Growth suppression of HER2-overexpressing breast cancer cells by berberine via modulating the HER2/PI3K/Akt signaling pathway

Han-Peng Kuo[a], Tzu-Chao Chuang[b] , Ming-Han Yeh[a], Shih-Chung Hsu[c], Tzong-Der Way[d], Shan-Shue Wang[e], Yung-Hsien Chang[f], Ming-Ching Kao[d,g,*], Jah-Yao Liu[h,*]

[a]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan

[b]Department of Chemistry, Tamkang University, Tamsui, New Taipei, Taiwan

[c]Kang-Ning Junior College of Medical Care and Management, Taipei, Taiwan

[d]Department of Biological Science and Technology, China Medical University, Taichung, Taiwan

[e]Department of Chemical and Biochemical Engineering, Kao Yuan University, Kaohsiung, Taiwan

[f]Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan

[g]Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan

[h]Department of Obstetrics & Gynecology, Tri-Service General Hospital, Taipei, Taiwan, ROC

[*]Correspondence:

Ming-Ching Kao: mckao@mail.cmu.edu.tw; Tel: 886-4-22053366 ext. 2206; Fax: 886-4-22071507.

Jah-Yao Liu: jyliu@mail.ndmctsgh.edu.tw; Tel: 886-2-87923311 ext. 12828; Fax: 886-2-87918437.

Abstract

Berberine (BBR) is a natural alkaloid with significant anti-tumor activities against many types of cancer. In this study, we investigate the molecular mechanism behind the growth suppression of BBR in HER2-overexpressing breast cancer cells. The results reveal that BBR decreases cell viability and induces G1 arrest. The BBR-induced G1 arrest is mediated through a decrease in the expression of cyclins D1, and E. In addition, BBR also induces cell apoptosis through the induction of the mitochondria/caspases pathway in HER2-overexpressing breast cancer cells. Our data also indicate that the BBR-induced growth inhibition and cell apoptosis are caused 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. In conclusion, this study suggests that BBR could be a useful chemotherapeutic and chemosensitizing agent for adjuvant therapy against HER2-overexpressing breast cancer.

Keywords: Berberine, Breast cancer, HER2, PI3K/Akt signaling cascade, Apoptosis, Cell cycle arrest

Introduction

Breast carcinoma is the most frequent form of cancer affecting women and has become the second cause of cancer death in the United States (*1*). Gene amplification and/or protein overexpression of some oncogenes have been involved in the development of breast cancer. The human epidermal growth factor receptor 2 (HER2) is one among the most characterized oncogenes associated with an aggressive phenotype in breast cancer (*2*). Aberrant up-regulation of HER2 is found in around 30% of breast cancer (*2*, *3*) and diminishes the effectiveness of cancer treatment, increases cancer metastasis and finally leads to poor clinical outcomes (*4*). Therefore, it suggests that 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 was first identified in 1984 (*5*), and is expressed by a gene localized on chromosome 17q21 (*6*). It is a transmembrane receptor tyrosine kinase (RTK) with a molecular weight 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 hetero-dimerization causing self-phosphorylation (except for HER3) on tyrosine residues localized on the C-terminal domain of the HER receptor. The phosphorylated HER receptor (activated form) phosphorylates downstream substrates to activate a variety of signaling cascades, including the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)/Akt, which promote cell growth, survival and metastasis (*7*). EGFR, HER2 and HER3 are known to be involved in the progression and development of cancer, while the role of HER4 in oncogenesis is still undefined (*8*). The HER2/HER3 heterodimer is the most potent HER receptor pair, which is considered as an oncogenic unit with respect to strength of interaction, ligand-induced

tyrosine phosphorylation and downstream signaling (*8*).

Based on the role of HER2 in breast cancer, a number of HER2-targeting therapeutics have been developed in 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 two well-documented and clinically used HER2-targeting anti-cancer drugs (*9*). However, botanical products are currently considered to be safer natural compounds and/or nutrients and may be used as alternative therapeutic agents for cancer treatment in breast cancer with HER2 overexpression (*10-12*).

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 anti-arrhythmic (*13*), anti-bacterial (*14*), anti-cholinergic (*15*), anti-hypertensive (*16*), anti-inflammation (*17*), and anti-oxidative activities (*18*). It is also known that BBR has growth inhibition effects on various types of cancer. For example, BBR inhibits cell growth, induces cell cycle arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating CKI-cdk-cyclin cascade, disrupting mitochondrial membrane potential and cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP) (*19*). BBR also inhibits cell proliferation through inducing the mitochondria/caspases pathway in human hepatoma HepG2 cells (*20*) or activation of caspases-independent cell death in human pancreatic cancer BxPC3 cells (*21*). Although BBR has anti-tumor activities in many human cancer cells, the molecular mechanisms for the growth suppression effect on HER2-overexpressing breast cancer cells have yet to be explored.

In this study, the BBR-mediated growth inhibition and the related molecular mechanisms in HER2-overexpressing breast cancer cells were investigated. The suppression of

HER2-mediated signaling pathway by BBR leads to cell cycle arrest and apoptosis was presented. Furthermore, the effect of BBR in combination with anti-cancer drugs on HER2-overexpressing breast cancer cells was also discussed.

Materials and Methods

Chemicals and antibodies

Berberine (BBR), DMSO, heregulin (HRG) and antibodies for cytochrome c were obtained from Sigma (St. Louis, MO, USA). Antibodies for cyclins A, B1, D1 and E, Akt1, phospho-Akt (Ser308), Bax and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for PARP and caspase-9 were obtained from BioVision. Antibodies for Bcl-2 were purchased from UPSTATE. Antibodies for 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, USA). Antibodies for HER2 (Ab-3) were obtained from CALBIOCHEM. Antibodies for phospho-HER2 (Ab-18) were purchased from NeoMarkers. Antibodies for β-actin were purchased from CHEMICON. Taxol (paclitaxel) was purchased from Bristol-Myers Squibb (Bristol-Myers Squibb, Wallingford, CT), and doxorubicin (adriamycin) was purchased from Pharmacia (Pharmacia & Upjohn S.P.A. Milan, Italy).

Cell culture

Human breast carcinoma SKBR-3 and BT-474 cells overexpressed HER2 and were obtained from American Type Culture Collection (ATCC, Rockville, MD). The human breast carcinoma MCF-7/HER2 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 humidified under 5 % CO_2 at 37 ^{o}C.

