JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Berberine, an Isoquinoline Alkaloid, Inhibits the Metastatic Potential of Breast Cancer Cells via Akt Pathway Modulation

Han-Peng Kuo,[†] Tzu-Chao Chuang,[‡] Shih-Chang Tsai,[†,§] Hsiu-Hsueh Tseng,[∥,§] Shih-Chung Hsu,[⊥] Yu-Chang Chen,[#] Chao-Lin Kuo,[#] Yueh-Hsiung Kuo,[#,$] Jah-Yao Liu,[¶] and Ming-Ching Kao*[,†,&]

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

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

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

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

[#]Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan

[$]Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung, Taiwan

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

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

ABSTRACT: Berberine (BBR) is a natural alkaloid with significant antitumor activities against many types of cancer cells. In this study, we investigated the molecular mechanisms by which BBR repressed the metastatic potential of breast cancer cells. BBR was found to downregulate the enzymatic activities and expression levels of matrix metalloproteinases 2 and 9 (MMP2 and MMP9, respectively). The BBR-mediated suppression of MMP2 and MMP9 involved the inhibition of the Akt/nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) signaling pathways. Furthermore, BBR repressed the expression of the Akt protein by modulating the mRNA expression level and protein degradation of Akt. In conclusion, this study suggests that BBR can reduce the metastatic potential of highly metastatic breast cancer cells and may be a useful adjuvant therapeutic agent in the treatment of breast cancer by targeting the Akt pathway.

KEYWORDS: *berberine, breast cancer, Akt/PKB, matrix metalloproteinases, metastasis*

INTRODUCTION

Breast carcinoma is the most common cancer and the leading cause of cancer-related mortality among women worldwide, and it accounted for approximately 23% of total new cases and 14% of all cancer deaths in 2008.[1] In Taiwan, breast cancer is also the most frequent cancer diagnosed in women and the fourth cause of cancer deaths among females.[2] Moreover, from 1986 to 2010, an increasing death rate from breast carcinoma has been observed in Taiwan.[2] Although the detection and treatment of breast cancer has improved, approximately 40% of all patients still die from this disease. In most cases, the major cause of breast cancer death is associated with the dissemination and proliferation of breast cancer cells at a secondary site.[3] Thus, novel and useful therapies/agents against highly metastatic breast cancer need to be developed.

Tumor metastasis, a process by which cancer cells spread from the primary tumor site to a secondary site, consists of a complex cascade of steps including cell attachment, migration, invasion, proliferation, and vessel formation.[4] For tumor cells to manifest this metastatic potential, they must develop the ability to degrade both the basement membrane (BM) and the extracellular matrix (ECM), thereby facilitating the invasion of tumor cells that can result in the establishment of cancer metastasis.

Matrix metalloproteinases (MMPs), which are an important class of ECM-degrading enzymes, are frequently overexpressed in malignant tumors and are implicated in the processes of tumor growth, invasion, and metastasis.[5] Expression of MMPs is primarily regulated at the transcriptional level via the activation of multiple signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[6,7] The PI3K/Akt signaling pathway is associated with the regulation of cell proliferation, survival, angiogenesis, and metastasis in various types of cancer, including breast cancer. Activation of PI3K/Akt and its downstream effectors (e.g., NF-κB and AP-1) has been reported to increase metastatic ability by modulating the motility and invasion of cancer cells.[6−8] Therefore, suppression of the PI3K/Akt pathway may be an effective approach in the treatment of cancer cells with highly metastatic abilities.

Berberine (BBR), a natural alkaloid abundantly present in Goldenseal (*Hydrastis canadensis*), is a popular dietary supplement in the market. BBR is also found in a variety of herbs such as the *Berberis* species. It is currently known to have many pharmacological activities, such as antibacterial, anticholinergic, antihypertensive, anti-inflammatory, and antioxidative activities.[9] BBR also possesses a repressive effect on the proliferation of various cancer types via the induction of cell cycle arrest and cellular apoptosis.[10−12] Recently, the application of BBR has attracted considerable attention for its use as an antimetastatic agent, suggesting it is a useful antimetastatic agent.[13−16] For

Received: July 2, 2012
Revised: August 28, 2012
Accepted: September 3, 2012
Published: September 5, 2012

dx.doi.org/10.1021/jf302832n | *J. Agric. Food Chem.* 2012, 60, 9649−9658

example, BBR has been observed to repress cell migration and invasion by reducing the expression of focal adhesion kinase (FAK), inhibitor kappaB kinase (IKK), NF-κB, urokinase-type plasminogen activator (u-PA), MMP2, and MMP9 in tongue squamous cancer cells and the levels of cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), and PGE_2 receptors in melanoma cells.[13,14] BBR demonstrated the ability to inhibit both tumor necrosis factor-alpha (TNF-α)- and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced metastasis in breast cancer cells.[15,16] Although BBR has antimetastatic potential in various cancer types, the molecular mechanisms governing its repressive influence on breast cancer metastasis are still unclear and need to be explored.

In this study, we investigated the effects of BBR on the viability, adhesion, migration, and invasion of a highly metastatic breast cancer cell line, MDA-MB-231. We also elucidated the potential of BBR in repressing the enzymatic activity and expression of MMP2 and MMP9. In addition, the impact of BBR on the Akt pathway was also examined to further explore the underlying molecular mechanisms of the antimetastatic action of BBR in breast cancer.

MATERIALS AND METHODS

Chemicals and Antibodies. Berberine (>98% purity), cycloheximide (CHX), DMSO, and MTT were all obtained from Sigma-Aldrich (St. Louis, MO, USA). LY294002 (a PI3K inhibitor) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against cyclins A, B, D1, and E, p21, phospho-Akt (Ser308), Akt1, NF-κB p65, proliferating cell nuclear antigen (PCNA), and ubiquitin (Ub) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against β-actin was purchased from Chemicon International Inc. (Temecula, CA, USA).

Cell Culture. Human breast carcinoma cell lines MDA-MB-231, MCF-7, and T-47D were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cells were cultured in DMEM/F12 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT Assay. Cell viability was determined using an MTT assay as previously described.[10] Briefly, cells were seeded at a density of 6,000 cells/well into 96-well plates and incubated overnight in medium containing 10% FBS. After the cells adhered to the plate, various doses of BBR were applied to the cells and then the cultures were incubated at 37 °C for 24 or 48 h. After a 4 h incubation with MTT reagent (0.5 mg/mL), the relative viable cell number was calculated as being directly proportional to the production of formazan crystals solubilized by DMSO. The final solution was measured using a spectrophotometer at a wavelength of 545 nm against a reference wavelength of 690 nm.

Cell-Matrix Adhesion Assay. Cell attachment was determined using a cell-matrix adhesion assay with a slight modification as previously described.[17] Briefly, microtiter wells were coated with type IV collagen (BD Biosciences, San Diego, CA, USA) at 4 °C overnight and then blocked using 0.5% BSA in PBS for 30 min. The cells were incubated with BBR at various concentrations (0, 10, 50, or 100 μM) for 24 h before seeding. One hundred microliters of the treated cells (5×10^5 cells/mL in serum-free medium) was seeded onto the coated wells and incubated at 37 °C for 30 min. Nonadherent cells were removed using PBS washes. The adherent cells (remaining cells) were measured using an MTT assay.

Wound Healing Assay. Cell motility/migration was measured using a wound healing assay, as previously described, with slight modification.[17] Briefly, the cells were grown to a confluent monolayer in a six-well tissue culture dish. Cell monolayers were "wounded" using a P200 micropipet tip. The wounded monolayers were then washed twice with DPBS to remove cell debris and incubated in medium with 0.1% FBS in the absence or presence of BBR for 24 or 48 h. The migrating cells in the denuded zone were monitored under an inverted microscope equipped with a camera.

