Molecular Pharmacology Fast Forward. Published on May 30, 2012 as doi: 10.1124/mol.112.078402
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MOL #78402

A new 2-pyrone derivative, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one, suppresses stemness in glioma stem-like cells

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Molecular Pharmacology Fast Forward. Published on May 30, 2012 as DOI: 10.1124/mol.112.078402
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MOL #78402

Running Title: A new 2-pyrone derivative targets glioma-initiating cells

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Number of pages : 27
Number of tables : 0
Number of figures : 6
Number of references : 40
Number of words in the Abstract : 230
Number of words in the Introduction : 413
Number of words in the Discussion : 658

Abbreviations : BHP, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; siRNA, small interfering RNA; GSCs, glioma stem-like cells; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DMSO, dimethly sulfoxide; DMEM, Dulbecco's modified Eagles's medium; FBS, fetal bovine serum; FACS, fluorescence-activated cell-sorting; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline.

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ABSTRACT

Glioma cells with stem cell properties, termed glioma stem-like cells (GSCs) have been linked to tumor formation, maintenance and progression, and are also responsible for the failure of chemo- and radiotherapy. Because conventional glioma treatments often fail to eliminate GSCs completely, residual surviving GSCs are able to repopulate the tumor. Thus, compounds that target GSCs could be helpful in overcoming resistance to anticancer treatments in human brain tumors. In this study, we show that 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP), a new 2-pyrone derivative, suppresses maintenance of the GSC population in both a glioma cell line and patient-derived glioma cells. Treatment of GSCs with BHP effectively inhibited sphere formation and suppressed the $CD133^+$ cell population. Treatment with BHP also suppressed expression of the stemness-regulating transcription factors Sox2, Notch2, and β-catenin in sphere-cultured glioma cells. Moreover, treatment of GSCs with BHP significantly suppressed two fundamental characteristics of cancer stem cells: self-renewal and tumorigenicity. BHP treatment dramatically inhibited clone-forming ability at the single cell level and suppressed *in vivo* tumor formation. BHP markedly inhibited both PI3K-AKT and Ras-Raf-1-ERK signaling, suggesting that either or both of these pathways are involved in BHP-induced suppression of GSCs. In addition, treatment with BHP effectively sensitized GSCs to chemo- and radiotherapy. Taken together, these results indicate that BHP targets GSCs and enhances their sensitivity to anticancer treatments, suggesting that BHP treatment may be useful for treating brain tumors by eliminating GSCs.

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Introduction

Glioma, the most common primary brain tumor, has aggressive and highly invasive characteristics and is highly lethal, with a median survival of 12-15 months from initial diagnosis despite optimal therapy (Ohgaki and Kleihues, 2005; Wen and Kesari, 2008). The major limitations of glioma treatment are the prevalence of recurrence after surgery, infiltration into surrounding tissues, and intrinsic or acquired resistance to chemo- and radiotherapy (Bao et al., 2006; Eramo et al., 2006; Rich, 2007). Recent evidence suggests that gliomas display cellular hierarchies, including the presence of a population of tumor-initiating glioma stem-like cells (GSCs). GSCs are functionally defined by their extensive self-renewal, multilineage differentiation potential, and capacity to propagate tumors that recapitulate the tissue architecture and cellular hierarchy of the primary tumor (Park and Rich, 2009; Zhou et al., 2009). Because the presence of GSCs permits sustained tumor propagation, GSCs are considered novel therapeutic targets for glioma treatment. However, because GSCs are resistant to chemotherapy and radiotherapy (Bao et al., 2006; Eramo et al., 2006; Rich, 2007), conventional cancer treatments often fail to eradicate them completely, and residual surviving GSCs are able to repopulate the tumor, causing relapse (Liu et al., 2006; Singh et al., 2004a). Thus, novel therapeutic strategies that specifically target GSCs are u rgently needed to more effectively eradicate malignant tumors and reduce the risk of relapse.

2-Pyrones, six-membered cyclic unsaturated esters that are highly abundant in bacteria, plant and animal systems (Faulkner, 2001; Jensen and Fenical, 1994), have been implicated in many different biological processes, including defense against other organisms (McGlacken and Fairlamb, 2005). Moreover, accumulating evidence indicates that microbial 2-pyrones, dihydro-2-pyrones, and secondary metabolites exhibit a wide range of antifungal, cytotoxic, neurotoxic, and phytotoxic properties (Dickinson, 1993). These compounds further display

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antitumor and HIV-inhibitory activities (Poppe et al., 1997; Thaisrivongs et al., 1996; Turner et al., 1998), reinforcing their potential medicinal importance. Additionally, an investigation on the anticancer properties of tricyclic 2-pyrones showed that these compounds prevent DNA synthesis and growth in human leukemic cells *in vitro* (Trachootham et al., 2008). Despite such *in vitro* evidences for the anticancer properties of 2-pyrones, the potential of these compounds to modulate sensitivity to anticancer therapy and/or stemness in human solid cancer cells remain unknown.

Here, we show that BHP, a new 2-pyrone derivative, suppresses stemness and malignancy of GSCs, and enhances the sensitivity of GSCs to anticancer therapies. We suggest that BHP may form the basis of a novel therapeutic strategy for eradicating malignant tumors by specifically targeting cancer stem cells.

Materials and Methods

Construction of 2-pyrone derivative, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one

A new 2-pyrone derivative, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP) was constructed as described earlier (Fig. 1A) (McGlacken and Fairlamb, 2005).

Chemical Reagents and Antibodies

Polyclonal antibodies to phospho-AKT (Ser-473), phospho-AKT (Thr-308), phospho-ERK1/2 and ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies to CD133 were purchased from Abcam and polyclonal antibodies to Nestin, Musashi-1 and Notch2 were purchased from Millipore (Billerica, MA). Polyclonal antibodies to AKT and Raf-1 were purchased from Santa Cruz (Santa Cruz, CA). 4, 6-

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diamidino-2-phenylindole (DAPI), epidermal growth factor (EGF) and monoclonal antibodies to β-actin were obtained from Sigma (St. Luis, MO). Basic fibroblast growth factor (bFGF) was purchased from R&D Systems. Anti-mouse Alexa Fluor 488, anti-rabbit Alexa Flour 488 and B27 were purchased from Invitrogen. CD133 (directly conjugated with phycoerythrin, clone 293C3) and mIgG2b-PE were purchased from Miltenyi Biotec Ltd. LY294002 was obtained from Calbiochem (San Diego, CA). Temozolomide was purchased from Santa Cruz (Santa Cruz, CA).

