Abstract. Accumulating experimental and clinical evidence has indicated that tumor-initiating or cancer stem-like cells are a sub-population of tumor cells capable of initiating and driving tumor growth, and cancer stem-like cells are resistant to most current cancer therapies, including chemo- and radiation therapy. More effective targeted-therapeutic approaches are urgently needed to eliminate cancer stem-like cells. Here, we report that broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree (broussonetia papyrifera), exhibited potent growth inhibitory activity towards estrogen receptor (ER)-negative breast cancer SK-BR-3 cells at sub-micromolar concentrations. Broussoflavonol B more potently inhibited growth and induced differentiation of stem-like SK-BR-3 cells compared to the anti-estrogen tamoxifen. In addition, broussoflavonol B treatment also reduced the steady-state levels of the Human epidermal growth factor receptor-2 (HER2) and ER-α36, a variant of ER-α. Our results, thus, indicate that broussoflavonol B is a potent growth inhibitor of ER-negative breast cancer stem-like cells and provide a rationale for pre-clinical and clinical evaluation of broussoflavonol B for breast cancer therapy.

Later, aldehyde dehydrogenase (ALDH)-1 expression and its activity were identified to be a marker for breast cancer stem/progenitor cells; fewer ALDH1-positive tumor cells than CD44+/CD24−/low tumor cells are required to generate tumors in vivo (7). Breast cancer with ALDH1high cancer stem-like cells are associated with more aggressive tumor phenotypes such as these with estrogen receptor negativity, high histological grade, Human epidermal growth factor receptor-2 (HER2) positivity, as well as poor prognosis (8).

Accumulating evidence has indicated that cancer stem-like cells are resistant to many current cancer therapies, including chemo- and radiation therapy as well as hormone therapy (9-13). This suggests that many cancer therapies, while killing the bulk of tumor cells, may eventually fail since they do not eliminate cancer stem-like cells that survive to regenerate new tumors. Thus, novel and effective therapeutic agents that target cancer stem-like cells are urgently needed.

Previously, we identified and cloned a 36-kDa variant of ER-α, ER-α36, that is mainly expressed at the plasma membrane and in the cytoplasm, and mediates non-genomic estrogen signaling (14, 15). 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 (15). ER-α36 is generated from a promoter located in the first intron of the ER-α66 gene (16), 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 and established ER-negative breast cancer cells that lack ER-α66 expression (17, 18). ER-α36 is highly expressed in ER-negative SK-BR-3 breast cancer cells and positively regulates HER2 expression in these cells (19). ER-α36 expression is required for maintenance of the ALDH1-positive stem-like SK-BR3 cells; knockdown of ER-α36 expression with the short hairpin RNA (shRNA) method dramatically reduced the population of ALDH1-positive cells (19). Thus, ER-α36-mediated signaling plays an important role in maintenance of ER-negative breast cancer stem-like cells, and down-regulation of ER-α36 expression may provide a novel approach to inhibit proliferation of ER-negative breast cancer stem-like cells.
Recently, we reported that several flavonoid derivatives purified from the bark of the Paper Mulberry tree (Broussonetia papyrifera) (L.) were able to down-regulate ER-α36 expression (20). In the present study, we examined the growth inhibitory activity of the most potent ER-α36 down-regulator broussoflavonol B (5,7,3',4'-tetrahydroxy-3-methoxy-6,8-diprenylflavone) from the bark of the Paper Mulberry tree on SK-BR-3 cells.

Materials and Methods

Chemicals and reagents. Broussoflavonol B (99.8% pure) was obtained from Beijing Shenogen Pharma Group (Beijing, China). Antibody to HER2 was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibody to ER-α36 was generated and characterized as described elsewhere (18). Antibodies to β-actin, CD10 (H-321), and cytokeratin-18 (DC-10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD10 (H-321), and cytokeratin-18 (DC-10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. SK-BR-3 cells were purchased from the American type culture collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% antibiotic-antimycotic from Invitrogen (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) for 24 h. All cells were maintained at 37°C and in 5% CO2 in a humidified incubator. The ALDEFLUOR kit (StemCell UT, USA) for 24 h. All cells were maintained at 37˚C and in 5% antimycotic from Invitrogen (Carlsbad, CA, USA). Before

Cell growth assay. Cells in phenol red-free medium were seeded into 35 mm dishes at 5×10^4 cells/dish. After 24 h, broussoflavonol B (0.1 to 1 μM) or tamoxifen (0.1 to 1 μM) were added and cells incubated for another seven days. The vehicle dimethyl sulfoxide (DMSO) was used as a control. Cells were then trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Soul, Korea). Three dishes were used for each concentration tested and the experiments were repeated three times.

