER-α and these patients are suitable for hormonal therapy that blocks estrogen stimulation of breast cancer cells. However, ER-negative breast cancer that accounts for about one third of breast cancers diagnosed is often more malignant and aggressive than ER-positive breast cancer. In addition, ER-negative breast cancer patients respond poorly to antiestrogen therapy, and current therapeutic modalities for ER-negative breast cancer patients are usually associated with strong toxicity and side effects. Less toxic and more effective targeted therapeutic approaches are urgently needed to treat this type of breast cancer.

Previously, we identified and cloned a 36 kDa variant of ER-α, ER-α36, that is mainly expressed outside of the cell nucleus and mediates non-genomic estrogen signaling (Wang et al., 2005, 2006). ER-α36 lacks both transcription activation function domains AF-1 and AF-2 of the full-length 66 kDa ER-α (ER-α66), consistent with the fact that ER-α36 has no intrinsic transcriptional activity (Wang et al., 2006). ER-α36 is generated from a promoter located in the first intron of the ER-α66 gene (Zou et al., 2009), indicating that ER-α36 expression is regulated differently from ER-α66, consistent with the findings that ER-α36 is expressed in specimens from ER-negative breast cancer patients and established ER-negative breast cancer cells that lack ER-α66 expression (Pelekanou et al., 2012; Shi et al., 2009; Vranic et al., 2011; Wang et al., 2006). ER-α36 is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011); ER-negative breast cancer MDA-MB-231 and -436 cells with knocked-down concentrations of ER-α36 protein failed to form xenograft tumors in nude mice. Thus, ER-α36-mediated signaling plays an important role in development and progression of ER-negative breast cancer, and ER-α36 may be used as a target to develop novel and more

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effective therapeutic agents for treatment of ER-negative breast cancer.

Broussonetia papyrifera (Moraceae), also known as paper mulberry, grows naturally in Asia and Pacific countries. Its dried fruits have been used for the treatment of ophthalmic disorders and impotency (Lee et al., 2001). The leaves, twig roots and barks of this plant are widely used to treat gynecological bleeding, dropsy, dysentery diseases as a folk medicine in China (Feng et al., 2008). Various types of flavonoids are the major constituents of this plant and some of which exhibited strong tyrosinase inhibitory (Zheng et al., 2008), aromatase inhibitorory (Lee et al., 2001), antifungal (Takasugi et al., 1980, 1984), secretory phospholipase A-2 inhibitory (Kwak et al., 2003), PTP1B enzyme inhibitory (Chen et al., 2002; Nguyen et al., 2012), antimicrobial, cytotoxic (Sohn et al., 2004), antiplatelet (Lin et al., 1996), antioxidant and inducible nitric oxide synthase suppressing activities (Cheng et al., 2001). However, the effects and the underlying mechanisms of the flavonoids from *B. papyrifera* in human cancer have never been studied. Recently, we purified and identified two prenylflavone derivatives from *B. papyrifera* (Guo et al., 2013); one is a known compound Broussonol D (Zhang et al., 2011) that has been isolated from Broussonetia kazinoki and another is Broussoflavonol B (Matsumoto et al., 1985). We also found that both compounds were able to inhibit growth of ER-positive breast cancer MCF7 cells presumably through down-regulation of ER-α36 expression (Guo et al., 2013). However, Broussoflavonol B was more potent than Broussonol D in downregulation of ER-α36 expression (Guo et al., 2013). Since ER-α36 is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011), we decided to study the effects and underlying mechanisms of Broussoflavonol B (5,7,3′,4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) purified from the bark of *B. papyrifera* in growth of ER-negative breast cancer MDA-MB-231 cell.

In the present study, we demonstrated that Broussoflavonol B exhibited potent growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells at sub-micromolar (μM) concentrations. Broussoflavonol B treatment decreased the steady state levels of ER-α36 and EGFR proteins, and induced cell cycle arrest and cell apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Broussoflavonol B (99.5% purity) was obtained from Shenogen Parma Group, Ltd (Beijing, China). Anti-p16^{INK4a} (N-20), p19^{INK4D} (M-167), p21$^{WAF1/CIP1}$ (F-5), β-actin (I-19), c-Myc (9E10), caspase 3 (S-19), caspase 8 (H-134), caspase 9 (H-170), Cdc 25c (H-150), cyclin B1 (GNS1) and Cdc2 p34 (POH-1), cytokeratin 18 (DC-10), CD10 (H-321) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-EGFR antibody (1F4) and anti-vimentin (D21H3) antibody were from Cell Signaling Technology (Danvers, MA, USA). Polyclonal anti-ER-α36 antibody was generated and characterized as described before (Wang et al., 2006).

2.2. Cell culture

MDA-MB-231 cells were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution from Invitrogen Life Technologies Corporation (Carlsbad, CA, USA). Before experiments, cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA).

2.3. Cell growth and differentiation assays

Cells in the phenol red-free medium were seeded onto 35 mm dishes at 5×10^4 cells/dish. After 24 h, the indicated concentrations of vehicle dimethyl sulfoxide (DMSO), Broussoflavonol B or tamoxifen were added and incubated for seven days. Cells were trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Korea). Three dishes were used for each concentration point and experiments were repeated at least three times.