Cell and Sphere Culture

U87MG and U373MG (glioma cells) were obtained from the Korean Cell Line Bank (KCLB) and cultured in the recommended medium containing 10% FBS, and in this medium condition, glioma cells were grown in monolayer. Patient-derived glioma stem-like cells X01GB, X02GB and X03AOA were established from acutely resected human tumor tissues (Soeda et al., 2008; Soeda et al., 2009). The X01GB line was derived from a 68- year-old woman with a glioblastoma multiforme. The X02GB line originated from a man with glioblastoma multiforme. The X03AOA was derived from a woman with anaplastic oligoastrocytoma. Each patient provided written informed consent for tumor tissue.

U87MG, U373MG, X01GB, X02GB and X03AOA cells were resuspended in DMEM-F12 (Invitrogen) containing 20 ng/mL of EGF, bFGF, and B27 (1:50) as a stem cell–permissive medium. For treatment, glioma cells were cultured in the presence of BHP (10 μM) or DMSO (vehicle). Spheres were then collected after 5 days, and protein was extracted for Western blotting and kinase assay or dissociated with Accutase (Innovative Cell Technologies, Inc.) for expansion (Singh et al., 2003; Suslov et al., 2002).

FACS analysis

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Cells were dissociated and resuspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mmol/L EDTA. Cells were stained with PE-conjugated CD133 antibody (1:100, Miltenyi Biotech) or isotype control antibody (1:100, mIgG2b-PE, Miltenyi Biotech) and analyzed by BD FACSCalibur, using Cell Quest software.

Quantification of Cell Death

Cell death was measured by FACS analysis using propidium iodide staining. Cells were harvested by trypsinization, washed in phosphate-buffered saline, and then incubated in propidium iodide (50 ng/ml) for 5 min at room temperature. Cells (10,000 per sample) were analyzed by BD FACSCalibur, using Cell Quest software.

Sphere Colony Counting

Sphere-forming U373MG cells were first distributed into 96-well plates at a density of 0.2 cells per well. After 12 hours, individual wells were visually checked for the presence of a single cell. The clones were grown and analyzed the sphere formation. Spheres with a diameter > 20 μm were counted using an inverted microscope at days 1, 10 and 20.

Western blot Analysis

Cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline, and incubated with primary antibodies for overnight at 4°C. The Blots were developed with a peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham, Arlington Heights, IL), using

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the manufacturer's protocol.

PI3 kinase Assay

Cell lysates (300 μg in 500 μL) were subjected to immunoprecipitation using anti-p85 antibodies (Upstate Biotechnology). The precipitates were washed twice with 1% NP40/PBS, followed by PBS, 0.1 mol/L Tris-HCl (pH 7.5)/0.5 mol/L LiCl, and finally 25 m mol/L HEPES (pH 7.5)/100 mmol/L NaCl/1 mmol/L EDTA. The precipitates were then resuspended in 50 μL of presonicated phosphatidylinositol substrate and incubated for 10 min at room temperature. Each sample was labeled with 10 μCi [^{32}P]ATP for 10 min at room temperature. Reactions were stopped by adding 100 μL of chloroform/methanol/HCl (50:100:1). Lipids were extracted with 200 μL chloroform and after mixing and centrifugation, the lower "organic" phase was transferred to a new tube. The organic phase was washed once with 100 μL methanol-1 mol/L HCl (1:1) and the upper phase was discarded. The lipid fraction was then dried and resuspended in 20 μL of chloroform and applied to a silica gel thin layer chromatography (TLC) plate preimpregnated with 1% potassium oxalate. Phospholipids were resolved by TLC in freshly prepared buffer-chloroform/methanol/ammonia/water (43:38:5:7) for 45 min in a closed glass chamber at room temperature (Hyun et al., 2011).

Raf-1 Kinase Assay

Raf-1 kinase activity was measured by the ability of immunoprecipitated enzyme to Raf-1 substrate. Briefly, cell lysates were prepared by lysis buffer containing 1% (v/v) NP-40, 10% (v/v) glycerol, 137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 1% (v/v) Triton X-100, and protease inhibitors. Lysates were incubated with anti-Raf-1 antibodies for 2hr at 4 °C. The immunocomplex was then precipitated with protein A Sepharose. Kinase reactions were carried out with 20 mmol/L HEPES (pH 7.4), 5 mmol/L MnCl2, 10 mmol/L MgCl2, 1

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mmol/L DTT, 10 μCi of [γ-32P]ATP and 1 mg/mL MBP substrate. Proteins were separated on SDS-polyacrylamide gels, and bands were detected by autoradiography.

Activated Ras affinity precipitation assay

Activated Ras affinity precipitation assays were performed according to the manufacturer's protocol. Briefly, cell lysates were incubated with 5 μg of Raf-1 RBD agarose beads (upstate, Charlotsville, VA) for 30 min at 4 °C. After extensive washing of the agarose beads three times with washing buffer (25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM $Na3VO_4$, 10% glycerol, 10 μg/ml leupeptin, 10 μg /ml aprotinin, and 25 mM NaF), the activated Ras (GTP-Ras) bound to Raf-1 RBD argarose beads was released by addition of SDS-PAGE sample buffer. The amount of activated Ras was determined by immunoblotting with a monoclonal pan-Ras antibody.

Soft Agar Colony Formation Assay

To examine anchorage-independent growth, cells (2×10^4 cells/mL) were suspended in 0.4% agarose with serum-free DMEM-F12 supplemented with B27, bFGF, and EGF and seeded onto 60-mm dishes that are pre-coated with 0.8% agar in complete growth medium and incubated at 37 °C, 5% CO_2. After 12–30 days, colonies were photographed and counted in 4 randomly chosen fields and expressed as means of triplicates, representative of two independent experiments.

