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Growth Suppression of HER2-Overexpressing Breast Cancer Cells by Berberine via Modulation of the HER2/PI3K/Akt Signaling Pathway

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ABSTRACT: Berberine (BBR) is a natural alkaloid with significant antitumor activities against many types of cancer cells. This study investigated the molecular mechanisms by which BBR suppresses the growth of HER2-overexpressing breast cancer cells. The results show that BBR induces G1-phase cell cycle arrest by interfering with the expression of cyclins D1 and E and that it induces cellular apoptosis through the induction of a mitochondria/caspase pathway. The data also indicate that BBR inhibits cellular growth and promotes apoptosis by down-regulating the HER2/PI3K/Akt signaling pathway. Furthermore, it is also shown that a combination of taxol and BBR significantly slows the growth rate of HER2-overexpressing breast cancer cells. In conclusion, this study suggests that BBR could be a useful adjuvant therapeutic agent in the treatment of HER2-overexpressing breast cancer.

KEYWORDS: berberine, breast cancer, HER2, PI3K/Akt signaling cascade, apoptosis, cell cycle arrest

INTRODUCTION

Breast cancer is the most frequent form of cancer affecting women and has become the second most common cause of cancer-related death in the United States.[1] Gene amplification as well as protein overexpression of some oncogenes is involved in the development of breast cancer. Human epidermal growth factor receptor 2 (HER2) is one of the most characterized oncogenes associated with an aggressive breast cancer phenotype.[2] Aberrant up-regulation of HER2 is found in around 30% of breast cancers[2,3] and diminishes the effectiveness of cancer treatment, increases cancer metastasis, and leads to poor clinical outcomes.[4] Therefore, HER2 might play a critical role in the initiation, progression, and outcome of breast cancer. In fact, suppression of HER2 expression and/or inhibition of its activity may be an effective approach to the treatment of breast cancer with HER2 overexpression.

HER2 is expressed by a gene localized on chromosome 17q21.[5] It is a transmembrane receptor tyrosine kinase (RTK) with a molecular mass of 185 kDa belonging to the epidermal growth factor receptor (EGFR) family, which includes four homologous members: HER1/EGFR, HER2, HER3, and HER4. Ligand stimulation induces homo- or heterodimerization, which causes self-phosphorylation (except for HER3) on tyrosine residues localized on the C-terminal domain of the HER receptor. Phosphorylation of downstream substrates by phosphorylated HER receptors (activated form) activates a variety of signaling cascades, including the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)/Akt cascades, which in turn promote cell growth, survival, and metastasis.[6] EGFR, HER2, and HER3 are known to be involved in the progression and development of cancer. The role HER4 plays in oncogenesis, however, is still undefined.[7] The HER2/HER3 heterodimer is the most potent HER receptor pair and is considered to be an oncogenic unit with respect to strength of interaction, ligand-induced tyrosine phosphorylation, and downstream signaling.[7]

A number of HER2-targeting agents have been developed for the treatment of HER2-overexpressing breast cancer, including monoclonal antibodies and small-molecule tyrosine kinase inhibitors. For example, trastuzumab (also known as Herceptin) and lapatinib (also known as Tykerb) are the most well-documented HER2-targeting drugs clinically used to treat breast cancer with HER2 overexpression.[8] However, botanical products are currently considered to be safer compounds and may be used as alternative therapeutic agents for treatment of HER2-overexpressing breast cancer.[9−11]

Berberine (BBR), a natural alkaloid isolated from a variety of traditional Chinese herbs (such as *Berberis aquifolium*, *Berberis aristata*, *Berberis vulgaris*, and *Tinospora cordifolia*), has been used for medicinal purposes for many years. BBR has many pharmacological activities, such as antiarrhythmic,[12] antibacterial,[13] anticholinergic,[14] antihypertensive,[15] anti-inflammation,[16] and

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antioxidative activities.[17] It is also known that BBR has growth inhibition effects on various types of cancer. For example, BBR has been shown to inhibit cell growth and induce cell cycle arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating the CKI-cdk-cyclin cascade, disrupting mitochondrial membrane potential, and inducing the cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP).[18] BBR has also been demonstrated to inhibit cell proliferation by inducing the mitochondria/caspase pathway in human hepatoma HepG2 cells[19] and to activate caspase-independent apoptosis in human pancreatic cancer BxPC3 cells.[20] Although BBR has antitumor activities in many human cancer cells, the molecular mechanisms governing its suppressive effect on HER2-overexpressing breast cancer cell growth have yet to be explored.

In this study, we investigated the molecular mechanisms by which BBR suppresses the growth of HER2-overexpressing breast cancer cells. Our results indicate that BBR inhibits cellular growth and promotes apoptosis by down-regulating the HER2/PI3K/Akt signaling pathway. Furthermore, we also show that a combination of taxol and BBR significantly slows the growth rate of HER2-overexpressing breast cancer cells.

MATERIALS AND METHODS

Chemicals and Antibodies. Berberine (>98% purity), DMSO, heregulin (HRG), and antibodies against cytochrome *c* were obtained from Sigma (St. Louis, MO). Antibodies against cyclins A, B1, D1, and E, Akt1, phospho-Akt (Ser308), Bax, and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP and caspase-9 were obtained from BioVision. Antibodies against Bcl-2 were purchased from Upstate. Antibodies aganist Erk 1/2, phospho-Erk 1/2, horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, and the PI3K inhibitor (LY294002) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against HER2 (Ab-3) were obtained from Calbiochem. Antibodies against phospho-HER2 (Ab-18) were purchased from NeoMarkers. Antibodies against β-actin were purchased from Chemicon. Taxol (paclitaxel) was purchased from Bristol-Myers Squibb (Wallingford, CT), and doxorubicin (adriamycin) was purchased from Pharmacia (Pharmacia & Upjohn SpA, Milan, Italy).

Cell Culture. Human breast carcinoma cell lines SKBR-3 ($HER2^{high}$), BT-474 ($HER2^{high}$), T47D ($HER2^{low}$), and MDA-MB-231 ($HER2^{low}$) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The human breast carcinoma MCF-7/HER2 ($HER2^{high}$) cell line (MCF-7 of a HER2-transfected stable line) was kindly provided by Dr. M. C. Hung (Department of Molecular and Cellular Oncology, University of Texas, M. D. Anderson Cancer Center, Houston, TX). All cells were cultured in DMEM/F12 medium (Gibco BRL) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT Assay. Cell viability was determined using the MTT metabolic assay as described by Chuang et al.[21] In brief, cells were seeded at a density of 12000 cells/well into 96-well plates and incubated overnight in a medium containing 10% FBS. Cells were treated with vehicle or various concentrations of BBR (10, 25, 50, 75, and 100 μM) for 24, 48, or 72 h and then stained with MTT (0.5 mg/mL). After 4 h of incubation with MTT, the viable cell number was directly proportional to the production of formazan crystals, solubilized with DMSO. The final solution was measured spectrophotometrically at 545 nm against a reference at 690 nm. All experiments were performed in triplicate and repeated at least three times.

Western Blot Analysis. Western blot analysis was performed as described by Kao et al.[22] In brief, the cells were washed with PBS, trypsinized, and pelleted at 400*g* at 4 °C. The cell pellet was resuspended in lysis buffer (20 mM Hepes buffer, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, and protease inhibitors), and each sample was vortexed for at least 2 min. The cell lysates were centrifuged at 16000*g* for 10 min at 4 °C. The protein content of the supernatant was determined using the Bio-Rad protein assay kit. Protein (50 μg) was resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a poly(vinylidene fluoride) (PVDF) membrane. The membrane was subjected to blocking with 5% nonfat milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. After blocking, the PVDF membrane was incubated with primary antibodies for 1 h at room temperature, followed by incubation with HRP-conjugated goat anti-rabbit or anti-mouse IgG for 1 h at room temperature. Reactive signals were visualized with an ECL system (Amersham Biosciences, Arlington Heights, IL). Results were scanned and quantified using Adobe Photoshop 7.0 software.