To assess the effects of Broussoflavonol B on epidermal growth factor (EGF)-stimulated cell growth, cells (1×10^4/dish) in 60 mm dishes were maintained in phenol red-free medium with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 48 h. EGF (10 ng/ml) alone, together with Broussoflavonol B or Broussoflavonol B alone were added to cells and incubated for 72 h, and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea). Three dishes were used for each treatment and experiments were repeated more than three times.

For cancer stem-like cell growth, MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA, USA) at 10,000 cells/ml and cultured in phenol red-free DMEM/F12 medium (Invitrogen) supplemented with 1X B27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). The different concentrations of Broussoflavonol B or tamoxifen were

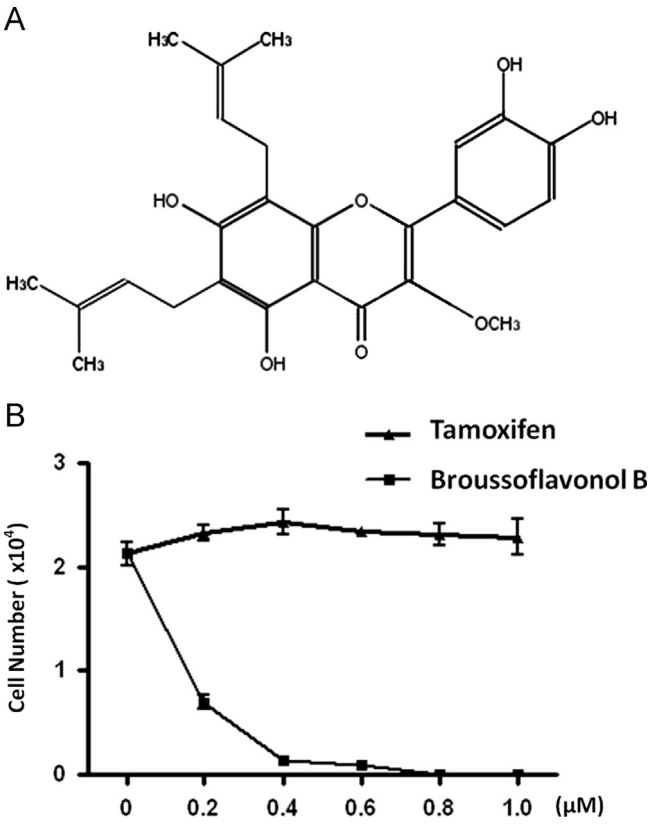

Fig. 1. Broussoflavonol B inhibits growth of ER-negative breast cancer MDA-MB-231 cells. (A) The chemical structure of Broussoflavonol B (5,7,3′,4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone). (B) Effects of Broussoflavonol B or tamoxifen on the growth of MDA-MB-231 cells. Cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with vehicle, DMSO (0) and 0.2, 0.4, 0.6, 0.8 and 1.0 μM of Broussoflavonol B or tamoxifen for seven days before cells were trypsinized and counted. Three dishes were used for each concentration in the experiments and all experiments were repeated three times. Each point represents mean ± S.D. of three independent experiments.

added and incubated for seven days. Cells were collected, washed with PBS, and incubated with Trypsin-EDTA (0.05%/0.5 mM) for 2 min at 37 °C, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

For the differentiation assay of cancer stem-like cells, MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 10 cm dishes (Corning Incorporated) at 10,000 cells/ml and cultured in the stem cell medium for seven days. Indicated concentrations of Broussoflavonol B were added and incubated for another three days. Cells were collected, washed with phosphate buffered saline (PBS) and cytospined onto slides. Cytospined slides were stained with indirect immunofluorescent staining using anti-CD10, vimentin or CK18 antibodies. Five hundred cells were assessed for vimentin or CK18 positivity under the fluorescent microscope (Nikon, Eclipss E600), and the percentage of cells positive for these markers were calculated.

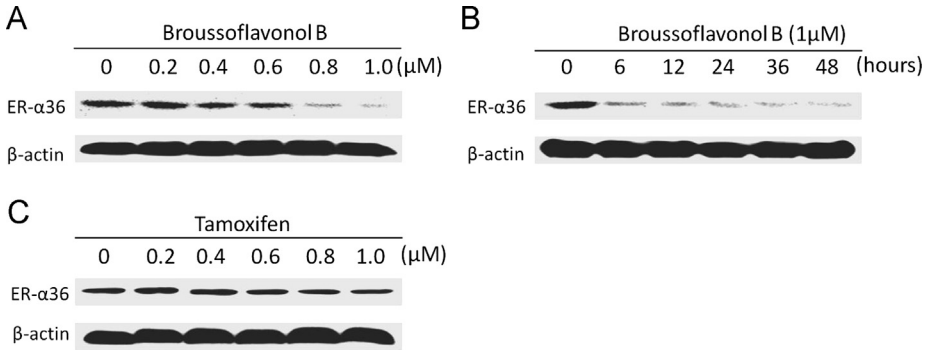

Fig. 2. Broussoflavonol B treatment downregulates ER-α36 expression. ((A) and (B)) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h or 1 μM of Broussoflavonol B for indicated time periods. Cell lysates were subjected to Western blot analysis with an antibody for ER-α36. (C) Cells were treated with DMSO (0) and indicated concentrations of tamoxifen. Cell lysates were subjected to Western blot analysis with the antibody for ER-α36. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

