FPTB, a novel CA-4 derivative, induces cell apoptosis of human chondrosarcoma cells through mitochondrial dysfunction and endoplasmic reticulum stress pathways

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Abstract

Chondrosarcoma is a malignant primary bone tumor that responds poorly to both chemotherapy and radiation therapy. The aim of this study was to elucidate the mechanism of the novel Combretastatin A-4 derivative, 2-(furanyl)-5-(pyrrolidinyl)-1-(3,4,5-trimethoxybenzyl)benzoimidazole (FPTB) -induced human chondrosarcoma cells apoptosis. FPTB induced cell apoptosis in human chondrosarcoma cell line but not primary chondrocytes. FPTB induced upregulation of Bax and Bak, downregulation of Bcl-2 and Bcl-XL and dysfunction of mitochondria in chondrosarcoma. FPTB also triggered endoplasmic reticulum (ER) stress, as indicated by changes in cytosol-calcium levels. We found that FPTB increased GRP78 but not GRP94 expression. In addition, treatment of cells with FPTB induced calpain expression and activity. Transfection of cells with GRP78 or calpain siRNA reduced FPTB-mediated cell apoptosis. Therefore, FPTB-induced apoptosis in chondrosarcoma cells through the mitochondria dysfunction and involves caspase-9 and caspase-3-mediated mechanism. FPTB also induced cell death mediated by increasing ER stress, GPR78 activation, and Ca^{2+} release, which subsequently triggers calpain, caspase-12 and caspase-3 activity, resulting in apoptosis.

Key words: Chondrosarcoma; mitochondria; ROS; ER; GRP78

Introduction

Chondrosarcoma is the third most common primary bone malignancy after myeloma and osteosarcoma, accounting for approximately 20% of bone sarcomas and mainly affecting the middle-aged population (Gelderblom *et al.*, 2008; Ozaki *et al.*, 1997). Chondrosarcoma is a malignant primary bone tumor with a poor response to currently-used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge (Terek *et al.*, 1998). Due to the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and therefore, it is important to explore a novel and adequate remedy (Yuan *et al.*, 2005).

Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen. Aerobic respiration coupled to the generation of ATP leads to the formation of the superoxide anion radical ($O2^-$). Superoxide anion radicals can then form other ROS, such as hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radicals (Feig *et al.*, 1994; Schumacker, 2006). Oxidative stress occurs when this critical balance is disrupted because of excess reactive oxygen species, antioxidant depletion, or both. Accumulating evidence indicates that chemotherapeutic agents may be selectively toxic to tumor cells because they increase oxidant stress and enhance these already stressed cells beyond their limit (Ham *et al.*, 2006; Moungjaroen *et al.*, 2006). Cytotoxic ROS signaling appears to be triggered by the activation of the mitochondrial-dependent cell death pathway through the proapoptotic Bcl-2 proteins Bax or Bak, with consequent mitochondrial membrane permeabilization and cell death (Feig *et al.*, 1994; Roos *et al.*, 2006).

Endoplasmic reticulum (ER) is a central organelle engaged in lipid synthesis, protein folding and maturation. A variety of toxic insults, including hypoxia, failure of protein synthesis, folding, transport or degradation, and Ca^{2+} overload, can disturb the ER function and result in ER stress (Abcouwer *et al.*, 2002; Soboloff *et al.*, 2002; Yung *et al.*, 2007). There is increasing evidence that ER stress plays a crucial role in the regulation of apoptosis. It have been reported that ER stress triggers several specific signaling pathways, including ER-associated protein degradation and

unfolded protein response (UPR) (Feldman et al., 2005; Moenner et al., 2007). Glucose-regulated proteins (GRP) are most abundant glycoprotein in ER and play a critical in regulation ER. On the other hand, ER also plays a direct role in activating a subset of caspase during activation of apoptosis that occurs during ER stress (Liu et al., 2005). Calpains are a family of Ca^{2+}-dependent intracellular cysteine proteases. Ubiquitously expressed calpain-I (μ-calpain) and calpain-II (m-calpain) proteases are implicated in development of apoptosis. A recent study has shown that ubiquitous calpains promote caspase-12 activation during ER stress-induced apoptosis (Orrenius et al., 2003).

