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BFPP, a phloroglucinol derivative, induces cell apoptosis in human chondrosarcoma cells through endoplasmic reticulum stress

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ABSTRACT

Chondrosarcoma is a malignant primary bone tumor that responds poorly to both chemotherapy and radiation therapy. This study is the first to investigate the anticancer effects of the new phloroglucinol derivative (2,4-bis(2-fluorophenylacetyl)phloroglucinol; BFPP) in human chondrosarcoma cells. BFPP induced cell apoptosis in two human chondrosarcoma cell lines, JJ012 and SW1353 but not in primary chondrocytes. BFPP triggered endoplasmic reticulum (ER) stress, as indicated by changes in cytosol calcium levels, and increased glucose-regulated protein 78 (GRP78) expression, but failed to show the same effects on GRP94 expression. BFPP also increased calpain expression and activity. Transfection of cells with GRP78 or calpain siRNA reduced BFPP-mediated cell apoptosis in JJ012 cells. Importantly, animal studies have revealed a dramatic 50% reduction in tumor volume after 21 days of treatment. This study demonstrates novel anticancer activity of BFPP against human chondrosarcoma cells and in murine tumor models.

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1. Introduction

Chondrosarcoma is the second most common primary malignancy of bone, a clinically aggressive tumor that responds poorly to currently available chemotherapy or radiation treatment, thereby complicating its management [1,2]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. In the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and therefore, it is important to explore novel and adequate remedies [3]. Aberrant regulation of cell growth and deregulation of apoptosis occur commonly in cancer cells and may play an essential role in tumor development. Accordingly, current anticancer agents act by inhibiting cell growth or inducing cell apoptosis [4].

The endoplasmic reticulum (ER) is a membranous synthesis and transport organelle that plays a central role in lipid synthesis, protein folding and maturation. A variety of toxic insults, including hypoxia, failure of protein synthesis, misfolding, transport or degradation, and Ca^{2+} overload, can disturb ER functioning and result in ER stress-related events [5–7]. 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, including ER-associated protein degradation and the unfolded protein response (UPR) [8,9]. Glucose-regulated proteins (GRP) are the most abundant glycoproteins in the ER and play a critical role in ER regulation. Overexpression, antisense and ribozyme approaches in tissue culture systems have directly demonstrated that GRP78 and GRP94 protect cells against oxidative injury [10,11]. The protective function of GRPs has also been observed in resistance to radiation in cervical cancer [12]. Their antiapoptotic function predicts that induction of GRPs in neoplastic cells may lead to cancer progression and drug resistance [13,14]. However, it has been reported that curcumin-induced apoptosis in human lung carcinoma cells occurs through GRP78 up-regulation [15]. Similarly, hepatitis C-virus induced cell apoptosis also occurs through the GRP78 up-regulation pathway. Therefore, GRP78 may also play a proapoptotic role in cell death [16]. The ER plays a direct role in activating a subset of caspase during activation of apoptosis that

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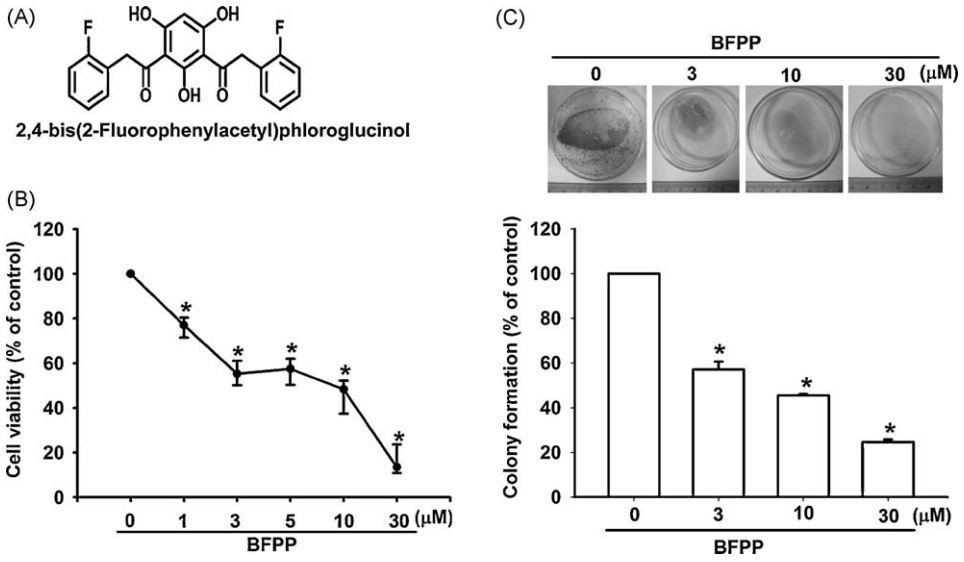


Fig. 1. The effects of BFPP on cell viability and colony formation in human chondrosarcoma cells. (A) Chemical structure of BFPP. (B) JJ012 cells were incubated with various concentrations of BFPP for 48 h, and the cell viability was examined by SRB assay ($n = 5$). (C) For the colony-forming assay, the clonogenic assay was performed as described under Section 2. The quantitative data are shown in the lower panel. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with controls.

occurs during ER stress [17]. Calpains are a family of Ca^{2+}-dependent intracellular cysteine proteases. The ubiquitously expressed calpain I (μ-calpain) and calpain II (m-calpain) proteases are implicated in the development of apoptosis. A recent study has shown that ubiquitous calpains promote caspase-12 and c-Jun N-terminal kinase (JNK) activation during ER stress-induced apoptosis [18]. Other evidence also suggests that GRP with Ca^{2+}-binding and antiapoptotic properties is a proteolytic target of calpain during etoposide-induced apoptosis [19].