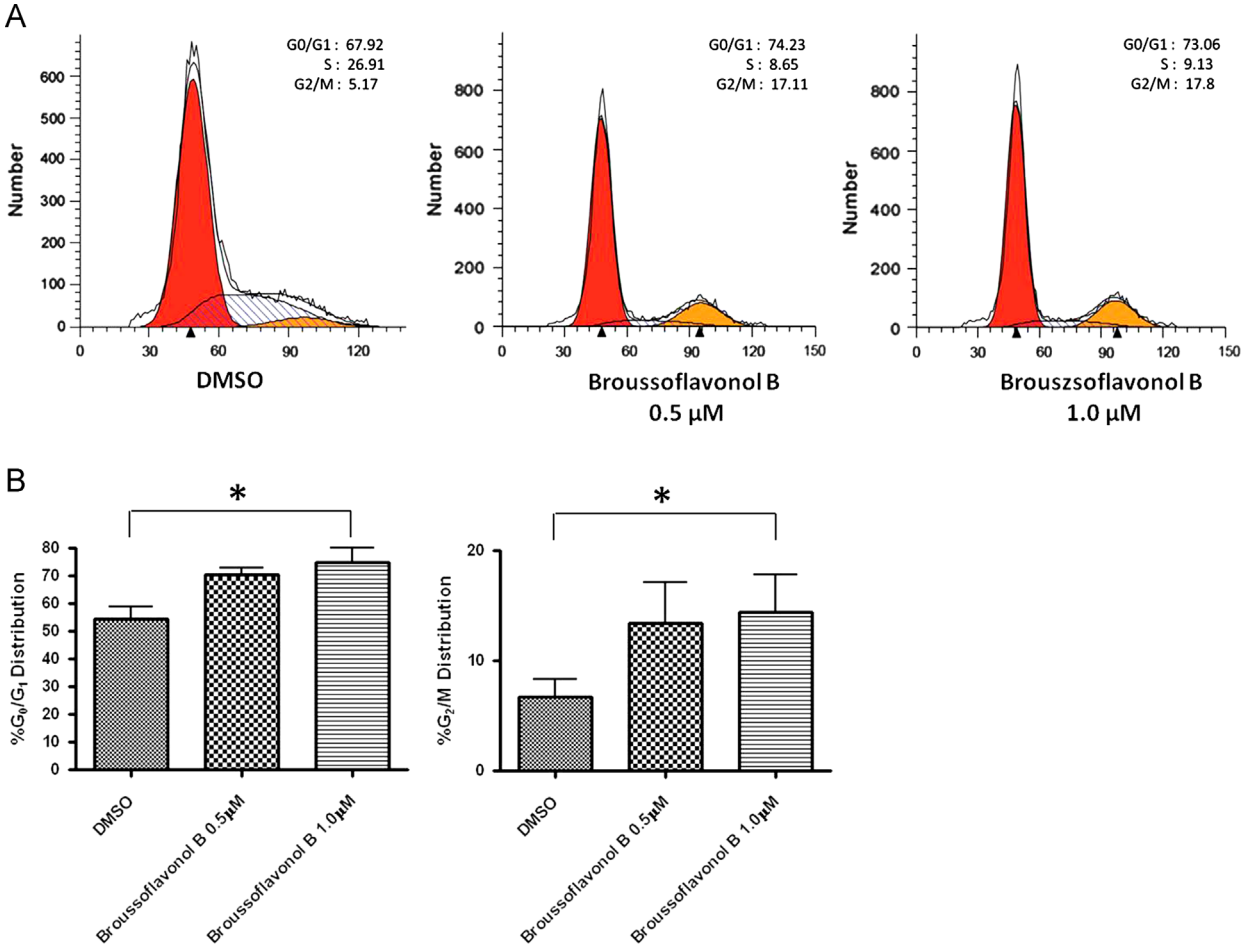

Fig. 3. Broussoflavonol B induces G_0/G_1 and G_2/M arrest of the cell cycle in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with DMSO (vehicle) or the indicated concentrations of Broussoflavonol B for 72 h. Cells were assayed with PI staining and flow cytometric analysis. All experiments were repeated three times and data from a representative experiment is shown. (B) Percentage of cells in the G_0/G_1 and G_2/M phases of the cell cycle in MDA-MB-231 cells treated with or without Broussoflavonol B. Each column represents mean ± S.D. of three independent experiments. $^{*}P < 0.05$.

2.4. Western blot assay

Cells were washed with cold PBS twice and lysed with the RIPA buffer containing 1% proteinase inhibitor and 1% phosphatase inhibitor cocktail solution (Sigma, St. Louis, MO, USA). The cell lysates were boiled for 5 min in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL, USA).

2.5. Cell cycle and cell death analysis

Cells at ~70% confluence were harvested and 1 ml of cold 70% ethanol was slowly added to the cell pellet while vortexing. Ethanol-fixed cells were treated with 100 μg/ml RNaseA and 50 μg/ml propidium iodide (PI) in PBS at room temperature for 30 min. Flow cytometry analysis of cell cycle distribution was performed using a FACSCalibur flow cytometer (BD-Biosciences).

Cell death was detected using the annexin V-FITC apoptosis kit (Invitrogen) according to the manufacturer's instruction. Data acquisition was performed with the CellQuest software and analyzed with the ModFit software.

2.6. Statistical analysis

Data were summarized as the means ± standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples *t*-test, or ANOVA followed by the Student–Newman–Keuls testing and the significance was accepted for *P* values less than 0.05.