Combretastatin A-4 (CA-4), a natural cis-stilbene product, strongly inhibits the tubulin assembly by binding to the colchicine site and prevents tubulin polymerization (Cenciarelli *et al.,* 2008; Kanthou *et al.*, 2004). It also displayed potent cytotoxic activities against a variety of human cancer cell lines (Lin et al., 1989; Pettit et al., 1995). Numerous studies on the structure–activity relationships (SAR) of CA-4 have confirmed that the cis-orientation between the diaryl groups was essential for its strong cytotoxicity (Tron *et al.*, 2005). Based on their SAR, the important 3,4,5-trimethoxyphenyl pharmacophore was retained and its olefinic bond was replaced with non-heterocyclic bridge according to the principal of bioisosterism (Wang *et al.*, 2002). In addition, ring B was replaced with substituted phenyl rings or heterodyclic rings (Sun *et al.*, 2007). Therefore, modification produces a series of newly designed and synthesized compounds of CA-4 analogues, some of which have been screened for their antitumor activity. Therefore, we synthesized the new CA-4 derivative 2-(furanyl)-5-(pyrrolidinyl)-1-(3,4,5-trimethoxybenzyl)benzoimidazole (FPTB; Fig. 1A) and investigated the anticancer activity in human chondrosarcoma cells. Our data provide evidences that FPTB induced cell apoptosis of chondrosarcoma cells through mitochondrial dysfunction and ER stress pathways.

Materials and Methods

Materials

FPTB was synthesized at the Graduate Institute of Pharmaceutical Chemistry, China Medical University (Taichung, Taiwan). Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for Bax, Bcl-XL, Bcl-2, Bak, GRP78, GRP94, calpain I, calpain II, GADD153, PARP, caspase 3, caspase 9 and caspase 12 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GRP78 (-132 to +7) luciferase plasmid was provided from Dr. Kazutoshi Mori (Kyoto University, Kyoto, Japan) (Yoshida *et al.*, 1998). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA)

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 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 (Chiu *et al.*, 2007). The cells were grown in plastic cell culture dishes in 95% air-5% CO_2 with DMEM which was supplemented with 20 mM HEPES and 10% FBS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Sulforhodamine B assay

Cells viability by FPTB was measured using the sulforhodamine B (SRB) assay. FPTB was added at a range of concentration for 48 hr. Cells were fixed with 50% trichloroacetic acid to terminate reaction, and 0.4% SRB in 1% acetic acid was added to each well. After 15-min incubation, the plates were washed, and dyes were

dissolved by 10 mM Tris buffer. Then, the 96-well plate was read by enzyme-linked immunosorbent assay reader (515 nm) to get the absorbance density values.

Colony assay

To determine long-term effects of FTBP, cells (1,000 per well) were treated with FTBP at various concentrations for 3 hr. After being rinsed with fresh medium, cells were allowed to form colonies for 7 day and then were stained with crystal violet (0.4 g/L). After washing with ddH_2O three times, acetic acid was added to final concentration of 33% (v/v), followed by measuring the absorbance at 550 nm.

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells, as previously described (Dijkers *et al.*, 2002). Cells were treated with vehicle or FPTB for indicated time intervals. After treatment, cells were washed twice with PBS, and resuspended in staining buffer containing 1 μg/ml PI and 0.025 μg/ml annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson).

Quantitative assessment of apoptotic cells was also assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert™ DNA Fragmentation Assay Kit. Briefly, cells were incubated with FPTB for the indicated times. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37 °C. The stained cells were then analyzed with flow cytometer.

DAPI staining

4’-6-diamidino-2-phenylindole (DAPI), a DNA-binding fluorescent dye, was used to determine whether the mechanism of growth inhibition after FPTB treatment is through apoptosis. After treatment with FPTB for 48 hr, the cells were washed three times with PBS, fixed in a 3.7% formaldehyde solution for 10 min, fixed once in 1ml of methanol and then stained with DAPI for 10 min. Results were determined by visual observation of nuclear morphology through fluorescence microscopy.

Determination of the mitochondrial membrane potential

JJ012 cells were plated at a density of 1 X 10^4 cells on cover slips. Mitochondrial activity was assessed using the fluorescent probe, 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen). JC-1 accumulates in mitochondria where its aggregation depends on mitochondrial transmembrane potential (ΔΨm). At low ΔΨm, JC-1 exists in monomeric form, it is excited at 490 nm and emits at 527 nm. At high ΔΨm, JC-1 forms aggregates, resulting in a shift in emission to 585 nm. Cells were incubated with FPTB and then incubated with JC-1 (10 μg/ml) in culture medium for 30 min at 37 °C. Staining was visualized with fluorescence microscope (Nikon, USA). To quantify JC-1 aggregate to monomer ratio, cells were incubated with FPTB for indicated times and then incubated with JC-1. Cells were trypsinized, washed twice with PBS and analyzed by flow cytometry with an argon ion laser at 488 nm.