Flow Cytometric Analysis. The distribution of the cell cycle was detected by flow cytometry as described previously with slight modifications.[21] In brief, cells were seeded at a density of 5×10^5 cells/well into a 6-well plate and treated with vehicle or BBR (25 or 50 μM) for 24 or 48 h. After washing the cells twice with phosphate-buffered saline (PBS) buffer, the cells were fixed with ice-cold 70% ethanol overnight at 4 °C. Prior to analysis, the cells were washed twice with PBS buffer and then incubated with propidium iodide (PI) solution (50 μg/mL PI in PBS plus 1% Tween-20 and 10 μg of RNase) for approximately 30 min. The stained cells were examined with a FACSCalibur Instrument (BD Bioscience, San Jose, CA). ModFit LT v3.0 software and FCS Express v2.0 software were used to analyze flow cytometric results.

DAPI Staining. Cells were seeded at a density of 5×10^4 cells/well into plastic 12-well culture plates and treated with vehicle or 50 μM BBR for 48 h. After the cells had been washed twice with PBS buffer, cells were fixed with 4% paraformaldehyde for 5 min at room temperature, followed by permeabilization with 0.1% Triton X-100 for 10 min at room temperature. Finally, the cells were individually stained with 200 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) for 10 min at room temperature and photographed using fluorescence microscopy (200×).

Determination of DNA Fragmentation. DNA fragmentation was detected using the method described by Hwang et al.[19] Briefly, cells were resuspended in 0.5 mL of lysis buffer (20 mM EDTA, 0.5% Triton X-100, and 5 mM Tris; pH 8.0). The cell lysates were centrifuged at 18000*g* for 10 min at 4 °C, and the genomic DNA in the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and once with chloroform. Finally, the genomic DNA was precipitated using 100% ethanol in the presence of 5 M NaCl overnight at −20 °C. The genomic DNA was centrifuged at 18000*g* for 10 min at 4 °C, and the DNA pellets were washed twice with 70% ethanol. Prior to measuring the content of DNA by spectrophotometry (260 nm), the DNA pellets were resuspended in Tris−EDTA buffer (pH 8.0) with 100 μg/mL RNase A and incubated at 56 °C for 2 h. Finally, an equal amount of DNA was electrophoresed on horizontal agarose gel (2%) at 1.5 V/cm for 3 h. The DNA stained with ethidium bromide (0.5 mg/mL) was visualized under UV light.

Release of Cytochrome *c*. The release of cytochrome *c* (Cyt-*c*) from the mitochondria to cytosol was detected as described by Hwang et al.[19] Briefly, cells were gently lysed in lysis buffer (1 mM EDTA, 20 mM Tris-HCl, pH 7.2, 250 mM sucrose, 1 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM KCl, 10 μg/mL leupeptin, 5 μg/mL pepstatin A, 2 μg/mL aprotinin). The cell lysates were centrifuged at 12000*g* at 4 °C for 10 min to obtain the pellets (the fractions that contained mitochondria) and the supernatants (cytosolic extracts free of mitochondria). The content of protein in the supernatant was determined by the Bio-Rad protein assay kit. Protein (40 μg) was resolved by SDS-PAGE (14%) and then transferred onto PVDF membranes for the detection of Cyt-*c*.

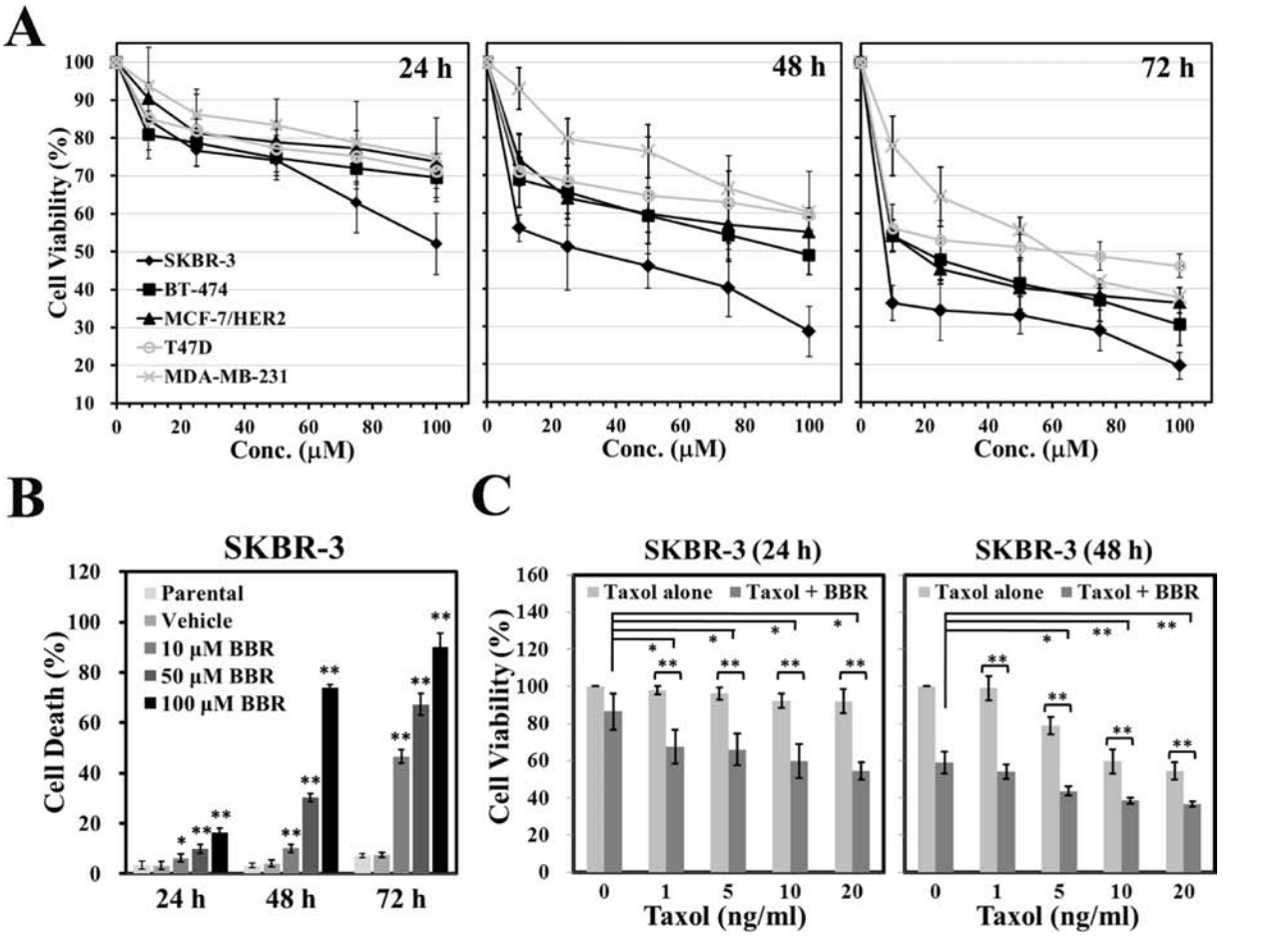

Figure 1. Growth suppression of HER2-overexpressing breast cancer cells by BBR. (A) SKBR-3 ($HER2^{high}$), BT-474 ($HER2^{high}$), MCF-7/HER2 ($HER2^{high}$), T47D ($HER2^{low}$), and MDA-MB-231 ($HER2^{low}$) cells were treated with either 0.2% DMSO (vehicle control) or BBR (10, 25, 50, 75, and 100 μM) for 24, 48, or 72 h. Cell viability was determined by the MTT assay as described under Materials and Methods. (B) SKBR-3 cells were treated with either vehicle control or BBR (10, 50, and 100 μM) for 24, 48, or 72 h. The cytotoxicity of BBR was examined using the trypan blue exclusion assay. (C) SKBR-3 cells were treated with various concentrations of taxol (0, 1, 5, 10, and 20 ng/mL) with or without 10 μM BBR for 24 or 48 h. Cell viability was determined by the MTT assay. All data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Statistical Analysis. All values are presented as the mean ± SD from three independent experiments. One-way ANOVA was used for statistical analysis. Significance was recognized as *, $P < 0.05$, and **, $P < 0.01$, relative to vehicle control.