3. Results

3.1. Broussoflavonol B exhibits growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells

Previously, we reported that ER-α36 plays an critical role in malignant growth of ER-negative breast cancer MDA-MB-231 cells (Zhang et al., 2011), suggesting the downregulation of ER-α36 expression may provide a novel approach to inhibit growth of ER-negative breast cancer cells. Recently, we found that a flavonoid, Broussoflavonol B (5, 7, 3′, 4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) (Fig. 1A) purified from the bark of *B. papyrifera* was able to downregulate ER-α36 expression and inhibit proliferation in ER-positive breast cancer MCF7 cells (Guo et al., 2013).

To examine the effects of this chemical on growth of ER-negative breast cancer cells, we used ER-negative breast cancer MDA-MB-231 cells as a model to perform cell growth inhibition assay. Cells were incubated with increasing concentrations of Broussoflavonol B or the classical anti-estrogen tamoxifen for seven days, and the cell numbers were then counted. Broussoflavonol B potently inhibited growth of MDA-MB-231 cells at sub-μM concentrations while the classical anti-estrogen tamoxifen had no effect (Fig. 1B). Our result thus suggested that Broussoflavonol B exhibited growth inhibitory activity in ER-negative breast cancer cells.

3.2. Broussoflavonol B downregulates ER-α36 expression in MDA-MB-231 cells

To probe the molecular mechanisms by which Broussoflavonol B inhibited growth of MDA-MB-231 cells, we assessed the effects of Broussoflavonol B on expression of ER-α36, a protein important for malignant growth of MDA-MB-231 cells (Zhang et al., 2011). Western blot analysis indicated that Broussoflavonol B treatment downregulated ER-α36 expression in a dose- and time-dependent manner (Fig. 2A and B) whereas the classic anti-estrogen tamoxifen was without any effect on ER-α36 expression (Fig. 2C). Thus, our data suggested that ER-α36 downregulation is a mechanism underlying Broussoflavonol B growth inhibitory activity in these cells.

3.3. Broussoflavonol B induces both the G_0/G_1 and G_2/M phase arrest in MDA-MB-231 cells

To further examine the mechanisms underlying Broussoflavonol B growth inhibitory activity, we also studied its effect on the cell cycle progression. Cell populations in the G_0/G_1, S and G_2/M phases of the cell cycle were determined with propidium iodide (PI) staining followed by flow cytometry. Broussoflavonol B treatment increased the population of MDA-MB-231 cells in both the G_0/G_1 and G_2/M phases accompanied with a dramatically reduced population of the S phase; for vehicle (DMSO), 0.5 and 1 μM Broussoflavonol B, respectively (Fig. 3A and B).

We also examined the effects of Broussoflavonol B on the expression of the proteins involved in regulation of the G_1/S transition of the cell cycle including c-Myc, $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. Western blot analysis showed that Broussoflavonol B treatment down-regulated the expression levels of the growth promoting protein c-Myc in a dose-dependent manner (Fig. 4A) while induced expression levels of the cell cycle inhibitory proteins $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ (Fig. 4A).

We then examined the effect of Broussoflavonol B on the expression of the proteins critical for the G_2/M transition including cyclin B1, cdc2 and cdc25C. Western blot analysis showed that

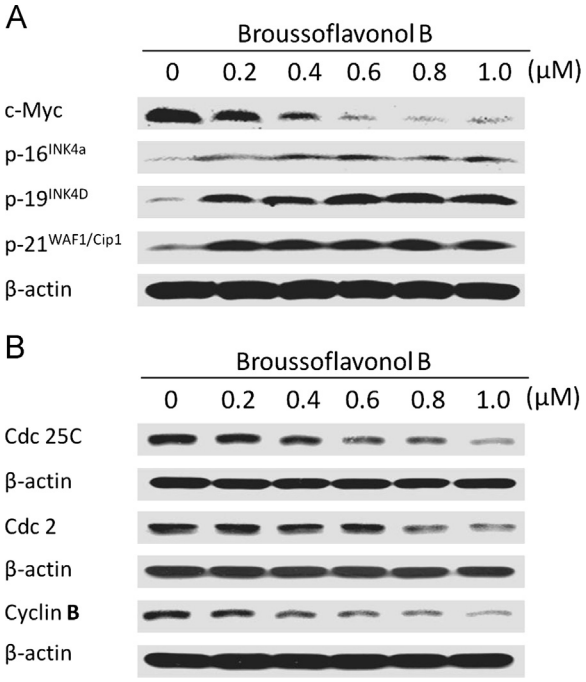

Fig. 4. Broussoflavonol B regulates expression levels of the cell cycle regulators in MDA-MB-231 cells. (A) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for c-Myc, $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. (B) MDA-MB-231 **c**ells were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for cyclin B1, cdc2 and cdc25C. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

Broussoflavonol B treatment down-regulated the expression levels of cyclin B1, cdc25C and cdc2 in a dose-dependent manner (Fig. 4B). Altogether, these results demonstrated that Broussoflavonol B treatment arrested ER-negative breast cancer MDA-MB-231 cells at both the G_0/G_1 and G_2/M phases of the cell cycle.