Measurements of reactive oxygen species

Levels of intracellular $O2^-$ and H_2O_2 were assessed spectrofluorimetrically by oxidation of specific probes: dihydroethidium (DHE) and H2DCFDA (Molecular Probes). Cells were plated at a density of 5 × 10^5, allowed to attach overnight, and exposed to FPTB for specified time intervals. The cells were stained with H2DCFDA (10 μM) and DHE (10 μM) for 10 min at 37 °C and the fluorescence intensity in cells was determined using the flow cytometry.

Detection of Ca^{2+} concentrations

Approximately 2×10^5 cells/well of JJ012 cells in 12-well plates were incubated with FPTB for 10, 30, 60 and 120 min to detect changes in Ca^{2+} levels. Cells were harvested and washed twice, and re-suspension in Fluo 3/AM (3 μg/ml) at 37 °C for 30 min and analyzed by flow cytometry.

Western blot analysis

The cellular lysates were prepared as described previously (Tang *et al.*, 2009). 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 Bax, Bak, Bcl-xL and Bcl-2 (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).

Reporter assay

The chondrosarcoma cells were transfected with reporter plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Twenty-Four hours after transfection, the cells were treated with FPTB or vehicle for 24 hr. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured (Tang *et al.*, 2009).

Caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzymesubstrate 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), 10mM dithiothreitol, 1mM 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.

Calpain activity assays

Suc-Leu-Leu-Val-Tyr-AMC is a calpain protease substrate. Quantitation of 7-amino-4-methylcoumarin (AMC) fluorescence permits the monitoring of enzyme hydrolysis of the peptide-AMC conjugate and can be used to measure enzyme activity. Cells were prepared and treated on 24-well Corning/Costar plates. Prior to addition of inhibitors cells were loaded with 40 M Suc-Leu-Leu-Val-Tyr-AMC (Biomol) and treated with FPTB for 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, Perkin Elmer) with filter settings of 360±20 nm for excitation and 460±20 nm for emission.

siRNA transfection

The siRNAs against human GRP78, calpain I, calpain II and control siRNA (for experiments using targeted siRNA transfection; each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased commercially from Santa Cruz Biotechnology. Cells were transfected with siRNAs (at a final concentration of 100 nM) using Lipofectamine 2000 (Invitrogen Life Technology) according to the manufacturer's instructions.

Statistics

The values given are means ± S.E.M. Statistical analysis between two samples was performed using Student's *t* test. Statistical comparisons of 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 as significant.

Results

FPTB induced cell apoptosis in human chondrosarcoma cells

To investigate the potential cell death of FPTB in human chondrosarocoma cells, we first examined the effect of FPTB on cell survival in human chondrosarcoma cells (JJ012). Treatment of JJ012 cells with FPTB-induced cell death in a concentration-dependent manner by using SRB assay (Fig. 1B). The IC_{50} value of FPTB was 4.8 μM for JJ012 cells. Next we investigated whether FPTB induced cell death in normal chondrocytes. However, FPTB (30 μM) did not affect the cell viability of normal chondrocytes (Fig. 1B). Colongenic assay correlate very well with in vivo assays of tumorigenicity in nude mice (Freedman *et al.*, 1974). JJ012 cells showed the ability to form clones in the un-treat control wells (Supplementary Fig. S1A). However, upon addition of FPTB, a dose-dependent inhibition in clonogenicity was observed, with a >60% inhibition at dosages as low as 3 μM FPTB (Supplementary Fig. S1A). The quantitative data are shown in Supplementary Fig. S1A; lower panel. We next investigated whether FPTB induces cell death through an apoptotic mechanism by DAPI staining, TUNEL and Annexin V/PI assay. Treatment of JJ012 cells with FPTB significantly increased the condensation of chromatin by DAPI staining using immunofluorescence microscopy (Supplementary Fig. S1B). In addition, compared with vehicle-treated JJ012 cells, FPTB-treated cells increased TUNEL fluorescence intensity (Fig. 2A). 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 FPTB (Fig, 2B&C). These data indicate that FPTB induced cell death through an apoptosis mechanism.

