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The novel phloroglucinol derivative BFP induces apoptosis of glioma cancer through reactive oxygen species and endoplasmic reticulum stress pathways

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ABSTRACT

Prenyl-phloroglucinol derivatives from hop plants have been shown to have anticancer activities. This study is the first to investigate the anticancer effects of the new phloroglucinol derivative (2,4-bis(4-fluorophenylacetyl)phloroglucinol; BFP). BFP induced cell death and anti-proliferation in three glioma, U251, U87 and C6 cells, but not in primary human astrocytes. BFP-induced concentration-dependently cell death in glioma cells was determined by MTT and SRB assay. Moreover, BFP-induced apoptotic cell death in glioma cells was measured by Hochest 33258 staining and fluorescence-activated cell sorter (FACS) of propidine iodine (PI) analysis. Treatment of U251 human glioma cells with BFP was also found to induce reactive oxygen species (ROS) generation, which was detected by a fluorescence dye used FACS analysis. Treatment of BFP also increased a number of signature endoplasmic reticulum (ER) stress markers glucose-regulated protein (GRP)-78, GRP-94, IRE1, phosphorylation of eukaryotic initiation factor-2α (eIF-2α) and up-regulation of CAAT/enhancer-binding protein homologous protein (CHOP). Moreover, treatment of BFP also increased the down-stream caspase activation, such as pro-caspase-7 and pro-caspase-12 degradation, suggesting the induction of ER stress. Furthermore, BFP also induced caspase-9 and caspase-3 activation as well as up-regulation of cleaved PARP expression. Treatment of antioxidants, or pre-transfection of cells with GRP78 or CHOP siRNA reduced BFP-mediated apoptotic-related protein expression. Taken together, the present study provides evidences to support that ROS generation, GRP78 and CHOP activation are mediating the BFP-induced human glioma cell apoptosis.

Introduction

Glioblastomas are one of the most lethal types of primary central nervous system tumors, and their biological features make successful treatment very difficult. Moreover, glioblastomas generally prove refractory to treatment by surgery, irradiation, and conventional chemotherapy. Their abnormal biological features lead to uncontrolled growth, invasiveness, and angiogenesis, and ultimately facilitate cell proliferation and survival (Amberger et al. 1998; Griscelli et al. 2000; Kleihues et al. 1995; Koul et al. 2006; Stupp et al. 2005). These dysregulated pathways provide the basis for designing molecular-targeted therapy for treatment of gliomas.

Endoplasmic reticulum (ER) is an organelle in the secretory pathways, and serves as a central role in lipid synthesis, protein folding and modification. However, protein folding in the ER is impaired under a variety of toxic insults, including hypoxia, failure of protein synthesis, protein misfolding, and Ca^{2+} overload, and can result in ER stress-related events (Abcouwer et al. 2002; Soboloff and Berger 2002). There is increasing evidence that ER stress plays a crucial role in the regulation of apoptosis. It has been reported that ER stress triggers several specific signaling pathways, such as ER-associated protein degradation and the unfolded protein response (UPR) (Feldman et al. 2005; Moenner et al. 2007). The UPR induces the expression of ER-resident chaperones, such as

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GRP(glucose-regulated protein)-78 and GRP-94 (Lee 2001). GRPs are the most abundant glycoproteins in the ER and play critical roles in ER regulation. The protective functions of GRP protein have also been observed in resistance to radiation in cancer cells (Kubota et al. 2005). On the other hand, several pro-apoptotic factors like CHOP/GADD153, and pro-apoptotic Bcl-2 family members like Bax, have been also shown to be involved in ER stress-induced cell death. CHOP/GADD153 is apparently a pro-apoptotic transcription factor induced during ER stress (Harding et al. 2002; McCullough et al. 2001; Rao et al. 2004; Wang et al. 1996). The eukaryotic translation initiation factor 2 alpha (eIF2α) phosphorylation is a highly conserved point of molecule that adapts cells to ER stress (Moenner et al. 2007; Wek et al. 2006). It provides stress resistance by arresting protein translation and induction of stress-inducible cytoprotective proteins. Furthermore, ROS generation appears to be triggered by the activation of the mitochondrial-dependent cell death pathway through the pro-apoptotic Bcl-2 proteins Bax or Bak, and is further transformed sequentially into more toxic ROS, like hydrogen peroxide reactive oxygen species, which with consequence induce cell death (Feig et al., 1994; Roos and Kaina, 2006).