3.4. Broussoflavonol B activates caspase-mediated cell apoptosis in MDA-MB-231 cells

During our experiments, we also noticed that there were floating cells in the MDA-MB-231 cells treated with Broussoflavonol B. We decided to determine whether Broussoflavonol B also induces cell apoptosis. MDA-MB-231 cells were treated with different concentrations of Broussoflavonol B for 48 h, and the annexin V-FITC and propidium iodide (PI) fluorescence assays were performed to examine the early stage apoptotic cells (annexin-positive/PI-negative), the late stage apoptotic cells (annexin-positive/PI-positive), and necrotic cells (annexin-positive/PI-positive). In MDA-MB-231 cells treated with 0.5 and 1 μM of Broussoflavonol B, cells were induced to apoptotic and/or necrotic cell death as shown as increased cell populations in groups of annexin V-positive/ PI-negative, annexin V-positive/PI-positive and annex V-negative/PI-positive (Fig. 5A and B).

We also examined whether the caspase cascades were activated in Broussoflavonol B treated cells. Western blot analysis revealed that Broussoflavonol B treatment resulted in a dose-dependent activation of the initiator caspases 8, 9 and the executor caspase 3. As shown in Fig. 5C, the levels of remaining procaspases 8, 9 and 3 in Broussoflavonol B treated cells were dramatically decreased compare to the control cells treated with DMSO vehicle. Taken together, our results demonstrated that Broussoflavonol B induces apoptotic as well as necrotic cell death in ER-negative breast cancer MDA-MB-231 cells.

3.5. Broussoflavonol B downregulates EGFR expression and inhibits EGF-stimulated growth of MDA-MB-231 cells

Recently, we reported that ER-α36 positively regulates the stability of the epidermal growth factor receptor (EGFR) protein; knockdown of ER-α36 expression destabilized EGFR protein (Zhang et al., 2011). We decided to examine whether down-regulated ER-α36 expression by Broussoflavonol B also down-regulates EGFR expression. We thus examined EGFR expression in MDA-MB-231 cells treated with Broussoflavonol B, and found that Broussoflavonol B also decreased the steady state levels of EGFR protein (Fig. 6A). MDA-MB-231 cells represent a typical triple-negative breast cancer that lacks expression of estrogen receptor, progesterone receptor and HER2, and often relies on EGFR signaling for malignant growth. We then decided to examine whether Broussoflavonol B is able to inhibit EGF-stimulated cell growth in MDA-MB-231 cells. In serum-starved cells, addition of

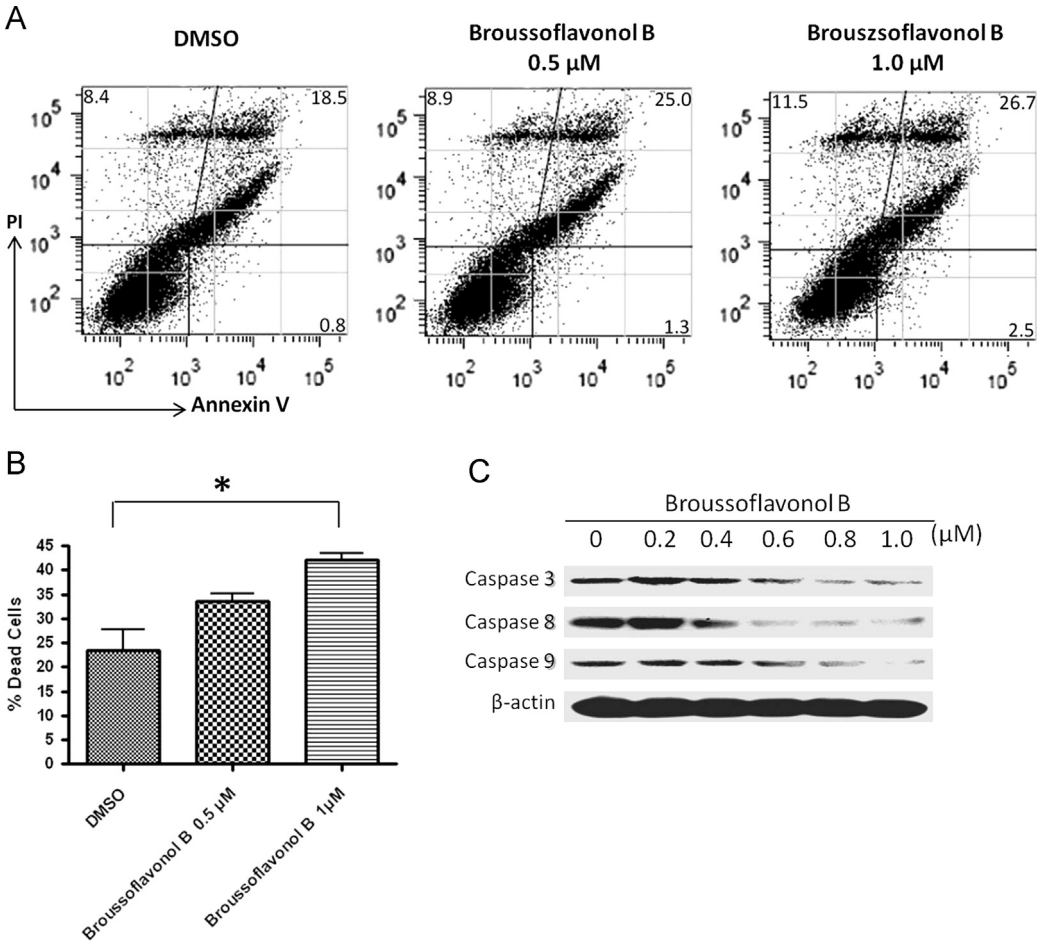

Fig. 5. Broussoflavonol B induces cell death in MDA-MB-231 cells. (A) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0) or 0.5 and 1 μM Broussoflavonol B for 48 h. Cells were collected and analyzed with flow cytometric analysis after annexin V and PI staining. The experiment was repeated three times and the results from a representative experiment were shown. (B) Percentage of dead cells in MDA-MB-231 cells treated with or without Broussoflavonol B. Each column represents mean ± S.D. of three independent experiments. * $P < 0.05$. (C) MDA-MB-231 cells were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for caspases 3, 8 and 9. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