Tumor xenografts on nude mice

Patient-derived X01GB cells (1×10^6 cells) were subcutaneously inoculated to the right flank of athymic Balb/c female nude mice (5 weeks of age; Charles River Laboratories). The mice were randomly distributed into two groups (each group, n=5) that were treated by

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intraperitoneal (i.p) injections every 3 d (three cycles) with DMSO or 15 μM (100 μl) BHP that corresponds to 1.32 x 10^{-2} mg BHP/kg weight of mouse. Tumor size was measured with a caliper (calculated volume = shortest diameter^2 × longest diameter/2) with three-day intervals. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute, which is an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility and abide by the Institute of Laboratory Animal Resources guide.

Irradiation

Cells were plated in 60 mm dishes and incubated at 37°C under humidified 5% CO2 in culture medium. Cells were exposed to γ-rays using a 137Cs γ-rays source (AtomicEnergy of Canada, Ltd, Mississauga, Canada) at a dose rate of 3.81 Gy min^{-1}.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Following cell fixation, cells were incubated with the appropriate primary antibodies in a solution of PBS with 1% bovine serum albumin and 0.1% Triton X-100 at 4°C overnight. Antibodies used were as follows: human anti-CD133 (rabbit polyclonal antibody, 1:200), -Nestin (rabbit polyclonal antibody, 1:200), -Musashi1 (rabbit polyclonal antibody, 1:200), -Sox2 (rabbit polyclonal antibody, 1:200) and -Tuj1 (mouse polyclonal antibody, 1:200). Stainings were visualized using anti-rabbit or anti-mouse Alexa Flour 488 (Molecular Probes). Nuclei were counterstained using 4, 6-diamidino-2-phenylindole (DAPI; Sigma). Stained cells were visualized with a fluorescence-microscope (Olympus IX71).

Immunohistochemistry

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Tumor tissues were fixed in Bouin's solution (Sigma, St. Louis, MO) for overnight, and processed for paraffin embedding. Sections (5 μm thick) were attached on poly-L-lysine coated slides and immunostaining was performed by using Vectastain Elite ABC kit (PK-6101, Vector Laboratories, Burlingame, CA). After deparaffination and rehydration, antigen retrieval was performed using 10 mM citrate buffer (pH 6.0) in autoclave at 120°C for 10 min and endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 min. After washing in PBS, slides were incubated with blocking solution (1.5% normal goat serum in PBS) for 30 min.

Human anti-Nestin (rabbit polyclonal antibody, Millipore, Billerica, USA) diluted 1:1000 in blocking solution was applied to the sections and incubated at 4°C for overnight. In the negative control, rabbit IgG (ab27478, Abcam) was used instead of primary antibody. After washing in PBS, 1:200 dilution of biotinylated goat anti-rabbit IgG antibody in blocking solution was applied to the sections and incubated for 30 min. After washing in PBS, ABC reagent was applied to the sections and incubated for 30 min. After washing in PBS, color reaction was performed with 3,3'-diaminobenzidine (Vector Laboratories) and slides were washed with PBS. After counterstaining with hematoxylin and clearing with graded ethanol series and xylene, the sections were mounted with Canada balsam. Observation and photography were conducted using IX71 microscope (Olympus, Tokyo, Japan) equipped with DP71 digital imaging system (Olympus).

Statistical analysis

All experimental data are reported as mean and the error bars represent the experimental standard error. Statistical analysis was performed by the non-parametric Student *t* test.

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RESULTS

BHP effectively suppresses the GSC population.

In previous studies, we showed that GSCs can be enriched by culturing glioma cells in serum-free medium supplemented with the growth factors epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Under these conditions, a fraction of glioma cells continue to proliferate and form spheres instead of a monolayer (Yoon et al., 2012). In the current study, we examined whether BHP selectively targets the stem-like cell population in glioma. To this end, we first determined the optimum concentration of BHP by treatment of patient-derived glioma cells (X01GB, X03AOA) cultured in sphere forming conditions with various concentrations of BHP from 0 to 20 μM, and analyzed sphere formation and cell death. Treatment of sphere-forming glioma cells with BHP below the concentration 10 μM most effectively suppressed sphere formation without significant cell death (Supplemental Figure 1). However, there was an increase of cell death at higher concentration than 15 μM (Supplemental Figure 1A). Thus, we determined to use 10 μM of BHP concentration in this study, though 5 μM of BHP also displayed marginal suppression of sphere formation without cell death. To determine whether BHP targets GSCs, we treated the glioma cell line U87 and patient-derived glioma cells (X01GB, X02GB, and X03AOA) with BHP (10 μM) under sphere-forming culture conditions and quantified sphere-forming ability by counting the number of spheres 7 days after treatment. Notably, treatment with BHP dramatically attenuated sphere formation in both the U87 glioma cell line and patient-derived glioma cells (X01GB, X02GB, and X03AOA) compared to treatment with DMSO (vehicle) or α-pyrone (Fig. 1A and B).

Because GSCs are enriched for a subpopulation of CD133-positive glioma cells with greater tumorigenic potential than CD133-negative cells (Singh et al., 2004b), we measured the

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CD133-positive cell population in U87, X01GB, and X03AOA cell lines after treatment with BHP. A fluorescence-activated cell-sorting (FACS) analysis showed that treatment of sphere-forming glioma cells with BHP significantly suppressed the CD133-positive cell population compared to DMSO or α-pyrone (Fig. 1C). Immunoblotting and immunocytochemical analyses also showed that treatment with BHP cause down-regulation not only of CD133 but also other stem cell markers, namely Nestin and Musashi-1 (Fig. 1D and E). Taken together, these results indicate that treatment with BHP effectively suppresses the stem-like cell population in glioma.

BHP suppresses self-renewal of GSCs.

Cancer stem cells are characterized by their strong tumorigenic properties and ability to self-renew. To further determine whether BHP treatment suppresses self-renewal in GSCs, we treated sphere-forming glioma cells with BHP, and monitored clone formation for 7 days using a single-cell clonogenic assay. Notably, treatment of patient-derived X01GB cells with BHP suppressed clone formation, dramatically reducing the size of clones compared to controls (Fig. 2A).