Numerous naturally occurring substances are recognized to be antioxidants, cancer preventive agents, or even antineoplastic agent such as paclitaxel (Taxol®) [20]. Four kinds of natural phloroglucinol derivatives have been identified as possessing anticancer activity [21–24]. Rottlerin-type compounds (drummondins) isolated from *Hypericum drummondii* have shown cytotoxic activity in human cancer cell lines [21], as has the acylphloroglucinol-type compound hyperforin, a polyphenol-derivative of St. John's wort (*Hypericum perforatum*) [22]. Bullatenone is the main cytotoxic compound in extracts of *Lophomyrtus bullata* [23]. Lastly, the long-chain acylated phloroglucinol thouvenol, isolated from the dried fruits of *Protorhus thouvenotii*, has shown *in vitro* cytotoxicity against ovarian cancer cells [24]. Although the effects of phloroglucinol derivatives on tumor apoptosis have been studied in various cancers [21–24], the cytotoxic activity of phloroglucinol derivatives in chondrosarcoma remains largely undefined. In this study, we synthesized the new phloroglucinol derivative 2,4-bis(2-fluorophenylacetyl)phloroglucinol (BFPP) (Fig. 1A) and investigated its anticancer activity in human chondrosarcoma cells. Our data indicate that BFPP reduces survival and tumor growth of human chondrosarcoma cells *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Phloroglucinol derivatives were synthesized at the Graduate Institute of Pharmaceutical Chemistry, China Medical University (Taichung, Taiwan) following the general procedure. To a stirred solution of phloroglucinol (1 mmol) and appropriate phenylacetonitrile (2 mmol) dissolved in borontrifluoride etherate (10 mL) was purged HCl gas for 8–12 h at room temperature. The solution was added distilled water (30 mL) and then refluxed for 2 h. After cooling the mixture solution was extracted with ethyl acetate (30 mL ×3). The organic layer was collected and concentrated in vacuum. The residue was subjected to chromatography on silica gel to afford related compound. BFPP was recrystalization from acetonitrile as a white crystal. mp 218.6–218.9 °C. Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for GRP78, GRP94, calpain I, calpain II, PARP, caspase 3, caspase 9, caspase 12 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human chondrosarcoma cell line JJ012 was kindly provided by Dr. Sean P Scully (University of Miami School of Medicine, Miami, FL, USA). The human chondrosarcoma cell line SW1353 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM/α-MEM supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Primary cultures of human chondrocytes were isolated from articular cartilage as previously described [25]. The cells were grown in plastic cell culture dishes in a humidified atmosphere of 95% air/5% CO_2 in DMEM supplemented with 20 mM HEPES and 10% FBS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.3. Sulforhodamine B (SRB) assay

Cell viability by BFPP was measured with the SRB assay. BFPP was added in a range of concentrations for 48 h. Cells were fixed with 50% trichloroacetic acid to terminate the reaction, and 0.4% SRB in 1% acetic acid was added to each well. After 15 min of incubation, the plates were washed and dyes were dissolved in 10 mM Tris buffer. The 96-well plate was subsequently read by an enzyme-linked immunosorbent assay reader (515 nm) to obtain absorbance density values.

2.4. Quantification of apoptosis by flow cytometry

Apoptosis was assessed by binding of Annexin V protein to exposed phosphatidylserine (PS) residues at the surface of cells undergoing apoptosis, as previously described [26]. Cells were treated with vehicle or BFPP for indicated time intervals. After treatment, cells were washed twice with PBS (pH 7.4) and re-suspended in staining buffer containing 1 μg/ml propidium iodide (PI) and 0.025 μg/ml annexin V–FITC. Double-labeling was performed at room temperature for 10 min in darkness before flow cytometric analysis. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson; Lincoln Park, NJ, USA).

Quantitative assessment of apoptotic cells was also conducted by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method, which examines DNA-strand breaks during apoptosis with the BD ApoAlert™ DNA Fragmentation Assay Kit (Lincoln Park, NJ, USA). Cells were incubated with BFPP for the indicated times, trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After undergoing washing, the cells were incubated with the reaction mixture for 60 min at 37 °C. The stained cells were then analyzed by flow cytometry [27].

2.5. Colony assay

To determine the long-term effects of BFPP, cells (1000 per well) were treated with BFPP at various concentrations for 3 h at a time. After undergoing rinsing with fresh medium, cells were allowed to form colonies for 7 days before being stained with crystal violet (0.4 g/L). After undergoing three washes with ddH_2O, acetic acid was added to a final concentration of 33% (v/v), which was achieved followed by measuring the absorbance at 550 nm.