MTT assay

Cell viability was determined using the MTT metabolic assay, as described by Chuang et al. (*22*). In brief, cells were seeded at a density of 12,000 cells/well into 96-well plates and incubated overnight in a media containing 10 % FBS. Cells were treated with the indicated condition, and then stained with MTT (0.5 mg/ml). After 4 h incubation with MTT, the viable cell number was directly proportional to the production of formazan crystals, solubilized with DMSO and 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. (*23*). In brief, the cells were washed with PBS, trypsinized from the culture dish, and pelleted at 2,000 rpm at 4 ^{o}C. The cell pellet was resuspended in lysis buffer (20 mM Hepes buffer, 10 mM KCl, 2 mM MgCl2, 0.5 % NP-40, and protease inhibitors) and each sample was vortexed for at least 2 min. The cell lysates were centrifuged at 13,000 rpm for 10 min at 4 ^{o}C. The protein content of the supernatant was determined using the Bio-Rad protein assay kit. Fifty micrograms of protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was subjected to blocking with 5 % non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. After blocking, the PVDF membrane was then 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 RT. 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 with a slight modification as described by Chuang et al. (*22*). In brief, cells were seeded at a density of 5×105 cells/well into a 6-well plate and treated under the indicated conditions. After washing the cells with phosphate buffered saline (PBS) buffer twice, the cells were fixed with ice-cold 70 % ethanol, and held at least overnight at 4 ^{o}C. Prior to analysis, the cells were washed with PBS buffer twice and then incubated with propidium iodide (PI) solution (50 μg/ml PI in PBS plus 1 % Tween-20 and 10 μg RNase) for approximately 30 min. The stained cells were analyzed with a FACS Calibur Instrument (BD Bioscience, San Jose, CA, USA).

DAPI staining

Cells were seeded at a density of 5×10^4 cells/well into 12-well plates and treated under the indicated conditions. After washing the cells with PBS buffer twice, cells were fixed with 4 % para-formaldehyde for 5 min at RT, followed by permeabilization with 0.1 % Triton X-100 for 10 min at RT. Finally, the cells were individually stained with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) and examined and photographed using fluorescence microscopy.

Determination of DNA fragmentation

DNA fragmentation was detected using the method described by Hwang et al. (*20*). Briefly, cells were resuspended in 0.5 ml lysis buffer (20 mM EDTA, 0.5 % Triton X-100, and 5 mM Tris; pH 8.0). The cell lysates were centrifuged at 14,000 rpm for 10 min at 4 ^{o}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 ^{o}C. The genomic DNA was centrifuged at 14,000 rpm for 10 min at 4 ^{o}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) in the presence of 100 μg/ml RNase A and incubated at 56 ^{o}C for 2 h. Finally, an equal amount of DNA was electrophoresed onto horizontal agarose gel (2.0 %) at 1.5 V/cm for 3 h. The DNA in the gel was visualized under UV light after staining with 0.5 μg/ml ethidium bromide.

Release of cytochrome c

The release of cytochrome c (Cyt-c) from the mitochondria to cytosol was detected as described by Hwang et al. (*20*). 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 12,000 x g at 4 ^{o}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. Forty micrograms of protein was resolved by SDS-PAGE (14 %). Proteins were then blotted onto PVDF membranes for the detection of Cyt-c.

Statistical analysis

All values were represented as the mean ± SD from three independent experiments. One-way ANOVA was used for statistical analysis. Significance was recognized as *, $P < 0.05$ and **, $P < 0.01$, through comparison with the vehicle control.

Results

BBR inhibits the growth of HER2-overexpressing breast cancer cells

To ascertain the growth inhibition effects of BBR on HER2-overexpressing breast cancer cells, we first determined the influence of BBR on cell viability and cytotoxicity. As shown in Figure 1A, BBR exerted dose- and time-dependent growth inhibition effects on SKBR-3, BT-474, and MCF-7/HER2 cells. After treating SKBR-3 cells with 50 μM BBR for 24, 48 and 72 h, the cell viability was reduced to 74.01 %, 46.03 % and 33.02 %, respectively. Moreover, the cytotoxic effects of BBR on SKBR-3 cells were presented in Figure 1B, showing the increases in cell death at 24, 48, and 72 h were from 6.22 to 46.58 % at 10 μM, 9.94 to 67.29 % at 50 μM, and 16.45 to 90.17 % at 100 μM, respectively. These results suggest that BBR is capable of suppressing the growth of breast cancer cells with HER2 overexpression.

Overexpression of HER2 negatively influences the response of breast cancer to chemotherapeutic agents (such as taxol and 5-fluorouracil) (*24, 25*). We next examined whether BBR enhanced the growth inhibition effects of anti-cancer drugs in HER2-overexpressing breast cancer cells. As illustrated in Figure 1C, a combination of taxol with BBR significantly enhanced the growth inhibition effect on SKBR-3 cells. The growth inhibition efficiency at 24 h of using taxol (10 ng/ml) and BBR (10 μM) alone or in combination were 7.74 %, 13.61 %, and 37.39 %, respectively. While the growth inhibition efficiency at 48 h of using taxol (10 ng/ml) and BBR (10 μM) alone or in combination were 40.45 %, 41.02 %, and 61.54 %, respectively. These results clearly demonstrate that BBR is capable of chemosensitizing the HER2-overexpressing breast cancer cells to taxol.

3.2. BBR induces G1 arrest through down-regulation of cell cycle regulatory proteins

To verify whether the growth inhibition effect of BBR was due to the disruption of cell

cycle, flow cytometry was used to analyze the distribution of cell cycle in SKBR-3 cells. As shown in Figure 2A, BBR significantly induced G1 phase proportion (up to 10.02 and 12.26 % for 25 and 50 μM BBR, respectively) while concomitantly reducing the S phase (about 8.89 and 10.79 % for 25 and 50 μM BBR, respectively). These results suggest that BBR suppresses the growth of SKBR-3 cells by inducing G1 cell cycle arrest.

To clarify the molecular mechanism of BBR-induced G1 phase arrest, we assessed the effect of BBR on the expression of cell cycle regulatory proteins. The results indicated that BBR presented a marked dose-dependent effect on the suppression of cyclins A, D1, and E (Figure 2B). Similarly, a significant effect of BBR on the expression of cyclins A, D1 and E was also observed in both BT-474 and MCF-7/HER2 cells (Figure 2C). Therefore, these results suggest that BBR-induced G1 arrest was due to the down-regulation of cell cycle regulatory proteins in HER2-overexpressing breast cancer cells.