For the growth assay of cancer stem-like cells, SK-BR-3 cells were seeded onto Corning Ultra-Low Attachment 6-well plates (Corning Incorporated, Corning, NY, USA) at 10,000 cells/ml and cultured in the stem cell culture medium: 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). Different concentrations of broussoflavonol B or tamoxifen were added and cells then incubated for seven days. Cells were then collected, washed with phosphate buffered saline (PBS) and trypsinized to dissociate cells, then counted using the ADAM automatic cell counter (Digital Bio) or examined for ALDH1 positive cells using the ALDEFLUOR kit.

Indirect immunofluorescent staining. Treated cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and then permeabilized in 0.5% Triton X-100 for 15 min. After washing with PBS, cells were incubated for 1 h at room temperature with different primary antibodies followed by extensive washing with PBS. The cells were then incubated for over 1 h at room temperature with secondary antibody Alexa 488-conjugated rabbit anti-mouse (Molecular Probes, Carlsbad, CA, USA) diluted 1:100 in PBS. Cells were washed with PBS and mounted with 10 μg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) in aqueous mountant (Dako, Carpinteria, CA, USA) and photographed using a Zeiss fluorescence microscope (Carl Zeiss).

Western blot assay. Cells were washed with cold PBS twice and lysed with the radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma-Aldrich). The cell lysates were boiled for five minutes in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies, which were then visualized with the corresponding secondary antibodies and ECL kit (Thermo Scientific, Rockford, IL, USA).

Cell differentiation assay. For the differentiation assay of cancer stem-like cells, SK-BR-3 cells were seeded onto Corning Ultra-Low Attachment 10 cm dishes (Corning Incorporated) at 10,000 cells/ml and cultured in the stem cell culture medium for seven days. Broussoflavonol B or tamoxifen were then added and cells were incubated for another three days. Cells were collected, washed with PBS and cytospinned onto slides. Cytospinned slides were stained with indirect immunofluorescent staining using antibodies to CD10 and CK18. Five hundred cells were assessed for CD10 or CK18 positivity under fluorescence microscopy (Nikon, Eclipss E600, Melville, NY, USA), and the percentage of cells positive for these markers was calculated.

Statistical analysis. Data are summarized as the mean±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.

Results

Broussoflavonol B inhibits proliferation of ER-negative SK-BR-3 breast cancer cells. Recently, we reported that broussoflavonol B (Figure 1A) was able to down-regulate ER-α36 expression in ER-positive MCF7 breast cancer cells (20). Since ER-α36 plays an important role in malignant growth in ER-negative breast cancer SK-BR-3 cells (19), we decided to test whether broussoflavonol B influences their growth. SK-BR-3 cells were incubated with different concentrations of broussoflavonol B or the classical anti-estrogen tamoxifen for seven days, and the numbers of surviving cells were counted. We found that broussoflavonol B potently inhibited growth of SK-BR-3 cells, while tamoxifen had no effect (Figure 1B), consistent with the fact that anti-estrogens have less or no effect on the growth of ER-negative breast cancer cells.
Broussoflavonol B down-regulates expression of both ER-α36 and HER2 in SK-BR-3 cells. To probe the underlying mechanisms by which broussoflavonol B inhibits the growth of SK-BR-3 cells, we decided to determine whether broussoflavonol B influences ER-α36 expression in SK-BR-3 breast cancer cells. Western blot analysis indicated that broussoflavonol B treatment potently down-regulated ER-α36 expression in a dose- and time-dependent manner (Figure 2A and B), whereas the anti-estrogen tamoxifen modestly increased the levels of ER-α36 expression in SK-BR-3 cells (Figure 2C). Previously, we found that a positive regulatory loop between ER-α36 and HER2 is critical for growth of SK-BR-3 cells; they positively regulate each other’s promoter activity (19). We also found that HER2 expression was dramatically down-regulated in SK-BR-3 cells treated with broussoflavonol B (Figure 2D). Thus, our data indicate that disruption of the positive regulatory loop of ER-α36 and HER2 is one of the mechanisms by which broussoflavonol B inhibits growth of these cells.