Migration and Invasion Assay. The migration and invasion abilities of tumor cells were determined using an in vitro migration and invasion assay with a slight modification as previously described.[17] Briefly, cells were incubated with various concentrations of BBR (0, 10, 50, or 100 μM) for 24 h before seeding. For the migration assay, 100 μL of the treated cells (5×10^5 cells/mL in serum-free medium) was added to the upper part of the cell culture chambers (Millipore Corp., Billerica, MA, USA). For invasion assay, 100 μL of the treated cells (1×10^6 cells/mL in serum-free medium) was seeded onto the upper part of the chambers coated with Matrigel (BD Biosciences, San Diego, CA, USA). FBS (10%) was used as the chemoattractant in the bottom chambers. After incubation at 37 °C for 24 h, the nonmigrated or noninvaded cells were removed from the upper face of the transwell membrane using a cotton swab. The migrated or invaded cells were fixed with methanol (100%) and then stained using 2% crystal violet in methanol. The migrated or invaded cells were counted in randomly selected fields under a light microscope.

Gelatin Zymography. The cells (5×10^5) were incubated in serum-free medium for 48 h with or without BBR (10, 50, or 100 μM). Subsequently, the medium was collected and gelatin zymography was performed to determine the enzymatic activity of MMP2 and MMP9, as described previously, with slight modification.[17] After electrophoresis, the gels were washed twice in zymography washing buffer (2.5% Triton X-100 in double-distilled H_2O) and then incubated in zymography reaction buffer (40 mM Tris-HCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% NaN_3) at 37 °C for 24 h. The reaction was terminated and stained using 0.1% Coomassie blue R-250 for 1 h followed by destaining using a destaining solution (20% methanol, 10% acetic acid, and 70% double-distilled H_2O). The enzyme-digested regions were observed as being white (transparent), nonstaining bands against a blue background. The results were scanned and quantified using ImageJ software.

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using TRIzol solution (Invitrogen, San Diego, CA, USA). Two micrograms of total RNA was used for first-strand cDNA synthesis. The appropriate primers (Akt1 sense, 5′-GCTGGACGATAGCTTGGA-3′; Akt1 antisense, 5′-GATGACAGATAGCTGGTG-3′; MMP2 sense, 5′-TCTCCTGACATTGACCTTGGC-3′; MMP2 antisense, 5′-CAAGGTGCTGGCTGAGTAGATC-3′; MMP9 sense, 5′-TTGACAGCGACAAGAAGTGG-3′; MMP9 antisense, 5′-GCCATTCACGTCGTCCT TAT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH antisense, 5′-TCCACCACCCTGTTGCTGTA-3′) were used to perform the polymerase chain reaction (PCR). The PCR products were separated by electrophoresis on a 1.2% agarose gel and detected by ethidium bromide staining.

Real-Time Quantitative RT-PCR. The mRNA levels were analyzed using real-time qRT-PCR, as previously described.[18] Briefly, total RNA was isolated using TRIzol solution and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time qPCR was then performed using a Power SYBR Green Master Mix Kit (Applied Biosystems) with the following PCR profile: 1 cycle at 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s and 52 °C for 1 min. The sequences of the specific primers for each gene are as follows: 5′-GTGGACCAACGTGAGGCTC-3′ (Akt1 sense) and 5′-GAAGGTGCGTTCGATGACAG-3′ (Akt1 antisense); 5′-CACCTACTGAGTGGCCGTGTT-3′ (MMP2 sense) and 5′-CAGGGAGCAGAGATTCGGAC-3′ (MMP2 antisense); 5′-TGCCTGCAACGTGAACATCT-3′ (MMP9 sense) and 5′-CACTTGTCGGCGATAAG GAA-3′ (MMP9 antisense); 5′-AGGGCTGCTTTTAACTCTGGT-3′ (GAPDH sense) and 5′-CCCCACTTGATTTTGGAGGGA-3′ (GAPDH antisense). The amplification reaction, PCR product detection, and data analyses were performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems).

Immunoprecipitation and Western Blotting. Cells were lysed in lysis buffer (20 mM Hepes buffer, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, and protease inhibitors) as previously described.[10] The cell lysates were centrifuged at 16000*g* for 10 min at 4 °C. The protein content of

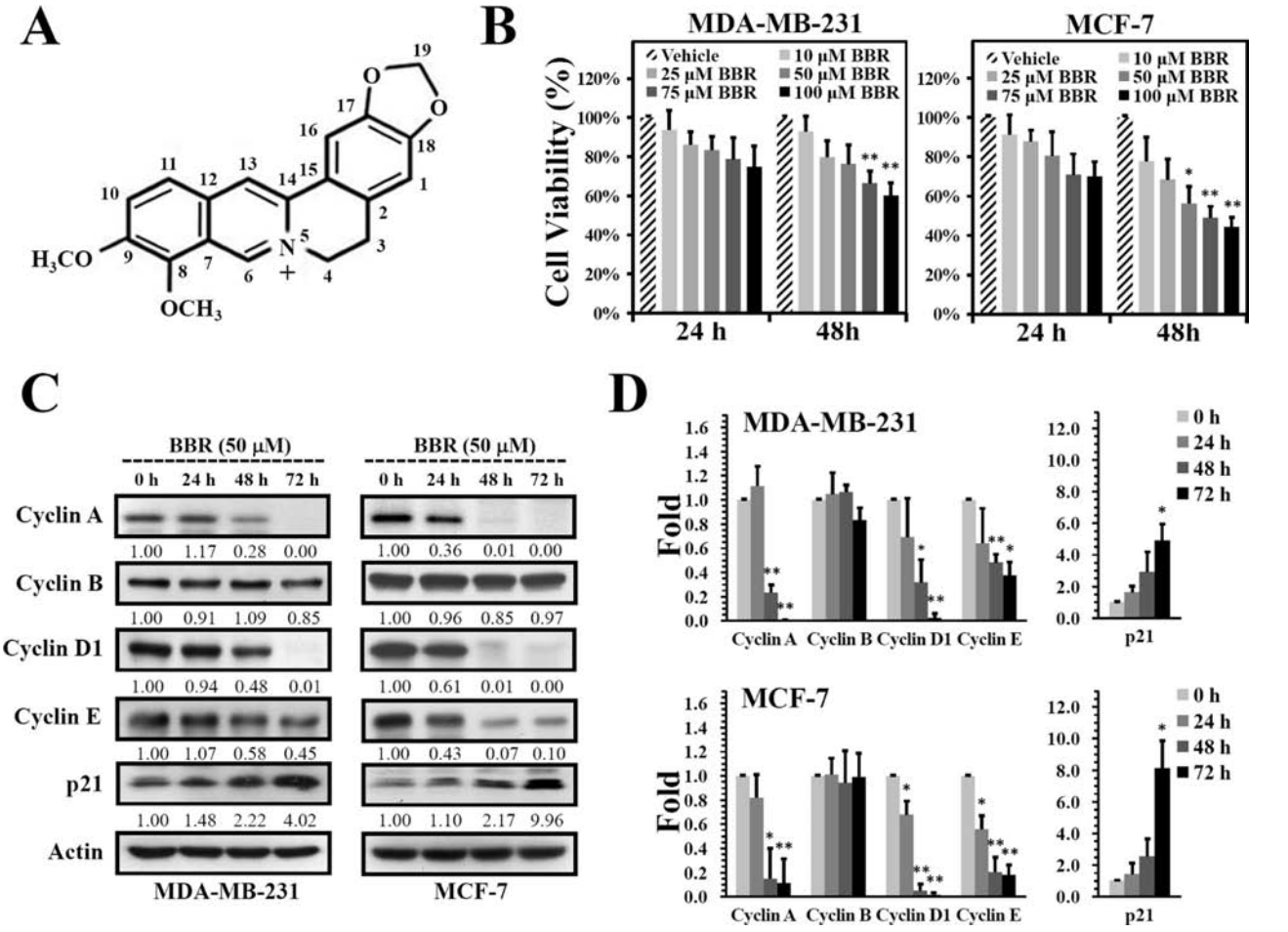

Figure 1. Effects of BBR on the viability of MDA-MB-231 and MCF-7 breast cancer cells. (A) Chemical structure of BBR. (B) MDA-MB-231 and MCF-7 cells were treated with 0.2% DMSO (vehicle control) or BBR (0, 10, 25, 50, 75, and 100 μM) for 24 or 48 h. Cell viability was determined using the MTT assay as described in Materials and Methods. (C) MDA-MB-231 and MCF-7 cells were treated with BBR (50 μM) for 24, 48, or 72 h. Expression levels of cell cycle-related proteins were determined by Western blotting as described in Materials and Methods. All data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$.

the supernatant was measured using the Bio-Rad protein assay kit. For immunoprecipitation, cell lysates containing 400 μg of total protein were incubated with anti-Akt1 antibody overnight at 4 °C, followed by protein A/G PLUS-Agarose (Santa Cruz) for 3 h at 4 °C. The precipitates were resolved using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and then transferred onto a polyvinylidene fluoride (PVDF) membrane. For Western blotting, cell lysates (40 μg) were directly subjected to SDS−PAGE and blotted onto the PVDF membrane. The membranes were blocked using 5% nonfat milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. After blocking, the PVDF membranes were incubated with primary antibodies for 1 h at room temperature followed by incubating with an HRP-conjugated or IRDye 800-conjugated secondary antibody. The reactive signals were visualized using the Enhanced Chemiluminescence Kit (Amersham Biosciences, Arlington Heights, IL) or the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, U.K.). The results were scanned and quantified using ImageJ software.