Numerous natural products are recognized to be cancer preventive agents or antineoplastic agents (Hsia et al. 2004; Lo et al. 2011; Weng et al. 2008). There are various kinds of natural phloroglucinol derivatives that have been identified as possessing anticancer activity. For example, prenyl-phloroglucinol derivative humulon from hop plants can induce cell apoptosis in human malignant glioblastoma cells. Moreover, it has also been reported that prenyl-phloroglucinol derivative lupulon from hop plants has apoptotic effect on colon cancer (Lamy et al. 2007, 2008, 2011). Bullatenone is a triketone phloroglucinol, the main cytotoxic component compound in *Lophomyrtus bullata* (Larsen et al. 2005). Thouvenol, the alkyl phloroglucinol, isolated from *Protorhus thouvenotii*, has shown cytotoxicity in ovarian cancer cells (Cao et al. 2004). Recently, we also reported that phloroglucinol derivates have anti-cancer activities in human colon cancer (Huang et al. 2011) and chondrosarcoma (Liu et al. 2011). However, the anti-cancer activity of phloroglucinol derivatives in human glioma remains unclear.

In this study, we synthesized the new phloroglucinol derivative 2,4-bis(4-fluorophenylacetyl)phloroglucinol (BFP) (Fig. 1A) and investigated its anticancer activity in human glioma cells. Our data indicate that BFP reduces tumor growth and survival of human glioma cells.

Materials and methods

Materials

The phloroglucinol derivative (Fig. 1; chemical purity ≥ 95%) was synthesized at the Graduate Institute of Pharmaceutical Chemistry, China Medical University (Taichung, Taiwan) following the general procedure. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and OPTI-MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA). Primary antibodies against cleaved caspase 3 and phosphorylation of eIF-2α were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies specific for calpain 1, IRE1, GRP78, GRP94, PARP, pro-caspase 3, pro-caspase 9, pro-caspase 7 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody against pro-caspase-12 was purchased from BD Bioscience (San Jose, CA). Propidium iodide (PI), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), staurosporine and other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

Cell culture

C6 cells originated from a rat brain glioma. U87 and U251 cells originated from a human brain glioma. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). C6 cells were maintained with F12 medium (Invitrogen Life Technologies, Carlsbad, CA), while U87 and U251 cells were maintained in 75 cm^2 flasks with DMEM.

Human astrocytes were purchased from Sciencell Research Laboratories (isolated from human cerebral cortex, Cat# 1800, Carlsbad, CA) and were cultured in human astrocyte medium (Sciencell, Cat# 1801) on poly-L-lysine coated tissue culture dishes. Media was changed every three days and cells were passaged once a week at a 1:5 ratio.

All cells were cultured in medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C, incubated in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Sulforhodamine B assay (SRB)

The SRB assay is based on the measurement of cellular protein content and the procedure was followed according to our previous report (Huang et al. 2011). After treatment with BFP for 24 h, cells were fixed with 10% trichloroacetic acid and stained by SRB at 0.4% (w/v) in 1% acetic acid for 30 min. Unbound SRB was washed out by 1% acetic acid and SRB-bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm using a microplate reader (Bio-Tek, Winooski, VT).

MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatment with BFP for 24 or 48 h, media were removed and washed with PBS. MTT (0.5 mg/ml) was then added to each well and the mixture was incubated for 2 h at 37 °C. MTT reagent was then replaced with DMSO (100 μl per well) to dissolve formazan crystals. After the mixture was shaken at room temperature for 10 min, absorbance was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT).

Hoechst 33258 staining

Hoechst 33258 is a DNA-binding fluorescent dye and being used to determine apoptotic nuclei. Cells were incubated with BFP for indicated time periods, and then stained with Hoechst 33258 (1 μg/ml) for 10 min. Results were determined by visual observation of nuclear morphology through fluorescence microscopy.

Quantification of apoptosis by flow cytometry

Cells were treated with various concentrations of BFP for 24 h and then washed twice with PBS. For apoptosis determination, cells were fixed by 70% ethanol at room temperature and then re-suspended in PBS containing 50 μg/ml propidium iodide (PI), 100 μg/ml RNase A and 0.1% Triton X-100 for 30 min. The apoptotic nuclei were determined by flow cytometry. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson, Lincoln Park, NJ).