Because BHP treatment decreased the self-renewal capacity of sphere-forming glioma cells and the population of cells positive for the stem cell markers CD133, Nestin and Musashi-1, we next examined the expression levels of stemness-regulating genes, Sox2, Notch-2, and β-catenin after treatment with BHP. As shown in Figure 2B, the expression levels of Sox2, Notch-2, and β-catenin were markedly down-regulated by BHP treatment (Fig. 2B). Immunocytochemical analyses also confirmed that BHP effectively suppresses Sox2 and Notch-2 expression (Fig. 2C). Taken together, these results indicate that treatment with BHP effectively suppresses self-renewal and expression of stemness-regulating genes in sphere-cultured glioma cells.

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BHP suppresses the tumorigenic properties of GSCs.

Tumorigenicity is the most important characteristic of GSCs. To determine whether BHP treatment suppresses the tumor-forming potential of GSCs, we examined anchorage independent colony-forming ability of patient-derived glioma cells (X01GB) after treatment with BHP. In contrast to normal cells, which do not proliferate without anchorage, tumorigenic cancer cells can grow under conditions that do not provide anchorage, such as in semisolid medium. Thus, colony-forming assays in soft agar are widely used to measure tumorigenic capacity *in vitro*, and are considered a good predictor of tumor-forming ability *in vivo*. As shown in Figure 3A, BHP treatment strongly inhibited colony formation in soft agar by sphere-derived cells. To further confirm the inhibitory effect of BHP on the tumorigenic properties of GSCs *in vivo*, we injected sphere-cultured X01GB glioma cells (1 x 10^6 cells per mouse) subcutaneously into nude mice, treated mice with BHP by i.p injection, and then examined tumor formation over the course of 30 days. As shown in Figure 3B, tumor formation by sphere-derived cells was significantly attenuated by BHP treatment. Since BHP treatment suppressed GSCs without significant cell death in vitro, we next examined whether the smaller sizes of tumor are due to the decrease of GSC population in mice treated with BHP. To this end, we performed immunohistochemistry with the tumors from BHP-treated and DMSO-treated mice. Importantly, Nestin protein levels, previously known GSC marker, were drastically lower in tumors derived from BHP-treated mice, compared to DMSO-treated mice (Fig. 3C). Taken together, these results suggest that BHP suppresses tumorigenic capacity by targeting of GSCs.

BHP inhibits PI3K-AKT and Ras-Raf-1-ERK signaling pathways.

Because phosphoinositol 3-kinase (PI3K)/AKT and Ras-Raf-1-ERK (extracellular signal-

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regulated kinase) signaling pathways have been shown to regulate glioma stemness (Bleau et al., 2009; Hambardzumyan et al., 2008; Sunayama et al., 2010), we next examined whether BHP acts by inhibiting these pathways. To this end, we treated sphere-cultured, patient-derived glioma cells (X01GB) with BHP and analyzed the phosphorylation status of AKT and ERK1/2. Notably, treatment with BHP attenuated phosphorylation of AKT at both Ser473 and Thr308. In addition, PI3 kinase assay was also performed by immunoprecipitating with a subunit of PI3K, P85 antibody. The activity was then measured by phospholipids generated in the presence of the substrate PIP using thin layer chromatography (TLC). As shown in Figure 4A, treatment of patient-derived X01GB cells with BHP decreased PI3K activity without altering PTEN (phosphatase and tensin homolog) expression levels. BHP treatment also decreased the phosphorylated AKT at Thr 308 and Ser 473. Consistent with these results, the kinase activity of AKT was attenuated by treatment with BHP (Fig. 4A). BHP treatment also inhibited Raf-1 and ERK activation as well as the interaction between Ras and Raf-1 in patient-derived X01GB cells without changing the expression levels of these proteins (Fig. 4B). Using a Raf-1 agarose assay to measure the active form of Ras, we found that activated Ras levels were decreased in BHP-treated X01GB cells, whereas the total amount of Ras was not altered. An *in vitro* kinase assay also showed that the kinase activity of Raf-1 was decreased by BHP treatment. Moreover, the kinase activity of ERK1/2 was suppressed by treatment with BHP, along with a decrease of active phosphorylated form of ERK1/2 (p-ERK1/2). Taken together, these results suggest that treatment with BHP attenuates both PI3K-AKT and Ras-Raf-1-ERK pathways and thereby suppresses the glioma stem-like cell population.

BHP sensitizes GSCs to anticancer treatments.

Upon initial diagnosis of glioblastoma multiforme, standard treatment consists of maximal

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surgical resection, radiotherapy, and concomitant and adjuvant chemotherapy with temozolomide or paclitaxel (Taxol). However, since GSCs are reported to endow resistance to chemo- and radiotherapy (Frosina, 2009; Zhang et al., 2010), those conventional therapy often fails to eliminate all self-renewing GSCs completely, and thus residual surviving GSCs are able to repopulate the tumor, causing relapse (Liu et al., 2006; Singh et al., 2004a). Accordingly, we next examined whether treatment with BHP could sensitize GSCs to chemotherapy and radiotherapy. To assess this possibility, we treated sphere-cultured, patient-derived X01GB and X03AOA glioma cells with BHP in combination with either ionizing radiation (10 Gy) or Taxol (500 nM), and evaluated the relative rates of cell death by FACS. As shown in Figure 5, treatment with either ionizing radiation (10 Gy) or Taxol (500 nM) alone did not cause significant cell death, indicating that sphere-cultured X01GB and X03AOA glioma cells are resistant to ionizing radiation and Taxol. Treatment with BHP alone also did not induce death of sphere-cultured X01GB and X03AOA glioma cells at the concentration of 10 μM. However, BHP in combination with ionizing radiation or Taxol dramatically increased cell death compared to treatment with either agent alone (Fig. 5). We next examined whether Bcl-2 family proteins are involved in BHP-induced sensitization of GSCs to ionizing radiation. As shown in Figure 5C, combined treatment with ionizing radiation and BHP led to a decrease of Bcl-2 and Bcl-xL, but not Mcl-1, while either single treatment with ionizing radiation or BHP did not alter the expression levels of Bcl-2, Bcl-xL and Mcl-1 in X01GB glioma cells. Taken together, these data suggest that treatment with BHP enhances the sensitivity of GSCs to ionizing radiation and Taxol through down-regulation of anti-apoptotic proteins, Bcl-2 and Bcl-xL.