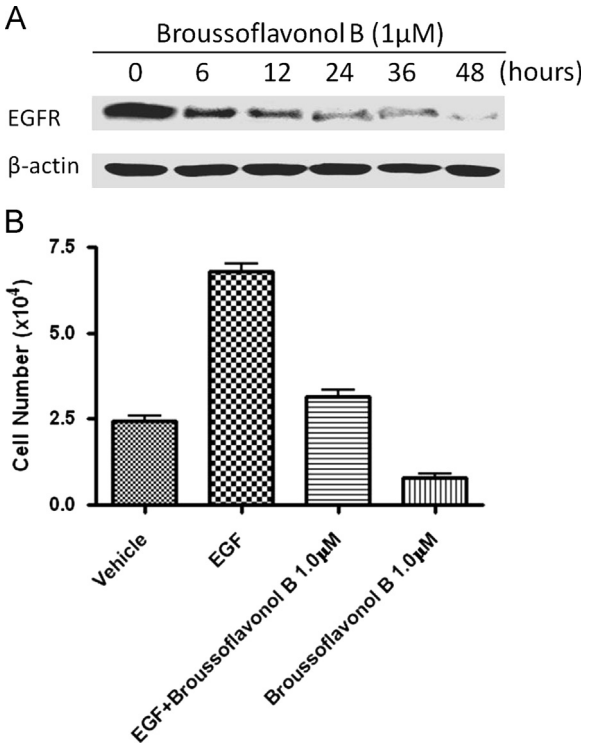

Fig. 6. Broussoflavonol B downregulates EGFR expression and attenuates mitogenic EGF signaling in MDA-MB-231 cells. (A) MDA-MB-231 **c**ells were treated with 1 μM of Broussoflavonol B for indicated time periods. Cell lysates were subjected to Western blot analysis with an antibody for EGFR. The membrane was stripped and re-probed with a β-actin antibody to ensure equal loading. (B) Cells maintained in phenol red-free medium with 2.5% charcoal-stripped FBS for 48 h. EGF (10 ng/ml) alone, together with 1 μM Broussoflavonol B or 1 μM Broussoflavonol B alone were added to cells and incubated for 72 h, and the cell numbers were determined. Three dishes were used for each treatment and experiments were repeated more than three times.

EGF (10 ng/ml) stimulated cell proliferation, which was inhibited by inclusion of Broussoflavonol B, indicating that Broussoflavonol B also attenuates mitogenic EGF signaling presumably via down-regulation of EGFR expression.

3.6. *Broussoflavonol B inhibits the growth of breast cancer stem-like cells in MDA-MB-231 cells*

Recently, we reported that ER-α36 is important in maintenance of the stem-like cells in ER-negative breast cancer SK-BR-3 cells (Kang et al., 2011). We decided to test the inhibitory effects of Broussoflavonol B on the stem-like cells in MDA-MB-231 cells. To this aim, we cultured MDA-MB-231 cells in a stem cell medium using ultralow-attachment dishes, which enriched the breast cancer stem-like cells. These stem-like cells were than treated with the increasing concentrations of tamoxifen or Broussoflavonol B for seven days. We found that Broussoflavonol B effectively inhibited the growth of these stem-like breast cancer cells while tamoxifen had less effect (Fig. 7A).

To examine whether Broussoflavonol B induces differentiation of breast cancer stem-like cells, we treated MDA-MB-231 cells cultured in the stem cell medium with indicated concentrations of Broussoflavonol B for three days. The cells were then examined for expression of differentiation markers including cytokeratin 18 (CK18) for luminal epithelial differentiation, CD10 for myoepithelial cell differentiation and vimentin for mecenchymal cell differentiation. We found that Broussoflavonol B treatment significantly increased the number of cells positive for CK18 while had no effect on the number of cells positive for vimentin (Fig. 7B). We did not observe CD10 staining in Broussoflavonol B treated cells (data not shown). Our results indicated that Broussoflavonol B is able to induce differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage and differentiation induction may be one of the mechanisms by which Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells.

4. Discussion

In this study, we investigated the growth inhibitory potential of a flavonoid derivative Broussoflavonol B from the Paper Mulberry tree (*B. papyrifera*). *B. papyrifera* has been used for cancer, dyspepsia, and pregnancy (Johnson, 1998). In mainland China, the fruits of *B. papyrifera* have been employed for impotency and ophthalmic disorders (Matsuda et al., 1995). Crude extracts or purified compounds from *B. papyrifera* have exhibited various biological activities, such as anti-proliferation, antioxidative, aromatase inhibitory, cytotoxic, glycosidase inhibitory, and platelet aggregation inhibitory effects (Lee and Kinghorn, 2003).

