1521-0111/12/8203-400–407$25.00
MOLECULAR PHARMACOLOGY
Vol. 82, No. 3
78402/3786727

Mol Pharmacol 82:400–407, 2012

A New 2-Pyrone Derivative, 5-Bromo-3-(3-hydroxyprop-1-ynyl)-2*H*-pyran-2-one, Suppresses Stemness in Glioma Stem-Like Cells[S]

Rae-Kwon Kim, Min-Jung Kim, Chang-Hwan Yoon, Eun-Jung Lim, Ki-Chun Yoo, Ga-Haeng Lee, Young-Heon Kim, Hyeonmi Kim, Yeung Bae Jin, Yoon-Jin Lee, Cheon-Gyu Cho, Yeong Seok Oh, Myung Chan Gye, Yongjoon Suh, and Su-Jae Lee

Department of Chemistry and Research Institute for Natural Sciences (R.-K.K., M.-J.K., C.-H.Y., E.-J.L., K.-C.Y., G.-H.L., Y.-H.K., H.K., C.-G.C., Y.S., S.-J.L.) and Department of Life Science, College of Natural Sciences (Y.S.O., M.C.G.), Hanyang University, Seoul, Korea; and Division of Radiation Effects, Korea Institute of Radiological and Medical Sciences, Seoul, Korea (Y.B.J., Y.-J.L.)

Received February 20, 2012; accepted May 30, 2012

ABSTRACT

Glioma cells with stem cell properties, termed glioma stem-like cells (GSCs), have been linked to tumor formation, maintenance, and progression and are responsible for the failure of chemotherapy and radiotherapy. Because conventional glioma treatments often fail to eliminate GSCs completely, residual surviving GSCs are able to repopulate the tumor. Compounds that target GSCs might be helpful in overcoming resistance to anticancer treatments in human brain tumors. In this study, we showed that 5-bromo-3-(3-hydroxyprop-1-ynyl)-2*H*-pyran-2-one (BHP), a new 2-pyrone derivative, suppressed the 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. 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 phosphoinositide 3-kinase/Akt and Ras/Raf-1/extracellular signal-regulated kinase signaling, which suggests that one or both of these pathways are involved in BHP-induced suppression of GSCs. In addition, treatment with BHP effectively sensitized GSCs to chemotherapy and radiotherapy. Taken together, these results indicate that BHP targets GSCs and enhances their sensitivity to anticancer treatments and suggest that BHP treatment may be useful for treating brain tumors by eliminating GSCs.

Introduction

Glioma, the most common primary brain tumor, has aggressive and highly invasive characteristics and is highly lethal, with median survival times of 12 to 15 months after 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 chemotherapy and radiotherapy (Bao et al., 2006; Eramo et al., 2006; Rich, 2007). 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,

This work was supported by the research fund of Hanyang University (HY-2012-N).

R.-K.K. and M.-J.K. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
http://dx.doi.org/10.1124/mol.112.078402.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: GSC, glioma stem-like cell; BHP, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2*H*-pyran-2-one; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; TLC, thin-layer chromatography; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; PE, phycoerythrin; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DMSO, dimethylsulfoxide; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline.

GSCs are considered novel therapeutic targets for glioma treatment. Because GSCs are resistant to chemotherapy and radiotherapy (Bao et al., 2006; Eramo et al., 2006; Rich, 2007), however, conventional cancer treatments often fail to eradicate them completely, and residual surviving GSCs are able to repopulate the tumor, causing relapse (Singh et al., 2004a; Liu et al., 2006). Novel therapeutic strategies that specifically target GSCs are urgently needed to eradicate malignant tumors more effectively and to reduce the risk of relapse.

2-Pyrones, which are six-membered, cyclic, unsaturated esters that are highly abundant in bacterial, plant, and animal systems (Jensen and Fenical, 1994; Faulkner, 2001), have been implicated in many different biological processes, including defense against other organisms (McGlacken and Fairlamb, 2005). 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 also display antitumor and HIV-inhibitory activities (Thaisrivongs et al., 1996; Poppe et al., 1997; Turner et al., 1998), which reinforces their potential medicinal importance. In addition, an investigation of the anticancer properties of tricyclic 2-pyrones showed that these compounds prevented DNA synthesis and growth in human leukemic cells in vitro (Trachootham et al., 2008). Despite such in vitro evidence 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 remains unknown.

Here we show that 5-bromo-3-(3-hydroxyprop-1-ynyl)-2*H*-pyran-2-one (BHP), a new 2-pyrone derivative, suppresses the 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 BHP. A new 2-pyrone derivative, BHP, was constructed as described earlier (Fig. 1A) (McGlacken and Fairlamb, 2005).

Chemical Reagents and Antibodies. Polyclonal antibodies to Ser473-phosphorylated Akt, Thr308-phosphorylated Akt, phosphorylated extracellular signal-regulated kinase (ERK) 1/2, and ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA). Polyclonal antibodies to CD133 were purchased from Abcam Inc. (Cambridge, MA), and polyclonal antibodies to nestin, Musashi-1, and Notch2 were purchased from Millipore Corp. (Billerica, MA). Polyclonal antibodies to Akt and Raf-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 4,6-Diamidino-2-phenylindole, epidermal growth factor (EGF), and monoclonal antibodies to β-actin were obtained from Sigma-Aldrich (St. Louis, MO). Basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). Alexa Fluor 488-conjugated anti-mouse IgG, Alexa Fluor 488-conjugated anti-rabbit IgG, and B27 supplement were purchased from Invitrogen (Carlsbad, CA). PE-conjugated anti-CD133 (clone 293C3) and PE-conjugated mouse IgG2b were purchased from Miltenyi Biotec Inc. (Auburn, CA). 2-(4-Morpholino)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), an inhibitor specific for PI3K, was obtained from Calbiochem (San Diego, CA). Temozolomide was purchased from Santa Cruz Biochemicals.