**Broussoflavonol B inhibits the growth of breast cancer stem/progenitor cells.** Accumulating evidence has demonstrated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (1-6). Breast cancer stem/progenitor cells are involved in resistance to chemo-and radiation therapies (9-13). Previously, we reported that ER-α36 expression is required for maintenance of the ALDH1-positive stem-like cells in SK-BR-3 cells; knockdown of ER-α36 expression with the short hairpin (sh) RNA method significantly reduced the population of ALDH1-positive SK-BR-3 cells (19). We decided to test the effects of broussoflavonol B on stem-like SK-BR-3 cells. With this aim, we cultured SK-BR-3 cells in a low-serum stem/progenitor cell culture medium and in ultralow-attachment dishes, which enriches the breast cancer stem-like cells. These stem-like cells were then treated with different concentrations of broussoflavonol B or tamoxifen for seven days and cell numbers were counted. We found that broussoflavonol B effectively inhibited the growth of these stem-like breast cancer cells while tamoxifen had a lesser effect (Figure 3A). We then tested the effects of broussoflavonol B on the ALDH1-positive SK-BR-3 cell population. ALDH expression or its activity has been used as a marker for breast cancer stem/progenitor cells (7). We first treated SK-BR-3 cells with 1 μM and 5 μM of broussoflavonol B or tamoxifen for seven days, and the ALDH1-positive cells from the remaining SK-BR-3 cells were analyzed, using the ALDEFLOUR kit and flow-cytometry. We found that treatment of SK-BR-3 cells with broussoflavonol B significantly reduced the population of ALDH1-positive cells while tamoxifen at 1 μM weakly but significantly increased the ALDH1-positive cell population (Figure 3B). These results indicate that the ALDH-high cells, i.e., breast cancer stem-like cells, are resistant to the widely used anti-estrogen tamoxifen, and broussoflavonol B acts as a potent inhibitor of these breast cancer stem-like cells.

**Broussoflavonol B induces differentiation of breast cancer stem-like cells.** Based on the cancer stem cell model, tumors originate from transformed stem cells that are able to self-renew and give rise to relatively differentiated cells (cancer progenitor cells) through asymmetric division, thereby forming heterogeneous cell populations found in a tumor (21). Thus, induction of cancer stem cell differentiation or de-stemming of cancer stem cells provides a novel therapeutic option to eliminate cancer stem cells. To examine whether broussoflavonol B also induces differentiation of breast cancer stem cells, we treated SK-BR-3 cells cultured in stem

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**Figure 1.** Broussoflavonol B inhibits growth of estrogen receptor (ER)-negative SK-BR-3 breast cancer cells. A. The chemical structure of broussoflavonol B (5,7,3’-A’-tetrahydroxy-3-methoxy-6,8-diprenylflavone). B. Broussoflavonol B inhibits growth of SK-BR-3 breast cancer cells. Cells maintained in phenol red-free medium with 2.5% charcoal-stripped fetal calf serum were treated with vehicle dimethyl sulfoxide (DMSO, 0), 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 all of the experiments and all experiments were repeated three times. Each point represents the mean±S.D. of three independent experiments.
cell culture medium with broussoflavonol B and tamoxifen for three days. The cells were then examined for expression of different differentiation markers including CK18 for luminal epithelial differentiation and CD10 for myoepithelial cell differentiation. We found that broussoflavonol B treatment significantly increased the number of cells positive for CK18 and modestly, but significantly increased the number of cells positive for CD10 (Figure 4), suggesting 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. On the other hand, however, anti-estrogen tamoxifen had less or no effect on the differentiation of these cells (Figure 4), consistent with the previous hypothesis that cancer stem/progenitor cells are resistant to most cancer therapies (9-13).

Discussion

Anti-estrogen tamoxifen has been widely used to treat patients with ER-positive breast tumors, either as adjuvant therapy following surgery, or as first-line treatment for advanced disease. Tamoxifen was also approved as a chemopreventive agent for high-risk women who have a familial history of breast cancer. Although tamoxifen is effective as an adjuvant and chemopreventive agent for ER-positive breast cancer, the therapeutic efficacy of tamoxifen is dramatically reduced in ER-negative tumors. Thus, novel therapeutic agents are urgently needed for treatment of ER-negative breast cancer.