Western blot analysis

The protocol of whole cell protein lysis was followed according to our previous report (Huang et al. 2011). Briefly, cells were treated

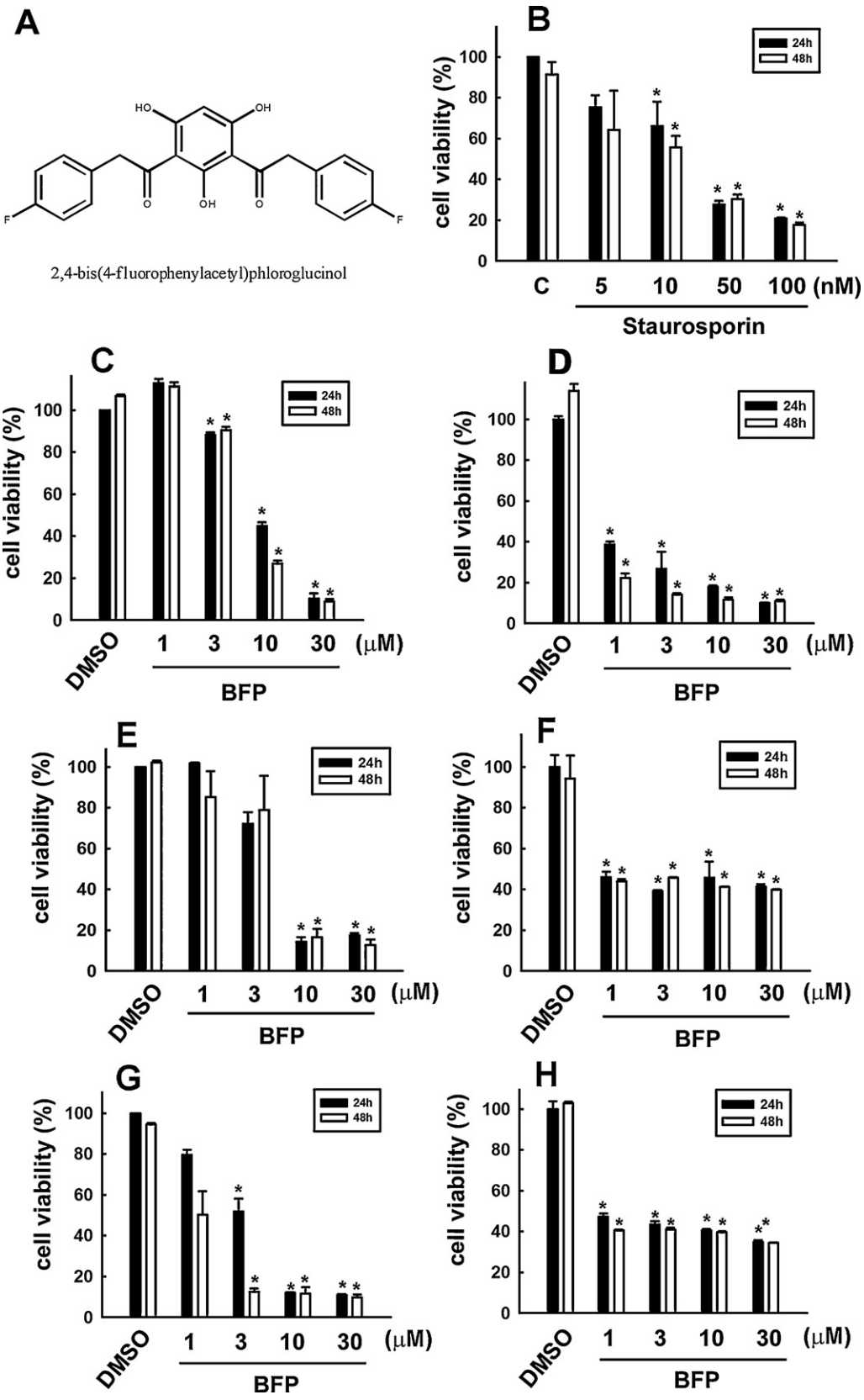

Fig. 1. BFP induces cancer cell death and anti-proliferation in glioma cells. (A) The structure of a novel phloroglucinol derivative 2,4-bis(4-fluorophenylacetyl)phloroglucinol; BFP. (B) Cells were incubated with various concentrations of staurosporine (5, 10, 50 or 100 nM), and cell viability was examined by MTT assay after 24 or 48 h treatment. Cells were treated with various concentrations of BFP (1, 3, 10 or 30 μM), and cell viability was examined by MTT assay and SRB assay after 24 or 48 h treatment in U251 (C and D), U87 (E and F) and C6 (G and H). Results are expressed as the means ± S.E.M. of at least three independent experiments. $^{*}p < 0.05$ compared with the vehicle treatment group.