RESULTS

BBR Inhibits the Growth of HER2-Overexpressing Breast Cancer Cells. To ascertain whether BBR inhibits growth of breast cancer cells with various degrees of HER2 expression, we first determined the viability of cells exposed to BBR by using the MTT assay. As shown in Figure 1A, $HER2^{high}$ cells were more sensitive to BBR than $HER2^{low}$ cells. Treatment of SKBR-3, a typical $HER2^{high}$ cell line, with 50 μM BBR for 24 h resulted in a 26% reduction in the number of viable cells, treatment for 48 h resulted in a 54% reduction in the number of viable cells, and treatment for 72 h resulted in a 67% reduction in the number of viable cells (Figure 1A). Moreover, we also determined the cytotoxic effect of BBR on SKBR-3 cells by using trypan blue exclusion assay. As shown in Figure 1B, exposure to 10 μM BBR for 24−72 h resulted in a 6−47% increase in the number of dead cells, exposure to 50 μM BBR for 24−72 h resulted in a 10−67% increase in the number of dead cells, and exposure to 100 μM BBR for 24−72 h resulted in a 16−90% increase in the number of dead cells. These results suggest that BBR is capable of suppressing the proliferation of HER2-overexpressing breast cancer cells.

Overexpression of HER2 negatively influences the response of breast cancer to chemotherapeutic agents (such as taxol and 5-fluorouracil).[23,24] We therefore examined whether BBR enhances the growth inhibitory effects of anticancer drugs in HER2-overexpressing breast cancer cells by incubating that cell line with both taxol and BBR. As illustrated in Figure 1C, BBR significantly enhanced the growth inhibitory effect of taxol on SKBR-3 cells. We found that the number of viable cells reduced by 8% in cells exposed to taxol (10 ng/mL) alone for 24 h, by 14% in cells exposed to BBR (10 μM) alone for 24 h, and by 37% in cells exposed to both agents for 24 h, relative to the control. After 48 h of exposure, taxol (10 ng/mL) alone resulted in a 40% reduction in the number of viable cells, BBR (10 μM) alone resulted in a 41% reduction in the number of viable cells, and combined treatment led to a 62% reduction in the number of viable cells, relative to control cells. A similar inhibitory effect on the growth of BT-474 ($HER2^{high}$) cells was also observed with a combination of taxol plus BBR (data not shown). These results clearly

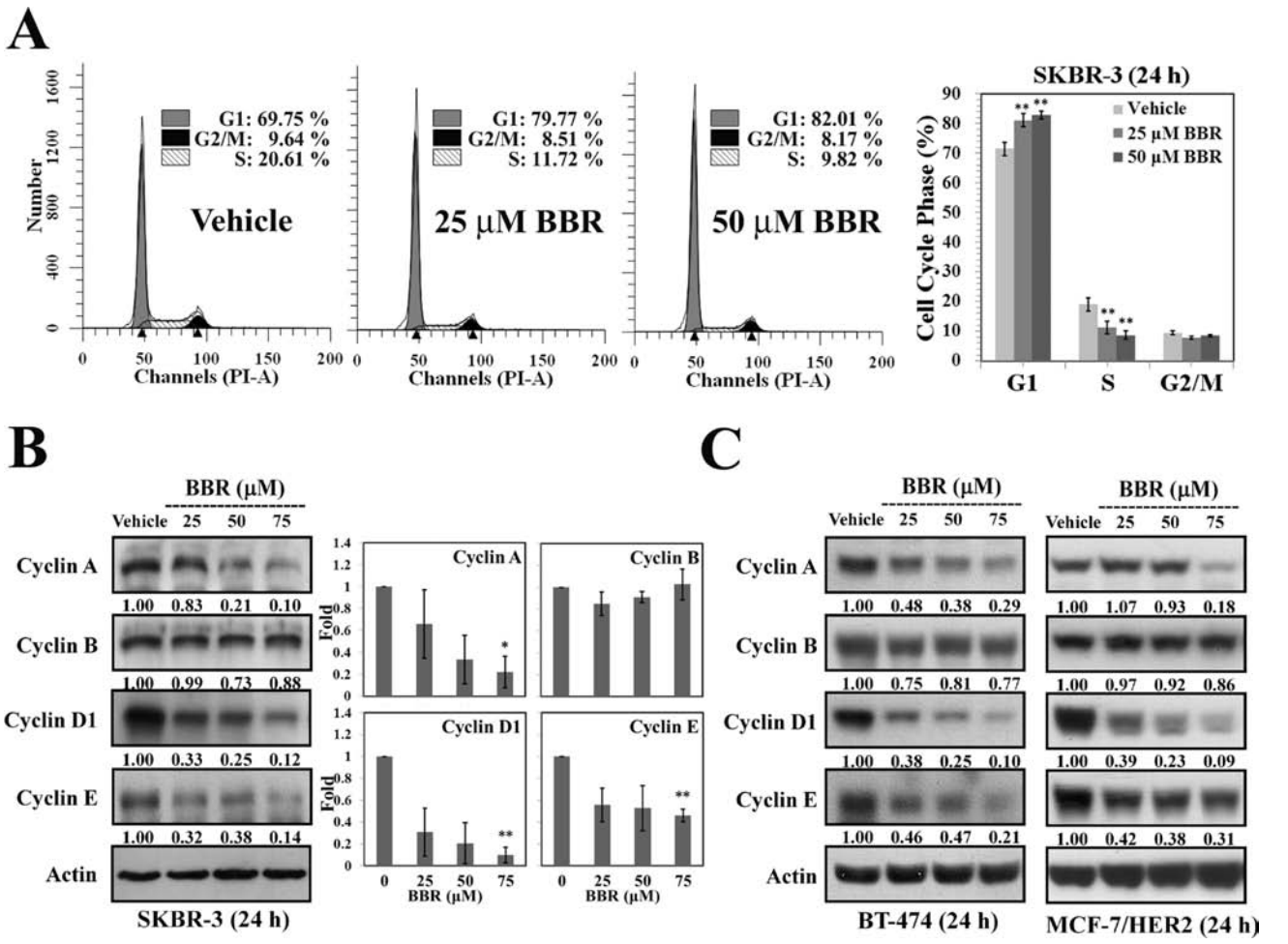

Figure 2. Perturbation of cell cycle by BBR in HER2-overexpressing breast cancer cells. (A) SKBR-3 cells were treated with various concentrations of BBR (0, 25, 50 μM) for 24 h. Cell cycle distribution was measured by flow cytometry as described under Materials and Methods. (B) SKBR-3 cells were treated with various concentrations of BBR (0, 25, 50, and 75 μM) for 24 h. Cell cycle related protein expression was determined by Western blotting as described under Materials and Methods. (C) BT-474 and MCF-7/HER2 cells were treated with various concentrations of BBR (0, 25, 50, and 75 μM) for 24 h. Cell cycle related protein expression was determined by Western blotting. All data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

demonstrate that BBR is capable of chemosensitizing HER2-overexpressing breast cancer cells to taxol.