FPTB caused mitochondrial dysfunction

To explore whether FPTB-induced cell apoptosis is mediated through mitochondrial dysfunction, we determined the mitochondrial membrane potential with the mitochondria-sensitive dye, JC-1, using fluorescence microscope and flow cytometry. Control cells showed heterogeneous staining of the cytoplasm with both red and green fluorescence coexisting in the same cell. Treatment of JJ012 cells with FPTB induced marked changes in ΔΨm as evident from the disappearance of red fluorescence or the increase of green fluorescence. Some cells were devoid of red fluorescence, which is an indication of the loss of ΔΨm and the severity of cell damage (Supplementary Fig. S1C). Next we determined the mitochondrial membrane potential with JC-1, using flow cytometry. As shown in Fig. 3A, treatment of JJ012 cells with FPTB induced the loss of the mitochondrial membrane potential in a dose-dependent manner (Fig. 3A). To further determine whether FPTB induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of cytochrome c and Bcl-2 family proteins. Mitochondrial and cytosolic proteins were isolated from FPTB-treated JJ012 cells, respectively, then these proteins were subjected to Western blotting. Equal protein loading was confirmed by immunodetection of VDAC for mitochondrial protein or β-actin for cytosolic protein. As shown in Fig. 3B, FPTB a greatly increased the cytosolic cytochrome c and notably decreased the mitochondrial cytochrome c as compared with the control group. On the other hand, treatment of JJ012 cells with FPTB induced Bax and Bak protein levels (Fig. 3C). In addition, FPTB decreased the expression of Bcl-XL and Bcl-2, which led to an increase in the proapoptotic/antiapoptotic Bcl-2 ratio (Fig. 3C). These data suggest that FPTB induced cell apoptosis through mitochondrial dysfunction.

Reactive oxygen species and Ca^{2+} release are involved in FPTB-induced apoptosis in human chondrosarcoma cells

It has been reported that ROS generation plays an important role in the proapoptotic activities of various anticancer agents (Zhang *et al.*, 2004). Therefore, we next examined whether the ROS accumulation is involved in FPTB-induced cell death. DHE-based FACS detection revealed that intracellular $O2^-$ level was increased in JJ012 cells following treatment with FPTB (Supplementary Fig. S2A). On the other hand, FPTB also induced an increase in intracellular H_2O_2 levels, as shown by H2DCFDA-based FACS detection assay (Supplementary Fig. S2B). Pretreatment of cells with catalase (H_2O_2 scavenging enzyme), vitamin C (scavenger of oxygen-free radicals) and N-acetylcysteine (NAC is a thiol compound that can act as a cysteine source for the repletion of intracellular glutathione and act as a direct scavenger of ROS) reduced FPTB-increased cell apoptosis in human chondrosarcoma cells (Fig. 3D). Therefore, ROS release is involved in FPTB-medicated cell death in human chondrosarcoma cells. Depletion of luminal ER calcium stores is believed to reflect ER stress, which can promote induction of the ER stress (Benali-Furet et al., 2005). We assessed the effect of FPTB on the mobilization of Ca^{2+}. When JJ012 cells were treated with FPTB, Ca^{2+} levels were significantly increased as compared with the vehicle-treated group. The results demonstrated that FPTB promoted the Ca^{2+} release in a time-dependent manner (Supplementary Fig. S2C). On the other hand, BAPTA (Ca^{2+} chelator) also reduced FPTB-increased cell apoptosis in human chondrosarcoma cells (Fig. 3D). Therefore, Ca^{2+} release and ER stress are involved in FPTB-mediated cell apoptosis.

FPTB increased GRP78/GADD153 and calpain activity

GRP, a glucose-regulated protein, is a major ER chaperone and plays a critical role in regulating ER homeostasis (Yoshida *et al.*, 1998). We examined the effects of FPTB on the expressions of GRP78 and GRP94 in human chondrosarcoma cells. FPTB markedly increased the levels of GRP78 in a time-dependent manner, but GRP94 levels are not affected (Fig. 4A). To determine whether FPTB increased GRP78 through a transcriptional mechanism, JJ012 cells were transiently transfected

with GRP78 luciferase plasmid as an indicator of GRP78 activation. As shown in Fig. 4B, FPTB treatment of JJ012 cells for 24 h increased GRP78 luciferase activity. In addition, FPTB also increased mRNA expression of GRP78 (Fig. 4C). To further investigate whether FPTB induced cell apoptosis through the GRP78 activation, GRP78 siRNA was used. Transfection of cells with GRP78 siRNA specifically inhibited GRP78 expression (Fig. 4D, upper panel; Supplementary Fig. S3). On the other hand, GRP78 siRNA also reduced FPTB-induced cell apoptosis (Fig. 4D). It has been reported that increased GADD153 protein concentration is the major marker of ER stress (Moenner *et al.*, 2007). Stimulation of cells with FPTB also increased GADD153 expression (Fig. 4A). Therefore, GRP78/GADD153 expression is mediated FPTB-induced cell apoptosis. We next determined whether the activity of calpain (calcium-dependent thiol proteases) would be induced by FPTB in chondrosarcoma cells. As shown in Fig. 5A, FPTB increased calpain I and II expression in a time-dependent manner. Furthermore, FPTB also enhanced calpain activity dose- and time-dependently (Supplementary Fig. S2D). Transfection of cells with calpain I or II siRNA markedly reduced FPTB-mediated cell apoptosis (Fig. 5B). Therefore, our data suggest that calpain activation is involved in FPTB-mediated cell death.