with BFP for various time periods and then lysed with radioimmunoprecipitation assay buffer. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinyldifluoride (PVDF) membranes. The blots were probed with primary antibody for 1 h at room temperature and subsequently incubated with a secondary antibody for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The blots were subsequently stripped through incubation in stripping buffer and reprobed for β-actin as a loading control.

siRNA transfection

The siRNAs against human GRP78, CHOP and control siRNA were purchased commercially from Santa Cruz Biotechnology. Cells were transiently transfected with the siRNA by Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the siRNA (at a final concentration of 100 nM) and LF2000 were premixed in OPTI-medium for 20 min and then applied to the cells. An equal volume of medium containing 20% FBS was added 6 h later. After transfection for 24 h, LF2000-containing medium was replaced with fresh serum-free medium and treated with BFP for another 24 h.

Reverse Transcriptase-PCR (RT-PCR)

Total RNA was extracted from cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Reverse transcription reaction was performed using 1 μg of total RNA which converted into cDNA using the RT kit (Promega, Madison, WI), then amplified using oligonucleotide primers:

GRP78: 5′-GCTCGACTCGAATTCCAAAG-3′ and 5′-TTTGTCAGGGGTCTTTCACC-3′;
GRP94: 5′-CAGTTTTGGATCTTGCTGTGG-3′ and 5′-CAGCTGTAGATTCCTTTGC-3′;
GAPDH: 5′-TGGGCTACACTGAGCACCAG-3′ and 5′-GGGTGTCGCTGTTGAAGTCA-3′.

Each PCR cycle was carried out for 30 s at 95 °C, 1 min at 55 °C, and 1 min at 65 °C. PCR products were then separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. The band intensity was quantified with a densitometric scanner and presented as the relative level of GAPDH.

Reactive oxygen species (ROS) assay

The production of ROS was assessed spectrofluorimetrically by oxidation of specific probes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Cells were plated at 6 well-plates and exposed to BFP for another 2 h. The cells were incubated with DHE (10 μM) or H_2DCFDA (10 μM) for 30 min at 37 °C. The fluorescence intensity was measured with excitation filter of 488 and 525 nm emission wavelengths using the flow cytometry.

Statistics

The values given are means ± S.E.M. The significance of difference between the experimental group and control group was assessed by the Student's *t*-test. The difference was significant if the p value was <0.05.

Results

BFP induces cell apoptosis in human glioma cells

As shown in Fig. 1B, staurosporine induced glioma cell death in a concentration-dependent manner. Staurosporine were used as a positive control to induce cell apoptosis (IC50 ≈ 30 nM). To investigate the cytotoxicity of 2,4-bis(4-fluorophenylacetyl)phloroglucinol (BFP, Fig. 1A) in glioma cells, we examined the effects on cell viability in three different glioma cells, U251, U87 and C6. Cells were incubated with various concentrations of BFP (1, 3, 10 or 30 μM), and the cell viability was examined by MTT assay and SRB assay for 24 or 48 h. BFP significantly induced cell death in all three types of glioma cells (Fig. 1C–H). Furthermore, the SRB assay determined that BFP-induced cell death for 24 and 48 h in glioma cells U251 (IC50 < 1 μM), U87 (IC50 < 3 μM) and C6 (IC50 < 10 μM), but not in primary human astrocytes (IC50 > 30 μM; data not shown). Next, we further confirmed that whether BFP induces cell death through an apoptotic mechanism. Nuclear shrinkage, chromatin condensation, and nuclear fragmentation are hallmarks of cell apoptosis. As shown in Fig. 2A, treatment with BFP (3 μM) for 4 h induced nuclear shrinkage and nuclear condensation, and for 8 h obviously revealed nuclear condensation and fragmentation. Furthermore, BFP-induced cell apoptosis was examined by evaluating the sub-G1 group using propidium iodide (PI) which was analyzed by flow cytometry. BFP induced concentration-dependent sub-G1 arrest in U251 human glioma cells (Fig. 2B). To determine whether BFP induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of Bcl-2 family proteins. Treatment of U251 cells with BFP induced Bax protein up-regulation significantly but did not affect Bcl-2 protein expression (Fig. 2C). Treatment of BFP also increased procaspase-3 degradation and caspase-3 cleaved form expression in U251 cells (Fig. 2D). Upstream procaspase-9 is also degraded and cleaved-caspase-9 increased upon BFP treatment in U251 cells (Fig. 2D). Notably, BFP also increased cleaved-PARP expression time-dependently (Fig. 2E).