Cell and Sphere Cultures. U87MG and U373MG glioma cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in the recommended medium containing 10% fetal bovine serum; with these medium conditions, glioma cells were grown as monolayers. Patient-derived glioma stem-like cell lines X01GB, X02GB, and X03AOA were established from acutely resected human tumor tissues (Soeda et al., 2008, 2009). The X01GB line originated from a 68-year-old woman with glioblastoma multiforme. The X02GB line originated from a man with glioblastoma multiforme. The X03AOA line was derived from a woman with anaplastic oligoastrocytoma. Each patient provided written informed consent for the use of tumor tissue.

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

FACS Analyses. Cells were dissociated and resuspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mM EDTA. Cells were stained with PE-conjugated anti-CD133 antibody (1:100; Miltenyi Biotech) or isotype control antibody (PE-conjugated mouse IgG2b, 1:100; Miltenyi Biotech) and were analyzed with a FACSCalibur system (BD Biosciences, San Jose, CA) with CellQuest software.

Quantification of Cell Death. Cell death was measured through FACS analysis using propidium iodide staining. Cells were harvested through trypsinization, washed with PBS, and then incubated with propidium iodide (50 ng/ml) for 5 min at room temperature. Cells (10,000 cells per sample) were analyzed with a FACSCalibur system with CellQuest software.

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

Western Blot Analyses. Cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.1% Nonidet P-40] supplemented with protease inhibitors. Proteins were separated with SDS-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and were incubated with primary antibodies overnight at 4°C. The blots were developed with peroxidase-conjugated secondary antibody, and proteins were observed by using enhanced chemiluminescence procedures (GE Healthcare), according to the manufacturer's protocol.

PI3K Assays. Cell lysates (300 μg in 500 μl) were subjected to immunoprecipitation with anti-p85 antibodies (Millipore). The precipitates were washed twice with 1% Nonidet P-40 in PBS, followed by PBS, 0.1 M Tris-HCl, pH 7.5, and 0.5 M LiCl, and finally 25 mM HEPES, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The precipitates were resuspended in 50 μl of presonicated phosphatidylinositol substrate solution and were incubated for 10 min at room temperature. Each sample was labeled with 10 μCi of [^{32}P]ATP for 10 min at room temperature. Reactions were stopped with the addition of 100 μl of chloroform/methanol/HCl (50:100:1). Lipids were extracted with 200 μl of 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 of methanol/1 M HCl (1:1), and the upper phase was discarded. The lipid fraction was dried, resuspended in 20 μl of chloroform, and applied to a silica gel thin-layer chromatography (TLC) plate impregnated with 1% potassium oxalate. Phospho-