Preparation of Nuclear Extracts. Nuclear proteins were extracted from the pelleted nuclei as previously described.[7] Briefly, each nuclear pellet was resuspended in extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 M NaCl, and 25% glycerol). The nuclear suspension was incubated on ice for 20 min and then centrifuged at 13000*g* for 5 min. The supernatant (the soluble nuclear fraction) was collected and used to detect NF-κB (p65) and AP-1 (c-Fos and c-Jun).

Statistical Analyses. All data are presented as the mean ± SD from three independent experiments. A one-way ANOVA was used to determine statistical significance. Significance was recognized as p values of *$p < 0.05$ or **$p < 0.01$ relative to the vehicle control.

RESULTS

BBR Inhibits the Viability of MDA-MB-231 and MCF-7 Breast Cancer Cells. We first determined the effect of BBR on the viability of MDA-MB-231 and MCF-7 cells. BBR treatment (10−100 μM) resulted in a dose- and time-dependent suppression of cell viability (Figure 1B), accounting for a 5−25% reduction at 24 h and a 7−40% reduction at 48 h in the number of viable MDA-MB-231cells. Similar results were found for MCF-7 cells, accounting for a 6−28% reduction at 24 h and a 14−52% reduction at 48 h in the number of viable MCF-7 cells (Figure 1B). The IC_{50} of BBR for MDA-MB-231 and MCF-7 cells at 24 h was approximately 210 μM and 190 μM, respectively. At 48 h, the IC_{50} values were approximately 130 μM and 95 μM for the MDA-MB-231 and MCF-7, respectively. In addition, the data also showed that the viability of MDA-MB-231, a highly metastatic cancer cell line, was not significantly affected by BBR at 50 μM (Figure 1B). Therefore, 50 μM BBR was predominantly chosen for the subsequent metastasis-related experiments.

A variety of regulator proteins, such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs), are

dx.doi.org/10.1021/jf302832n | J. Agric. Food Chem. 2012, 60, 9649−9658

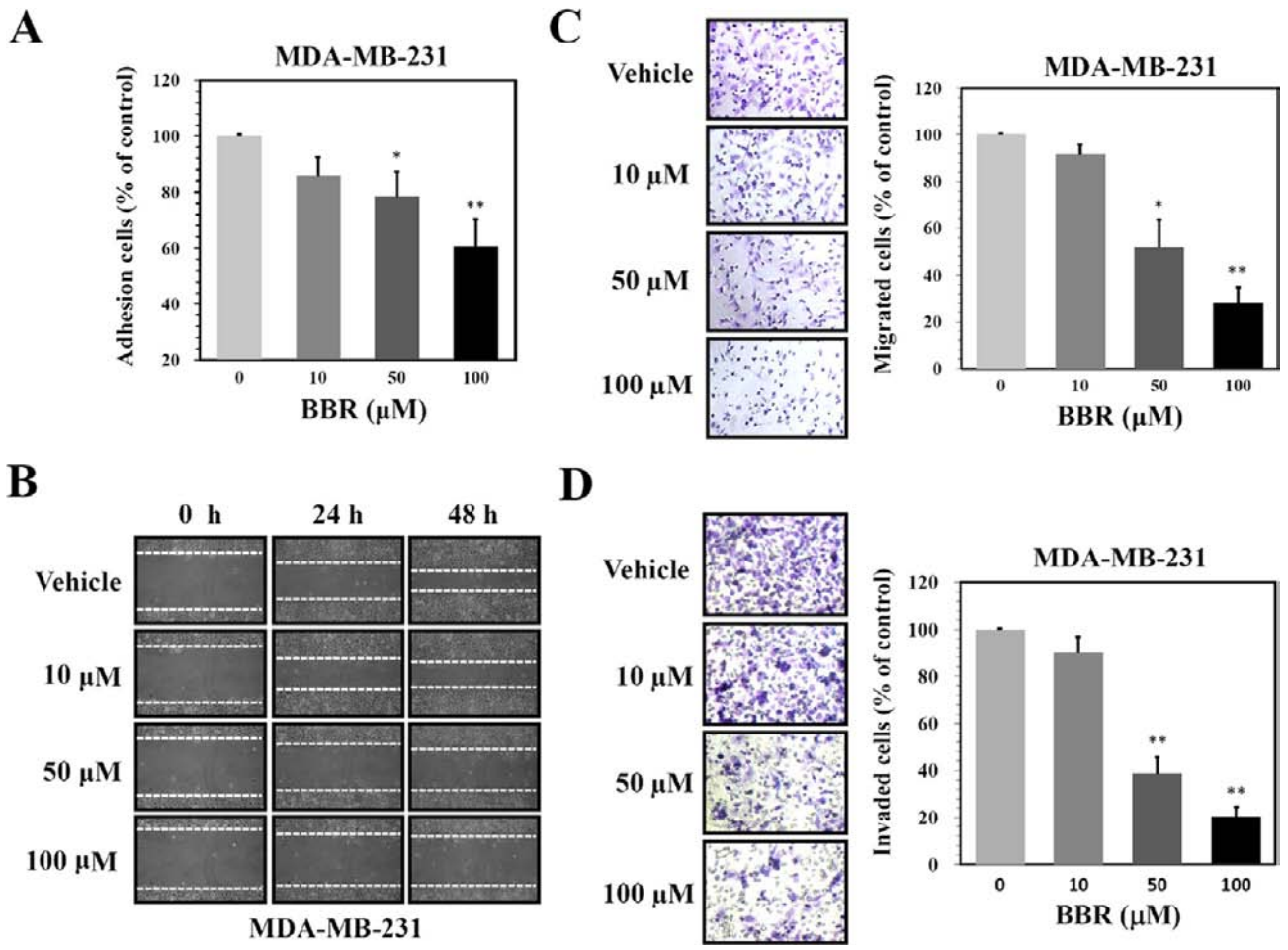

Figure 2. Effects of BBR on the adhesion, migration, and invasion abilities of highly metastatic MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 24 h and then subjected to analysis for cell adhesion as described in the Materials and Methods. (B) Confluent monolayers of MDA-MB-231 cells were wounded using a plastic tip and then incubated in medium with 0.1% FBS in the presence of 0, 10, 50, and 100 μM BBR. At 0, 24, and 48 h after wounding, the cells were photographed under a phase contrast microscope (100×) equipped with a CCD camera. (C) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 24 h and then subjected to analysis for cell migration as described in the Materials and Methods. (D) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 24 h and then subjected to analysis for cell invasion as described in Materials and Methods. All data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$.

involved in multiple cellular pathways that tightly promote and/or inhibit cell growth via regulation of cell cycle progression.[19] To clarify the mechanisms of BBR-inhibited cell viability, we assessed the influence of BBR on the expression of cell cycle regulators. We found that treatment with BBR (50 μM) had an inhibitory influence on the protein expression levels of cyclins A, D1, and E and an induction effect on p21 protein levels in both breast cancer cell lines with more responses observed in MCF-7 cells (Figure 1C). These results suggest that BBR inhibits cell growth by modulating the expression of cell cycle regulatory proteins in breast cancer cells.