BFP induces reactive oxygen species generation and ER stress in U251 human glioma cells

It has been reported that oxidative stress has been implicated in the pro-apoptotic activities of cancer therapy (Lau et al. 2008; Mates and Sanchez-Jimenez 2000). Therefore, we next examined whether the ROS accumulation is involved in BFP-induced apoptosis. BFP induced an increase in intracellular H_2O_2 levels, as shown by H_2DCF-DA staining which were observed by a fluorescence microscope (Fig. 3A) and analyzed by FACS detection assay (Fig. 3B). ER stress is generally characterized by up-regulation of IRE1, GRP78, GRP94, calpain 1 and CHOP, and phosphorylation of eukaryotic initiation factor-2α (eIF2α). BFP exposure caused a significant increase in the expression of GRP 78 and GRP 94 protein levels (Fig. 4A) and mRNA levels (Fig. 4B). We next determined whether the activity of calpain (calcium-dependent thiol proteases) would be induced by BFP in glioma cells. As shown in Fig. 4C, BFP also increased calpain 1 expression in U251 cells. Moreover, BFP also induced IRE1 (Fig. 4D), CHOP expression and the phosphorylation of eIF2α at Ser51 (Fig. 4E) in U251 cells. One of the hallmarks of the apoptotic process is the caspases activation, which represents both initiators and executors of death signals. BFP also triggered the pro-caspase-7 and pro-caspase-12 degradation in a time-dependent manner (Fig. 4F). These results indicate that the occurrence of ER stress is involved in BFP-induced cell death.