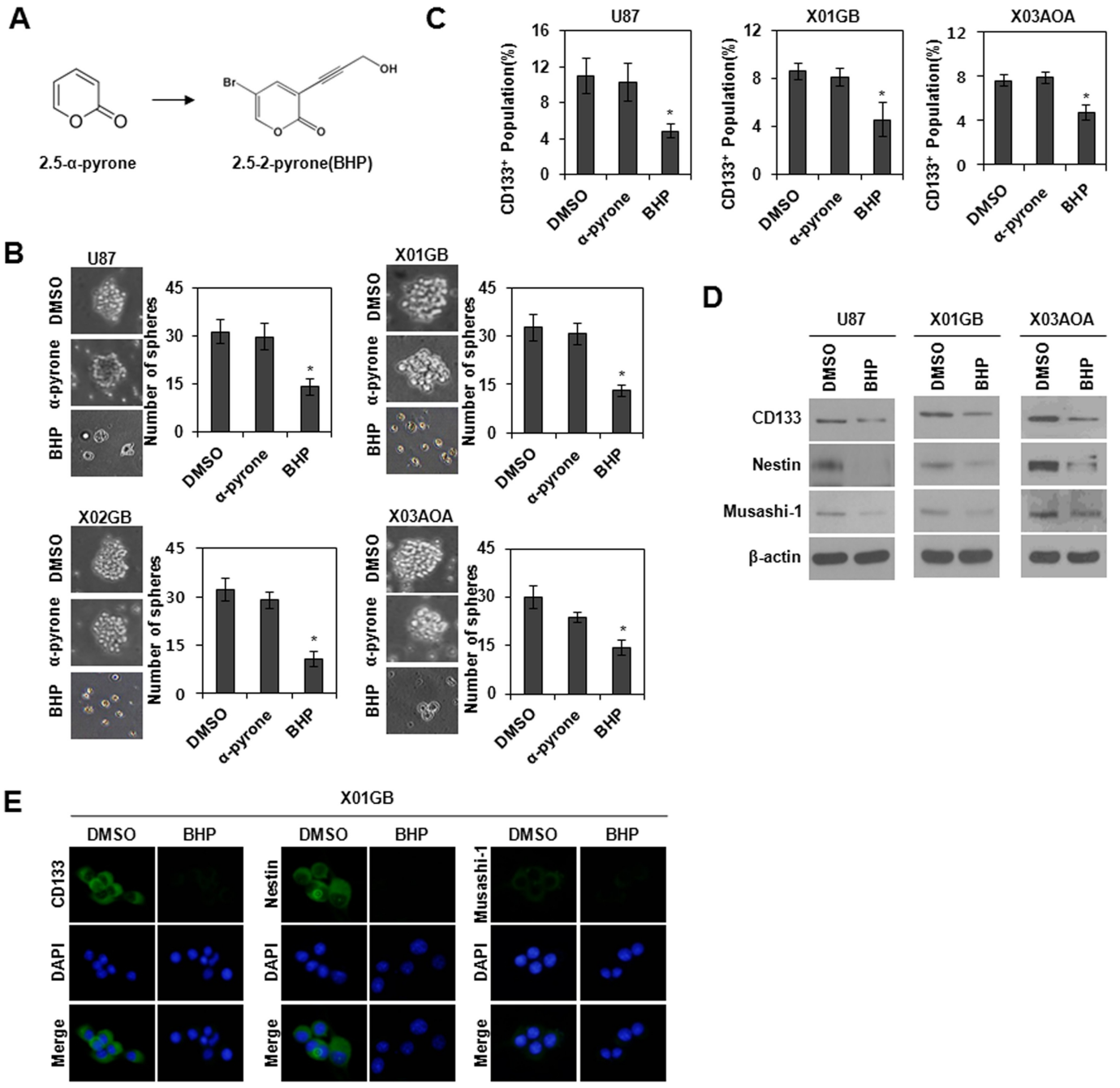

Fig. 1. BHP suppresses sphere-forming glioma cells expressing GSC markers. A, chemical structures of 2-pyrone 1α and BHP. B, sphere formation in the presence of BHP for 7 days in the U87 glioma cell line and patient-derived glioma cells (X01GB, X02GB, and X03AOA). C, quantification through FACS analysis of $CD133^+$ cell populations in U87 glioma cells and patient-derived glioma cells (X01GB and X03AOA) after treatment with BHP for 5 days. D, immunoblot analyses of CD133, nestin, and Musashi-1 in U87 glioma cells and patient-derived glioma cells (X01GB and X03AOA) after treatment with BHP for 5 days. β-Actin was used as the loading control. E, immunocytochemical analyses of CD133, nestin, and Musashi-1 in patient-derived glioma cells (X01GB) after treatment with BHP for 5 days. DAPI, 4,6-diamidino-2-phenylindole. Data are the mean ± S.D. of triplicate samples. ∗, $p < 0.01$, one-way analysis of variance.

lipids were resolved through TLC in a freshly prepared chloroform/methanol/ammonia/water (30:23.5:1:5.65) solution for 45 min at room temperature, in a closed glass chamber (Hyun et al., 2011).

Raf-1 Kinase Assays. Raf-1 kinase activity was measured as the ability of immunoprecipitated enzyme to phosphorylate Raf-1 substrate. Cell lysates were prepared in lysis buffer containing 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% (v/v) Triton X-100, and protease inhibitors. Lysates were incubated with anti-Raf-1 antibodies for 2 h at 4°C, and the immunocomplexes were precipitated with protein A-Sepharose. Kinase reactions were performed with 20 mM HEPES, pH 7.4, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μCi of [γ-^{32}P]ATP, and 1 mg/ml myelin basic protein (substrate). Proteins were separated on SDS-polyacrylamide gels, and bands were detected autoradiographically.

Activated Ras Affinity Precipitation Assays. Activated Ras affinity precipitation assays were performed according to the manufacturer's protocol. Cell lysates were incubated with 5 μg of Raf-1 RBD agarose beads (Millipore) for 30 min at 4°C. After three extensive washes of the agarose beads 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 Na_3VO_4, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM NaF], activated Ras (GTP-Ras) bound to Raf-1 RBD agarose beads was released with the addition of SDS-polyacrylamide gel electrophoresis sample buffer. The amount of activated Ras was determined through immunoblotting with a monoclonal pan-Ras antibody.

Soft-Agar Colony Formation Assays. To examine anchorage-independent growth, cells (2×10^4 cells per ml) were suspended with 0.4% agarose in serum-free Dulbecco's modified Eagle's medium/F12 medium supplemented with B27, bFGF, and EGF, were seeded onto 60-mm dishes precoated with 0.8% agar in complete growth medium, and were incubated at 37°C in 5% CO_2. After 12 to 30 days, colonies were photographed and counted in four randomly chosen fields; results are expressed as means of triplicate determinations that were representative of two independent experiments.

Tumor Xenografts in Nude Mice. Patient-derived X01GB cells (1×10^6 cells) were injected subcutaneously into the right flank of athymic BALB/c female nude mice (5 weeks of age; Charles River Laboratories, Wilmington, MA). The mice were randomly distributed into two groups ($n = 5$ in each group), which were treated with intraperitoneal injections of DMSO or 100 μl of 15 μM BHP (i.e., 1.32×10^{-2} mg of BHP per kg of mouse weight) every 3 days for three cycles. Tumor sizes were measured with calipers (calculated volume = shortest diameter^2 × longest diameter/2) at 3-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 abides by the Institute of Laboratory Animal Resources guide.

Irradiation. Cells were plated in 60-mm dishes and incubated in culture medium at 37°C in humidified 5% CO_2. Cells were exposed to γ-rays by using a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Mississauga, Canada), at a dose rate of 3.81 Gy/min.