DISCUSSION

Gliomas have cellular hierarchies that contain a tumor-initiating GSC population that

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displays self-renewal and drives tumor formation, maintenance and progression (Schatton and Frank, 2008), and contributes to resistance to chemo- and radiotherapy (Bao et al., 2006; Eramo et al., 2006; Rich, 2007). Thus, conventional glioma treatment has limitations with respect to completely eradicating all self-renewing GSCs, and may allow residual surviving GSCs to repopulate another tumor and cause relapse. Accordingly, novel therapeutic strategies that specifically target GSCs have the potential to eradicate malignant tumors more effectively than conventional treatments, and thereby reduce the risk of relapse.

In this study, we assessed the effect of BHP on GSCs. We found that BHP treatment effectively suppresses sphere formation as well as the $CD133^+$ cell population in sphere-cultured glioma cells without causing cell death. Treatment with BHP also suppressed expression of the GSC markers, CD133, Nestin and Musashi-1, in sphere-cultured glioma cells, and enhanced the sensitivity of GSCs to chemotherapy and radiotherapy. These results indicate that BHP may form the basis of a n ovel therapeutic strategy that focuses on eradicating malignant tumors by specifically targeting cancer stem cells. Recent studies have shown that Sox2, a transcription factor that regulates the stemness of several types of endogenous stem cells, in particular neural stem cells (He et al., 2009; Pevny and Nicolis, 2010), is also critical for the stemness of GSCs in gliomas (Gangemi et al., 2009; He et al., 2009; Pevny and Nicolis, 2010). Consistent with these studies, we found that BHP treatment leads to a marked reduction in Sox2 levels in sphere-cultured glioma cells. In addition, we found that BHP suppresses the expression and activation of Notch2 and β-catenin, which play important roles in the maintenance of cancer stem cells in several human cancers such as glioma, colorectal and breast cancers (Farnie and Clarke, 2007; Hudson, 2004; Kanwar et al., 2010; Wang et al., 2010). Collectively, these results suggest that BHP treatment suppresses the GSC population through down-regulation of the stemness-regulating transcription factors, Sox2, Notch2, and β-catenin.

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Previous studies have shown that PI3K-AKT and Ras-Raf-1-ERK signaling pathways are activated in GSCs and are involved in the survival and maintenance of stemness in GSCs (Bleau et al., 2009; Hambardzumyan et al., 2008; Sunayama et al., 2010). These pathways have also been implicated in the resistance of malignant cancer cells to anticancer treatments (Hjelmeland et al., 2010; Tang et al., 2007). In this study, we found that BHP treatment effectively inhibited both PI3K-AKT and Ras-Raf-1-ERK pathways in GSCs. Treatment with BHP led to a decrease in the activity of both PI3K and AKT, and markedly inhibited the interaction between Ras and Raf-1 as well as activation of Raf-1 and ERK in sphere-cultured glioma cells. These results suggest that BHP suppresses GSCs by blocking both PI3K-AKT and Ras-Raf-1-ERK signaling pathways. By previous studies, 2-pyrone derivatives have been implicated in biological processes such as defense against other organisms, as key biosynthetic intermediates, and as metabolites (McGlacken and Fairlamb, 2005). However, its molecular targets are largely unknown. Previously, Zhu *et al* suggested that a 2-pyrone derivative, 4-(hydroxymethly)-5-hydroxy-2H-pyran-2-one is a ligand for GPR12 (Probable G-protein coupled receptor 12) (Lin et al., 2008). In this study, we also observed that a new 2-pyrone derivative, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP), inhibits the interaction of Ras, a small GTP-binding protein with Raf and suppresses GSCs. Thus, our observation together with the previous study suggests that a 2-pyrone derivative may act as an antagonizing ligand for GTP-binding protein.

In this study, BHP-mediated suppression of GSCs was not restricted to the glioma cell line U87MG, but was also observed in patient-derived X01GM and X03AOA glioma cells. Consistent with these findings, BHP treatment dramatically inhibited tumor formation *in vivo*, suggesting its clinical relevance. Collectively, our study shows that BHP suppresses stemness and malignancy of GSCs and enhances the sensitivity of GSCs to anticancer treatments.

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These results suggest that BHP treatment may form the basis of novel therapeutic strategies that seek to eradicate malignant tumors by targeting GSCs.

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Acknowledgments

The authors thank Dr Akio Soeda (Department of Neurological Surgery, University of Virginia, USA) for providing patient-derived glioma cells.

Authorship Contributions

Participated in research design : R.-K. Kim, S.-J. Lee

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Footnotes

This work was supported by the National Nuclear Technology Program [2008-2003935]; and the Converging Research Center Program [2011K000877] in the Ministry of Education, Science and Technology.

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Figure Legends

Fig. 1. BHP suppresses sphere forming glioma cells expressing GSC markers. (A) The chemical structure of 2-pyrone 1α and BHP. (B) Sphere formation in the presence of BHP for 7 days in U87 glioma cell line and patient-derived glioma cells (X01GB, X02GB, and X03AOA). (C) Quantification of CD133 positive cell population by FACS after treatment with BHP for 5 d ays in U87 glioma cells and patient-derived glioma cells (X01GB, X03AOA). (D) Immunoblot analysis of CD133, Nestin and Musashi-1 in U87 gli oma cells and patient-derived glioma cells (X01GB, X03AOA) after treatment with BHP for 5 days. β-actin was used as the loading control. (E) Immunocytochemistry of CD133, Nestin and Musashi-1 in patient-derived glioma cells X01GB after treatment with BHP for 5 days.
Error Bars represent mean ± S.D. of triplicate samples. * $p<0.01$ by one-way ANOVA.