2.6. Detection of Ca^{2+} concentrations

JJ012 cells were seeded at approximately 2×10^5 cells/well in 12-well plates and incubated with BFPP for 0, 1, 2, 4 and 6 h to detect any changes in Ca^{2+} levels. Cells were harvested and washed twice, and re-suspended in Indo 1/AM (3 μg/ml) at 37 °C for 30 min and analyzed by flow cytometry.

2.7. Western blot analysis

The cellular lysates were prepared as described previously [27]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against GRP78 or GRP94 (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA).

2.8. Caspase activity

The assay was based on the ability of the active enzyme to cleave the chromophore from the enzyme substrates LEHD-pNA (for caspase-9) and Ac-DEVD-pNA (for caspase-3). The cell lysates were prepared and incubated with specific anti-caspase-9 and caspase-3 antibodies. Immunocomplexes were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.4) for 2 h at 37 °C. The release of p-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

2.9. Calpain activity assay

Calpain activity was assayed using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC). Cells were prepared and treated on 24-well plates, then loaded with 40 M Suc-Leu-Leu-Val-Tyr-AMC (Biomol; Plymouth Meeting, PA, USA) and treated with BFPP for the indicated timing at 37 °C in a humidified 5% CO_2 incubator. Proteolysis of the fluorescent probe was monitored using a fluorescent plate reading system (HTS-7000 Plus Series BioAssay, PerkinElmer; Stockholm, Sweden) using filter settings of 360 ± 20 nm for excitation and 460 ± 20 nm for emission.

2.10. siRNA transfection

The siRNAs against human calpain I, calpain II and control siRNA were purchased commercially from Santa Cruz Biotechnology. The ON-TARGET *smart pool* siRNA of GRP78 and scrambled siRNA were obtained from Dharmacon (Lafayette, CO, USA). Cells were transfected with siRNAs (at a final concentration of 100 nM) using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions.

2.11. Quantitative real time PCR (qPCR)

The qPCR analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, Foster City, CA, USA). One hundred nanogram of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (β-actin was used as the internal control) (Applied Biosystems, CA, USA). Quantitative RT-PCR assays were carried out in triplicate on the StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT).

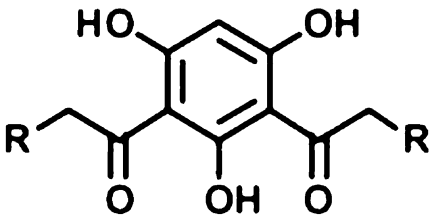
2.12. In vivo tumor xenograft study

Male SCID mice [6 weeks old; BALB/cA-nu (nu/nu)] were purchased from the National Science Council Animal Center (Taipei, Taiwan) and maintained in pathogen-free conditions. JJ012 cells were injected subcutaneously into the flanks of these SCID mice (1×10^6 cells in 200 μl) and tumors were allowed to develop for ~14 days until they reached a size of approximately 100 mm^3, when treatment was initiated. The mice were treated with vehicle, 0.5 or 1.5 mg/kg BFPP every day for 21 days (10 mice/group). The volume of the implanted tumor in dorsal side of mice was measured twice a week with a caliper, using the formula $V = (LW2)\,\pi/6$: where V, volume (mm3); L, biggest diameter (mm); W, smallest diameter (mm). All protocols complied with institutional guidelines and were approved by the Animal Care Committee of China Medical University.

2.13. Statistics

The values given are means ± S.E.M. Statistical analysis between two samples was performed using the Student's t-test. Statistical comparisons involving more than two groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. In all cases, $P < 0.05$ was considered to be significant.

Table 1
Phloroglucinol derivatives induced apoptosis of human chondrosarcoma cells.



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#	R	JJ012	SW1353	Chondrocyte
1	4-Fluorophenyl	>30	>30	>30 (μM)
2	3-Fluorophenyl	8.5	0.4	>30
3	2-Fluorophenyl	8.2	0.65	>30
4	4-Chlorophenyl	21.6	2.05	>30
5	3-Chlorophenyl	17.4	0.65	>30
6	2-Chlorophenyl	>30	>30	>30
7	3,4-Dichlorophenyl	9.5	0.33	>30
8	4-Methoxyphenyl	18.2	3	>30
9	3,4-Dimethoxyphenyl	16.7	0.58	>30
10	4-Notirophenyl	17.7	0.35	>30
11	4-Methylphenyl	28.3	2.17	>30
12	Phenyl	12.4	0.41	>30
13	3,4-Methylenedioxy	24.7	3	>30
14	Thiophenyl	11.4	0.3	>30
15	1-Naphthyl	9.87	0.35	>30

JJ012, SW1353 and primary chondrocytes were incubated with various concentrations of phloroglucinol derivatives for 48 h, the cell viability was examined by SRB assay. The IC50 values of different cell lines were examined.