BBR induces cell apoptosis by activating the mitochondria/caspases pathway

As mentioned above, our results showed that BBR caused a significant induction of cell death (about 30.25 % for 50 μM BBR at 48 h) in SKBR-3 cells (Figure 1B), we next determined whether BBR-induced cell death was due to an induction of cell apoptosis. As shown in Figure 3A, treatment of SKBR-3 cells with 50 μM BBR for 48 h resulted in a significant condensation of chromatin under DAPI staining. Moreover, to assess the effect of BBR on the fragmentation of DNA, SKBR-3 cells were treated with various concentrations of BBR and analyzed for the status of genomic DNA by gel electrophoresis. The results in Figure 3B indicated that BBR exerted dose-dependent effects on the induction of DNA fragmentation in SKBR-3 cells. In addition, to further confirm the BBR-induced apoptosis effect, we used flow cytometry to examine the distribution of the sub-G1 phase in cell cycle. Figure 3C clearly demonstrated that the percentage of sub-G1 phase was significantly higher

in the BBR-treated group than the control group (38.67 % vs. 3.23 %). These observations suggest that BBR induces cell apoptosis in HER2-overexprssing breast cancer cells.

To unravel the underlying molecular mechanism behind BBR-induced cell apoptosis, we examined the effect of BBR on the protein expression of Bcl-2 family controlling the activation of mitochondria/caspases pathway (*26*). As shown in Figure 4A, the anti-apoptotic Bcl-2 protein was dramatically reduced following BBR-treatment for 48 h, but no significant changes with the pro-apoptotic Bax protein were observed in SKBR-3 cells. Moreover, we analyzed the effects of BBR on the release of Cyt-c and found that BBR caused a dose-dependent increase of Cyt-c in the cytosolic fractions (Figure 4B). In addition, after treatment with BBR, significant increases of the cleaved forms of caspase-9, -3 and PARP were observed in SKBR-3 cells (Figure 4C). These findings demonstrate that BBR induces cell apoptosis by activating the mitochondria/caspases pathway in HER2-overexpressing breast cancer cells.

BBR induces growth suppression via the HER2/PI3K/Akt signaling pathway

Because HER2 signaling pathway is associated with cell proliferation and survival (*10, 22, 27*), we next examined the effect of BBR on HER2 and its downstream PI3K/Akt and Ras/MAPK signaling cascades (*28*). 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 a similar repression effect on phospho-HER2 and phospho-Akt in other HER2-overexpressed BT-474 and MCF-7/HER2 cells (Figure 5B). These data clearly demonstrate that BBR exerts inhibitory effects on the HER2/PI3K/Akt signaling pathway in breast cancer cells with HER2 overexpression.

The above results were validated by using the PI3K-specific inhibitor, LY294002, and the ligand of the HER2/HER3 receptor, heregulin (HRG), to test whether BBR-induced growth

suppression effect was through the HER2/PI3K/Akt signaling pathway. The results elucidated that LY294002 not only suppressed cell growth (Figure 6A), but also similar to BBR down-regulated the phospho-Akt, and cyclins D1 and E levels (Figure 6B) via blocking the PI3K/Akt signaling cascade in SKBR-3 cells. In contrast, HRG not only restored BBR-inhibited cell viability from 63.33 % to 78.33 % (Figure 6A), but also overcome BBR-induced down-regulation of the phospho-Akt, and cyclins D1 and E levels in SKBR-3 cells (Figure 6C). Furthermore, as shown in Figure 6D, HRG also significantly decreased the BBR-induced cell apoptosis from 31.48 % to 13.67 % in SKBR-3 cells at 48 h. These data indicate that BBR-induced growth inhibition and cell apoptosis are due to suppression of the HER2/PI3K/Akt signaling pathway in breast cancer cells with HER overexpression (Figure 7).

Discussion

Approximately 30 % of breast cancers overexpressing HER2 were correlated with increased cancer metastasis and poor response to anticancer therapies (*4*). Fortunately, the development of drugs, such as trastuzumab and lapatinib (*9*), that specifically target HER2-overexpressed breast cancer has improved clinical outcomes. In addition, a number of natural products may be considered as alternative therapeutic agents for treatment of HER2-overexpressing breast cancer (*10-12*). Here, we demonstrate for the first time that BBR, a natural alkaloid, has a marked inhibitory effect on the cell growth 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 (*29*). Furthermore, the cell cycle arrest of cancer cells also provides an occasion for cells to either undergo repair mechanisms or undergo apoptosis. Several natural products have marked inhibitory effects on cancer cells

with HER2-overexpression via perturbing cell cycle progression and/or inducing cell apoptosis (*10, 27*). For instance, the plant flavonoid apigenin inhibits growth of HER2-overexpressing breast cancer cells via inducing cell apoptosis (*10*), while the natural triterpenoid corosolic acid (CRA) inhibits cell growth through inducing cell cycle arrest and apoptosis in gastric cancer cells with HER2 overexpression (*27*). Here, our *in vitro* data demonstrate that treatment of SKBR-3 cells with the natural alkaloid BBR induces G1 cell cycle arrest (Figure 2A). Therefore, one of the molecular mechanisms by which BBR causes growth suppression of SKBR-3 cells is via disruption of cell cycle progression. Furthermore, we examine the effects of BBR on the expression of cell cycle regulators and reveal that the BBR-induced G1 arrest is involved in a down-regulation of cyclins D1 and E in breast cancer SKBR-3, BT-474 and MCF-7/HER2 cells (Figure 2B-D). Although down-regulation of cyclin A is also observed in SKBR-3, BT-474 and MCF-7/HER2 cells (Figure 2B-D), the decrease of cyclin A may be involved in or caused by the G1 arrest. These observations suggest that BBR-induced G1 cell cycle arrest is mediated by regulating cyclins D1 and E levels.

Many anti-cancer drugs exert their anti-tumor activities via inducing cell 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 find that BBR not only disrupts cell cycle progression, but also induces cell apoptosis in HER2-overexpressing breast cancer cells (Figure 3A-3C). Moreover, we also show that treatment of SKBR-3 cells with BBR results in a significant decrease in Bcl-2 protein (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 an activation or cleavage of caspases-9,-3 and PARP (Figure 4C). In contrast, BBR-induced cell apoptosis is implicated in an up-regulation of Bax protein and a down-regulation of Bcl-2 protein in many human cancer cells (*19, 30, 31*). However, we do not observe a significant effect on

Bax protein level in $HER2^+$ breast cancer cells, which is similar to the finding in a recent report by Patil *et al.* in HER2 low-expressing breast cancer cells (*32*). In addition, some natural products are reported to have an induction effect on cell apoptosis without affecting the protein expression of both Bcl-2 and Bax. For example, 3, 3'-diindolylmethane induces cell apoptosis through inhibiting HER2/Ras/Erk signaling pathway in HER2-overexpressing breast cancer cells without altering the expression of Bax protein (*33*), while lupeol induces apoptosis through inhibition of Ras signaling pathway in pancreatic cancer cells without a change on the Bcl-2 protein level (*34*). 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 and/or a result from up/down-regulation of different upstream regulatory molecules.