BBR Inhibits Adhesion, Migration, and Invasion in Highly Metastatic MDA-MB-231 Cells. Cancer metastasis, a complex and multistep process, is highly related to cell adhesion, motility, and invasion abilities of cancer cells.[4] To determine the influence of BBR on breast cancer metastasis, we examined the effect of BBR on the adhesion, migration, and invasion abilities of highly metastatic MDA-MB-231 cells. The cell-matrix adhesion assay determined that BBR exhibited a dose-dependent repressive effect on the cell adhesion ability of MDA-MB-231 cells. We found that the number of adhesive cells was reduced by 15%, 22%, and 40% in cells exposed to 10 μM, 50 μM, or 100 μM BBR, respectively (Figure 2A).

We next assessed the effect of BBR on cell motility using the wound-healing and cell migration assays. BBR treatment resulted in a dose-dependent inhibitory effect on the motility of MDA-MB-231 cells (Figures 2B and 2C). Based on a quantitative assessment, exposure to 10 μM BBR resulted in an 8% reduction in the number of migrated cells, exposure to 50 μM BBR resulted in a 48% reduction in the number of migrated cells, and exposure to 100 μM BBR resulted in a 72% reduction in the number of migrated cells (Figure 2C). Furthermore, to test the influence of BBR on the invasive ability of MDA-MB-231 cells, cell culture inserts coated with Matrigel were used in a dose escalation experiment. As illustrated in Figure 2D, BBR treatment resulted in a noticeable inhibitory effect on the invasion of MDA-MB-231 cells, accounting for a 10%, 61%, and 80% reduction in the number of invaded cells after treatment with 10 μM, 50 μM, and 100 μM BBR, respectively. These data demonstrate that BBR exerts inhibitory effects on the adhesion, migration, and invasion abilities of highly metastatic MDA-MB-231 cells.

BBR Inhibits the Enzymatic Activity and Expression Levels of MMP2 and MMP9 in Highly Metastatic MDA-

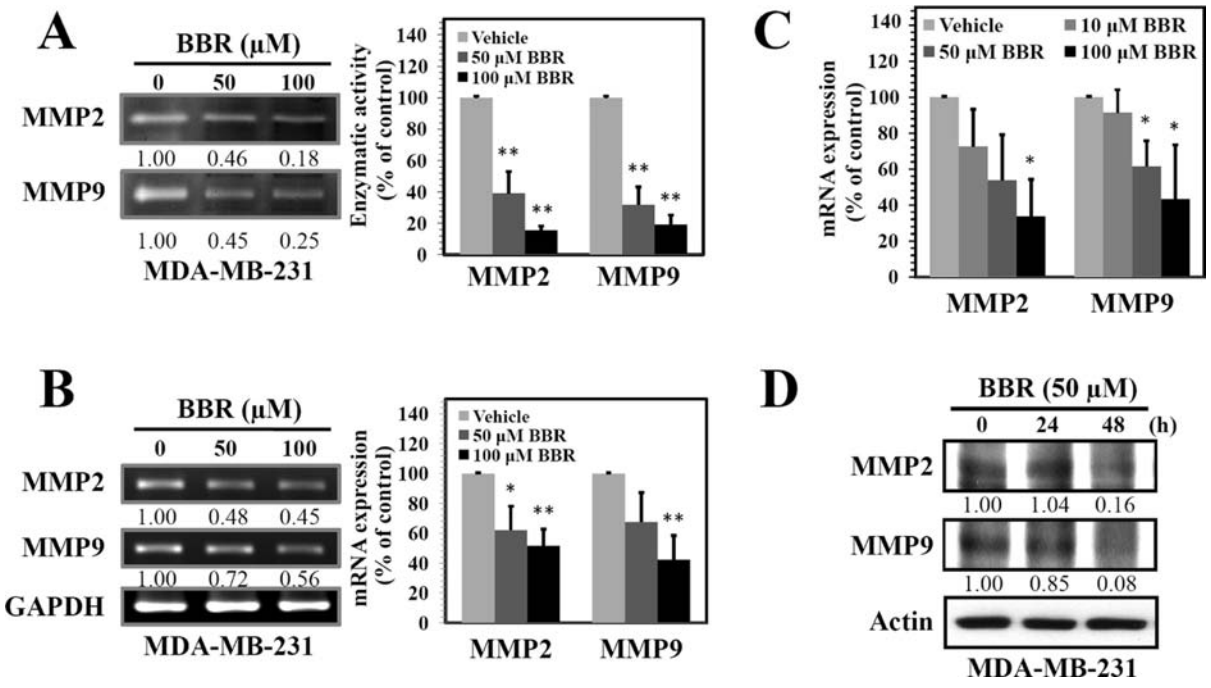

Figure 3. Effects of BBR on the activity and expression levels of MMP2 and MMP9 in highly metastatic MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with BBR (0, 50, or 100 μM) for 48 h. The culture medium of the cells after treatment was subjected to gelatin zymography to analyze the activities of MMP2 and MMP9. (B) MDA-MB-231 cells were treated with BBR (0, 50, or 100 μM) for 48 h. The expression of MMP2 and MMP9 mRNA was measured using semiquantitative RT-PCR as described in Materials and Methods. (C) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 48 h. The expression of MMP2 and MMP9 mRNA was measured using real-time RT-PCR as described in Materials and Methods. (D) MDA-MB-231 cells were treated with 50 μM BBR for 24 and 48 h. The protein expression levels of MMP2 and MMP9 were determined by Western blotting. All data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$.

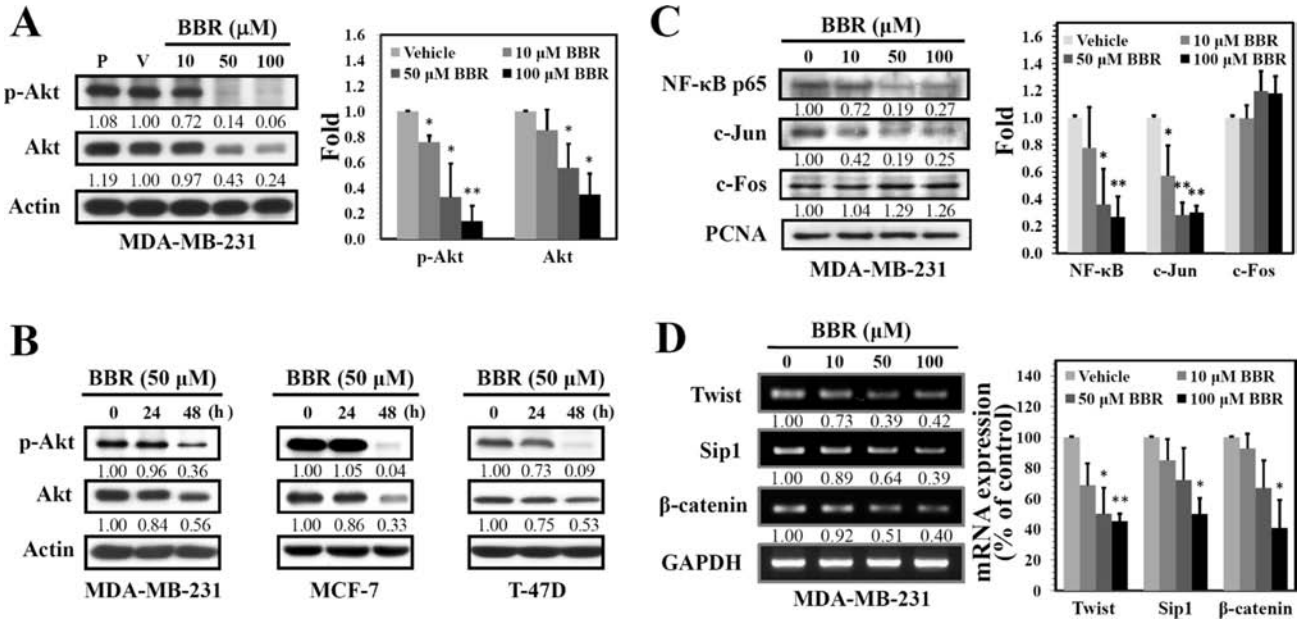

Figure 4. Effects of BBR on the Akt pathway in highly metastatic MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 24 h. Protein expression levels of phospho-Akt and Akt were measured by Western blotting. (B) MDA-MB-231, MCF-7, and T-47D cells were treated with BBR (50 μM) for 24 and 48 h. Protein expression levels of phospho-Akt and Akt were measured by Western blotting. (C) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 48 h. Protein levels of NF-κB (p65) and AP-1 from the nuclear fraction were measured by Western blotting. The PCNA was used as loading control. (D) MDA-MB-231 cells were treated with BBR (0, 10, 50, or 100 μM) for 48 h. The mRNA expression levels of Twist, Sip1, and β-catenin were measured using semiquantitative RT-PCR. All data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$.