BBR Induces G1-Phase Arrest by Down-regulating the Expression of Cell Cycle Regulatory Proteins. As mentioned above, our results showed a growth suppression effect of BBR on breast cancer cells, especially in HER2-overexpressing breast cancer SKBR-3 cells (Figure 1A). To verify whether the growth inhibition effect of BBR was due to the disruption of the cell cycle, flow cytometry was used to analyze the cell cycle distribution of SKBR-3 cells. As shown in Figure 2A, treatment of SKBR-3 cells with BBR resulted in a 10% increase in the number of cells in the G1 phase at a concentration of 25 μM and a 12% increase in the number of cells in the G1 phase at a concentration of 50 μM. A similar BBR-mediated cell cycle distribution pattern was observed in BT-474 cells (data not shown). These results suggest that BBR suppresses the growth of HER2-overexpressing breast cancer cells by inducing G1 cell cycle arrest.

To clarify the molecular mechanism governing BBR-induced G1 phase arrest, we assessed the effect of BBR on the expression of cell cycle regulatory proteins. We found that treatment with BBR had a marked dose-dependent effect on the suppression of cyclins A, D1, and E (Figure 2B). Similarly, BBR significantly affected the expression of cyclins A, D1, and E in BT-474 and MCF-7/HER2 cells (Figure 2C). The results suggest that BBR induces G1 phase cell cycle arrest by down-regulating cell cycle regulatory proteins in HER2-overexpressing breast cancer cells.

BBR Induces Cell Apoptosis by Activating the Mitochondria/Caspase Pathway. On the basis of the preliminary results there was a significant cytotoxicity effect of BBR on SKBR-3 cells (Figure 1B), we next determined whether the increase in cell death of SKBR-3 cells by BBR was due to an induction of apoptosis. As shown in Figure 3A, treatment of SKBR-3 cells with 50 μM BBR for 48 h resulted in marked condensation of chromatin. To assess the effect of BBR on the fragmentation of DNA, SKBR-3 cells were treated with various concentrations of BBR, and genomic DNA was analyzed by gel electrophoresis. As shown in Figure 3B, BBR exerted a dose-dependent effect on the induction of DNA fragmentation in SKBR-3 cells. In addition, to further confirm the influence of BBR on inducing cellular apoptosis, we used flow cytometry to examine the distribution of cells in the sub-G1 phase. As shown in Figure 3C, the percentage of cells in the sub-G1 phase in the BBR-treated group

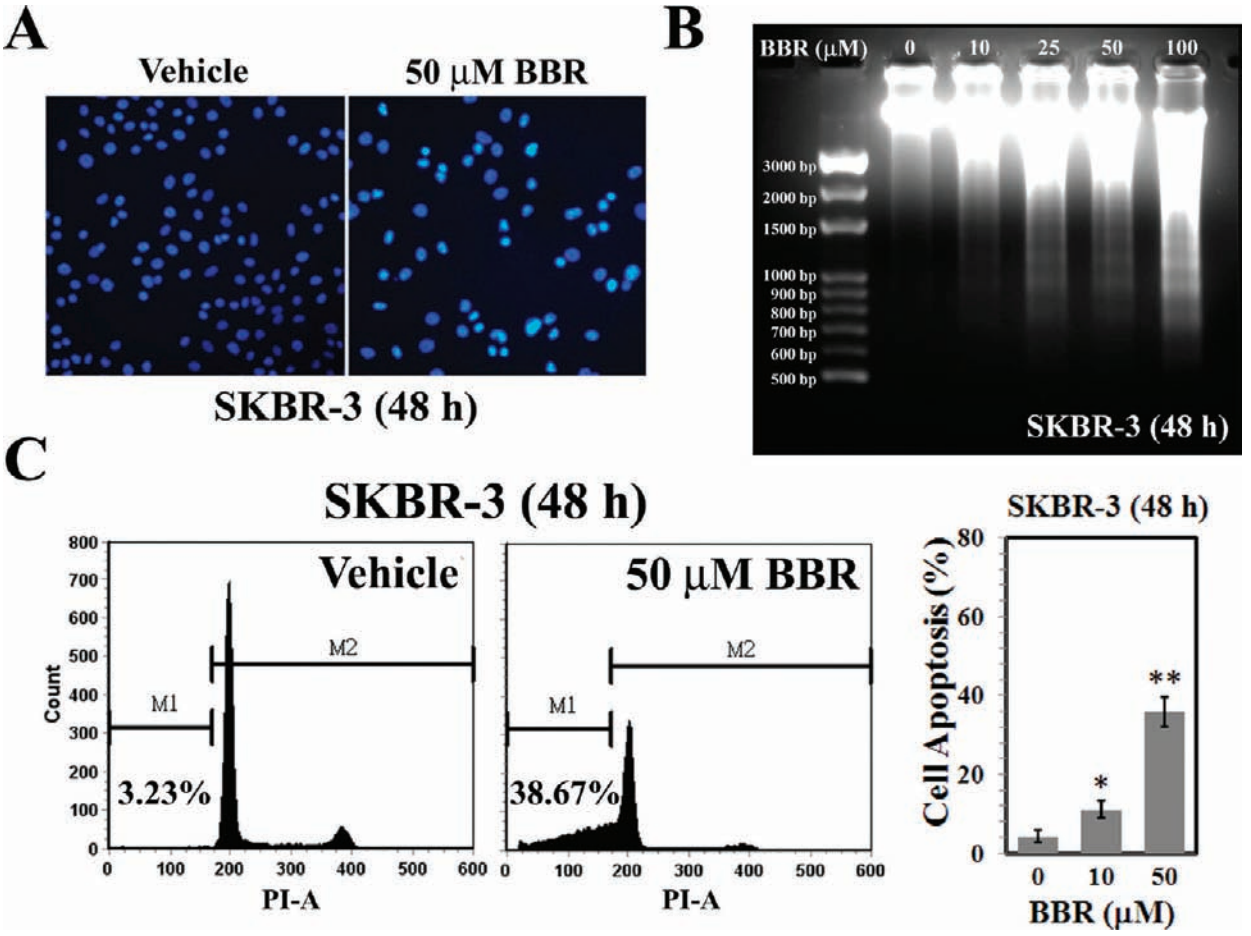

Figure 3. BBR induces cellular apoptosis in HER2-overexpressing breast cancer SKBR-3 cells. (A) SKBR-3 cells were treated with 50 μM BBR for 48 h. BBR influenced chromatin condensation via DAPI staining assay as described under Materials and Methods. (B) SKBR-3 cells were treated with various concentrations of BBR (0, 10, 25, 50, and 100 μM) for 48 h. BBR caused DNA fragmentation as determined by agarose gel electrophoresis. (C) SKBR-3 cells were treated with 10 or 50 μM BBR for 48 h. BBR increased the proportion of cells in the sub-G1 phase as determined by flow cytometry. All data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

was significantly higher than that in the control group (39 vs 3%). These observations suggest that BBR induces cell apoptosis in HER2-overexprssing breast cancer SKBR-3 cells.

To unravel the underlying molecular mechanisms behind BBR-induced cell apoptosis, we examined the effect of BBR on the protein expression of members of the Bcl-2 family that controls the activation of the mitochondria/caspase pathway.[25] As shown in Figure 4A, BBR treatment for 48 h resulted in a marked reduction in protein expression of the anti-apoptotic protein Bcl-2 in SKBR-3 cells; however, treatment did not result in significant changes in protein levels of the pro-apoptotic Bax protein. Moreover, we analyzed the effects of BBR on the release of Cyt-*c* and found that BBR caused a dose-dependent increase in Cyt-*c* in the cytosolic fractions (Figure 4B). In addition, treatment with BBR resulted in significant increases in the cleaved forms of caspase-9, caspase-3, and PARP in SKBR-3 cells (Figure 4C). These findings demonstrate that BBR induces cellular apoptosis by activating the mitochondria/caspase pathway in HER2-overexpressing breast cancer SKBR-3 cells.