3. Results

3.1. BFPP induced cell apoptosis in human chondrosarcoma cells

To investigate the cytotoxicity of phloroglucinol derivatives in human chondrosarocoma cells, we synthesized 15 phloroglucinol derivatives and examined the effects on cell survival (Table 1). The SRB assay confirmed that these compounds induced cell death in human chondrosarcoma cell lines JJ012 and SW1353 but not primary chondrocytes (Table 1). As shown in Table 1, 2,4-bis(2-fluorophenylacetyl)phloroglucinol (BFPP; compound 3) showed the greatest cytoxicity. The IC_{50} values of BFPP were 8.2 and 0.65 μM for JJ012 and SW1353 cells, respectively (Table 1). BFPP (30 μM) did not affect cell viability in primary chondrocytes, as according to the MTT assay (Supplementary Fig. S1). We therefore focused on the anticancer effects of BFPP in human chondrosarcoma cells. Treatment of JJ012 cells with BFPP induced cell death in a concentration-dependent manner, as assessed by SRB assay (Fig. 1B). The anticancer activities of BFPP were also assessed by clonogenic assays, which correlate very well with *in vivo* assays of tumorigenicity in nude mice [28]. JJ012 cells showed the ability to form clones in the untreated control wells (Fig. 1C; upper panel). However, upon addition of BFPP, a dose-dependent inhibition in clonogenicity was observed; quantitative data are shown in the lower panel of Fig. 1C. We next investigated whether BFPP induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. As compared to vehicle-treated cells, a high proportion of Annexin V^+ labeling was detected in cells treated with BFPP (Fig. 2A and B). We then investigated the effect of BFPP-induced apoptosis by using the TUNEL assay. Compared with vehicle-treated JJ012 cells, those treated with BFPP showed significant cell apoptosis (Fig. 2C). These data indicate that BFPP induces cell apoptosis in human chondrosarcoma cells.

3.2. BFPP-induced Ca^{2+} release in chondrosarcoma cells

Depletion of luminal ER calcium stores is believed to reflect ER stress, which can promote induction of ER stress [29]. We assessed the effect of BFPP on Ca^{2+} mobilization. When JJ012 cells were treated with BFPP, Ca^{2+} levels were significantly increased as compared with the vehicle-treated group. The results demonstrated that BFPP promoted Ca^{2+} productions in a time-dependent manner (Fig. 3A). In contrast, pretreatment of cells with the Ca^{2+} chelator BAPTA reduced BFPP-induced cell apoptosis (Fig. 3B). Thus, increased Ca^{2+} flux is involved in BFPP-mediated cell death in human chondrosarcoma cells.

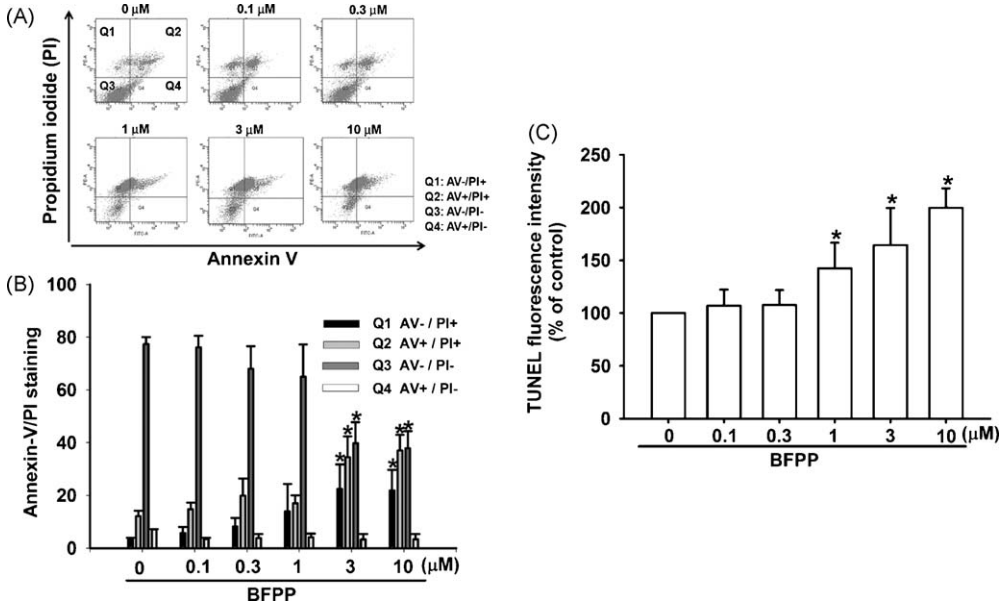

Fig. 2. BFPP induced apoptosis of human chondrosarcoma cells. JJ012 cells were treated with vehicle or BFPP for 48 h. The percentage of apoptotic cells was analyzed by flow cytometry of Annexin V/PI double staining ($n = 4$) (A and B). Cells were treated with vehicle or BFPP for 48 h. The TUNEL positive cells were examined by flow cytometry ($n = 4$) (C). Results are expressed as the mean ± S.E. $^*p < 0.05$ compared with controls.