Two major approaches to the suppression of HER2 signaling pathway in breast cancer with HER2 overexpression are down-regulation of HER2 and suppression of HER2 tyrosine kinase activity/phosphorylation (*35*). In this study, we find that BBR inhibits both phospho-HER2 and HER2 in SKBR-3, BT-474 and MCF-7/HER2 cells (Figure 5A and 5B). The suppression effect on phospho-HER2 levels by BBR may be caused by depleting the HER2 protein level. This phenomenon in which BBR down-regulates both of phospho-HER2 and HER2 levels is very similar to that of several natural compounds (*10, 11*). For example, Way *et al.* indicate that apigenin inhibits the tyrosine phosphorylation of HER2 by depleting the HER2 protein level (*10*), whereas Jeong *et al.* reveal that quercetin suppresses HER2 tyrosine kinase activity via down-regulating HER2 protein level (11). These observations suggest that BBR may be a useful HER2-targeting agent for the treatment of breast cancer with HER2 overexpression.

Although the PI3K/Akt and Ras/MAPK signaling cascades are the two main intracellular signaling pathways activated by receptor tyrosine kinase (such as HER2) (*7, 28*), our data show that BBR dramatically inhibits the PI3K/Akt, but not the Ras/MAPK, signaling

cascades in HER2-overexpressing breast cancer cells (Figure 5A and 5B). Moreover, LY294002 (a PI3K-specific inhibitor) and HRG (a ligand of the HER2/HER3 receptor) are used to confirm that BBR-induced growth suppression effect is through the HER2/PI3K/Akt signaling pathway (Figure 6A-6D). Interestingly, BBR is found to have an inhibitory effect on Akt protein (Figure 5A and 5B), suggesting that Akt may be a target of BBR in HER2-overexpressing human breast cancer cells. However, whether BBR-induced down-regulation of Akt protein is due to inhibition of its gene expression and/or protein stability remains to be clarified.

Overexpression of HER2 negatively influences the response of breast cancer to chemotherapeutic agents (*24, 25*), whereas the HER2-targeting therapeutics such as trastuzumab potentiate the anti-tumor activity of those chemotherapeutic agents in the treatment of HER2-overexpressing breast cancers (*36, 37*). In addition, some natural products are reported to even have more enhanced anti-proliferation effects (*33, 38*). For example, combination of epigallocatechin-3-gallate with taxol, and 3, 3'-diindolylmethane with taxol significantly elucidate synergistic growth suppression on HER2-overexpressing breast cancer cells (*33, 38*). Similarly, we have also demonstrated that BBR enhances the chemotherapeutic efficacy of taxol (Figure 1C) and doxorubicin (data not shown) against SKBR-3 cells. These results indicate that BBR performs in the same manner as other HER2 inhibitors or natural products through suppressing the HER2 signaling pathway in HER2-overexpressing breast cancer cells.

In conclusion, we have demonstrated that BBR induces G1 cell cycle arrest and cell apoptosis through inhibiting the HER2/PI3K/Akt signaling pathway in breast cancer cells with HER2 overexpression. 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. This approach provides potential benefits for enhancing the effectiveness of

chemotherapy and chemosensitization in the treatment of breast cancer with HER2 overexpression using BBR.

Abbreviations

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

Acknowledgments

This study was supported by the grant from China Medical University, Taichung, Taiwan. We also wish to thank Dr. M. C. Hung (Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center, Houston, TX) for providing the human breast carcinoma MCF-7/HER2 cell line.

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), 177-82.
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), 290-7.
4. Yu, D.; Hung, M. C., Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. *Oncogene* **2000,** 19, (53), 6115-21.
5. Schechter, A. L.; Stern, D. F.; Vaidyanathan, L.; Decker, S. J.; Drebin, J. A.; Greene, M. I.; Weinberg, R. A., The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature* **1984,** 312, (5994), 513-6.
6. 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), 230-4.
7. Yarden, Y.; Sliwkowski, M. X., Untangling the ErbB signalling network. *Nat Rev Mol*

Cell Biol **2001,** 2, (2), 127-37.

8. Baselga, J., Treatment of HER2-overexpressing breast cancer. *Ann Oncol* **2010,** 21 Suppl 7, vii36-vii40.
9. Baselga, J.; Swain, S. M., Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat Rev Cancer* **2009,** 9, (7), 463-75.
10. 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), 4479-89.
11. 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), 585-95.
12. 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), 1884-91.
13. Wang, Y. X.; Zheng, Y. M., Ionic mechanism responsible for prolongation of cardiac action-potential duration by berberine. *J Cardiovasc Pharmacol* **1997,** 30, (2), 214-22.
14. Amin, A. H.; Subbaiah, T. V.; Abbasi, K. M., Berberine sulfate: antimicrobial activity, bioassay, and mode of action. *Can J Microbiol* **1969,** 15, (9), 1067-76.
15. 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, 116-31.
16. 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), 318-23.
17. 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), 133-41.
18. 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), 664-70.
19. 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), 2018-27.
20. 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.
21. 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), 1155-61.

22. 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), 653-9.
23. 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), 547-54.
24. 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), 1359-65.
25. Miles, D. W.; Harris, W. H.; Gillett, C. E.; Smith, P.; Barnes, D. 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), 354-9.
26. Cory, S.; Adams, J. M., The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2002,** 2, (9), 647-56.
27. 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), 931-7.
28. Hynes, N. E.; Lane, H. A., ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* **2005,** 5, (5), 341-54.
29. 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), 211-9.
30. 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), 4063-70.
31. 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), 558-62.
32. 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), 70-8.
33. 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), 208-13.
34. 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), 1956-64.
35. Imai, K.; Takaoka, A., Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* **2006,** 6, (9), 714-27.
36. 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), 783-92.
37. Bullock, K.; Blackwell, K., Clinical efficacy of taxane-trastuzumab combination regimens for HER-2-positive metastatic breast cancer. *Oncologist* **2008,** 13, (5), 515-25.
38. 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), 3486-91.

Figure legends

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

Figure 2. Perturbation of cell cycle by BBR in HER2-overexpressing breast cancer cells. **(A)** SKBR-3 cells were treated with varying concentrations of BBR (0, 25, 50 μM) for 24 h.