Immunocytochemical Studies. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. After cell fixation, cells were incubated overnight at 4°C with the appropriate primary antibodies in a solution of PBS with 1% bovine serum albumin and 0.1% Triton X-100. The antibodies used were as follows: anti-human CD133 (rabbit polyclonal antibody, 1:200), anti-nestin (rabbit polyclonal antibody, 1:200), anti-Musashi-1 (rabbit polyclonal antibody, 1:200), anti-Sox2 (rabbit polyclonal antibody, 1:200), and anti-Tuj1 (mouse polyclonal antibody, 1:200). Staining was observed by using Alexa Fluor 488-conjugated anti-rabbit IgG or anti-mouse IgG (Invitrogen). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Stained cells were observed with a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

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

Anti-human nestin (rabbit polyclonal antibody; Millipore) diluted 1:1000 in blocking solution was applied to the sections and incubated overnight at 4°C. For the negative control samples, rabbit IgG (ab27478; Abcam) was used instead of primary antibody. After washing with PBS, a 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 with PBS, avidin/biotin complex reagent was applied to the sections and incubated for 30 min. After washing with PBS, the color reaction was developed with 3,3′-diaminobenzidine (Vector Laboratories) and the slides were washed with PBS.After counterstaining with hematoxylin and clearing with a graded ethanol series and xylene, the sections were mounted with Canada balsam. Observation and photography were performed with a IX71 microscope (Olympus) equipped with a DP71 digital imaging system (Olympus).

Statistical Analyses. All experimental data are reported as means, and error bars represent experimental S.E. values. Statistical analysis was performed with the nonparametric Student's t test.

Results

BHP Effectively Suppresses the GSC Population. In previous studies, we showed that GSCs could be enriched by culturing glioma cells in serum-free medium supplemented with the growth factors EGF and bFGF. Under such conditions, a fraction of glioma cells continued to proliferate and formed 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 gliomas. We first determined the optimal concentration of BHP by treating patient-derived glioma cells (X01GB and X03AOA) cultured under sphere-forming conditions with various concentrations of BHP (0–20 μM) and analyzing sphere formation and cell death. Treatment of sphere-forming glioma cells with BHP at <10 μM most effectively suppressed sphere formation without producing significant cell death (Supplemental Fig. 1). Increased rates of cell death were observed with concentrations of >15 μM (Supplemental Fig. 1A). Therefore, we decided to use 10 μM BHP in this study, although 5 μM BHP also yielded 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. Treatment with BHP dramatically attenuated sphere formation in both the U87 glioma cell line and patient-derived glioma cells (X01GB, X02GB, and X03AOA), compared with treatment with DMSO (vehicle) or α-pyrone (Fig. 1, A and B).

Because GSCs are enriched for a subpopulation of $CD133^+$ glioma cells with greater tumorigenic potential than $CD133^-$ cells (Singh et al., 2004b), we measured the $CD133^+$ cell populations in U87, X01GB, and X03AOA cell lines after treatment with BHP. FACS analysis showed that treatment of sphere-forming glioma cells with BHP significantly suppressed the $CD133^+$ cell population, compared with DMSO or α-pyrone (Fig. 1C). Immunoblotting and immunocytochemical analyses showed that treatment with BHP caused down-regulation of not only CD133 but also other stem cell markers, namely, nestin and Musashi-1 (Fig. 1, D and E). Taken together, these results indicate that treatment with BHP effectively suppresses the stem-like cell population in gliomas.

BHP Suppresses Self-Renewal of GSCs. Cancer stem cells are characterized by their strong tumorigenic properties and ability to self-renew. To determine whether BHP treatment suppresses self-renewal in GSCs, we treated sphere-forming glioma cells with BHP and monitored clone formation for 7 days by using a single-cell clonogenic assay. Treatment of patient-derived X01GB cells with BHP sup-

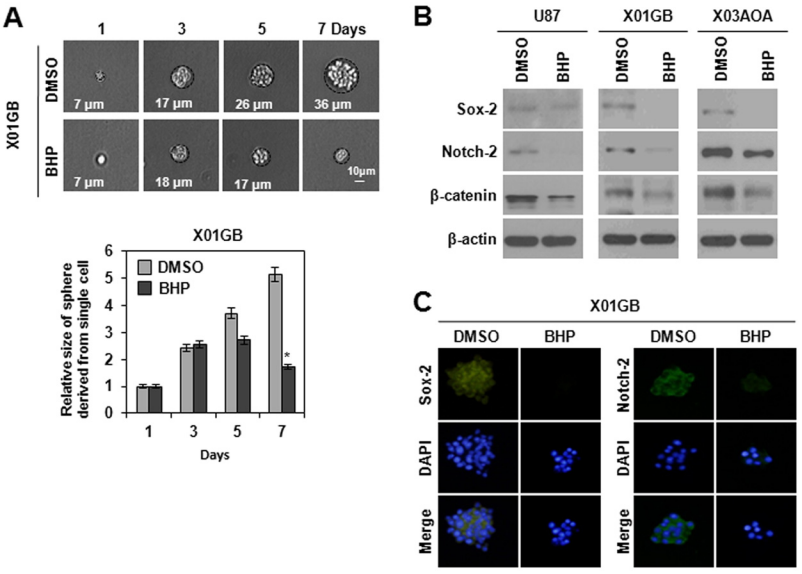

Fig. 2. BHP suppresses self-renewal of GSCs. A, clonal analysis of patient-derived glioma cells (X01GB) at the single-cell level, after treatment with BHP for 7 days. Top, changes in sphere sizes in the presence of DMSO or BHP (original magnification, 200×). Bottom, numbers of spheres with diameters of >20 μm in the presence of DMSO or BHP, counted microscopically. B, immunoblot analyses of Sox2, Notch2, and β-catenin. β-Actin was used as the loading control. C, immunocytochemical analyses of Sox2, Notch2, and β-catenin in patient-derived glioma cells (X01GB) after treatment with DMSO or BHP. DAPI, 4,6-diamidino-2-phenylindole. Data represent the mean ± S.D. of triplicate samples. *, $p < 0.01$, one-way analysis of variance.

pressed clone formation, dramatically reducing the size of clones, compared with control samples (Fig. 2A).