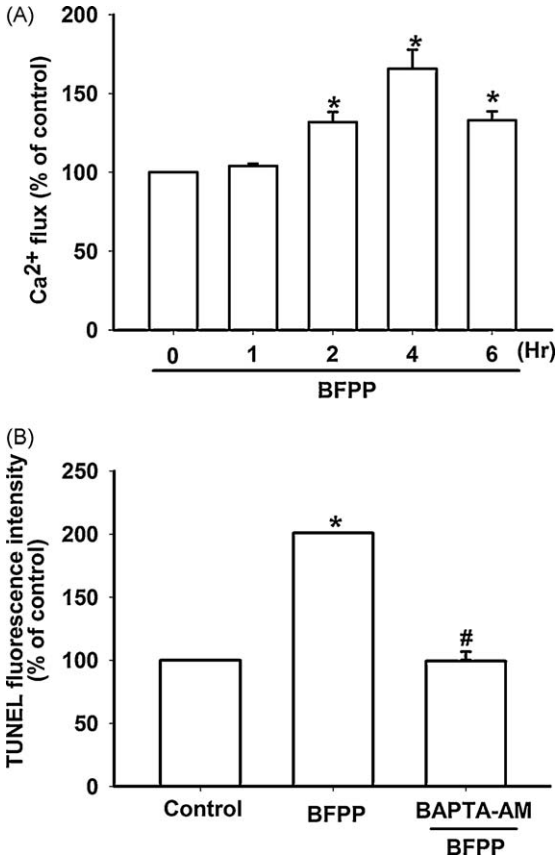

Fig. 3. BFPP induced Ca^{2+} release in chondrosarcoma cells. (A) JJ012 cells were incubated with BFPP (10 μM) for different time intervals. The Ca^{2+} flux was examined by flow cytometry ($n = 4$). (B) JJ012 cells were pretreated for 30 min with BATA-AM (10 μM) followed by stimulation with BFPP (10 μM) for 24 h. The percentage of apoptotic cells was the analyzed by flow cytometry of TUNEL-stained cells. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with controls; $^{\#}p < 0.05$ compared with the BFPP-treated group.

3.3. BFPP increased GRP78 expression and calpain activity

GRP is a major ER chaperone that plays a critical role in regulating ER homeostasis [30]. BFPP markedly increased the levels of GRP78, but not GRP94, in a time-dependent manner (Fig. 4A). Stimulation of cells with BFPP also increased GRP78 mRNA expression in concentration-dependent manner (Fig. 4B). To further investigate whether BFPP induced cell apoptosis through GRP78 activation, cell were transfected with GRP78 siRNA, which specifically inhibited GRP78 expression (Fig. 4C; upper panel), but also reduced BFPP-induced cell apoptosis (Fig. 4C; lower panel). We next determined whether the calpain activity is induced by BFPP in chondrosarcoma cells. As shown in Fig. 5A, BFPP increased calpain I and II expression in a time-dependent manner. Furthermore, BFPP also enhanced calpain activity does- and time-dependently (Fig. 5B). Transfection of cells with calpain I and II siRNA reduced calpain I and II expression, respectively (Fig. 5C; upper panel) and markedly reduced BFPP-mediated cell apoptosis (Fig. 5C; lower panel). Thus, our data suggest that GRP78 and calpain activation are involved in BFPP-mediated cell deaths.

3.4. BFPP increased caspase 3, 9 and 12 expression in chondrosarcoma cells

One of the hallmarks of the apoptotic process is the activation of cysteine proteases (caspases), which represent both initiators and executors of death signals. BFPP increased the expression and activation of caspase-3 in JJ012 cells (Fig. 6A and B). Pretreatment of cells with the specific caspase-3 inhibitor z-DEVD-FMK reduced the BFPP-induced cell death (Fig. 6D). Notably, BFPP also increased cleaved-PARP expression (Fig. 6A). Upstream caspase-9 activities increased significantly upon treatment with BFPP in JJ012 cells (Fig. 6A and C). Pretreatment of cells with caspase-9 inhibitor z-LEHD-FMK reduced BFPP-mediated cell apoptosis (Fig. 6D). It has been reported that calpains promote caspase-12 activation during ER stress-induced apoptosis [18], we found that BFPP decreased the expression of pro-caspase-12 in chondrosarcoma cells (Fig. 6A).