Currently, seven broussoflavonols were purified from *B. papyrifera* and named as broussoflavonols A through G (Lee and Kinghorn 2003). Broussoflavonols E and F exhibited platelet aggregation inhibitory activity partially due to an inhibitory effect on cyclooxygenase (Lin et al., 1996). Both Broussoflavonols F and G potently inhibited Fe^{2+}-induced lipid oxidation in rat-brain homogenate and significantly inhibited the proliferation of rat vascular smooth muscle cells (Ko et al., 1997). Recently, we reported that Broussoflavonol B significantly inhibited growth of ER-positive breast cancer cells (Guo et al., 2013). Here, we demonstrated that Broussoflavonol B also potently inhibited growth of triple-negative and basal-like breast cancer MDA-MB-231 cells through down-regulation of ER-α36 and EGFR expression and induction of the G_0/G_1 and G_2/M arrest of the cell cycle as well as cell death. We also showed that Broussoflavonol B reduced the population of breast cancer stem-like cells.

The ER-α variant, ER-α36, is highly expressed in ~40% of ER-negative breast cancer (Shi et al., 2009) and its expression is significantly correlated with expression of members of the EGFR family such as EGFR and HER2 (Shi et al., 2009; Zhang et al., 2011). Recently, we reported the existence of a positive feedback loop between EGFR and ER-α36 expression in ER-negative breast cancer cells, which is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011). Here, we reported that Broussoflavonol B potently downregulated ER-α36 expression at sub-μM while antiestrogen tamoxifen was without any effects. In addition, Broussoflavonol B also downregulated the levels of EGFR protein since ER-α36 protein is important for stability of EGFR protein (Zhang et al., 2011), and attenuated the mitogenic EGF signaling that is critical for malignant growth of MDA-MB-231 cells. Thus, our results suggested that disruption of the positive regulatory loop between ER-α36 and EGFR through down-regulation of ER-α36 provides an effective approach to inhibit growth of ER-negative breast cancer cells.

Eukaryotic cell cycle progression involves sequential activation of Cdks, which are controlled by a complex of proteins, including the cyclins. Here, we found that Broussoflavonol B treatment also arrested MDA-MB-231 cells mainly at the G_2/M phase of the cell cycle, which was accompanied with down-regulation of the expression levels of the proteins pivotal for the G_2/M transition. We also found that Broussoflavonol B modestly arrested the cell cycle at the G_0/G_1 phase. Cell-cycle progression involves sequential activation of cyclins and cyclin-dependent kinases (CDKs). To prevent abnormal proliferation, cyclin-CDK complexes are negatively regulated by cell cycle inhibitors (Sherr and Roberts, 1999). Here, we found that in Broussoflavonol B treated MDA-MB-231