Because BHP treatment decreased the self-renewal capacity of sphere-forming glioma cells and the populations of cells positive for the stem cell markers CD133, nestin, and Musashi-1, we examined the expression levels of the stemness-regulating genes Sox2, Notch2, and β-catenin after treatment with BHP. As shown in Fig. 2B, the expression levels of Sox2, Notch2, and β-catenin were markedly downregulated by BHP treatment (Fig. 2B). Immunocytochemical analyses confirmed that BHP effectively suppressed Sox2 and Notch2 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.

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. Therefore, colony-forming assays in soft agar are widely used to measure tumorigenic capacity in vitro, and results are considered good predictors of in vivo tumor-forming ability. As shown in Fig. 3A, BHP treatment strongly inhibited colony formation by sphere-derived cells in soft agar. To confirm the inhibitory effect of BHP on the tumorigenic properties of GSCs in vivo, we injected sphere-cultured X01GB glioma cells (1×10^6 cells per mouse) subcutaneously into nude mice, treated mice with BHP administered intraperitoneally, and then examined tumor formation over the course of 30 days. As shown in Fig. 3B, tumor formation by sphere-derived cells was significantly attenuated with BHP treatment. Because BHP treatment suppressed GSCs without yielding significant cell death in vitro, we next examined whether the smaller tumor sizes were attributable to the decrease in the GSC population in mice treated with BHP. We performed immunohistochemical analyses of the tumors from BHP-treated and DMSO-treated mice. Levels of the protein nestin, a previously known GSC marker, were drastically lower in tumors derived from BHP-treated mice, compared with DMSO-treated mice (Fig. 3C). Taken together, these results suggest that BHP suppresses tumorigenic capacity by targeting GSCs.

BHP Inhibits PI3K/Akt and Ras/Raf-1/ERK Signaling Pathways. Because PI3K/Akt and Ras/Raf-1/ERK signaling pathways were shown to regulate glioma stemness (Hambardzumyan et al., 2008; Bleau et al., 2009; Sunayama et al., 2010), we examined whether BHP acts by inhibiting these pathways. We treated sphere-cultured, patient-derived glioma cells (X01GB) with BHP and analyzed the phosphorylation status of Akt and ERK1/2. Treatment with BHP attenuated the phosphorylation of Akt at both Ser473 and Thr308. The PI3K assay was performed by immunoprecipitating a subunit of PI3K with anti-p85 antibody. Activity was then measured on the basis of phospholipids generated in the presence of the substrate phosphatidylinositol, by using TLC. As shown in Fig. 4A, treatment of patient-derived X01GB cells with BHP decreased PI3K activity without altering phosphatase and tensin homolog expression levels. BHP treatment also decreased the phosphorylation of Akt at Thr308 and Ser473. 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 with BHP treatment. Moreover, the kinase activity of ERK1/2 was suppressed by treatment with BHP, with decreased levels of the active phosphorylated form of 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

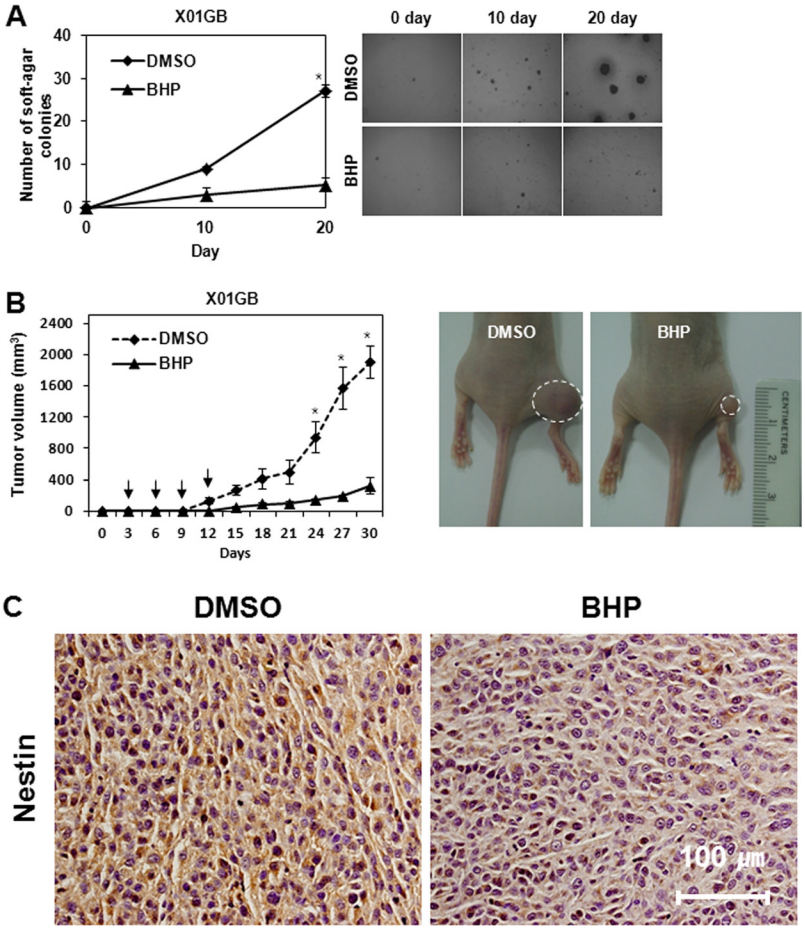

Fig. 3. BHP suppresses the tumorigenic capacity of GSCs. A, colony formation in soft agar by patient-derived X01GB cells in the presence of DMSO or BHP. B, tumor formation in xenograft mice ($n = 5$ in each group). DMSO or BHP was administered four times, at 3-day intervals, after implantation of glioma X01GB cells (10^6 cells), as indicated. Representative mouse photographs obtained 30 days after implantation are shown. C, immunohistochemical staining of nestin in tumor tissues derived from DMSO-treated and BHP-treated mice. Data represent the mean ± S.D. of triplicate samples. *, $p < 0.01$.