Fig. 2. BHP suppresses self-renewal of GSCs. (A) Clonal analysis of patient-derived glioma cells (X01GB) at single cell level after treatment with BHP for 7 days. (Upper) Images represent changes in sphere sizes in the presence of DMSO or BHP (magnification 200×). (Lower) Number of spheres with a diameter > 20 μm was counted under microscopy in the presence of DMSO or BHP. (B) Immunoblot analysis of Sox2, Notch2 and β-catenin. β-actin was used as the loading control. (C) Immunocytochemistry of Sox2, Notch2 and β-catenin in patient-derived glioma cells X01GB after treatment with DMSO or BHP. Error Bars represent mean ± S.D. of triplicate samples. * $p<0.01$ by one-way ANOVA.

Fig. 3. BHP suppresses the tumorigenic capacity of GSCs. (A) Colony formation in soft-agar in the presence of DMSO or BHP in patient-derived X01GB. (B) Tumor formation in xenograft mice (each group, n=5). DMSO or BHP was administered four times with three days interval after implantation of glioma cells X01GB ($1x10^6$ cells) as indicated.

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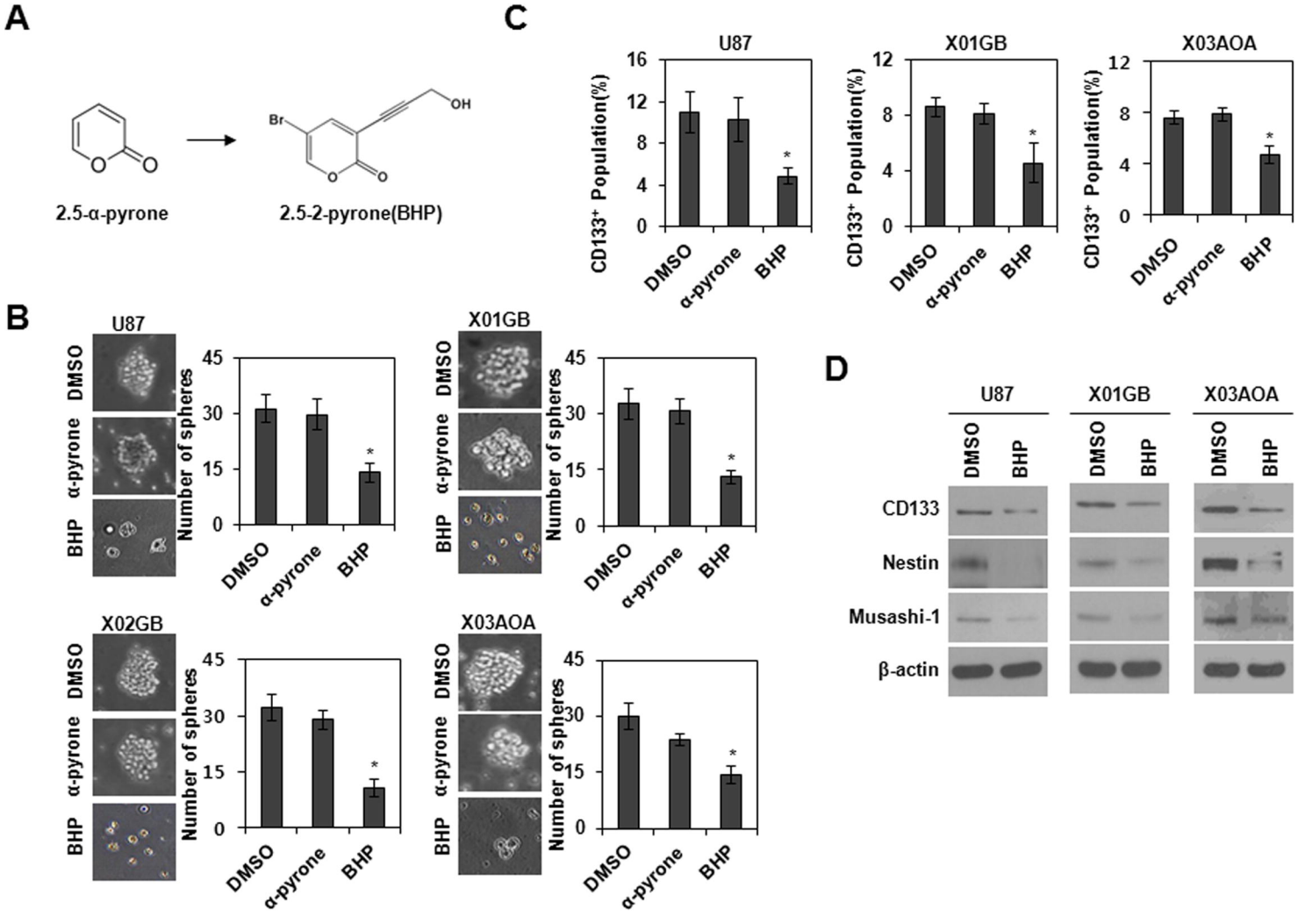
Representative mouse photos are shown at 30 days after implantation (right). (C) Immunohistochemical staining of Nestin in tumor tissues derived from DMSO-treated and BHP-treated mice. Error Bars represent mean ± S.D. of triplicate samples. * $p<0.01$.

Fig. 4. BHP inhibits PI3K-AKT and Ras-Raf-1-ERK signaling pathways. (A) PI3 and AKT kinase assay and immunoblot analysis of PTEN, total form of AKT and phosphorylated form of AKT at Ser473 or Thr308 after treatment with DMSO or BHP. PIP and GSK3β indicate the substrates for kinase assay. (B) Activated Ras affinity precipitation assay using Raf-1 agarose beads and Raf-1 and ERK kinase assay after treatment with DMSO or BHP. Immunoblot analysis of Pan-Ras, Raf-1, ERK1/2 and phosphorylated ERK1/2 after treatment with DMSO or BHP. MBP indicates the substrate for kinase assay. β-actin was used as the loading control.