BBR Suppresses Growth of HER2-Overexpressing Cells by Modulating the HER2/PI3K/Akt Signaling Pathway. The HER2 signaling pathway is associated with cell proliferation and survival;[9,21,26] therefore, we examined the effect of BBR on HER2 and its downstream PI3K/Akt and Ras/MAPK signaling cascades.[27] As shown in Figure 5A, BBR exhibited dose- and time-dependent effects on the suppression of phospho-HER2 and phospho-Akt without a significant reduction in phospho-Erk1/2 in SKBR-3 cells. Moreover, BBR showed similar repressive effects on phospho-HER2 and phospho-Akt in other HER2-overexpressing cell lines, namely, BT-474 and MCF-7/HER2 (Figure 5B). These data clearly demonstrate that BBR exerts inhibitory effects on the HER2/PI3K/Akt signaling pathway in breast cancer cells that overexpress HER2.

We tested the validity of our results by incubating SKBR-3 cells with the PI3K-specific inhibitor LY294002 and the ligand of the HER2/HER3 receptor heregulin (HRG). We found that LY294002 not only suppressed the proliferation of SKBR-3 cells (Figure 6A) but also down-regulated the levels of phospho-Akt and cyclins D1 and E in SKBR-3 cells (Figure 6B). In other words, LY294002 exhibited a suppression effect similar to that of BBR on SKBR-3 cells. In contrast, HRG not only protected against the BBR-induced suppression of cell proliferation (Figure 6A) but also attenuated the BBR-induced down-regulation of phospho-Akt, cyclin D1, and cyclin E in SKBR-3 cells (Figure 6C). Furthermore, as shown in Figure 6D, HRG also significantly attenuated the BBR-induced cell apoptosis in SKBR-3 cells at

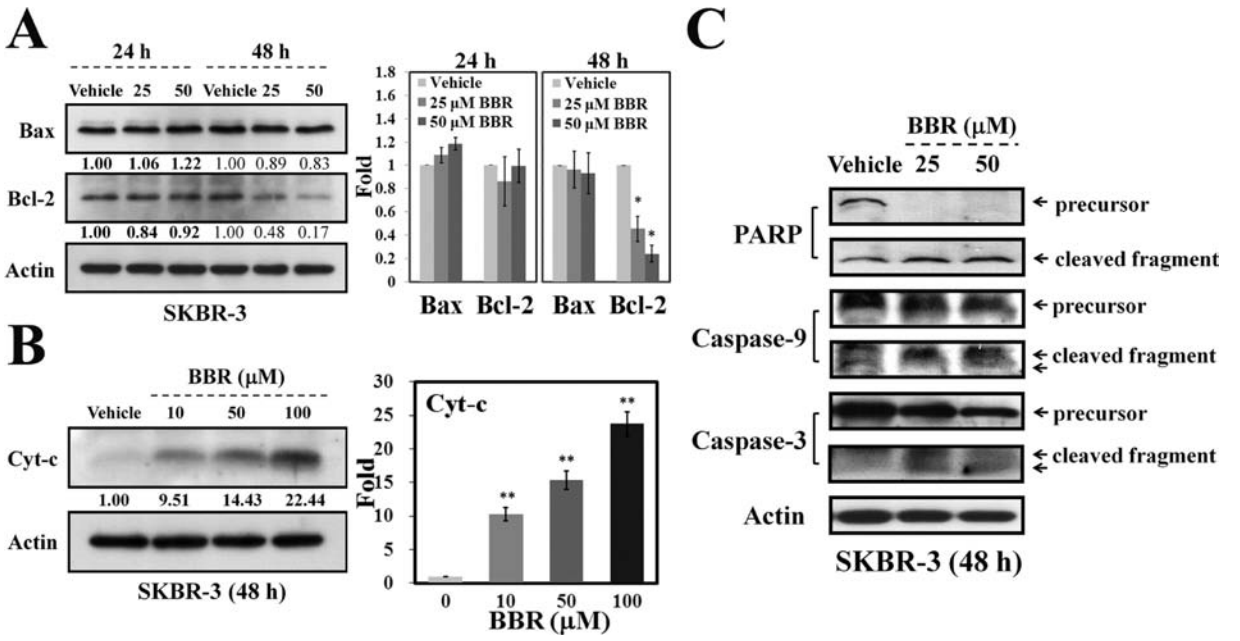

Figure 4. BBR activates the mitochondria/caspase pathway in HER2-overexpressing breast cancer SKBR-3 cells. (A) SKBR-3 cells were treated with various concentrations of BBR (0, 25, and 50 μM) for 24 or 48 h. Protein expression of Bcl-2 and Bax was measured by Western blotting. (B) SKBR-3 cells were treated with various concentrations of BBR (0, 10, 50, and 100 μM) for 48 h. The release of cytochrome *c* (Cyt-c) into cytoplasm was measured by Western blotting. (C) SKBR-3 cells were treated with various concentrations of BBR (0, 25, 50 μM) for 48 h. The cleavage of caspase-9, caspase-3, and PARP was measured by Western blotting. All data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

48 h. These data indicate that BBR inhibits cell proliferation and induces apoptosis by suppressing the HER2/PI3K/Akt signaling pathway in breast cancer cells that overexpress HER2 (Figure 7).

DISCUSSION

HER2-overexpressing breast cancer is associated with increased risk for metastasis and poor response to anticancer therapies.[4] Fortunately, treatment with agents that specifically target breast cancer cells that overexpress HER2, such as trastuzumab and lapatinib, has improved clinical outcomes.[8] In addition to those agents, a number of natural products have been shown to be effective alternative therapeutic agents for the treatment of HER2-overexpressing breast cancer.[9−11] In this study, we demonstrate for the first time that BBR, a natural alkaloid, has a marked inhibitory effect on the proliferation of SKBR-3, BT-474, and MCF-7/HER2 cells (Figure 1A).

Disruption of cell cycle progression, which is controlled by cell cycle regulators, in cancer cells is a useful strategy to halt tumor growth.[28] Furthermore, the cell cycle arrest of cancer cells also provides an occasion for cells to undergo either repair mechanisms or apoptosis. Several natural products have marked inhibitory effects on cancer cells with HER2 overexpression via perturbing cell cycle progression and/or inducing cell apoptosis.[9,26] For instance, the plant flavonoid apigenin inhibits the growth of HER2-overexpressing breast cancer cells via inducing cell apoptosis,[9] whereas the natural triterpenoid corosolic acid (CRA) inhibits cell growth by inducing cell cycle arrest and apoptosis in gastric cancer cells with HER2 overexpression.[26] Herein, our in vitro data demonstrate that treatment of SKBR-3 (Figure 2A) and BT-474 (data not shown) cells with the natural alkaloid BBR induces G1 cell cycle arrest. Therefore, one of the molecular mechanisms by which BBR suppresses the growth of HER2-overexpressing breast cancer cells is via disruption of cell cycle progression. Furthermore, we examined the effects of BBR on the expression of cell cycle regulators and found that BBR induces G1-phase arrest by down-regulating the expression of cyclins D1 and E in SKBR-3, BT-474, and MCF-7/HER2 cells (Figure 2B−C). Although down-regulation of cyclin A was also observed in those cell lines (Figure 2B−C), it is not clear whether the decrease in cyclin A is involved in the arrest of cells in the G1 phase or is a consequence of G1 arrest. Therefore, our findings suggest that BBR induces G1 cell cycle arrest by regulating the expression of cyclins D1 and E and, possibly, cyclin A.