MB-231 Cells. The activity and expression of MMPs, particularly MMP2 and MMP9, has been associated with high metastatic potential in breast cancer.[5] To determine whether the anti-invasive effect of BBR was correlated with the suppression of MMP2 and MMP9 in MDA-MB-231 cells, we first analyzed the influence of BBR on the enzymatic activity of MMP2 and MMP9

dx.doi.org/10.1021/jf302832n | J. Agric. Food Chem. 2012, 60, 9649−9658

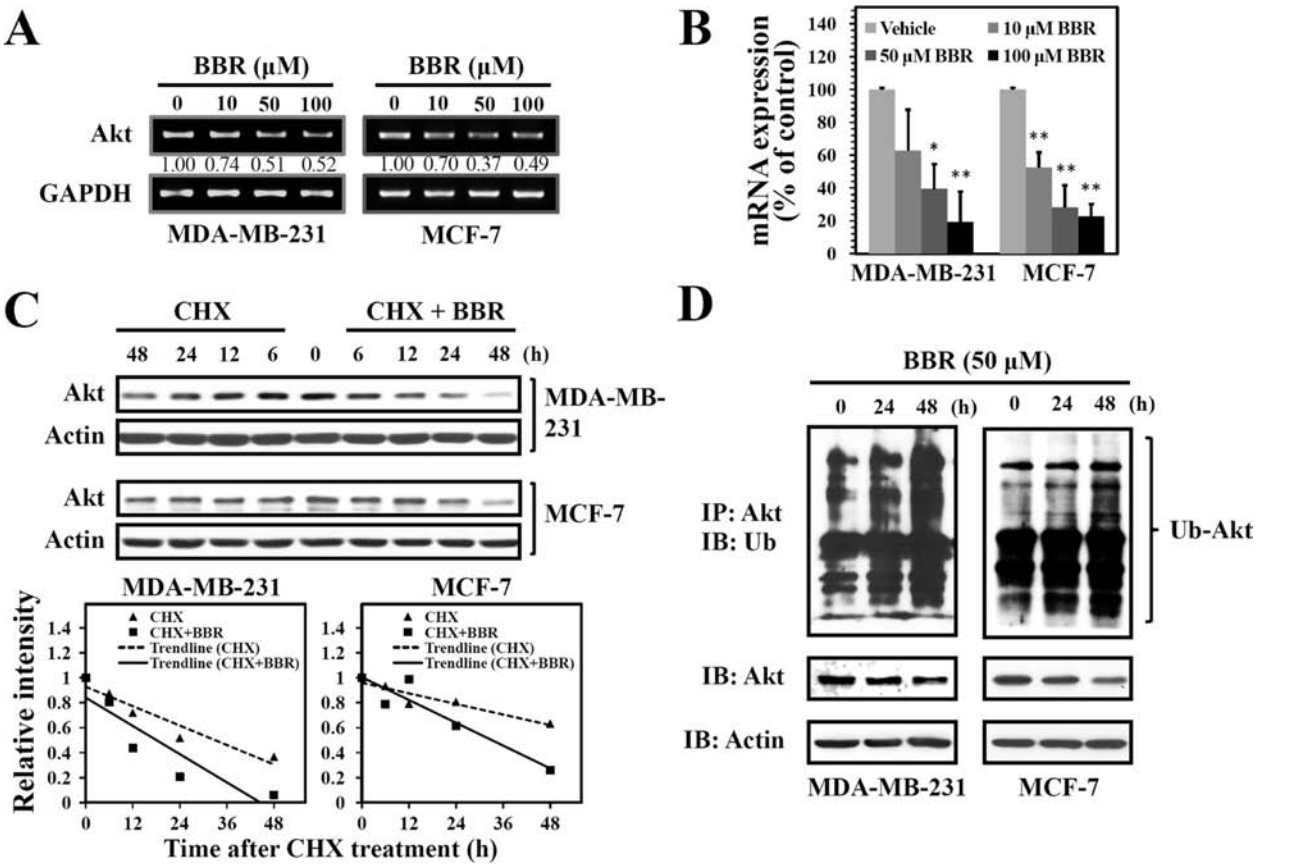

Figure 5. Effects of BBR on the mRNA expression level and protein stability of Akt. (A) MDA-MB-231 and MCF-7 cells were treated with BBR (0, 10, 50, or 100 μM) for 24 h. The mRNA expression level of Akt was measured using semiquantitative RT-PCR. (B) Cells were treated with BBR (0, 10, 50, or 100 μM) for 24 h. The mRNA expression level of Akt was measured using real-time RT-PCR. (C) Cells were pretreated with 20 μg/mL of cycloheximide (CHX) for 30 min and then treated with or without BBR (50 μM). Akt stability was determined by measuring the protein's half-life. (D) Cells were treated with BBR (50 μM) for 12, 24, and 48 h. To detect polyubiquitinated Akt (Akt-Ub$_{(n)}$), Akt was immunoprecipitated and subjected to Western blot analysis using ubiquitin antibody. The total Akt and actin protein levels in the whole-cell extracts were also detected by Western blotting. All data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$.

using gelatin zymography. The gelatinolytic data illustrated that the enzymatic activities of MMP2 and MMP9 were reduced by 61% and 68%, respectively, in cells exposed to 50 μM BBR. Furthermore, the enzymatic activities of MMP2 and MMP9 were reduced by 84% and 81%, respectively, in cells exposed to 100 μM BBR (Figure 3A).

We next examined the effect of BBR on the mRNA levels of MMP2 and MMP9 using semiquantitative RT-PCR. BBR significantly inhibited the mRNA expression levels of both MMP2 and MMP9 (Figure 3B). Additionally, real-time qRT-PCR was performed to assess the mRNA levels of MMP2 and MMP9 in cells treated with various concentrations of BBR (0, 10, 50, and 100 μM). As displayed in Figure 3C, BBR treatment resulted in an evident inhibitory effect on the mRNA levels of both MMP2 and MMP9, accounting for a 27−66% decrease in the mRNA levels of MMP2 and a 9−57% decrease in the expression of MMP9 mRNA after treatment with various concentrations of BBR (10−100 μM) on MDA-MB-231 cells (Figure 3C). In addition, the protein expression of MMP2 and MMP9 was downregulated in cells treated with BBR (Figure 3D). These results suggest that the antimetastatic potential of BBR is associated with the suppression of enzymatically degradative processes during metastasis in highly metastatic MDA-MB-231 cells.

BBR Inhibits the Akt Signaling Pathway in Highly Metastatic MDA-MB-231 Cells. To unravel the underlying molecular mechanisms of the BBR-mediated antimetastatic effect observed in the highly metastatic MDA-MB-231 cells, we determined the effect of BBR on the Akt signaling pathway, which is a mediator of metastatic potential in cancer cells.[20,21] The treatment of MDA-MB-231 cells with BBR resulted in a marked dose- and time-dependent repressive effect on the expression of phospho-Akt and Akt (Figures 4A and 4B). Similarly, BBR also significantly affected the protein levels of phospho-Akt and Akt in other breast cancer cell lines, specifically, MCF-7 and T-47D (Figure 4B).