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

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

Figure 4. BBR activates the mitochondria/caspases pathway in HER2-overexpressing breast cancer cells. (A) SKBR-3 cells were treated with varying concentrations of BBR (0, 25, 50 μM) for 24 and 48 h. Bcl-2 and Bax protein expressions were measured by Western blotting. **(B)** SKBR-3 cells were treated with varying concentrations of BBR (0, 10, 50, 100 μM) for 48 h. The release of cytochrome c (Cyt-c) to cytoplasm was measured by Western blotting. **(C)** SKBR-3 cells were treated with varying concentrations of BBR (0, 25, 50 μM) for 48 h. The cleavage of caspase-9, -3 and PARP was measured by Western blotting. All data

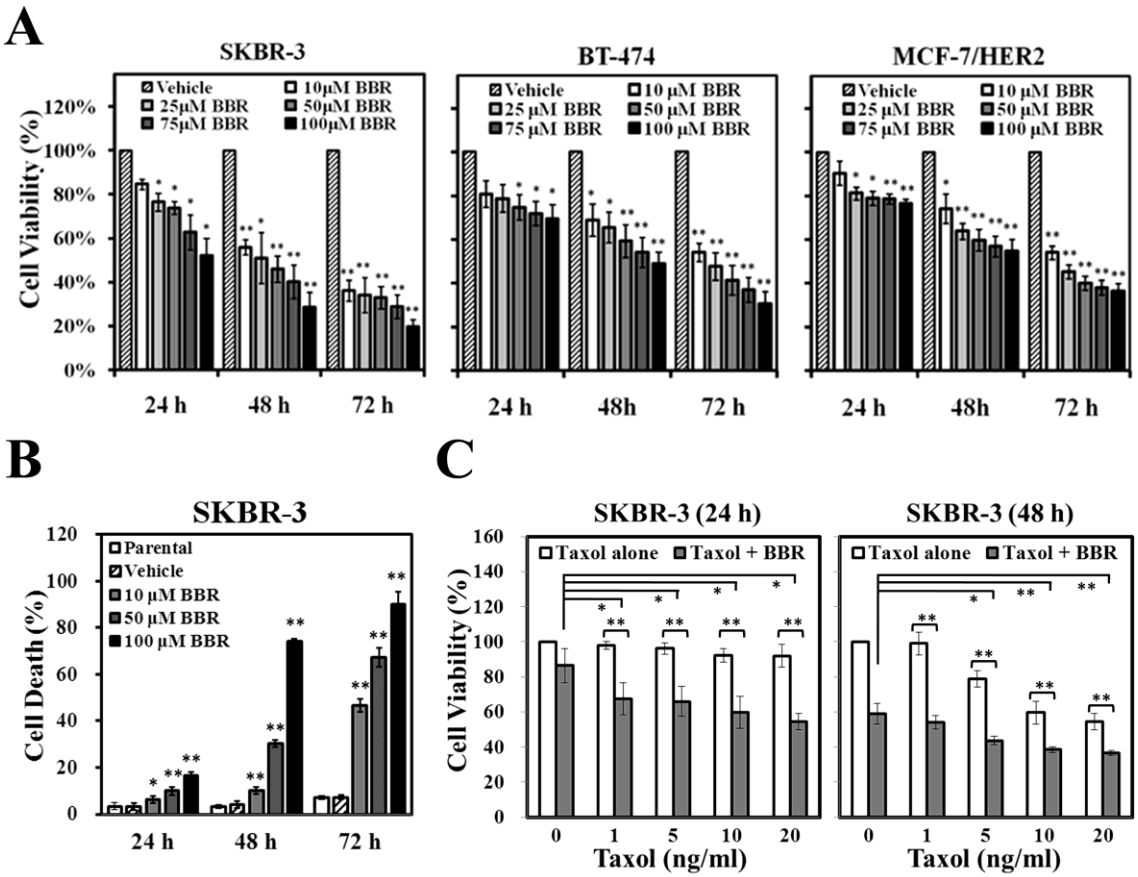
were expressed as the mean ± SD of three independent experiments. *, *P<0.05*; **, *P<0.01*.

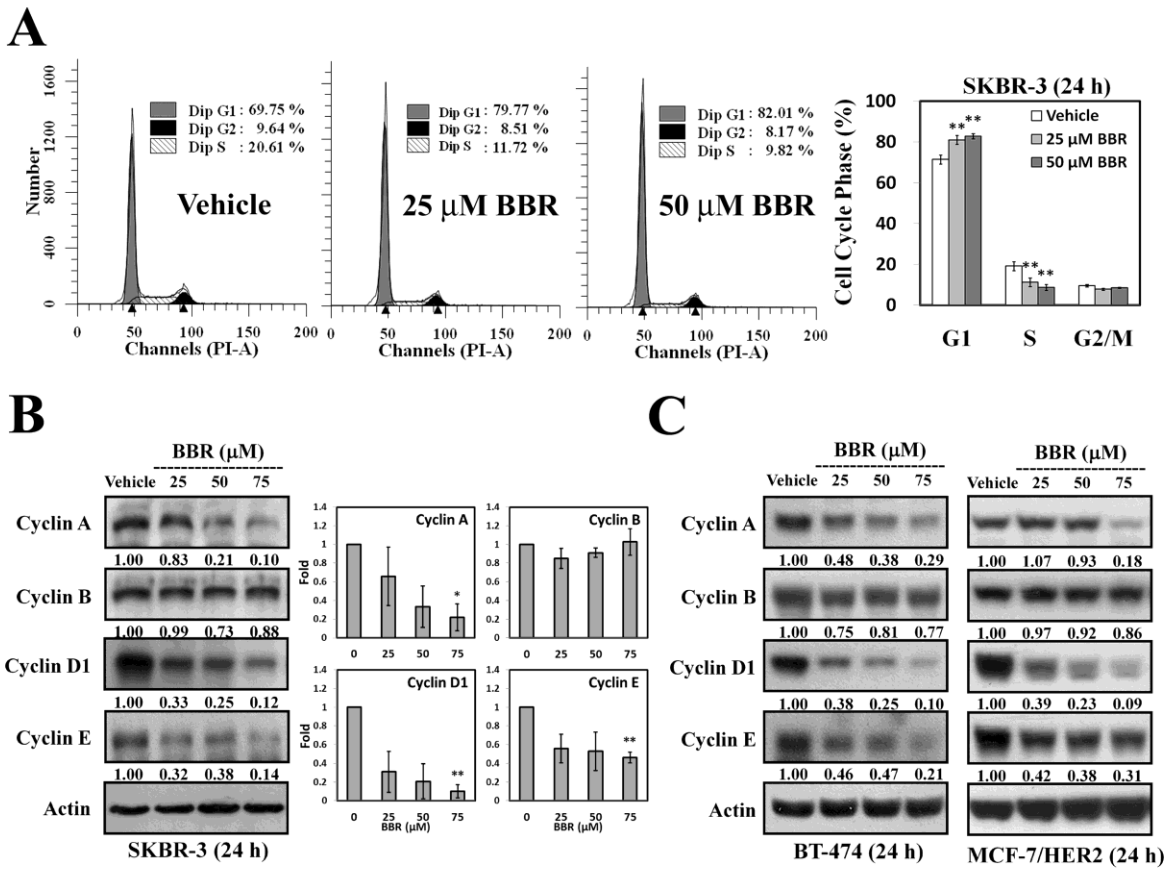
Figure 5. BBR inhibits the HER2/PI3K/Akt signaling pathway in HER2-overexpressing breast cancer cells. (A) Suppression of HER2/PI3K/Akt, but not HER2/Erk, signaling pathways by treating SKBR-3 cells with BBR (0, 25 and 50 μM) for 24 and 48 h. **(B)** Suppression of HER2/PI3K/Akt signaling pathways by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