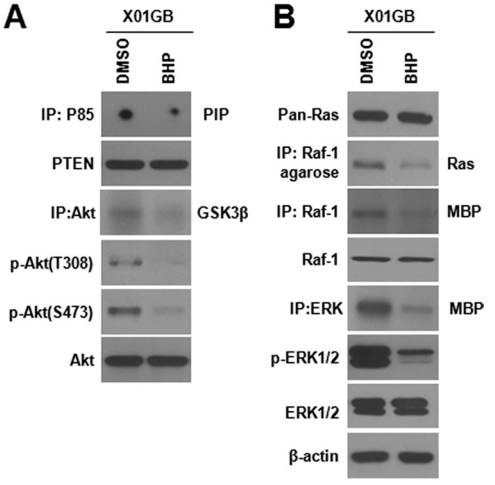

Fig. 4. BHP inhibits PI3K/Akt and Ras/Raf-1/ERK signaling pathways. A, PI3K and Akt kinase assays and immunoblot analyses of phosphatase and tensin homolog (PTEN), total Akt, and Akt phosphorylated at Ser473 or Thr308 after treatment with DMSO or BHP. Phosphatidylinositol (PIP) and glycogen synthase kinase-3β (GSK-3β) are substrates for the kinase assay. IP, immunoprecipitation. B, activated Ras affinity precipitation assay using Raf-1-agarose beads, Raf-1 and ERK kinase assays after treatment with DMSO or BHP, and immunoblot analyses of pan-Ras, Raf-1, ERK1/2, and phosphorylated ERK1/2 after treatment with DMSO or BHP. MBP, myelin basic protein (kinase assay substrate). β-Actin was used as the loading control.

treatment consists of maximal surgical resection, radiotherapy, and concomitant adjuvant chemotherapy with temozolomide or paclitaxel (Taxol). Because GSCs are reported to exhibit resistance to chemotherapy and radiotherapy (Frosina, 2009; Zhang et al., 2010), however, such conventional therapy often fails to eliminate all self-renewing GSCs; residual surviving GSCs are able to repopulate the tumor, causing relapse (Singh et al., 2004a; Liu et al., 2006). We 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 paclitaxel (500 nM) and evaluated the relative rates of cell death through FACS analysis. As shown in Fig. 5, treatment with either ionizing radiation (10 Gy) or paclitaxel (500 nM) alone did not cause significant cell death, which indicated that sphere-cultured X01GB and X03AOA glioma cells were resistant to ionizing radiation and paclitaxel. Treatment with BHP alone at 10 μM also did not induce the death of sphere-cultured X01GB and X03AOA glioma cells. BHP in combination with ionizing radiation or paclitaxel dramatically increased cell death rates, however, compared with treatment with either agent alone (Fig. 5). We next examined whether Bcl-2 family proteins were involved in BHP-induced sensitization of GSCs to ionizing radiation. As shown in Fig. 5C, combined treatment with ionizing radiation and BHP led to decreases in the levels of Bcl-2 and Bcl-xL but not Mcl-1, whereas single treatment with either ionizing radiation or BHP did not alter 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

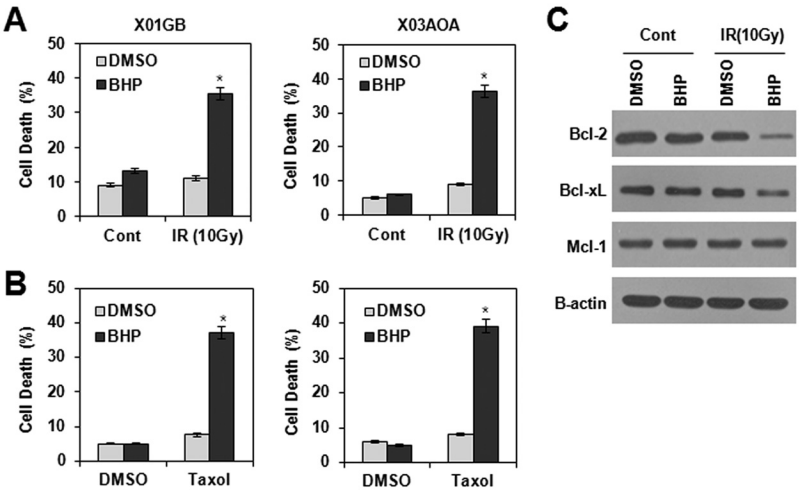

Fig. 5. BHP sensitizes GSCs to paclitaxel and ionizing radiation. A and B, quantification of cell death through FACS analysis with propidium iodide staining. BHP treatment sensitized GSCs to ionizing radiation (IR) (10 Gy) (A) and paclitaxel (Taxol; 500 nM) (B) in patient-derived glioma cells (X01GB and X03AOA). Single treatment with BHP, ionizing radiation (10 Gy), or paclitaxel (500 nM) did not cause significant cell death. Cont, control. C, immunoblot analyses of the 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. Data represent the mean ± S.D. of triplicate samples. *, $p < 0.001$.

paclitaxel through down-regulation of the antiapoptotic proteins Bcl-2 and Bcl-xL.