In this study, we investigated the growth-inhibitory potential of a flavonoid derivative broussoflavonol B from the Paper Mulberry tree that grows naturally in Asian and Pacific countries. Crude extracts from this plant exhibit various activities such as anti-platelet activity, inhibition of aromatizing enzymes, anti-oxidant, anti-microbial, anti-inflammatory, inhibition of PTP1B and cytotoxicity. Here, we demonstrated, to our knowledge, for the first time that broussoflavonol B potently inhibited growth of ER-negative SK-BR-3 breast cancer cells, presumably through down-regulation of ER-α36 and HER2 expression. We also demonstrated that broussoflavonol B induced differentiation of breast cancer stem-like cells and restricted the population of ALDH1-positive SK-BR-3 cells.

ER-α36 is highly expressed in ~40% of ER-negative breast cancer cases and its expression is significantly correlated with HER2 expression (18). Recently, we reported the existence of a positive feedback loop between HER2 and ER-α36 expression in SK-BR-3 cells; HER2 signaling activates the promoter activity of ER-α36 and ER-α36 signaling induces HER2 promoter (19). In addition, we also found that ER-α36
that broussoflavonol B potently down-regulated ER-α36 expression at sub-micromolar concentrations while tamoxifen had no effect, suggesting that broussoflavonol B is an ER-α36 down-regulator that may inhibit the non-genomic estrogen signaling mediated by ER-α36. In addition, we also found that broussoflavonol B down-regulated the steady-state levels of HER2 protein. Thus, our results strongly suggest that disruption of the positive feedback loop between HER2 and ER-α36 attenuates mitogenic signaling and restricts the malignant growth of ER-negative SK-BR-3 cells.

Accumulating evidence indicates that many types of cancer, including breast cancer, originate from and are maintained by a small population of cancer stem/progenitor cells (23). In this study, we showed that ER-negative breast cancer stem-like cells were also resistant to the anti-estrogen tamoxifen, consistent with the previous reports that cancer stem/progenitor cells are resistant to many current cancer therapies including chemotherapeutic and radiation therapy (9-13). However, broussoflavonol B inhibited growth of ALDH1-positive breast cancer stem-like cells and induced their differentiation.

According to the cancer stem cell model, tumors originate from cancer stem cells that are able to differentiate into non-cancer cells (1, 3). Thus, it was postulated that induction of cancer stem cell terminal differentiation or de-stemming of cancer stem cells may provide with a novel therapeutic option to eliminate cancer stem cells (24). Recently, it was shown that bone morphogenic protein-4 enhanced terminal differentiation, apoptosis and chemosensitization of colorectal cancer stem cells (25), suggesting the possibility of ligand-induced differentiation therapy. Here, we demonstrated that broussoflavonol B was able to inhibit the growth of these stem-like cells, suggesting that broussoflavonol B may induce terminal differentiation of ER-negative breast cancer stem-like cells. Our results strongly indicate that induction of cancer stem-like cell terminal differentiation is a feasible therapeutic approach to eradicate human breast cancer by eliminating cancer stem cells.

Acknowledgements

We thank Perry Greg for his technical support for flow-cytometry. This work was supported by Department of Defense grant DAMD 11-1-0497 and the Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-606 and LB692) to Z.Y. Wang.

References

Figure 4. Broussoflavonol B induces differentiation of ER-negative breast cancer stem-like cells. SK-BR-3 cells maintained in stem cells culture medium and ultra-low attachment dishes were treated with dimethyl sulfoxide (DMSO, vehicle), or 1 μM of broussoflavonol B (BB) or tamoxifen (Tam) for three days. Cells were then cytospun onto slides, stained with antibodies against CK18 or CD10 and appropriate secondary antibodies, and photographed using a Zeiss fluorescence microscope. Five hundred cells were then assessed for CD10 and CK18 positivity under a fluorescence microscope and the percentage of cells positive for these markers was calculated. The experiment was repeated three times. Each column represents the mean±S.D. of three independent experiments. *p<0.01.