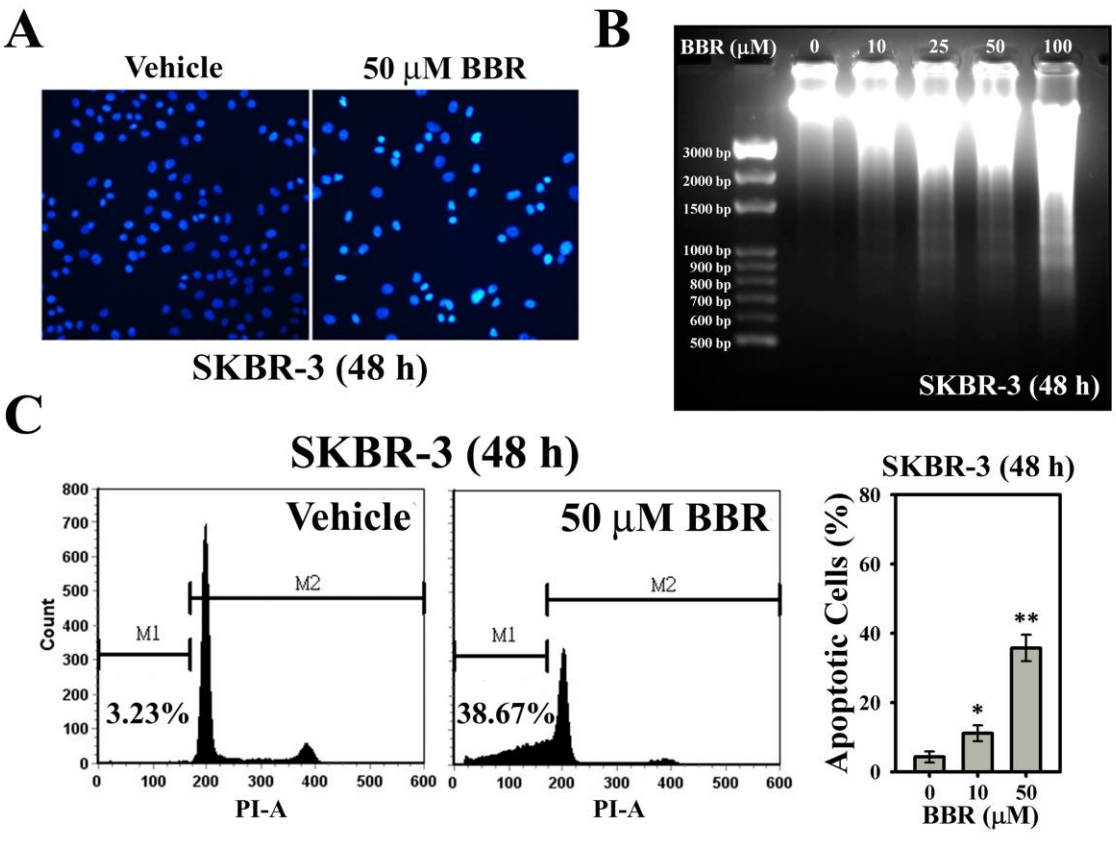
Figure 6. Validation of BBR-induced growth suppression effects are through the HER2/PI3K/Akt signaling pathway. (A) SKBR-3 cells were treated with 20 μM LY294002 (LY, a PI3K inhibitor), 100 ng/ml HRG (an activator of HER2/HER3 pathway) alone or in combination with 50 μM BBR for 24 h. Cell viability was determined by MTT assay. **(B)** LY, similar to BBR, led to down-regulation of p-Akt, and 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 were 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 were expressed as the mean ± SD of three independent experiments. *, *P<0.05*; **, *P<0.01*.

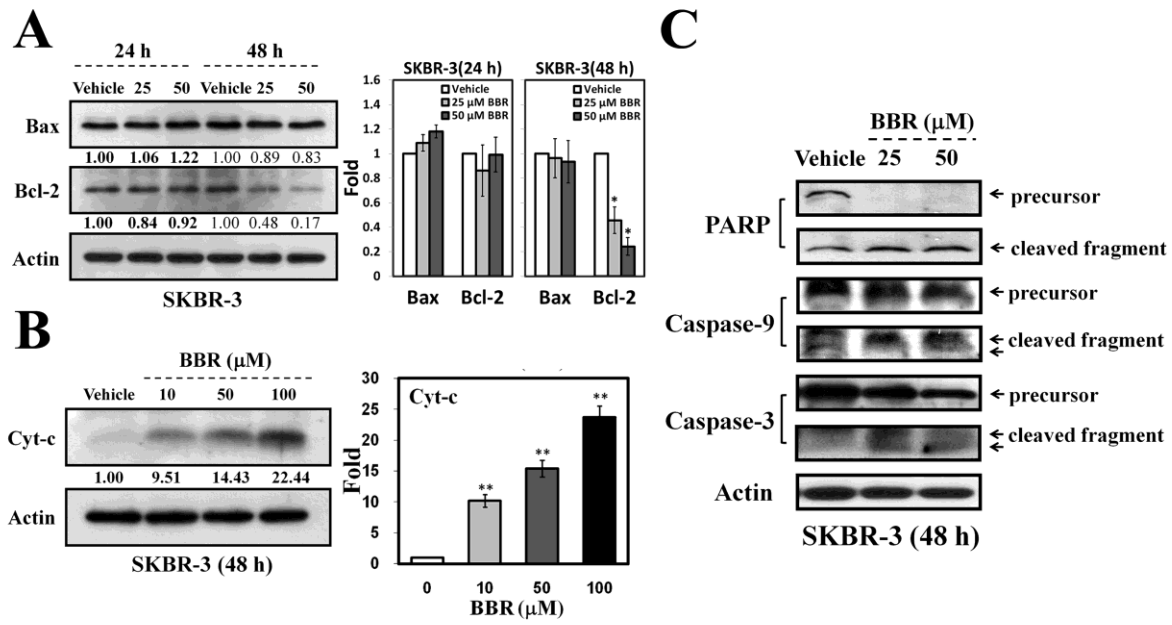
Figure 7. A schematic model for BBR-induced growth suppression and apoptosis in HER2-overexpressing breast cancer cells. Ligand (heregulin, HRG) stimulation induces hetero-dimerization of HER2 with HER3 resulting in self-phosphorylation at tyrosine residues located on the C-terminal domain of HER2 protein. The phosphorylated HER2 then