Fig. 5. BHP sensitizes GSCs to taxol and ionizing radiation. (A-B) Quantification of cell death by FACS using propidium iodide staining. BHP treatment sensitizes GSCs to ionizing radiation (10 Gy) (A) and Taxol (500 nM) (B) in both patient-derived glioma cells X01GB and X03AOA. Single treatment with either BHP or ionizing radiation (10 Gy) or Taxol (500 nM) did not cause significant cell death. (C) Immunoblot analysis for Bcl-2 family proteins Bcl-2, Bcl-xL and Mcl-1 after single treatment with BHP or ionizing radiation, or combined treatment with BHP and ionizing radiation in X01GB cells. β-actin was used as the loading control. Error Bars represent mean ± S.D. of triplicate samples. * $p<0.001$.

Fig. 1



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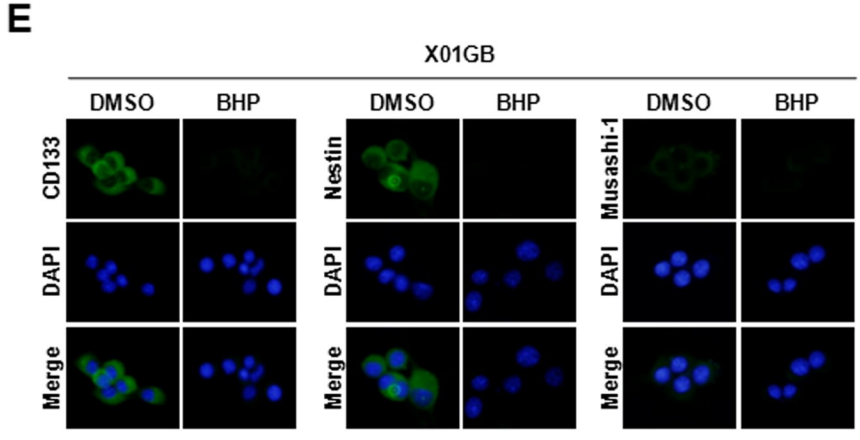