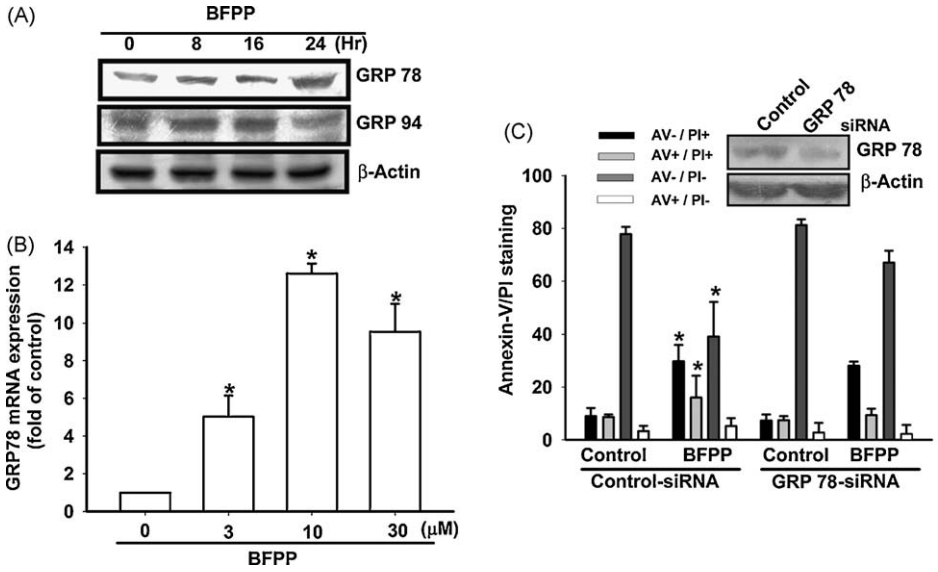

Fig. 4. GRP78 activation is involved in BFPP-mediated cell apoptosis in human chondrosarcoma cells. (A) JJ012 cells were incubated with BFPP (10 μM) for different time intervals. GRP78 and GRP94 expression was examined by Western blot analysis. (B) JJ012 cells were incubated with BFPP for 24 h. mRNA expression of GRP78 was examined by qPCR analysis. Cells were transfected with GRP78 or control siRNA for 24 h. GRP78 expression was examined by Western blot analysis (C; upper panel). Cells were transfected with GRP78 or control siRNA for 24 h, before incubation with or without BFPP for 24 h. The percentage of apoptotic cells was also analyzed by flow cytometry of Annexin V/PI double staining (C; lower panel). Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with controls.

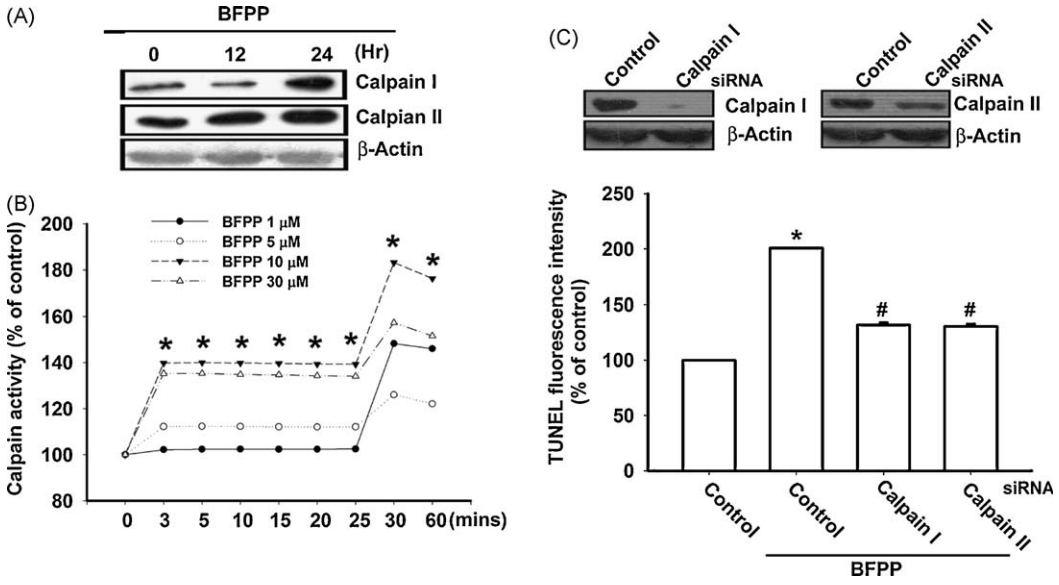

Fig. 5. Calpain activation is involved in BFPP-mediated cell apoptosis in human chondrosarcoma cells. (A) JJ012 cells were incubated with BFPP (10 μM) for different time intervals. Calpain I and II expression was examined by Western blot analysis. (B) JJ012 cells were incubated with BFPP and calpain activity was measured with the fluorescent calpain substrate. (C) Cells were transfected with calpain I, calpain II or control siRNA for 24 h, before incubation with BFPP (10 μM) for 24 h. The percentage of apoptotic cells was the analyzed by flow cytometry analysis of TUNEL-stained cells. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with controls; $^{\#}p < 0.05$ compared with the BFPP-treated group.

3.5. BFPP inhibits tumor growth in the mouse xenograft model

To determine whether BFPP possesses antitumor activities *in vivo*, we established xenografts of JJ012 cells in SCID mice. When the tumors reached 100 mm^3 in size, the mice were divided into three groups and treated with either vehicle or BFPP (0.5 or 1.5 mg/kg/day). BFPP dose-dependently inhibited of tumor growth (Fig. 7B). The average tumor volume in mice treated with BFPP 1.5 mg/kg/day was statistically significantly lower than the average tumor volume of vehicle-treated controls (Fig. 7A). *Ex*