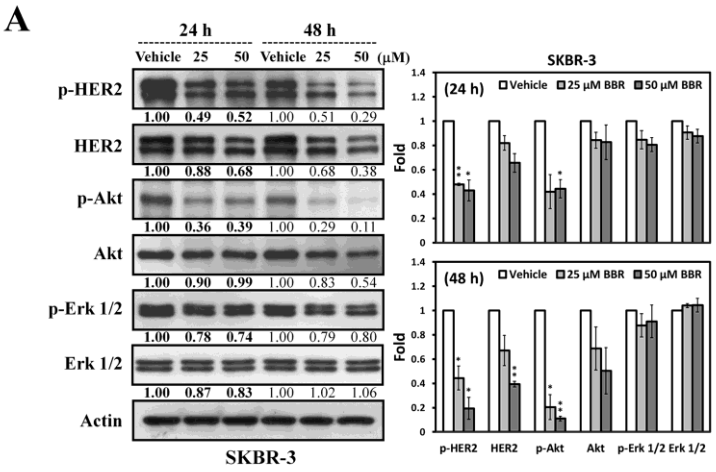
activates downstream PI3K/Akt signaling cascade (inhibited by LY294002) which promotes cell growth and survival. After BBR treatment, cell growth is inhibited because of induction of cell cycle arrest and apoptosis. The BBR-induced growth suppression and cell apoptosis is due to down-regulation of the HER2/PI3K/Akt signaling pathway.

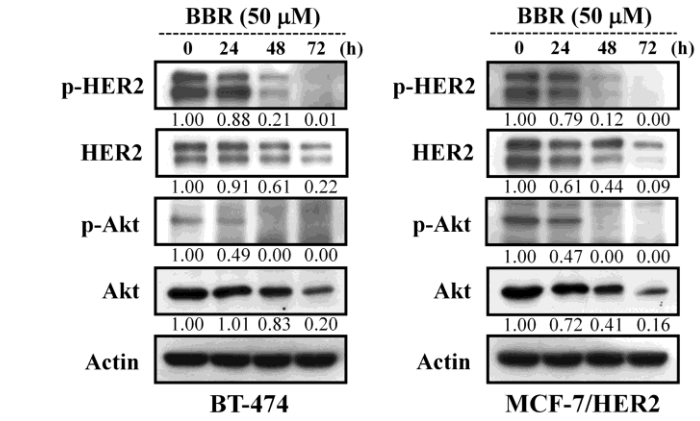

A
SKBR-3
BT-474
MCF-7/HER2
Vehicle
10μM BBR
25μM BBR
50μM BBR
75μM BBR
100μM BBR
10 μM BBR
25 μM BBR
50 μM BBR
75 μM BBR
100 μM BBR
Cell Viability (%)
120%
100%
80%
60%
40%
20%
0%
24 h
48 h
48h
72 h
B
SKBR-3
Parental
Vehicle
10 μM BBR
50 μM BBR
100 μM BBR
Cell Death (%)
120
100
80
60
40
20
0
24 h
48 h
72 h
C
SKBR-3 (24 h)
SKBR-3 (48 h)
Taxol alone
Taxol + BBR
Cell Viability (%)
160
140
120
100
80
60
40
20
0
0
1
5
10
20
Taxol (ng/ml)