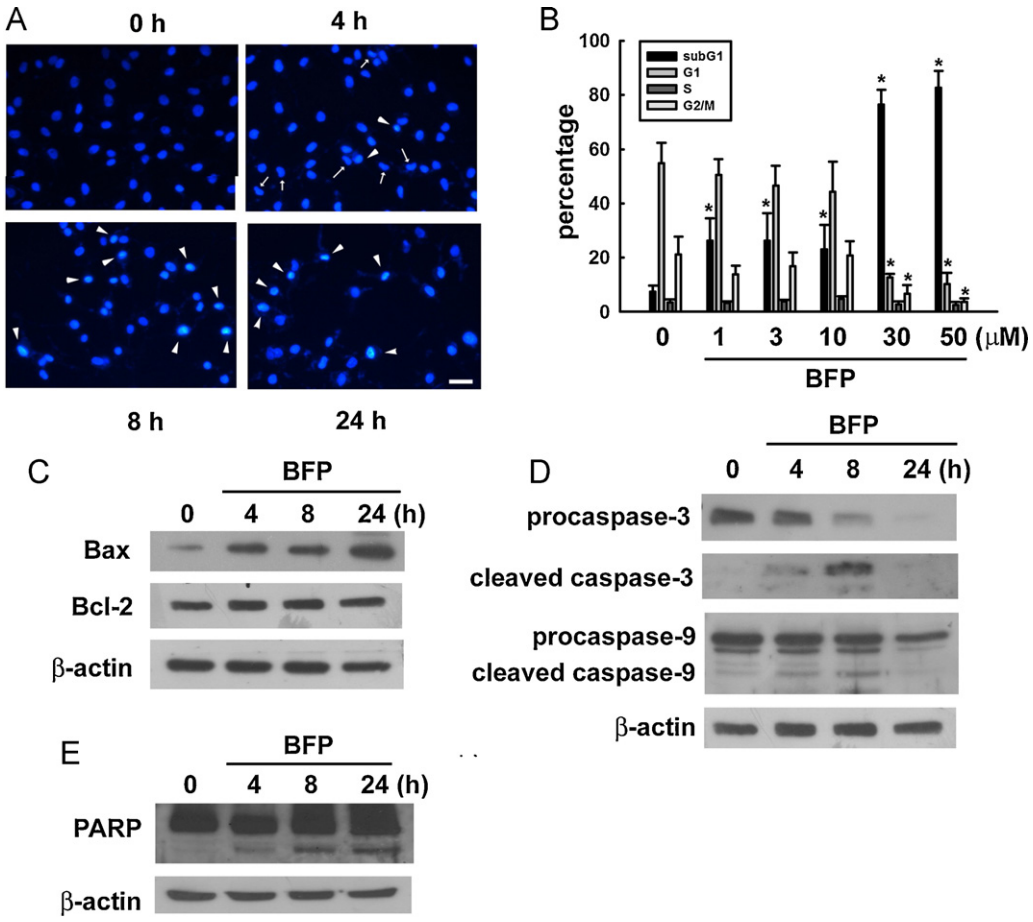

Fig. 2. BFP induces cell apoptosis in U251 human glioma cells. (A) Cells were treated with BFP for indicated time periods. Hochest staining was visualized by florescence imaging. BFP treatment induced nuclear shrinkage (indicated in arrowhead), condensation and fragmentation (indicated in arrows). (B) The percentage of apoptotic cells was analyzed by flow cytometry of PI staining after treatment with various concentrations of BFP for 24 h. Results are expressed as the means ± S.E.M. (n = 3–4). Cells were incubated with BFP (3 μM) for different time periods, levels of apoptotic proteins (C–E) were examined by Western blot analysis. Results are the representative of three independent experiments.

BFP-induced caspase-3 and caspase-9 activation are mediated by ROS generation, GRP78 and CHOP expression

These results suggest that BFP triggers ER stress and induces cancer cell apoptosis in U251 cancer cells. We next further determined whether the ROS generation is involved in BFP-induced ER stress in glioma cells. As shown in Fig. 5A, BFP-induced IRE1 increase, and procaspase-3 and procaspase-9 degradation were reversed by treatment with two different antioxidants apocynin (10 μM) and N-acetylcysteine (NAC, 10 mM). Furthermore, pre-transfection with siRNA against GRP78 significantly reduced BRP-induced ER stress proteins

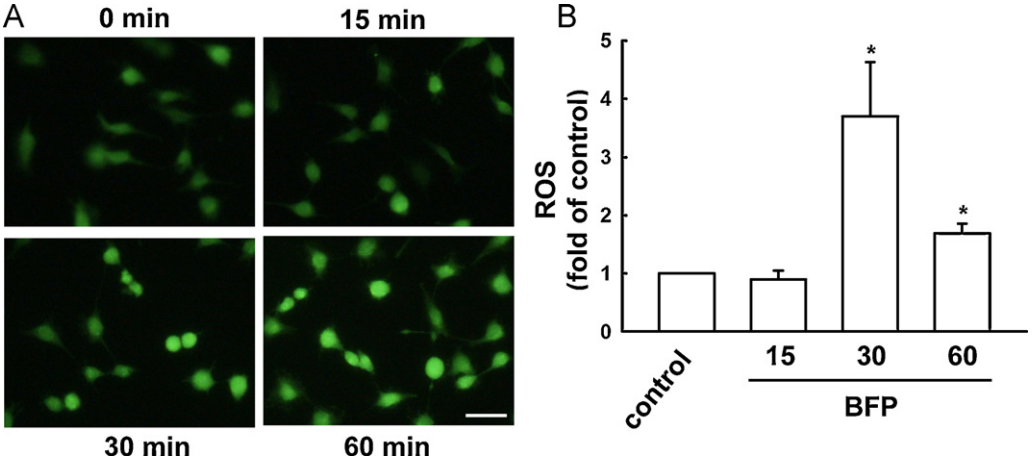

Fig. 3. BFP increases reactive oxygen species release in U251 human glioma cells. Cells were incubated with BFP (3 μM) for indicated time periods. The production of ROS was examined by fluorescence image (A) and quantitatived by flow cytometry (B). Results are expressed as the mean ± S.E.M. of three independent experiments. *, $p < 0.05$ compared with the control group.