Many anticancer drugs exert their antitumor activities by inducing apoptosis of cancer cells. Resistance to apoptosis therefore results in the failure of chemotherapy, which is caused by a decrease in the sensitivity of cancer cells to drugs. In this study, we found that BBR not only disrupted cell cycle progression but also induced cell apoptosis in HER2-overexpressing SKBR-3 cells (Figure 3). Moreover, we also found that treatment of SKBR-3 cells with BBR resulted in a significant decrease in protein levels of Bcl-2 (Figure 4A). This alteration may be responsible for the concomitant execution phase of apoptosis including an increase in the release of cytochrome *c* to cytosol (Figure 4B) and activation or cleavage of caspase-9, caspase-3, and PARP (Figure 4C). In contrast, BBR has been shown to induce apoptosis by up-regulating the expression of Bax protein and by down-regulating the protein expression of Bcl-2 in many human cancer cells.[18,29,30] In our study, however, BBR did not have a significant effect on Bax protein level in breast cancer cells that overexpress HER2. Patil et al. reported a similar finding in HER2 low-expressing breast cancer cells.[31] In addition, some natural products have been reported to induce cell apoptosis without affecting the protein expression of Bcl-2 or Bax. For example, McGuire et al. showed that 3,3′-diindolylmethane

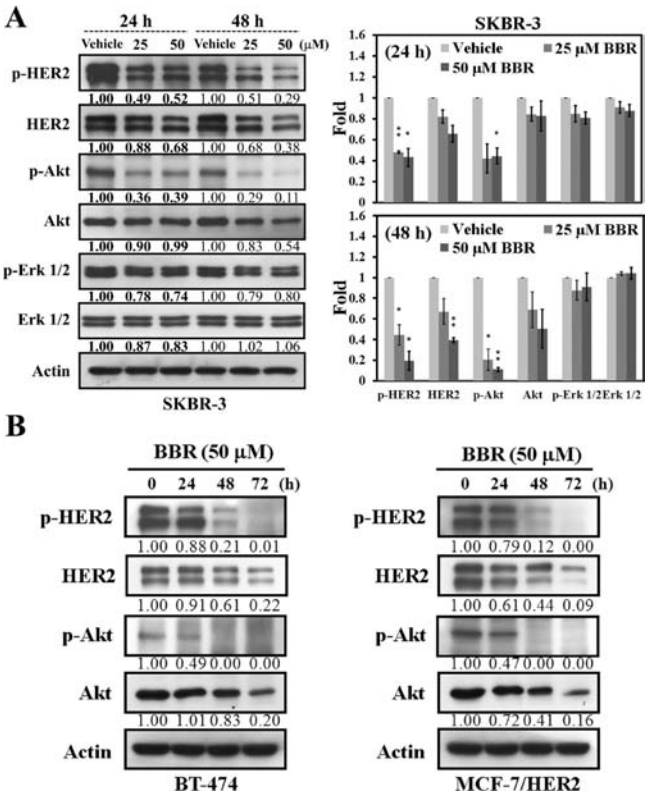

Figure 5. BBR inhibits the HER2/PI3K/Akt signaling pathway in HER2-overexpressing breast cancer cells. (A) Treatment of SKBR-3 cells with BBR (0, 25, or 50 μM) for 24 or 48 h suppressed the HER2/PI3K/Akt, but not the HER2/Erk, signaling pathway. (B) Treatment of BT-474 and MCF-7/HER2 cells with BBR (50 μM) for 24, 48, or 72 h also suppressed the HER2/PI3K/Akt signaling pathway. All data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

induced cellular apoptosis by inhibiting the HER2/Ras/Erk signaling pathway in HER2-overexpressing breast cancer cells without altering the expression of Bax protein,[32] whereas Saleem et al. demonstrated that lupeol induced apoptosis by inhibiting the Ras signaling pathway in pancreatic cancer cells without affecting the protein expression of Bcl-2.[33] Therefore, the various effects of natural products including BBR on the protein expression of Bcl-2 and Bax may be due to cell-type specificity or result from up/down-regulation of different upstream regulatory molecules.

The two primary therapeutic approaches to treat HER2-overexpressing breast cancer involve agents that down-regulate the expression of the HER2 receptor and agents that suppress HER2 tyrosine kinase activity/phosphorylation.[34] In this study, we found that BBR inhibits both phospho-HER2 and HER2 in SKBR-3, BT-474, and MCF-7/HER2 cells (Figure 5). The suppressive effect of BBR on phospho-HER2 levels may be governed by its ability to inhibit the expression of HER2 protein. Other natural compounds have also been shown to be able to down-regulate the protein levels of both phospho-HER2 and HER2.[9,10] For example, Way et al. reported that apigenin inhibited the tyrosine phosphorylation of HER2 by depleting the HER2 protein level,[9] and Jeong et al. showed that quercetin suppressed HER2 tyrosine kinase activity by down-regulating the level of HER2 protein expression.[10] Those observations suggest that BBR may be a useful HER2-targeting agent for the treatment of HER2-overexpressing breast cancer.

Although the PI3K/Akt and Ras/MAPK signaling cascades are the two main intracellular signaling pathways activated by receptor tyrosine kinases, such as HER2,[6,27] our data show that BBR dramatically inhibits the PI3K/Akt, but not the Ras/MAPK, signaling cascade in breast cancer cells that overexpress HER2 (Figure 5). Moreover, we incubated HER2-overexpressing cells with LY294002, a PI3K-specific inhibitor, and HRG, a ligand of the HER2/HER3 receptor, to confirm that BBR suppresses cellular proliferation by modulating the HER2/PI3K/Akt signaling pathway (Figure 6). Interestingly, we found that BBR attenuated the expression of the Akt protein (Figure 5), a finding that suggests that Akt may be a target of BBR in breast cancer cells that overexpress HER2. Whether BBR down-regulates the gene expression of Akt or interferes with the stability of Akt at the protein level remains to be clarified.

Previous studies have shown that BBR inhibits the proliferation of breast cancer cells with HER2 low expression;[31,35,36] however, to the best of our knowledge, our study is the first to investigate whether BBR can suppress the growth of HER2-overexpressing breast cancer cells. We found that although BBR suppresses the growth of breast cancer cells with various levels of HER2 expression, cells that overexpress HER2 were more sensitive to BBR than cell lines with low levels of HER2 expression (Figure 1A). In addition, although we found that the HER2 signaling pathway, which plays an important role in the proliferation and survival of $HER2^{high}$ but not $HER2^{low}$ cancer cells,[37−39] is inhibited by BBR (Figure 5), our data suggest that BBR may also affect cellular proliferation by modulating non-HER2-mediated pathways. For example, treatment with HRG, a ligand of HER2/HER3 receptors, protected against BBR-induced growth inhibition, down-regulation of cyclin D1, and cell apoptosis (Figure 6A,C,D); however, HRG was not capable of completely masking the effects of BBR. Therefore, we suspect that non-HER2-mediated pathways are involved in the growth inhibitory effects of BBR on breast cancer cells that overexpress HER2. The presence of non-HER2-mediated pathways inhibited by BBR may explain why BBR also has a growth suppression influence on breast cancer cells with HER2 low expression.

Overexpression of HER2 negatively influences the response of breast cancer to chemotherapeutic agents,[23,24] and down-regulation of HER2 pathway by HER2-targeting therapeutic agents such as trastuzumab potentiates the antitumor activity of those chemotherapeutic agents in the treatment of HER2-overexpressing breast cancers.[40,41] Some natural products have been reported to be even more effective than synthetic agents at inhibiting cellular proliferation.[32,42] For example, treatment comprising epigallocatechin-3-gallate combined with taxol and treatment comprising 3,3′-diindolylmethane combined with taxol have been shown to have a synergistic effect on growth suppression of HER2-overexpressing breast cancer cells.[32,42] Similarly, we demonstrated that BBR enhances the chemotherapeutic efficacy of taxol against SKBR-3 cells (Figure 1C) and BT-474 (data not shown) cells. These results indicate that BBR performs in a manner similar to that of other HER2 inhibitors or natural products by suppressing the HER2 signaling pathway in HER2-overexpressing breast cancer cells.