Fig. 2

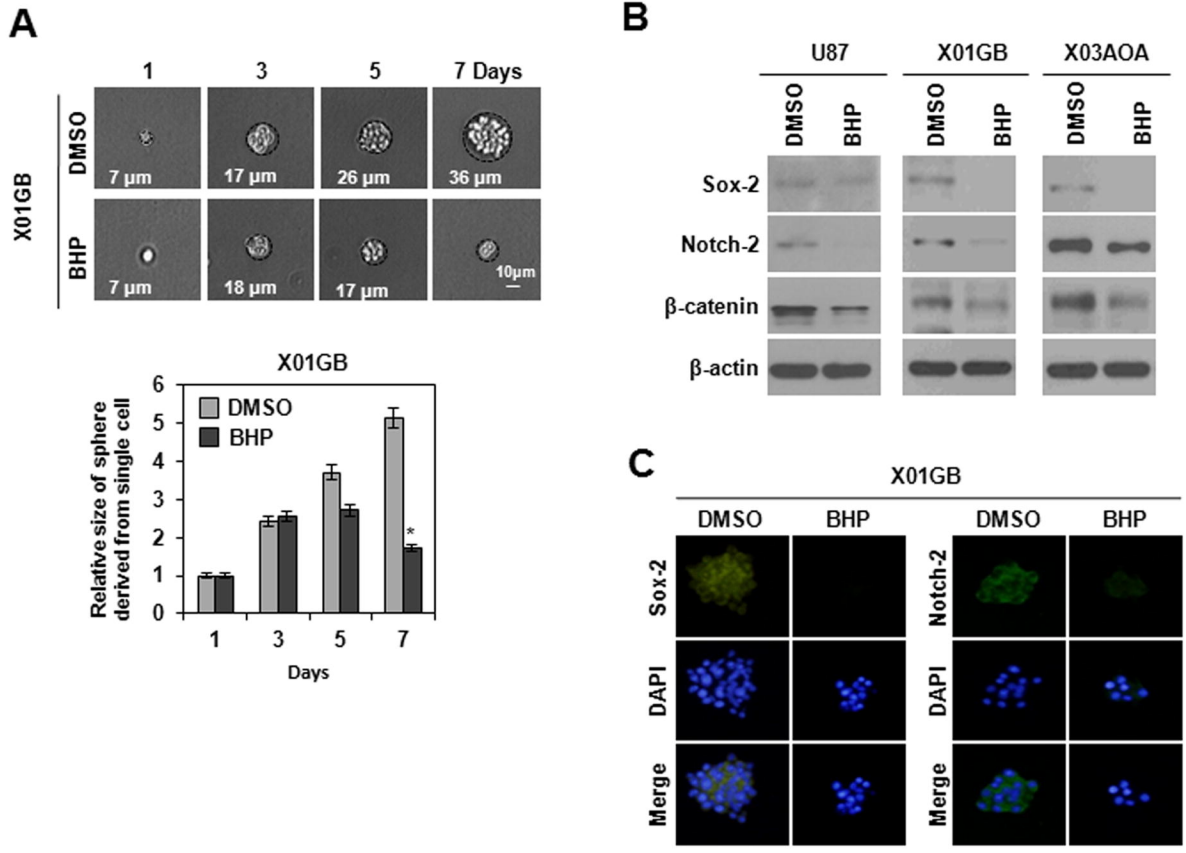

Fig. 3

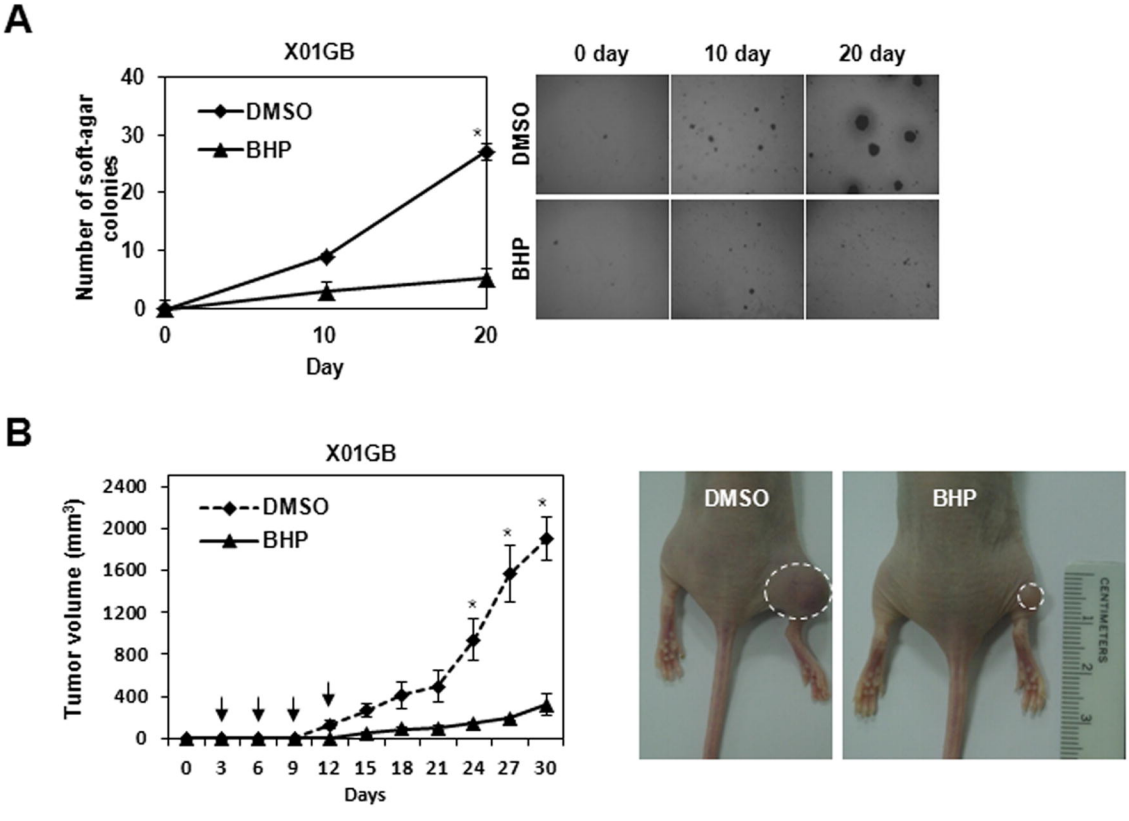

Fig. 3-1

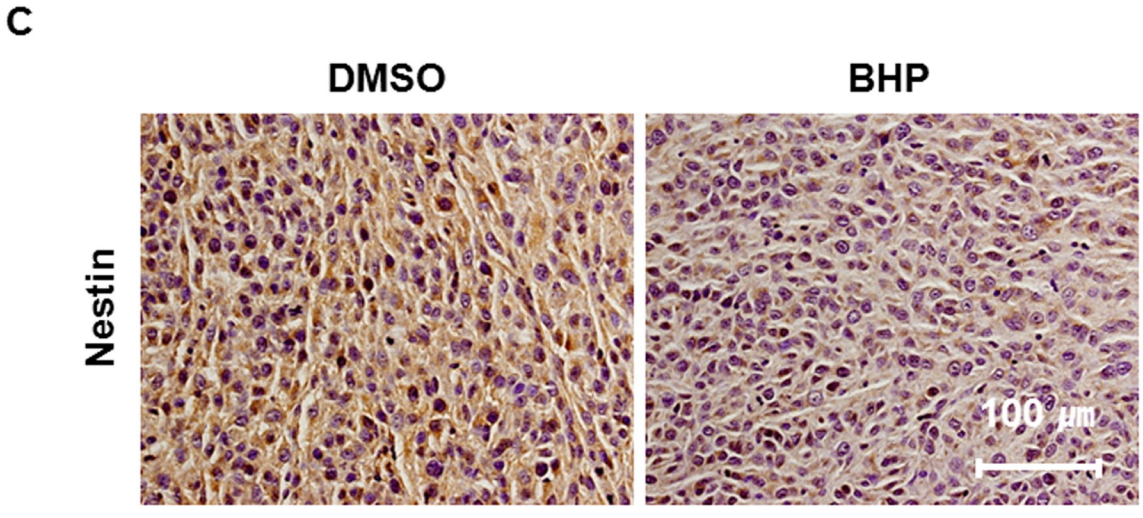

Fig. 4

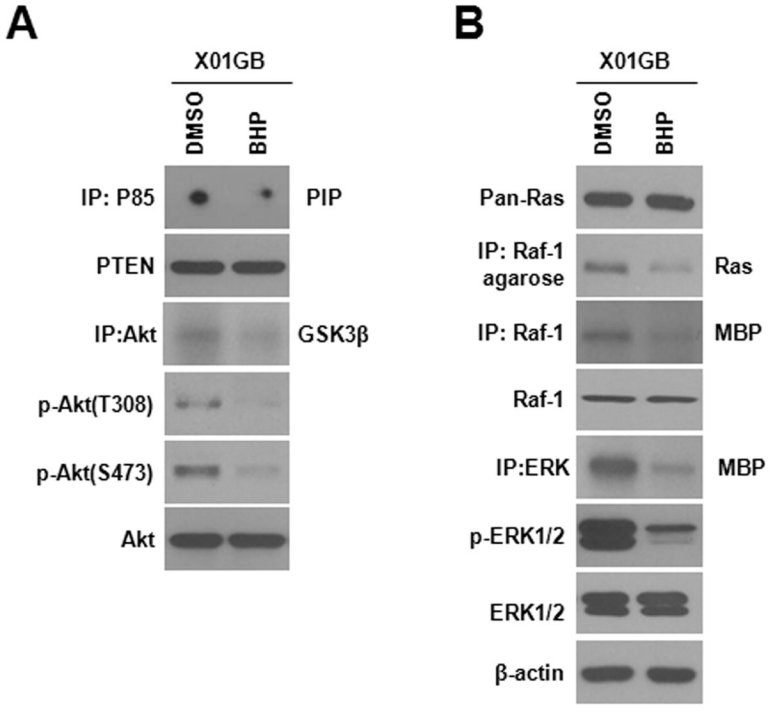

Fig. 5