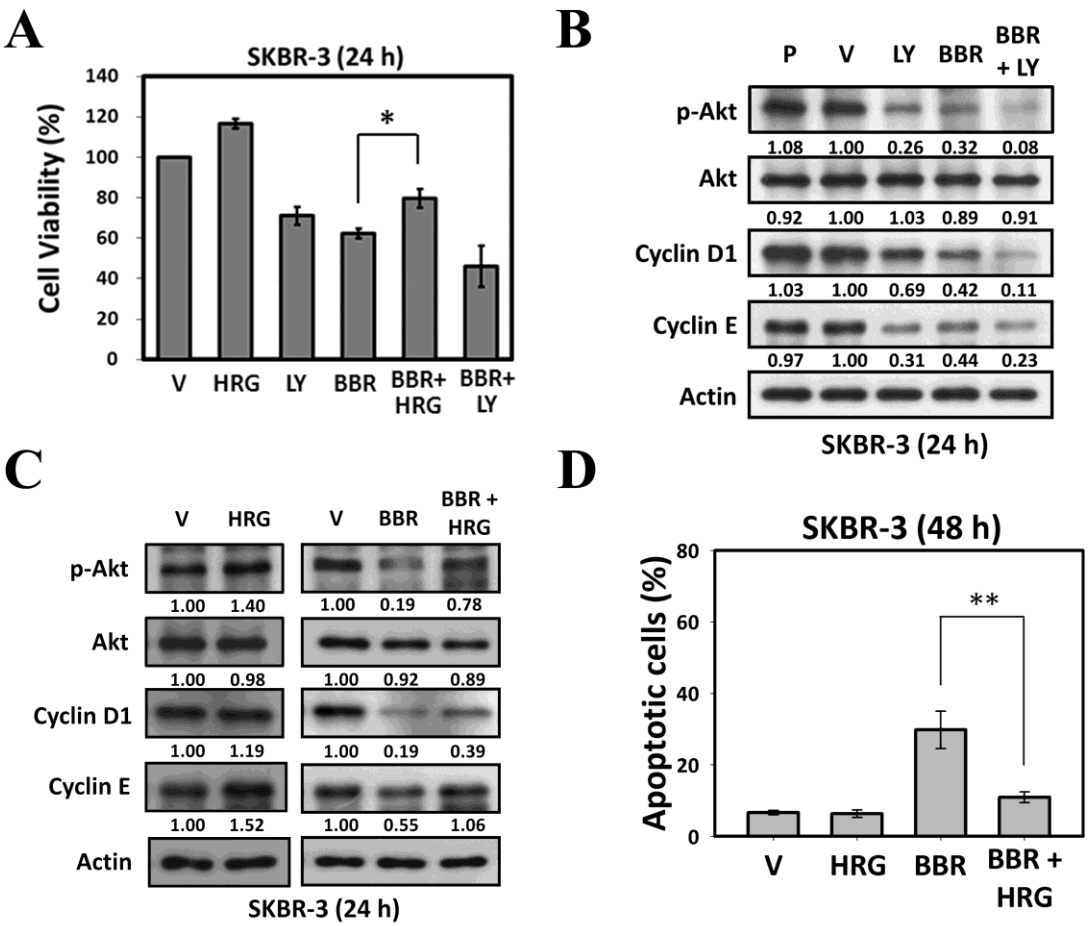

A
Dip G1 : 69.75 %
Dip G2 : 9.64 %
Dip S : 20.61 %
Vehicle
Dip G1 : 79.77 %
Dip G2 : 8.51 %
Dip S : 11.72 %
25 μM BBR
Dip G1 : 82.01 %
Dip G2 : 8.17 %
Dip S : 9.82 %
50 μM BBR
Number
Channels (PI-A)
SKBR-3 (24 h)
Vehicle
25 μM BBR
50 μM BBR
Cell Cycle Phase (%)
G1
S
G2/M
B
BBR (μM)
Vehicle 25 50 75
Cyclin A
Cyclin B
Cyclin D1
Cyclin E
Actin
1.00 0.83 0.21 0.10
1.00 0.99 0.73 0.88
1.00 0.33 0.25 0.12
1.00 0.32 0.38 0.14
Fold
SKBR-3 (24 h)
C
1.00 0.48 0.38 0.29
1.00 0.75 0.81 0.77
1.00 0.38 0.25 0.10
1.00 0.46 0.47 0.21
1.00 1.07 0.93 0.18
1.00 0.97 0.92 0.86
1.00 0.39 0.23 0.09
1.00 0.42 0.38 0.31
BT-474 (24 h)
MCF-7/HER2 (24 h)

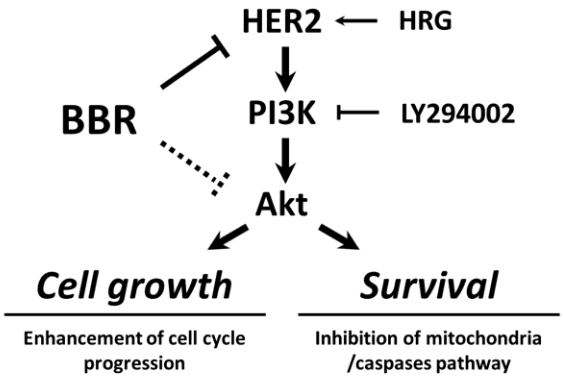

A
Vehicle
50 μM BBR
SKBR-3 (48 h)
B
BBR (μM)
0
10
25
50
100
3000 bp
2000 bp
1500 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
SKBR-3 (48 h)
C
SKBR-3 (48 h)
Vehicle
50 μM BBR
M1
M2
3.23%
38.67%
Count
PI-A
SKBR-3 (48 h)
Apoptotic Cells (%)
BBR (μM)
*
**


A
24 h
48 h
Vehicle 25 50 Vehicle 25 50
Bax
1.00 1.06 1.22 1.00 0.89 0.83
Bcl-2
1.00 0.84 0.92 1.00 0.48 0.17
Actin
SKBR-3
SKBR-3(24 h)
SKBR-3(48 h)
Vehicle
25 μM BBR
50 μM BBR
Fold
Bax Bcl-2 Bax Bcl-2
B
BBR (μM)
Vehicle 10 50 100
Cyt-c
1.00 9.51 14.43 22.44
Actin
SKBR-3 (48 h)
Cyt-c
BBR (μM)
C
BBR (μM)
Vehicle 25 50
PARP
precursor
cleaved fragment
Caspase-9
precursor
cleaved fragment
Caspase-3
precursor
cleaved fragment
Actin
SKBR-3 (48 h)


A
24 h
48 h
Vehicle 25 50 Vehicle 25 50 (μM)
p-HER2
1.00 0.49 0.52 1.00 0.51 0.29
HER2
1.00 0.88 0.68 1.00 0.68 0.38
p-Akt
1.00 0.36 0.39 1.00 0.29 0.11
Akt
1.00 0.90 0.99 1.00 0.83 0.54
p-Erk 1/2
1.00 0.78 0.74 1.00 0.79 0.80
Erk 1/2
1.00 0.87 0.83 1.00 1.02 1.06
Actin
SKBR-3
SKBR-3
(24 h) Vehicle 25 μM BBR 50 μM BBR
(48 h) Vehicle 25 μM BBR 50 μM BBR
Fold
p-HER2 HER2 p-Akt Akt p-Erk 1/2 Erk 1/2


B
BBR (50 μM)
0 24 48 72 (h)
p-HER2
1.00 0.88 0.21 0.01
HER2
1.00 0.91 0.61 0.22
p-Akt
1.00 0.49 0.00 0.00
Akt
1.00 1.01 0.83 0.20
Actin
BT-474
BBR (50 μM)
0 24 48 72 (h)
p-HER2
1.00 0.79 0.12 0.00
HER2
1.00 0.61 0.44 0.09
p-Akt
1.00 0.47 0.00 0.00
Akt
1.00 0.72 0.41 0.16
Actin
MCF-7/HER2


A
SKBR-3 (24 h)
Cell Viability (%)
140
120
100
80
60
40
20
0
*
V
HRG
LY
BBR
BBR+ HRG
BBR+ LY
B
P
V
LY
BBR
BBR + LY
p-Akt
1.08 1.00 0.26 0.32 0.08
Akt
0.92 1.00 1.03 0.89 0.91
Cyclin D1
1.03 1.00 0.69 0.42 0.11
Cyclin E
0.97 1.00 0.31 0.44 0.23
Actin
SKBR-3 (24 h)
C
V
HRG
V
BBR
BBR + HRG
p-Akt
1.00 1.40 1.00 0.19 0.78
Akt
1.00 0.98 1.00 0.92 0.89
Cyclin D1
1.00 1.19 1.00 0.19 0.39
Cyclin E
1.00 1.52 1.00 0.55 1.06
Actin
SKBR-3 (24 h)
D
SKBR-3 (48 h)
Apoptotic cells (%)
80
60
40
20
0
**
V
HRG
BBR
BBR + HRG


HER2
HRG
BBR
PI3K
LY294002
Akt
Cell growth
Survival
Enhancement of cell cycle progression
Inhibition of mitochondria /caspases pathway