In conclusion, we have demonstrated that BBR induces G1 cell cycle arrest and apoptosis of HER2-overexpressing breast cancer cells by inhibiting the HER2/PI3K/Akt signaling pathway. In addition, we have also shown that a combination of BBR with taxol exerts an enhanced growth suppression effect on HER2-overexpressing breast cancer cells. Our findings suggest that BBR could be a useful adjuvant therapeutic agent in the treatment of HER2-overexpressing breast cancer.

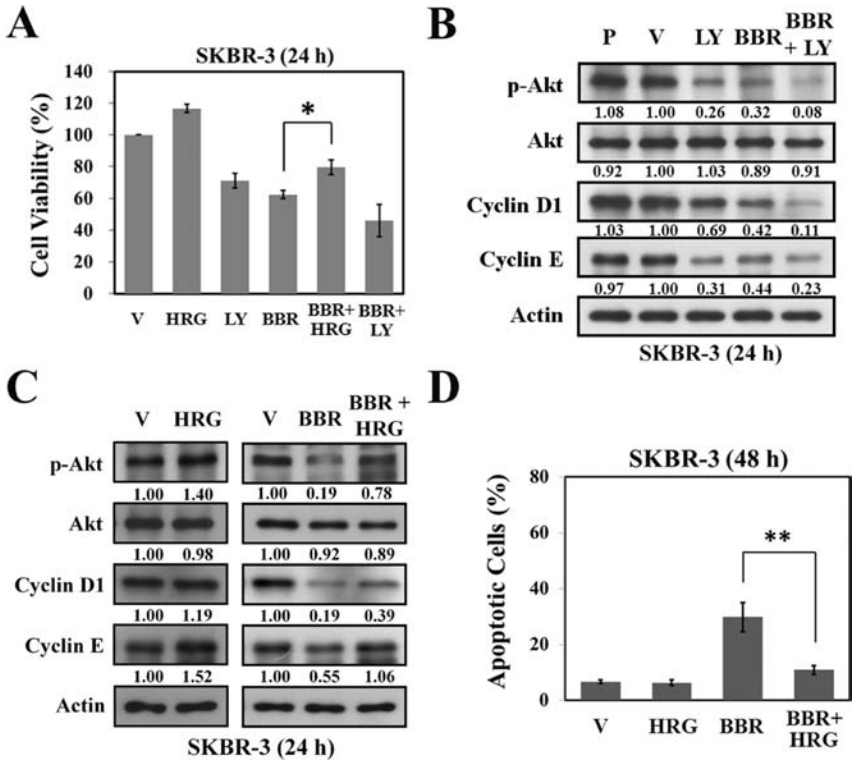

Figure 6. Confirmation that BBR suppresses cellular proliferation by modulating the HER2/PI3K/Akt signaling pathway. (A) SKBR-3 cells were treated with 20 μM LY294002 (LY, a PI3K inhibitor) and 100 ng/mL HRG (an activator of HER2/HER3 pathway) alone or in combination with 50 μM BBR for 24 h. Cell viability was determined by the MTT assay. (B) LY, similar to BBR, led to down-regulation of p-Akt and down-regulation of cyclins D1 and E. SKBR-3 cells were treated with 20 μM LY alone or in combination with 50 μM BBR for 24 h. (C) BBR-induced down-regulation of p-Akt and cyclins D1 and E was reversed by HRG treatment. SKBR-3 cells were treated with HRG (100 ng/mL) alone or in combination with 50 μM BBR for 24 h. (D) BBR-induced cell apoptosis was reversed by HRG treatment. SKBR-3 cells were treated with HRG (100 ng/mL) alone or in combination with 50 μM BBR for 48 h. P, parental SKBR-3 cells; V, vehicle control. All data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

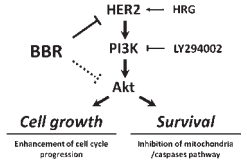

Figure 7. Schematic model for BBR-induced growth suppression and apoptosis in HER2-overexpressing breast cancer cells. Ligand (heregulin, HRG) stimulation induces heterodimerization of HER2 with HER3 resulting in self-phosphorylation at tyrosine residues located on the C-terminal domain of HER2 protein. The phosphorylated HER2 then activates downstream PI3K/Akt signaling cascade (inhibited by LY294002), which in turn promotes cell proliferation and survival. After BBR treatment, cell growth is inhibited because of induction of cell cycle arrest and apoptosis. The BBR-induced growth suppression and cell apoptosis is due to down-regulation of the HER2/PI3K/Akt signaling pathway.

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ABBREVIATIONS USED

BBR, berberine; HER2, human epidermal growth factor receptor-2; PARP, poly-(ADP-ribose) polymerase; Cyt-*c*, cytochrome *c*; HRG, heregulin.

REFERENCES

(1) Jemal, A.; Siegel, R.; Xu, J.; Ward, E. Cancer statistics, 2010. *CA Cancer J. Clin.* **2010**, *60* (5), 277–300.

(2) Slamon, D. J.; Clark, G. M.; Wong, S. G.; Levin, W. J.; Ullrich, A.; McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **1987**, *235* (4785), 177–182.

(3) Witton, C. J.; Reeves, J. R.; Going, J. J.; Cooke, T. G.; Bartlett, J. M. Expression of the HER1−4 family of receptor tyrosine kinases in breast cancer. *J. Pathol.* **2003**, *200* (3), 290–297.

(4) Yu, D.; Hung, M. C. Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. *Oncogene* **2000**, *19* (53), 6115–6121.

(5) Yamamoto, T.; Ikawa, S.; Akiyama, T.; Semba, K.; Nomura, N.; Miyajima, N.; Saito, T.; Toyoshima, K. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **1986**, *319* (6050), 230–234.

(6) Yarden, Y.; Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.* **2001**, *2* (2), 127–137.

(7) Baselga, J. Treatment of HER2-overexpressing breast cancer. *Ann. Oncol.* **2010**, *21* (Suppl. 7), vii36–vii40.

(8) Baselga, J.; Swain, S. M. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat. Rev. Cancer* **2009**, *9* (7), 463–475.

(9) Way, T. D.; Kao, M. C.; Lin, J. K. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. *J. Biol. Chem.* **2004**, *279* (6), 4479–4489.

(10) Jeong, J. H.; An, J. Y.; Kwon, Y. T.; Li, L. Y.; Lee, Y. J. Quercetin-induced ubiquitination and down-regulation of Her-2/neu. *J. Cell Biochem.* **2008**, *105* (2), 585–595.

(11) Hong, R. L.; Spohn, W. H.; Hung, M. C. Curcumin inhibits tyrosine kinase activity of p185neu and also depletes p185neu. *Clin. Cancer Res.* **1999**, *5* (7), 1884–1891.

(12) Wang, Y. X.; Zheng, Y. M. Ionic mechanism responsible for prolongation of cardiac action-potential duration by berberine. *J. Cardiovasc. Pharmacol.* **1997**, *30* (2), 214–222.

(13) Amin, A. H.; Subbaiah, T. V.; Abbasi, K. M. Berberine sulfate: antimicrobial activity, bioassay, and mode of action. *Can. J. Microbiol.* **1969**, *15* (9), 1067–1076.

(14) Tsai, C. S.; Ochillo, R. F. Pharmacological effects of berberine on the longitudinal muscle of the guinea-pig isolated ileum. *Arch. Int. Pharmacodyn. Ther.* **1991**, *310*, 116–131.