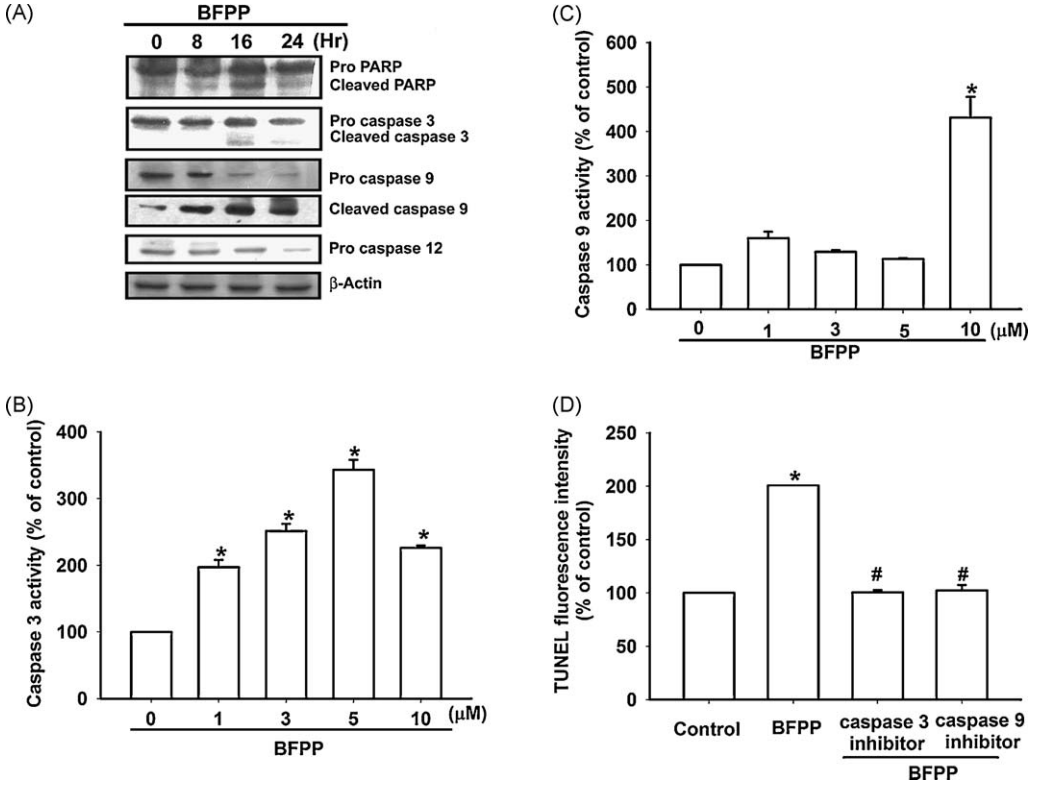

Fig. 6. BFPP induces the activation of caspases in human chondrosarcoma cells. (A) JJ012 cells were incubated with BFPP (10 μM) for different time intervals. Levels of PARP, caspase-3, caspase-9 and caspase-12 expressions were examined by Western blot analysis. JJ012 cells were incubated with BFPP for 24 h. Caspase-3 (B) and caspase-9 (C) activities were examined by caspase ELISA kit. (D) Cells were pretreated for 30 min with z-DEVD-FMK (caspase 3 inhibitor) or z-LEHD-FMK (caspase 9 inhibitor), followed by stimulation with BFPP for 24 h. The percentage of apoptotic cells was the analyzed by flow cytometry of TUNEL-stained cells. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with controls; $^{\#}p < 0.05$ compared with the BFPP-treated group.

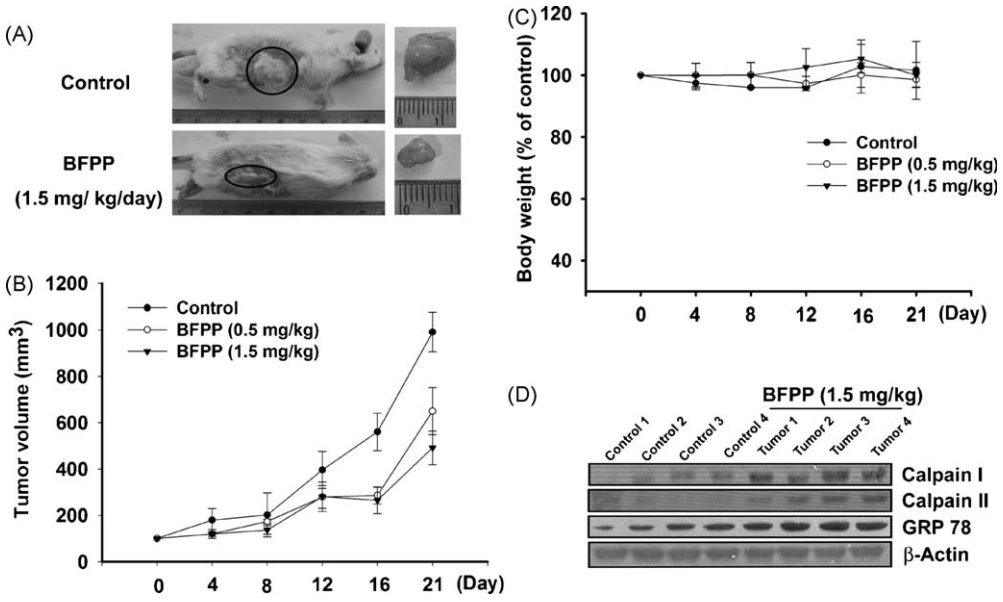

Fig. 7. BFPP inhibits tumor growth in SCID mice. (A and B) Mice were injected s.c. with JJ012 tumor cells. After the tumors reached 100 mm^3 in size, BFPP (0.5 or 1.5 mg/kg) or vehicle was administered daily for 3 weeks. Mean tumor volume was measured at the indicated number of days after implantation (n = 8–10). (C) Mean body weight was measured at the indicated number of days after implantation. (D) Western blot analysis determined levels of GRP78, calpain I and calpain II expression in tumors with and without BFPP treatment.

vivo analysis by Western blot of excised tumors showed significant increases in GRP78, calpain I and calpain II expression in the BFPP-treated groups compared with tumors from controls (Fig. 7D). These results suggest that BFPP inhibits tumor growth by inducing JJ012 cell apoptosis *in vivo*.