We next examined the effect of BBR on the expression of NF-κB and AP-1 (c-Fos and c-Jun), which are two critical downstream signaling molecules in the Akt pathway. NF-κB and AP-1 both play important roles in the regulation of MMP expression (e.g., MMP2 and MMP9).[6,7,20] BBR treatment resulted in a dose-dependent reduction in the protein expression levels of NF-κB (p65) and c-Jun in the nuclear fraction (Figure 4C). We also determined the effect of BBR on the DNA binding activity of NF-κB in MDA-MB-231 cells using an electrophoretic mobility shift assay (EMSA) and determined that BBR effectively suppressed the binding ability of NF-κB in a dose-dependent manner (data not shown). Furthermore, we examined the effect of BBR on the expression of other Akt downstream molecules

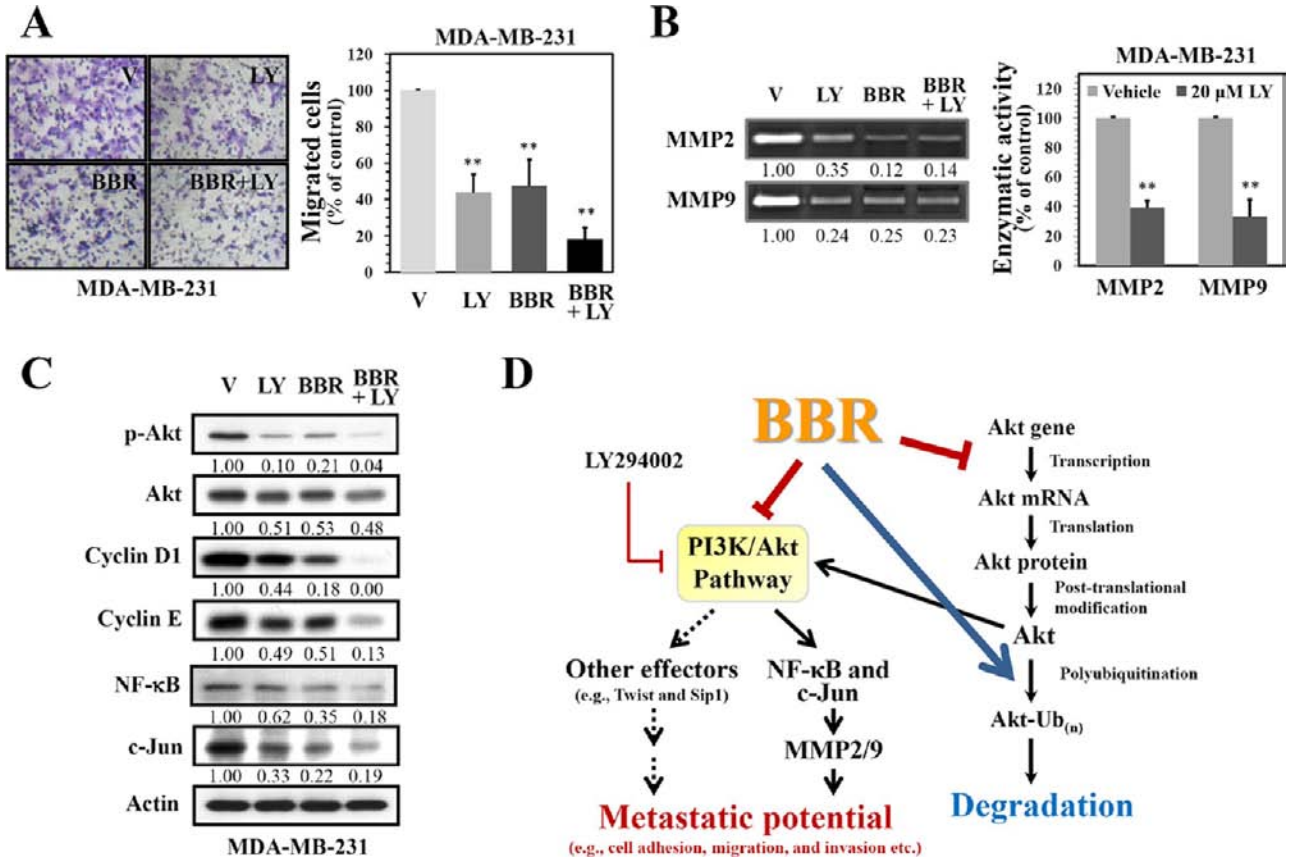

Figure 6. Confirmation that BBR inhibits the metastatic potential of highly metastatic MDA-MB-231 cells by modulating the Akt pathway. (A) MDA-MB-231 cells were treated with 20 μM LY294002 (LY, a PI3K inhibitor) alone or in combination with BBR (50 μM) for 24 h and then subjected to cell migration analysis. (B) MDA-MB-231 cells were treated with LY (20 μM) alone or in combination with BBR (50 μM) for 48 h. The culture medium of the cells after treatment was subjected to gelatin zymography to analyze the enzymatic activity of MMP2 and MMP9. (C) MDA-MB-231 cells were treated with LY (20 μM) alone or in combination with BBR (50 μM) for 48 h. Protein expression levels of p-Akt, Akt, cyclin D1, cyclin E, NF-κB, and c-Jun were measured by Western blotting. (D) A schematic model for the BBR-inhibited metastatic potential of breast cancer cells. All data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$.

that associate with the regulation of cancer metastasis. The mRNA expression levels of Twist, Smad interacting protein 1 (Sip1), and β-catenin were noticeably reduced in cells exposed to BBR relative to the control (Figure 4D). These observations suggest that BBR inhibits the metastatic potential of highly metastatic MDA-MB-231 cells via a reduction in Akt pathway signaling.

BBR Downregulates Akt Protein Expression by Modulating the mRNA Level and Protein Stability of Akt. As mentioned above, our results showed a marked inhibitory effect of BBR on the protein expression of Akt in breast cancer cell lines (Figures 4A and 4B). To evaluate the underlying molecular mechanisms of the BBR-mediated down-regulation of Akt protein levels, we examined the influence of BBR on the expression of Akt mRNA using semiquantitative RT-PCR and real-time qRT-PCR. We found that BBR treatment caused a dose-dependent decrease in the mRNA level of Akt in MDA-MB-231 cells (Figures 5A and 5B). Similarly, BBR also had a significant repressive effect on the expression of Akt mRNA in another breast cancer cell line, MCF-7 (Figures 5A and 5B). These results clearly demonstrate that BBR is capable of reducing the mRNA level of Akt in MDA-MB-231 and MCF-7 breast cancer cells.

An overall reduction in protein stability may also be responsible for the observed reduced Akt protein levels. To test this possibility, we examined the effect of BBR on the Akt protein and found that the protein half-life of Akt was significantly shortened by BBR treatment in MDA-MB-231 as well as in MCF-7 cells (Figure 5C).

Many proteins, such as Akt, are tagged with polyubiquitin and then degraded by the ubiquitin-proteasome system (UPS). Therefore, we determined whether BBR-induced instability of Akt protein was associated with the upregulation of the UPS. Our data illustrate that the number of polyubiquitinated Akt (Akt-$Ub_{(n)}$) proteins were evidently augmented in MDA-MB-231 and MCF-7 cells exposed to 50 μM BBR for 24 or 48 h (Figure 5D). These findings suggest that the depletion of Akt by BBR may also occur via the induction of Akt protein instability/degradation.

BBR Inhibits the Metastatic Potential of Highly Metastatic MDA-MB-231 Breast Cancer Cells via Modulation of the Akt Pathway. To investigate the validity of our results, we first tested whether interfering with the Akt pathway by a specific PI3K-Akt inhibitor (LY294002) suppressed the metastatic potential of MDA-MB-231 cells. The inhibition of the Akt pathway by LY294002 (20 μM) resulted in a 56% decrease in the number of migrated cells (Figure 6A). LY29002 treatment also resulted in a marked repressive effect on the enzymatic activity of MMP2 and MMP9, accounting for a 61% decrease in the activity of MMP2 and a 67% decrease in the activity of MMP9 after treatment with LY294002 on MDA-MB-231 cells (Figure 6B). Furthermore, we examined the influence of LY294002 on the levels of proteins (e.g., cyclins D1 and E) affected by BBR, as

dx.doi.org/10.1021/jf302832n | J. Agric. Food Chem. 2012, 60, 9649−9658

mentioned above. We observed that the protein levels of cyclin D1, cyclin E, NF-κB (p65), and c-Jun were reduced in cells exposed to LY294002 (Figure 6C). Therefore, LY294002 exhibited a suppressive effect that was similar to that of BBR on highly metastatic MDA-MB-231 cells. These results indicate that BBR inhibits the metastatic potential of highly metastatic MDA-MB-231 breast cancer cells through the modulation of the Akt pathway (Figure 6D).