Discussion

Gliomas have cellular hierarchies that contain a tumor-initiating GSC population that displays self-renewal, drives tumor formation, maintenance, and progression (Schatton and Frank, 2008), and contributes to resistance to chemotherapy and radiotherapy (Bao et al., 2006; Eramo et al., 2006; Rich, 2007). Conventional glioma treatment has limitations with respect to eradicating all self-renewing GSCs completely and may allow residual surviving GSCs to repopulate the tumor, causing relapse. Novel therapeutic strategies that specifically target GSCs have the potential to eradicate malignant tumors more effectively than conventional treatments, thereby reducing the risk of relapse.

In this study, we assessed the effect of BHP on GSCs. We found that BHP treatment effectively suppressed sphere formation and 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 novel therapeutic strategy that focuses on eradicating malignant tumors by specifically targeting cancer stem cells. Studies showed that Sox2, a transcription factor that regulates the stemness of several types of endogenous stem cells, particularly 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 those studies, we found that BHP treatment led to a marked reduction in Sox2 levels in sphere-cultured glioma cells. We also found that BHP suppressed 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 gliomas, colorectal cancers, and breast cancers (Hudson, 2004; Farnie and Clarke, 2007; 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.

Previous studies showed 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 (Hambardzumyan et al., 2008; Bleau et al., 2009; Sunayama et al., 2010). These pathways have also been implicated in the resistance of malignant cancer cells to anticancer treatments (Tang et al., 2007; Hjelmeland et al., 2010). 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 decreases in the activities 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. In previous studies, 2-pyrone derivatives were implicated as factors in biological processes such as defenses against other organisms, as key biosynthetic intermediates, and as metabolites (McGlacken and Fairlamb, 2005); however, the molecular targets are largely unknown. It was suggested previously that a 2-pyrone derivative, 4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one, is a ligand for probable G-protein-coupled receptor 12 (Lin et al., 2008). In this study, we observed that a new 2-pyrone derivative, BHP, inhibited the interaction of Ras (a small GTP-binding protein) with Raf and suppressed GSCs. Our observations, together with those of the previous study, suggest that a 2-pyrone derivative may act as an antagonizing ligand for GTP-binding proteins.

In this study, BHP-mediated suppression of GSCs was not restricted to the glioma cell line U87MG but also was observed with patient-derived X01GM and X03AOA glioma cells. Consistent with these findings, BHP treatment dramatically inhibited tumor formation in vivo, which suggests its clinical relevance. Our study shows that BHP suppresses the stemness and malignancy of GSCs and enhances the sensitivity of GSCs to anticancer treatments. These results suggest that BHP treatment may form the basis of novel therapeutic strategies that seek to eradicate malignant tumors by targeting GSCs.

Acknowledgments

We thank Dr. Akio Soeda (Department of Neurological Surgery, University of Virginia, Charlottesville, VA) for providing patient-derived glioma cells.

Authorship Contributions

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

Conducted experiments: R.-K. Kim, M.-J. Kim, Yoon, Lim, Yoo, G.-H. Lee, Y.-H. Kim, and H. Kim.

Contributed new reagents or analytic tools: Jin, Y.-J. Lee, and Oh.

Performed data analysis: R.-K. Kim, M.-J. Kim, Cho, Gye, Suh, and S.-J. Lee.

Wrote or contributed to the writing of the manuscript: Suh and S.-J. Lee.

References

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, and Rich JN (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444:**756–760.

Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, and Holland EC (2009) PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell* **4:**226–235.

Dickinson JM (1993) Microbial pyran-2-ones and dihydropyran-2-ones. *Nat Prod Rep* **10:**71–98.

Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, Pilozzi E, Larocca LM, Peschle C, and De Maria R (2006) Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* **13:**1238–1241.

Farnie G and Clarke RB (2007) Mammary stem cells and breast cancer—role of Notch signalling. *Stem Cell Rev* **3:**169–175.

Faulkner DJ (2001) Marine natural products. *Nat Prod Rep* **18:**1–49.

Frosina G (2009) DNA repair and resistance of gliomas to chemotherapy and radiotherapy. *Mol Cancer Res* **7:**989–999.

Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, Ravetti GL, Zona GL, Daga A, and Corte G (2009) SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. *Stem Cells* **27:**40–48.

Hambardzumyan D, Becher OJ, Rosenblum MK, Pandolfi PP, Manova-Todorova K, and Holland EC (2008) PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma in vivo. *Genes Dev* **22:**436–448.

He S, Nakada D, and Morrison SJ (2009) Mechanisms of stem cell self-renewal. *Annu Rev Cell Dev Biol* **25:**377–406.

Hjelmeland AB, Wu Q, Wickman S, Eyler C, Heddleston J, Shi Q, Lathia JD, Macswords J, Lee J, McLendon RE, et al. (2010) Targeting A20 decreases glioma stem cell survival and tumor growth. *PLoS Biol* **8:**e1000319.

Hudson DL (2004) Epithelial stem cells in human prostate growth and disease. *Prostate Cancer Prostatic Dis* **7:**188–194.

Hyun KH, Yoon CH, Kim RK, Lim EJ, An S, Park MJ, Hyun JW, Suh Y, Kim MJ, and Lee SJ (2011) Eckol suppresses maintenance of stemness and malignancies in glioma stem-like cells. *Toxicol Appl Pharmacol* **254:**32–40.

Jensen PR and Fenical W (1994) Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. *Annu Rev Microbiol* **48:**559–584.