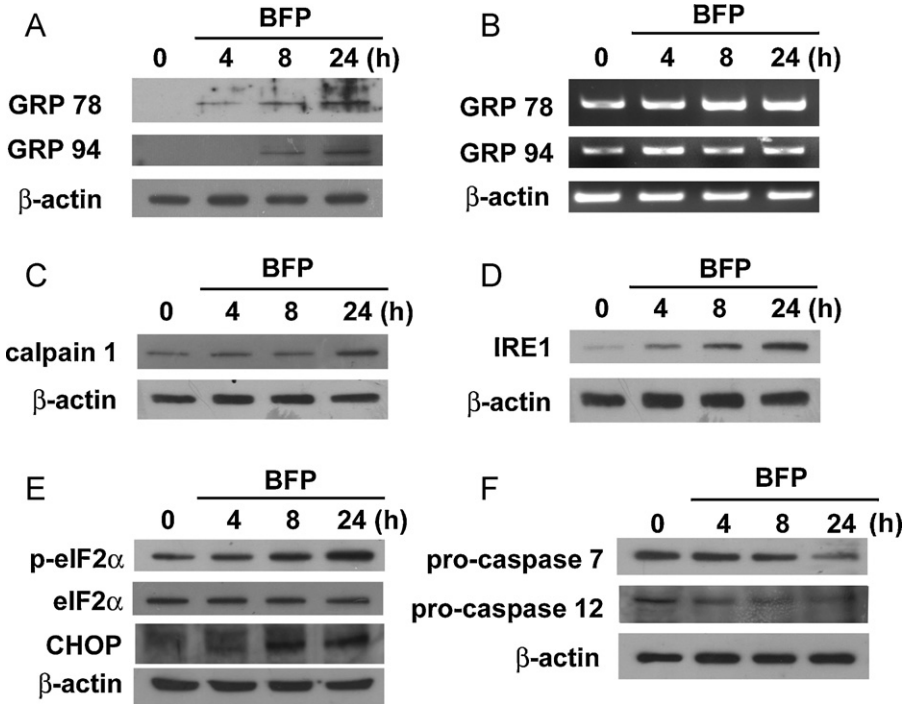

Fig. 4. BFP increases ER stress-related proteins activation in U251 human glioma cells. Cells were incubated with BFP (3 μM) for indicated time periods. ER stress-related protein expressions were examined by Western blot analysis. Results are the representative of three independent experiments. Cells were incubated with BFP for indicated time periods, mRNA expression of GRP78 and GRP94 were examined by RT-PCR analysis (B). Results are the representative of three independent experiments.

expression such as IRE1 and CHOP expression (Fig. 5B). Moreover, pre-transfection with GRP78 siRNA also reduced BFP-mediated cell apoptosis proteins expression, such as cleaved PARP, procaspase-9 degradation and cleaved caspase-3 up-regulation (Fig. 5C). On the other hands, pre-transfection with siRNA against CHOP also reduced BFP-mediated cell apoptosis (Fig. 5D).

Discussion

Apoptosis has been described as multiple pathways converging from numerous different initiating events and insults. Increasing evidence shows that efficacy of antitumor agents and cancer preventive agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis. In this study,