4. Discussion

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, in which long-term survival has increased dramatically with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to the absence of an effective adjuvant therapy [31]. Novel therapeutic agents targeting the malignant behavior of chondrosarcoma cells are needed to improve the prognosis. It has previously been reported that phloroglucinol derivatives induce antimitotic and antitumor effects in many human cancer cell lines [21–24]. However, the antitumor effects of phloroglucinol derivatives on chondrosarcoma cells are mostly unknown. In this study, we synthesized a new phloroglucinol derivative and examined its anticancer effects in human chondrosarcoma cells. We found that this compound induced cell death in human chondrosarcoma cell lines, but not primary chondrocytes. When we further examined the selectivity of BFPP in chondrosarcoma cells and primary chondrocytes, we found that BFPP did not affect the protein levels of GRP78, calpain I and calpain II in primary chondrocytes (Supplementary Fig. S2). It appears that these molecules have greater resistant to apoptosis compounds and that BFPP shows greater anticancer potential in human chondrosarcoma cells.

ER is the primary site for protein synthesis, folding, and trafficking [32]. Under a variety of stressful conditions, the accumulation of unfolded or misfolded proteins in the ER results in the onset of ER stress [32]. Elevation of cytosolic calcium levels or depletion of ER calcium stores represents typical responses of cells to various stimuli. Our study found that BFPP induces a number of ER stress markers, including elevated cytosolic calcium levels and activation of caspase 12. The calcium chelator BAPTA-AM blocked BFPP-induced cell apoptosis in human chondrosarcoma cells. These findings indicate that BFPP induces apoptotic cell death through stimulation of ER stress in human chondrosarcoma cells.

GRP78 up-regulation is believed to increase the capacity to buffer against stressful insults initiating from the ER [33]. It has been reported that GRP78 plays antiapoptotic roles and its considered to be a target for cancer chemotherapy [10]. Notably, we demonstrated in this study that BFPP increased GRP78 expression but not GRP94 expression. Stimulation of cells with BFPP also increased GRP78 mRNA expression and promoter activity. These results indicate that BFPP increases GRP78 transcription activity. In addition, pretreatment of cells with actinomycin D (transcriptional level inhibitor) or cycloheximide (translational level inhibitor) reduced BFPP-induced GRP78 mRNA expression (Supplementary Fig. S3). Therefore, induction of GRP78 by BFPP depends on *de novo* protein synthesis. Furthermore, the GRP78 siRNA antagonized BFPP-mediated potentiation of cell apoptosis, suggesting that GRP78 expression is an obligatory event in BFPP-induced cell death in these cells. A similar function has been reported with curcumin-induced apoptosis in human lung carcinoma cells through GRP78 up-regulation [15]. In addition, hepatitis C virus-induced cell apoptosis operates through the GRP78 up-regulation pathway [16]. Moreover, Hsu and colleagues have previously reported that dehydrocostuslactone induces GRP78 (Bip) expression and cell apoptosis in liver cancer cells [34]. Therefore, GRP78 may play a proapoptotic role in BFPP-induced cell death. Calpains and caspases are two families of cysteine proteases that are involved in the regulation of pathological cell death [19]. These proteases share several death-related substrates including the caspases themselves, cytoskeletal proteins, and proapoptotic proteins Bax and Bid [35]. Calpain-mediated proteolysis proceeds in a limited manner, without requiring a specific amino acid residue that is needed by caspases. Although both calpain and caspase have been proposed to play important roles in regulating pathological cell death, the interactions of these two families of proteases under pathological conditions remain unclear. In the present study, we found BFPP increased calpain I and II expression. Treatment of cells with BFPP also increased calpain activity. Knockdown approaches have contributed significantly to our knowledge of calpain biology,

particularly with respect to its specific function on cell apoptosis, which suggests that caspase 12 is downstream from calpain in mediating BFPP-induced chondrosarcoma cell apoptosis.

In conclusion, our data indicate that the novel phloroglucinol derivative BFPP induces cell death in human chondrosarcoma cells *in vitro* and *in vivo*. BFPP-induced cell death is mediated by increasing ER stress, GPR78 activation and Ca^{2+} release, which subsequently trigger calpain, caspase 12, caspase 9 and caspase 3 activity, resulting in apoptosis. We hope that our proposed working model for the molecular basis will provide valuable insights for the development of effective chemotherapy by targeting appropriate signal transducers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.01.002.

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