Kanwar SS, Yu Y, Nautiyal J, Patel BB, and Majumdar AP (2010) The Wnt/beta-catenin pathway regulates growth and maintenance of colonospheres. *Mol Cancer* **9:**212.

Lin A, Lu X, Fang Y, Zhu T, Gu Q, and Zhu W (2008) Two new 5-hydroxy-2-pyrone derivatives isolated from a marine-derived fungus *Aspergillus flavus*. *J Antibiot* (*Tokyo*) **61:**245–249.

Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL, and Yu JS (2006) Analysis of gene expression and chemoresistance of CD133$^+$ cancer stem cells in glioblastoma. *Mol Cancer* **5:**67.

McGlacken GP and Fairlamb IJ (2005) 2-Pyrone natural products and mimetics: isolation, characterisation and biological activity. *Nat Prod Rep* **22:**369–385.

Ohgaki H and Kleihues P (2005) Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. *J Neuropathol Exp Neurol* **64:**479–489.

Park DM and Rich JN (2009) Biology of glioma cancer stem cells. *Mol Cells* **28:**7–12.

Pevny LH and Nicolis SK (2010) Sox2 roles in neural stem cells. *Int J Biochem Cell Biol* **42:**421–424.

Poppe SM, Slade DE, Chong KT, Hinshaw RR, Pagano PJ, Markowitz M, Ho DD, Mo H, Gorman RR 3rd, Dueweke TJ, et al. (1997) Antiviral activity of the dihydropyrone PNU-140690, a new nonpeptidic human immunodeficiency virus protease inhibitor. *Antimicrob Agents Chemother* **41:**1058–1063.

Rich JN (2007) Cancer stem cells in radiation resistance. *Cancer Res* **67:**8980–8984.

Schatton T and Frank MH (2008) Cancer stem cells and human malignant melanoma. *Pigment Cell Melanoma Res* **21:**39–55.

Singh SK, Clarke ID, Hide T, and Dirks PB (2004a) Cancer stem cells in nervous system tumors. *Oncogene* **23:**7267–7273.

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, and Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. *Cancer Res* **63:**5821–5828.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, and Dirks PB (2004b) Identification of human brain tumour initiating cells. *Nature* **432:**396–401.

Soeda A, Inagaki A, Oka N, Ikegame Y, Aoki H, Yoshimura S, Nakashima S, Kunisada T, and Iwama T (2008) Epidermal growth factor plays a crucial role in mitogenic regulation of human brain tumor stem cells. *J Biol Chem* **283:**10958–10966.

Soeda A, Park M, Lee D, Mintz A, Androutsellis-Theotokis A, McKay RD, Engh J, Iwama T, Kunisada T, Kassam AB, et al. (2009) Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α. *Oncogene* **28:**3949–3959.

Sunayama J, Matsuda K, Sato A, Tachibana K, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, et al. (2010) Crosstalk between the PI3K/mTOR and MEK/ERK pathways involved in the maintenance of self-renewal and tumorigenicity of glioblastoma stem-like cells. *Stem Cells* **28:**1930–1939.

Suslov ON, Kukekov VG, Ignatova TN, and Steindler DA (2002) Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proc Natl Acad Sci USA* **99:**14506–14511.

Tang C, Ang BT, and Pervaiz S (2007) Cancer stem cell: target for anti-cancer therapy. *FASEB J* **21:**3777–3785.

Thaisrivongs S, Romero DL, Tommasi RA, Janakiraman MN, Strohbach JW, Turner SR, Biles C, Morge RR, Johnson PD, Aristoff PA, et al. (1996) Structure-based design of HIV protease inhibitors: 5,6-dihydro-4-hydroxy-2-pyrones as effective, nonpeptidic inhibitors. *J Med Chem* **39:**4630–4642.

Trachootham D, Lu W, Ogasawara MA, Nilsa RD, and Huang P (2008) Redox regulation of cell survival. *Antioxid Redox Signal* **10:**1343–1374.

Turner SR, Strohbach JW, Tommasi RA, Aristoff PA, Johnson PD, Skulnick HI, Dolak LA, Seest EP, Tomich PK, Bohanon MJ, et al. (1998) Tipranavir (PNU-140690): a potent, orally bioavailable nonpeptidic HIV protease inhibitor of the 5,6-dihydro-4-hydroxy-2-pyrone sulfonamide class. *J Med Chem* **41:**3467–3476.

Wang J, Wakeman TP, Lathia JD, Hjelmeland AB, Wang XF, White RR, Rich JN, and Sullenger BA (2010) Notch promotes radioresistance of glioma stem cells. *Stem Cells* **28:**17–28.

Wen PY and Kesari S (2008) Malignant gliomas in adults. *N Engl J Med* **359:**492–507.

Yoon CH, Kim MJ, Kim RK, Lim EJ, Choi KS, An S, Hwang SG, Kang SG, Suh Y, Park MJ, et al. (2012) c-Jun N-terminal kinase has a pivotal role in the maintenance of self-renewal and tumorigenicity in glioma stem-like cells. *Oncogene* http://dx.doi.org/10.1038/onc.2011.634.

Zhang M, Atkinson RL, and Rosen JM (2010) Selective targeting of radiation-resistant tumor-initiating cells. *Proc Natl Acad Sci USA* **107:**3522–3527.

Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, and Dirks PB (2009) Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. *Nat Rev Drug Discov* **8:**806–823.

Address correspondence to: Su-Jae Lee, Laboratory of Molecular Biochemistry, Department of Chemistry, Hanyang University, 17 Haengdang-Dong, Seongdong-Ku, Seoul 133-791, Korea. E-mail: sj0420@hanyang.ac.kr