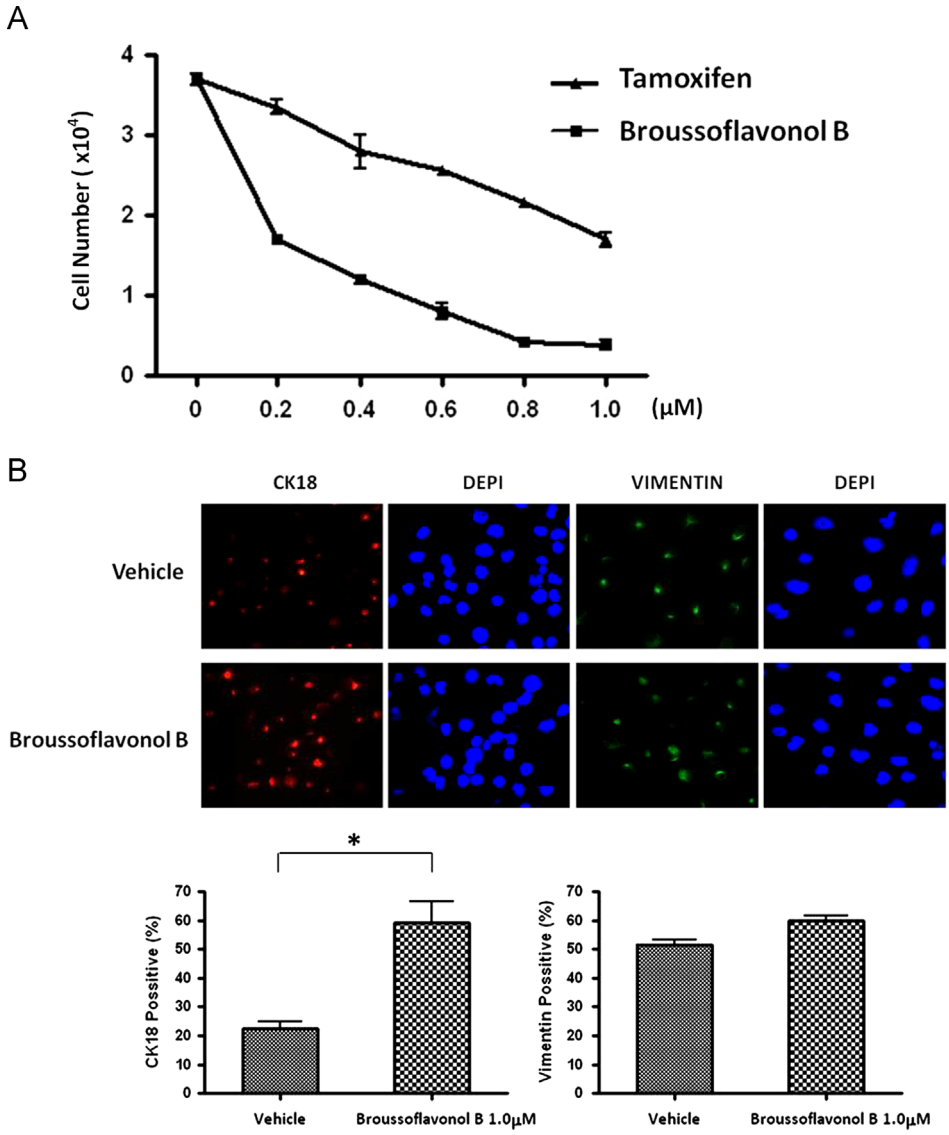

Fig. 7. Broussoflavonol B inhibits growth of ER-negative breast cancer stem/progenitor cells. (A) MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 6-well plate and cultured in stem cell medium. Different concentrations of Broussoflavonol B or tamoxifen were added and incubated for seven days. Cells were counted using the ADAM automatic cell counter. Three dishes were used for each treatment and experiments were repeated three times. Each point represents mean ± S.D. of three independent experiments. (B) MDA-MB-231 cells were cultured in the stem cell medium for seven days. Indicated concentrations of Broussoflavonol B were added and incubated for another three days. Cells were collected and cytospined onto slides. Cytospined slides were stained with indirect immunofluorescent staining using anti-CK18 and vimentin antibodies (upper panels). Five hundred cells were assessed for vimentin or CK18 positivity under the fluorescent microscope and the percentage of cells positive for these markers were calculated (lower panels). The experiments were repeated three times. $^{*}P < 0.01$.

cells, the G_0/G_1 arrest of the cell cycle was accompanied with down-regulation of the growth-promoting protein c-Myc and induction of the cell cycle inhibitors including $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. Thus, The G_0/G_1 phase arrest is also involved in Broussoflavonol B inhibitory function in growth of ER-negative MDA-MB-231 cells.

Furthermore, flow cytometric analysis using Annexin V/PI staining demonstrated that Broussoflavonol B could dose-dependently induced apoptosis in MDA-MB-231 cells. Caspases are the principal effectors of apoptosis involved in pathways such as caspase 8 regulated extrinsic and caspase 9-regulated intrinsic pathways. The caspase 9 pathway links mitochondrial damage to caspase activation, and serves as an index of damage in mitochondrial membrane function (Bao and Shi, 2007). In addition, the downstream member caspase 3 is an executor of DNA fragmentation (Bao and Shi, 2007). As expected, we observed that Broussoflavonol B treatment induced activation of caspases 8, 9 and 3. Thus, proteolytic processing of the initiator caspases as well as the executor caspase, and subsequent apoptosis contributed to growth inhibitory activity of Broussoflavonol B.

Accumulating evidence indicated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (Liu et al., 2005; Charafe-Jauffret et al., 2009). These cancer stem/progenitor cells are resistant to most therapeutic approaches currently used (Dean, 2006; Diehn and Clarke, 2006; O'Brien et al., 2009; Hambardzumyan et al., 2006; Shafee et al., 2008). In this study, we showed that ER-negative breast cancer stem-like cells were resistant to anti-estrogen tamoxifen, consistent with the concept that cancer stem/progenitor cells are resistant to current cancer therapies. Thus, the development of novel drugs that are able to selectively attack the cancer stem cells is of the greatest priority. Recently a large scale screening was conducted to seek agents selectively kill epithelial cancer stem cells, and salnomycin was identified as a potent agent specifically targeting breast cancer stem cells (Gupta et al., 2009). More recently, dietary chemopreventive agents sulforaphane and benzyl isothiocyanate were

reported to be able to inhibit growth of breast cancer stem cells both in vitro and in vivo (Li et al., 2010; Kim et al., 2013). It is worth noting that we have previously reported that phenethyl isothiocyanate acted more potently than the "pure" antiestrogen ICI 182,780 to down-regulates ER-α36 expression and to inhibit breast cancer cell growth (Kang and Wang, 2010). Together with our current finding, these results suggested that down-regulation of ER-α36 and function is a novel approach to target breast cancer stem/progenitor cells.

In this study, we also found that Broussoflavonol B not only inhibited growth of ER-negative breast cancer stem-like cells but also induced differentiation of these cells, suggesting that differentiation induction may be one of the mechanisms by which Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells. Based on the cancer stem cell model, tumors are originated from malignantly transformed stem cells that are able to self-renew (Clarke et al., 2006). Thus, induction of cancer stem cell differentiation or "destemming" cancer stem cells provides a novel therapeutic option to eliminate cancer stem cells. Thus, our results demonstrated that induction of cancer stem-like cell terminal differentiation or "destemming" cancer stem cells is a feasible therapeutic approach to eradicate human breast cancer by eliminating cancer stem cells.

5. Conclusion

Our results demonstrated that Broussoflavonol B from the Paper Mulberry tree possesses potent anti-growth activity; inducing the arrest of the cell cycle and cell death in ER-negative breast cancer cells. Broussoflavonol B also effectively downregulates the steady state levels of ER-α36 and EGFR proteins, indicating that Broussoflavonol B acts like a selective estrogen receptor down-regulator (SERD) and a disruptor of the positive regulatory loop consisted of ER-α36 and EGFR in ER-negative breast cancer cell. More importantly, our results also indicated that Broussoflavonol B restricts growth of breast cancer stem-like cells. Thus, our results provide experimental evidence for the hypothesis that ER-α36 can serve as a target to develop novel and effective therapeutic approaches for ER-negative breast cancer.

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