DISCUSSION

Aberrant proliferation and tumor metastasis are thought to be important biological characteristics of cancers. Cancer cell metastasis consists of a complex, multistep process that includes cell adhesion, migration, invasion, proliferation, and vessel formation.[4] Tumor metastasis can be linked to the cause of approximately 90% of all cancer deaths.[22] Therefore, an agent that could disrupt one or more steps of the metastatic process would be a useful candidate in the treatment of cancer by decreasing cancer mortality. A number of natural products have been shown to be effective antimetastatic agents for the treatment of breast cancer. For instance, the natural alkaloid berbamine and the plant flavonoid baicalein have been reported to have repressive effects on the metastatic process of the highly metastatic MDA-MB-231 breast cancer cells.[17,23] In this study, our in vitro data clearly demonstrate that the natural alkaloid berberine (BBR) has significant suppressive effects on the proliferation, adhesion, migration, and invasion of MDA-MB-231 cells (Figures 1 and 2).

Degradation of the basement membrane and extracellular matrix, a process known to be dependent on MMPs such as MMP2 and MMP9, is crucial for the initial step of cancer invasion, thus enabling cancer cells to break out of their primary site into the circulation, which can result in the establishment of metastases at distant sites.[4,5] These degradation events are positively correlated with the expression of MMPs and the potential of cancer invasion. In fact, the suppression of MMP expression and/or suppression of the enzymatic activity of MMPs may be an effective approach for antimetastatic therapy.

Many studies have indicated that BBR could exert its anticancer effect partly by repressing cancer cell migration, invasion, and metastasis in many types of cancer cells. For example, BBR inhibited metastatic ability via suppression of MMP2 and MMP9 expression levels in SCC-4 oral cancer cells;[13] through the repression of MMP1, MMP2, and MMP9 expression in SNU-5 gastric cancer cells;[24] and by reducing MMP2 expression in A549 lung cancer cells.[25] In MDA-MB-231 breast cancer cells, BBR suppressed the TNF-α-induced MMP9 expression [15] and TPA-induced MMP1 and MMP9 expression.[16] Similarly, in the present study, we demonstrate that BBR downregulates the enzymatic activities of MMP2 and MMP9 by decreasing the amount of MMP2 and MMP9 mRNA/protein levels in MDA-MB-231 cells (Figures 3A−3D). Taken together, BBR may be a useful antimetastatic agent in the treatment of patients with breast cancer via repression of metastasis markers, such as MMP2 and MMP9.

The PI3K/Akt signaling pathway is linked to the regulation of cell growth, survival, angiogenesis, and metastasis in breast cancer. Inhibition of the PI3K/Akt and its downstream substrates by some natural products has been shown to be a mechanism for suppressing metastatic potential via the modulation of several important factors, such as MMPs.[6−8] Although BBR has been reported to inhibit TNF-α- and TPA-induced expression of MMPs in breast cancer cells,[15,16] the molecular mechanisms underlying the regulation of MMPs by BBR are not well understood. In this study, we demonstrate for the first time that BBR distinctly inhibits the protein levels of both phospho-Akt and Akt in MDA-MB-231, MCF-7, and BT-474 breast cancer cells (Figures 4A and 4B). Our data also reveal that the protein expression of NF-κB (p65) and c-Jun, a major component of AP-1 in the nuclear fraction, and the mRNA levels of other Akt downstream molecules (such as Twist, Sip1, and β-catenin), are noticeably reduced in cells exposed to BBR (Figures 4C and 4D). Moreover, LY294002, a PI3K/Akt pathway inhibitor, is used to validate our results and confirm that the suppressive effect of BBR on the metastatic process in breast cancer cells involves the modulation of the Akt signaling pathway (Figures 6A−6C). These results suggest that BBR may be a useful antimetastatic agent for the inhibition of breast cancer metastasis of by targeting the Akt signaling pathway.

Interestingly, our data also indicate that BBR attenuates Akt protein expression, suggesting that the Akt protein may be a target of BBR in breast cancer cells (Figures 4A and 4B). To further clarify the underlying mechanisms of BBR-induced downregulation of the Akt protein, we examine the influence of BBR on the mRNA level of Akt. Our results reveal that BBR decreases the mRNA level of Akt in MDA-MB-231 and MCF-7 breast cancer cells (Figures 5A and 5B). We have shown that the Akt mRNA level is downregulated in cells treated with BBR; however, the mechanism of this downregulation is unclear. The regulation of Akt mRNA synthesis at the transcriptional level is still elusive in any given tissue type. Nevertheless, a few reports elucidate that Akt gene transcription is regulated by some transcription factors and/or protein molecules, such as signal transducer and activator of transcription 3 (STAT3) and cyclic-AMP response element-binding protein (CREB).[26−28] Therefore, further studies are needed to clarify the molecular basis by which Akt's mRNA levels are regulated to ultimately help us design better strategies for the treatment of breast cancer.

The regulation of Akt protein stability/degradation is another possible mechanism involved in the expression of Akt protein. It is firmly established that the Akt protein can be degraded by two mechanisms: a ubiquitin-proteasome-dependent pathway or caspase-mediated cleavage.[29] Our data show that the half-life of the Akt protein is significantly reduced by BBR in MDA-MB-231 and MCF-7 cells (Figure 5C). This suppressive effect of BBR on Akt stability may be governed by its ability to induce Akt polyubiqitination (Figure 5D), which leads to its degradation by the proteasome complex instead of an induction of caspase-mediated cleavage (data not shown). In addition, several molecules, such as heat shock protein 90 (Hsp90) and peptidyl-prolyl cis/trans isomerase 1 (Pin1), have been reported to be required for the maintenance of Akt stability and activation.[30,31] It would be worthwhile to study whether these molecules are involved in the BBR-induced degradation/instability of the Akt protein.

In conclusion, we provide a schematic presentation of possible mechanisms for the repressive effects of BBR on the adhesion, migration, and invasion abilities of highly metastatic MDA-MB-231 breast cancer cells (Figure 6D). Our data demonstrate that BBR inhibits the activities and mRNA expression levels of MMP2 and MMP9 via the modulation of the Akt signaling pathway, thereby leading to a reduction in the metastatic ability of MDA-MB-231 cells. We have also shown that the downregulation of the Akt signaling pathway by BBR involves a reduction in the expression of Akt mRNA and an increase in the degradation of Akt protein. These in vitro data shed light on the research of BBR

in breast cancer metastasis in vivo. Taken together, our findings suggest that BBR may be a useful candidate agent for the prevention and treatment of breast cancer metastasis.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mckao@mail.cmu.edu.tw. Tel: 886-4-22053366 ext 2206. Fax: 886-4-22071507.

Author Contributions

§These authors contributed equally to this work.

Funding

This study was supported by a grant from the China Medical University (CMU99-COL-29), Taichung, Taiwan.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank members of the Medical Research Core Facilities Center (Office of Research & Development, China Medical University, Taichung, Taiwan, ROC) and Chiao-Che Chen for helpful technical assistance.

ABBREVIATIONS USED

BBR, berberine; MMPs, matrix metalloproteinases; CDKs, cyclin-dependent kinases; CKIs, CDK inhibitors; PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor kappa B; AP-1, activator protein-1

REFERENCES

(1) Jemal, A.; Bray, F.; Center, M. M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *CA—Cancer J. Clin.* **2011**, *61* (2), 69−90.

(2) Department of Health, Executive Yuan, Taiwan, R.O.C., Health and vital statistics, 2011. Available from http://www.doh.gov.tw.

(3) Lacroix, M. Significance, detection and markers of disseminated breast cancer cells. *Endocr.-Relat. Cancer* **2006**, *13* (4), 1033−67.

(4) Bellahcene, A.; Castronovo, V.; Ogbureke, K. U.; Fisher, L. W.; Fedarko, N. S. Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. *Nat. Rev. Cancer* **2008**, *8* (3), 212−26.

(5) Duffy, M. J.; Maguire, T. M.; Hill, A.; McDermott, E.; O'Higgins, N. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Res.* **2000**, *2* (4), 252−7.