(15) Bova, S.; Padrini, R.; Goldman, W. F.; Berman, D. M.; Cargnelli, G. On the mechanism of vasodilating action of berberine: possible role of inositol lipid signaling system. *J. Pharmacol. Exp. Ther.* **1992**, *261* (1), 318–323.

(16) Akhter, M. H.; Sabir, M.; Bhide, N. K. Anti-inflammatory effect of berberine in rats injected locally with cholera toxin. *Indian J. Med. Res.* **1977**, *65* (1), 133–141.

(17) Hwang, J. M.; Wang, C. J.; Chou, F. P.; Tseng, T. H.; Hsieh, Y. S.; Lin, W. L.; Chu, C. Y. Inhibitory effect of berberine on *tert*-butyl hydroperoxide-induced oxidative damage in rat liver. *Arch. Toxicol.* **2002**, *76* (11), 664–670.

(18) Mantena, S. K.; Sharma, S. D.; Katiyar, S. K. Berberine inhibits growth, induces G1 arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating Cdki-Cdk-cyclin cascade, disruption of mitochondrial membrane potential and cleavage of caspase 3 and PARP. *Carcinogenesis* **2006**, *27* (10), 2018–2027.

(19) Hwang, J. M.; Kuo, H. C.; Tseng, T. H.; Liu, J. Y.; Chu, C. Y. Berberine induces apoptosis through a mitochondria/caspases pathway in human hepatoma cells. *Arch. Toxicol.* **2006**, *80* (2), 62–73.

(20) Pinto-Garcia, L.; Efferth, T.; Torres, A.; Hoheisel, J. D.; Youns, M. Berberine inhibits cell growth and mediates caspase-independent cell death in human pancreatic cancer cells. *Planta Med.* **2010**, *76* (11), 1155–1161.

(21) Chuang, T. C.; Lee, Y. J.; Liu, J. Y.; Lin, Y. S.; Li, J. W.; Wang, V.; Law, S. L.; Kao, M. C. EBNA1 may prolong G(2)/M phase and sensitize HER2/neu-overexpressing ovarian cancer cells to both topoisomerase II-targeting and paclitaxel drugs. *Biochem. Biophys. Res. Commun.* **2003**, *307* (3), 653–659.

(22) Kao, M. C.; Liu, G. Y.; Chuang, T. C.; Lin, Y. S.; Wuu, J. A.; Law, S. L. The N-terminal 178-amino-acid domain only of the SV40 large T antigen acts as a transforming suppressor of the HER-2/neu oncogene. *Oncogene* **1998**, *16* (4), 547–554.

(23) Yu, D.; Liu, B.; Tan, M.; Li, J.; Wang, S. S.; Hung, M. C. Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. *Oncogene* **1996**, *13* (6), 1359–1365.

(24) Miles, D. W.; Harris, W. H.; Gillett, C. E.; Smith, P.; BarnesD. M. Effect of c-erbB(2) and estrogen receptor status on survival of women with primary breast cancer treated with adjuvant cyclophosphamide/methotrexate/fluorouracil. *Int. J. Cancer* **1999**, *84* (4), 354–359.

(25) Cory, S.; Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2002**, *2* (9), 647–656.

(26) Lee, M. S.; Cha, E. Y.; Thuong, P. T.; Kim, J. Y.; Ahn, M. S.; Sul, J. Y. Down-regulation of human epidermal growth factor receptor 2/neu oncogene by corosolic acid induces cell cycle arrest and apoptosis in NCI-N87 human gastric cancer cells. *Biol. Pharm. Bull.* **2010**, *33* (6), 931–937.

(27) Hynes, N. E.; Lane, H. A. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* **2005**, *5* (5), 341–354.

(28) Grana, X.; Reddy, E. P. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* **1995**, *11* (2), 211–219.

(29) Ho, Y. T.; Lu, C. C.; Yang, J. S.; Chiang, J. H.; Li, T. C.; Ip, S. W.; Hsia, T. C.; Liao, C. L.; Lin, J. G.; Wood, W. G.; Chung, J. G. Berberine induced apoptosis via promoting the expression of caspase-8, -9 and -3, apoptosis-inducing factor and endonuclease G in SCC-4 human tongue squamous carcinoma cancer cells. *Anticancer Res.* **2009**, *29* (10), 4063–4070.

(30) Eom, K. S.; Hong, J. M.; Youn, M. J.; So, H. S.; Park, R.; Kim, J. M.; Kim, T. Y. Berberine induces G1 arrest and apoptosis in human glioblastoma T98G cells through mitochondrial/caspases pathway. *Biol. Pharm. Bull.* **2008**, *31* (4), 558–562.

(31) Patil, J. B.; Kim, J.; Jayaprakasha, G. K. Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway. *Eur. J. Pharmacol.* **2010**, *645* (1−3), 70–78.

(32) McGuire, K. P.; Ngoubilly, N.; Neavyn, M.; Lanza-Jacoby, S. 3,3′-Diindolylmethane and paclitaxel act synergistically to promote apoptosis in HER2/Neu human breast cancer cells. *J. Surg. Res.* **2006**, *132* (2), 208–213.

(33) Saleem, M.; Kaur, S.; Kweon, M. H.; Adhami, V. M.; Afaq, F.; Mukhtar, H. Lupeol, a fruit and vegetable based triterpene, induces apoptotic death of human pancreatic adenocarcinoma cells via inhibition of Ras signaling pathway. *Carcinogenesis* **2005**, *26* (11), 1956–1964.

(34) Imai, K.; Takaoka, A. Comparing antibody and small-molecule therapies for cancer. *Nat. Rev. Cancer* **2006**, *6* (9), 714–727.

(35) Kim, J. B.; Lee, K. M.; Ko, E.; Han, W.; Lee, J. E.; Shin, I.; Bae, J. Y.; Kim, S.; Noh, D. Y. Berberine inhibits growth of the breast cancer cell lines MCF-7 and MDA-MB-231. *Planta Med.* **2008**, *74* (1), 39–42.

(36) Liu, J.; He, C.; Zhou, K.; Wang, J.; Kang, J. X. Coptis extracts enhance the anticancer effect of estrogen receptor antagonists on human breast cancer cells. *Biochem. Biophys. Res. Commun.* **2009**, *378* (2), 174–178.

(37) Lewis, G. D.; Figari, I.; Fendly, B.; Wong, W. L.; Carter, P.; Gorman, C.; Shepard, H. M. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol. Immunother.* **1993**, *37* (4), 255–263.

(38) Roh, H.; Pippin, J.; Drebin, J. A. Down-regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. *Cancer Res.* **2000**, *60* (3), 560–565.

(39) Faltus, T.; Yuan, J.; Zimmer, B.; Kramer, A.; Loibl, S.; Kaufmann, M.; Strebhardt, K. Silencing of the HER2/neu gene by siRNA inhibits proliferation and induces apoptosis in HER2/neu-overexpressing breast cancer cells. *Neoplasia* **2004**, *6* (6), 786–795.

(40) Slamon, D. J.; Leyland-Jones, B.; Shak, S.; Fuchs, H.; Paton, V.; Bajamonde, A.; Fleming, T.; Eiermann, W.; Wolter, J.; Pegram, M.; Baselga, J.; Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **2001**, *344* (11), 783–792.

(41) Bullock, K.; Blackwell, K. Clinical efficacy of taxane-trastuzumab combination regimens for HER-2-positive metastatic breast cancer. *Oncologist* **2008**, *13* (5), 515–525.

(42) Masuda, M.; Suzui, M.; Lim, J. T.; Weinstein, I. B. Epigallocatechin-3-gallate inhibits activation of HER-2/neu and downstream signaling pathways in human head and neck and breast carcinoma cells. *Clin. Cancer Res.* **2003**, *9* (9), 3486–3491.