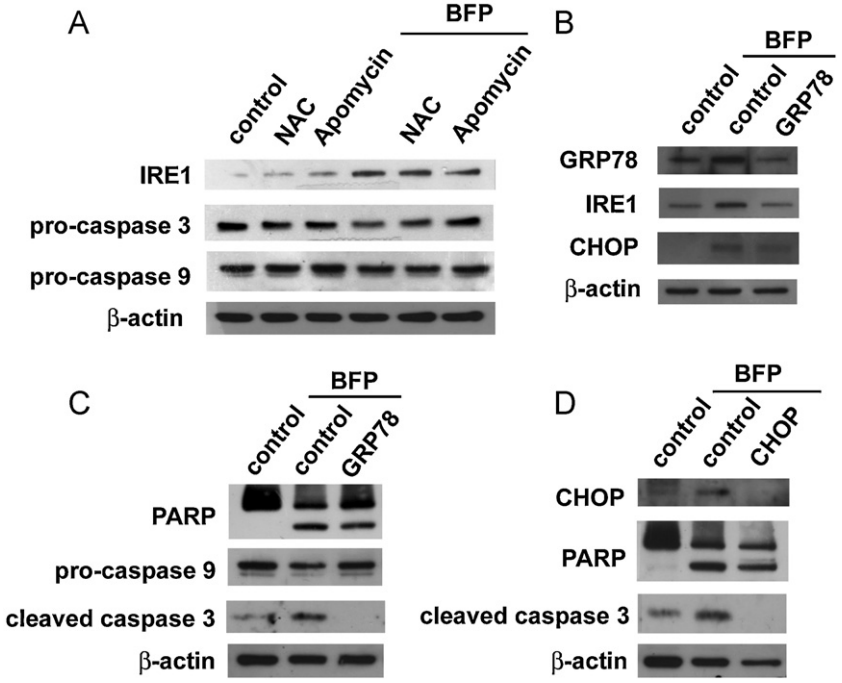

Fig. 5. Involvement of ROS generation, GRP78 and CHOP expression in BFP-induced caspase-9 and caspase-3 activation in U251 glioma cells. (A–D) Levels of apoptotic and ER stress-related proteins were examined by Western blot analysis. Results are the representative of at least three independent experiments.

we demonstrated that the molecular mechanism by which BFP triggered human glioma cells apoptosis. Our results demonstrated that BFP inhibited cancer cell growth and proliferation of three different glioma cells, such as U251, U87 and C6 cells. BFP also induced nuclear shrinkage (4 h), and nuclear condensation and fragmentation (8 and 24 h) as well as sub G1 arrest, the last phase of apoptosis, in U251 cancer cells. In conclusion, these observations suggest that BFP induces cancer cell death-mediated apoptosis. Indeed, treatment with BFP caused the activation of caspases including caspase-9 and caspase-3, associated with the degradation of PARP, which preceded the onset of apoptosis.

Our previous study (Huang et al. 2011; Liu et al. 2011) and presence data demonstrated that phloroglucinol derivatives induce human cancer cell death (IC50 < 10 μM) but not in primary human cells (IC50 > 30 μM). The fundamental finding in this study provides important evidence to support the involvement of ER stress in the induction of apoptosis by BFP in U251 glioma cells. The following experimental evidence in the present study reveals that the induction of ER stress-related proteins may be involved in BFP induced apoptosis: (i) BFP induced IRE1, GRP78, and GRP94 expression; (ii) BFP increased CHOP expression which is one of the highest inducible genes during ER stress; (iii) suppression of GRP78 and CHOP genes by specific siRNA attenuated BFP-induced caspase-9 and caspase-3 activation; (iv) BFP also induced phosphorylation of eIF-2α. Taken together, BFP induces up-regulation of IRE1, GRP78, GRP94 and CHOP as well as phosphorylation of eIF-2a, all of which are mediating ER stress.

Bcl-2 family proteins play an important role in cancer cells apoptosis (Cotter 2009; Leber et al. 2010). The Bcl-2 family can regulate mitochondrial membrane permeabilization. Bax protein mediates mitochondrial membrane permeabilization. Treatment of U251 cells with BFP induced Bax protein level increase but did not affect Bcl2 within a period of 4 h and prolonged Bax expression at 24 h, which may lead to an increase in the pro-apoptotic/anti-apoptotic Bcl-2 ratio. On the other hand, the mitochondrial apoptotic pathway has been described as an important downstream signal of ROS in apoptotic cell death (Iwamaru et al. 2007; Zu et al. 2005). High levels of ROS can also induce apoptosis by triggering mitochondrial permeability transition pore opening, release of pro-apoptotic factors and activation of caspase-9 and caspase-3 (Iwamaru et al. 2007; Zu et al. 2005). It has been reported that inhibition of ROS by a ROS scavenger prevents camptothecin-induced apoptosis (Wenzel et al. 2004). Here, we also found that FBP increased ROS generation in human glioma cells. Treatment with antioxidants such as NAC and apomycin both reduced BFP-induced IRE1 expression, pro-caspase-9 and pro-caspase-3 degradation. In agreement with these observations, we noted that the mitochondrial dysfunction and ROS generation may be involved in BFP-induced cell apoptosis of human glioma cells.

Our results showed that ROS generation, up-regulation of pro-apoptotic protein and activation of caspases may be involved in BFP-induced apoptotic cell death in human glioma, with its ability to cause ER stress. In conclusion, the novel phloroglucinol derivative BFP-induced human glioma cell death is mediated by ROS generation, which subsequently induces GPR78 and CHOP expression, increases caspases activity, such as caspase-9 and caspase-3, resulting in apoptosis. Present study on the molecular basis will provide valuable strategies of target signal transducers for the development of effective anti-tumor therapy.

Acknowledgments

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