(6) Lee, Y. C.; Lin, H. H.; Hsu, C. H.; Wang, C. J.; Chiang, T. A.; Chen, J. H. Inhibitory effects of andrographolide on migration and invasion in human non-small cell lung cancer A549 cells via down-regulation of PI3K/Akt signaling pathway. *Eur. J. Pharmacol.* **2010**, *632* (1−3), 23−32.

(7) Lee, Y. C.; Cheng, T. H.; Lee, J. S.; Chen, J. H.; Liao, Y. C.; Fong, Y.; Wu, C. H.; Shih, Y. W. Nobiletin, a citrus flavonoid, suppresses invasion and migration involving FAK/PI3K/Akt and small GTPase signals in human gastric adenocarcinoma AGS cells. *Mol. Cell. Biochem.* **2011**, *347* (1−2), 103−15.

(8) Hong, K. O.; Kim, J. H.; Hong, J. S.; Yoon, H. J.; Lee, J. I.; Hong, S. P.; Hong, S. D. Inhibition of Akt activity induces the mesenchymal-to-epithelial reverting transition with restoring E-cadherin expression in KB and KOSCC-25B oral squamous cell carcinoma cells. *J. Exp. Clin. Cancer Res.* **2009**, *28*, 28.

(9) Tan, W.; Li, Y.; Chen, M.; Wang, Y. Berberine hydrochloride: anticancer activity and nanoparticulate delivery system. *Int. J. Nanomed.* **2011**, *6*, 1773−7.

(10) Kuo, H. P.; Chuang, T. C.; Yeh, M. H.; Hsu, S. C.; Way, T. D.; Chen, P. Y.; Wang, S. S.; Chang, Y. H.; Kao, M. C.; Liu, J. Y. Growth suppression of HER2-overexpressing breast cancer cells by berberine via modulation of the HER2/PI3K/Akt signaling pathway. *J. Agric. Food Chem.* **2011**, *59* (15), 8216−24.

(11) Lin, C. C.; Yang, J. S.; Chen, J. T.; Fan, S.; Yu, F. S.; Yang, J. L.; Lu, C. C.; Kao, M. C.; Huang, A. C.; Lu, H. F.; Chung, J. G. Berberine induces apoptosis in human HSC-3 oral cancer cells via simultaneous activation of the death receptor-mediated and mitochondrial pathway. *Anticancer Res.* **2007**, *27* (5A), 3371−8.

(12) 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.

(13) Ho, Y. T.; Yang, J. S.; Li, T. C.; Lin, J. J.; Lin, J. G.; Lai, K. C.; Ma, C. Y.; Wood, W. G.; Chung, J. G. Berberine suppresses in vitro migration and invasion of human SCC-4 tongue squamous cancer cells through the inhibitions of FAK, IKK, NF-kappaB, u-PA and MMP-2 and -9. *Cancer Lett.* **2009**, *279* (2), 155−62.

(14) Singh, T.; Vaid, M.; Katiyar, N.; Sharma, S.; Katiyar, S. K. Berberine, an isoquinoline alkaloid, inhibits melanoma cancer cell migration by reducing the expressions of cyclooxygenase-2, prostaglandin E and prostaglandin E receptors. *Carcinogenesis* **2011**, *32* (1), 86−92.

(15) Kim, S.; Choi, J. H.; Kim, J. B.; Nam, S. J.; Yang, J. H.; Kim, J. H.; Lee, J. E. Berberine suppresses TNF-alpha-induced MMP-9 and cell invasion through inhibition of AP-1 activity in MDA-MB-231 human breast cancer cells. *Molecules* **2008**, *13* (12), 2975−85.

(16) Kim, S.; Han, J.; Lee, S. K.; Choi, M. Y.; Kim, J.; Lee, J.; Jung, S. P.; Kim, J. S.; Kim, J. H.; Choe, J. H.; Lee, J. E.; Nam, S. J., Berberine Suppresses the TPA-Induced MMP-1 and MMP-9 Expressions Through the Inhibition of PKC-alpha in Breast Cancer Cells. *J. Surg. Res.* **2011**.

(17) Wang, L.; Ling, Y.; Chen, Y.; Li, C. L.; Feng, F.; You, Q. D.; Lu, N.; Guo, Q. L. Flavonoid baicalein suppresses adhesion, migration and invasion of MDA-MB-231 human breast cancer cells. *Cancer Lett.* **2010**, *297* (1), 42−8.

(18) Yeh, M. H.; Tsai, T. C.; Kuo, H. P.; Chang, N. W.; Lee, M. R.; Chung, J. G.; Tsai, M. H.; Liu, J. Y.; Kao, M. C. Lentiviral short hairpin RNA screen of human kinases and phosphatases to identify potential biomarkers in oral squamous cancer cells. *Int. J. Oncol.* **2011**, *39* (5), 1221−31.

(19) 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.

(20) Qiao, M.; Sheng, S.; Pardee, A. B. Metastasis and AKT activation. *Cell Cycle* **2008**, *7* (19), 2991−6.

(21) Sheng, S.; Qiao, M.; Pardee, A. B. Metastasis and AKT activation. *J. Cell. Physiol.* **2009**, *218* (3), 451−4.

(22) Mehlen, P.; Puisieux, A. Metastasis: a question of life or death. *Nat. Rev. Cancer* **2006**, *6* (6), 449−58.

(23) Wang, S.; Liu, Q.; Zhang, Y.; Liu, K.; Yu, P.; Luan, J.; Duan, H.; Lu, Z.; Wang, F.; Wu, E.; Yagasaki, K.; Zhang, G. Suppression of growth, migration and invasion of highly-metastatic human breast cancer cells by berbamine and its molecular mechanisms of action. *Mol. Cancer* **2009**, *8*, 81.

(24) Lin, J. P.; Yang, J. S.; Wu, C. C.; Lin, S. S.; Hsieh, W. T.; Lin, M. L.; Yu, F. S.; Yu, C. S.; Chen, G. W.; Chang, Y. H.; Chung, J. G. Berberine induced down-regulation of matrix metalloproteinase-1, -2 and -9 in human gastric cancer cells (SNU-5) in vitro. *In Vivo* **2008**, *22* (2), 223−30.

(25) Peng, P. L.; Hsieh, Y. S.; Wang, C. J.; Hsu, J. L.; Chou, F. P. Inhibitory effect of berberine on the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2. *Toxicol. Appl. Pharmacol.* **2006**, *214* (1), 8−15.

(26) Misra, U. K.; Pizzo, S. V. Upregulation of AKT1 protein expression in forskolin-stimulated macrophage: evidence from ChIP analysis that CREB binds to and activates the AKT1 promoter. *J. Cell. Biochem.* **2007**, *100* (4), 1022−33.

(27) Park, S.; Kim, D.; Kaneko, S.; Szewczyk, K. M.; Nicosia, S. V.; Yu, H.; Jove, R.; Cheng, J. Q. Molecular cloning and characterization of the

human AKT1 promoter uncovers its up-regulation by the Src/Stat3 pathway. *J. Biol. Chem.* **2005**, *280* (47), 38932−41.

(28) Nemoto, T.; Kanai, T.; Yanagita, T.; Satoh, S.; Maruta, T.; Yoshikawa, N.; Kobayashi, H.; Wada, A. Regulation of Akt mRNA and protein levels by glycogen synthase kinase-3beta in adrenal chromaffin cells: effects of LiCl and SB216763. *Eur. J. Pharmacol.* **2008**, *586* (1−3), 82−9.

(29) Liao, Y.; Hung, M. C. Physiological regulation of Akt activity and stability. *Am. J. Transl. Res.* **2010**, *2* (1), 19−42.

(30) Sato, S.; Fujita, N.; Tsuruo, T. Modulation of Akt kinase activity by binding to Hsp90. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (20), 10832−7.

(31) Liao, Y.; Wei, Y.; Zhou, X.; Yang, J. Y.; Dai, C.; Chen, Y. J.; Agarwal, N. K.; Sarbassov, D.; Shi, D.; Yu, D.; Hung, M. C. Peptidyl-prolyl cis/trans isomerase Pin1 is critical for the regulation of PKB/Akt stability and activation phosphorylation. *Oncogene* **2009**, *28* (26), 2436−45.