One of the hallmarks of the apoptotic process is the activation of cysteine proteases, which represent both initiators and executors of cell death. FPTB increased the activation of caspase-3 in JJ012 cells (Fig. 6A&B). Pretreatment of cells with specific caspase-3 inhibitor (z-DEVD-FMK) reduced the FPTB-induced cell death (Fig. 6D). On the other hand, FPTB also increased cleaved-PARP (Fig. 6A). Upstream caspase-9 activities increased significantly, as shown by the observation that treatment with FPTB increased caspase-9 activity in JJ012 cells (Fig. 6A&C). It has been reported that calpains promote caspase-12 activation during ER stress-induced apoptosis (Orrenius *et al.*, 2003). FPTB also triggered the expression of cleaved caspase-12, which the magnitude of increase was proportional to the timing (Fig. 6A).

Discussion

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which are dramatic increase in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continue to have a poor prognosis due to absence of an effective adjuvant therapy (Fong *et al.,* 2007). Therefore, development of novel therapeutic agents targeting the malignant behavior of chondrosarcoma cells is important to improve the prognosis of patients. CA-4, a naturally occurring stilbene derived from the South African tree *Combretum caffrum* (Pettit *et al.*, 1989), shows potent cytotoxicity against a broad spectrum of human cancer cell lines. Therefore, the structural simplicity of CA-4 made this natural product an attractive lead compound in the development of new antitumor agents. Here, we synthesized the new CA-4 derivative 2-(furanyl)-5-(pyrrolidinyl)-1-(3,4,5-trimethoxybenzyl)benzoimidazole (FPTB). In this study, we identified FPTB as a potential lead base on anti-tumor activity in human chondrosarcoma cells with good pharmacological properties.

It has been reported that the enhancement of oxidative stress is associated with the apoptotic response induced by several anticancer agents (Haga *et al.,* 2005; Kallio *et al.*, 2005). ROS can cause apoptotic cell death via a variety of mechanism, among which is the activation of stress kinase. High levels of ROS can also induce apoptosis by triggering mitochondrial permeability transitionpore opening, release of proapoptotic factors and activation of caspase 9 (Iwamaru *et al.*, 2007; Zu *et al.*, 2005). O_2^- also has been shown to regulate Bcl-XL expression, and inhibition of O_2^- by O_2^- scavenger p-benzoquinone prevents camptothecin-induced apoptosis (Wenzel *et al.*, 2004). In this study, we found that FPTB mediated oxidative stress by increasing the production of O_2^- and H_2O_2. Treatment of cells with vitamin C (scavengers of oxygen-free radicals) and catalase (H_2O_2 scavenging enzyme) reduced the FPTB-induced cell death. Our data suggest that ROS accumulation contributes to FPTB-induced cell death in human chondrosarcoma cells.

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). Two major events have been noted in apoptosis involving mitochondrial dysfunction. One event is the change in the membrane permeability and subsequent loss of membrane potential (Zamzami *et al.*, 1998). The other is the release of apoptotic proteins including cytochrome *c* from the intermembrane space of mitochondria into the cytosol (Liu *et al.*, 1996). Here, we also found that FPTB reduced mitochondria membrane potential and increased the release of cytochrome c. Bcl-2 family proteins regulate mitochondria-dependent apoptosis with the balance of anti- and pro-apoptotic members arbitrating life-and-death decisions (Adams *et al.*, 2001). On the other hand, FPTB treatment results in a significant increase of Bax and Bak expression, and a decrease of Bcl-XL and Bcl-2, suggesting that changes in the ratio of proapoptotic and anti-apoptotic Bcl-2 family proteins might contribute to apoptosis-promotion activity of FPTB. In agreement of these observations, we noted that the mitochondrial dysfunction may be involved in FPTB-induced cell apoptosis of human chondrosarcoma cells.

ER is the primary site for protein synthesis, folding, and trafficking (Kaufman, 1999). Under a variety of stressful conditions, the accumulation of unfolded or misfolded proteins in the ER results in the onset of ER stress (Kaufman, 1999). Elevation of cytosolic-calcium levels or depletion of ER calcium stores represents typical responses of cells to various stimuli. Our study found that FPTB induced a number of ER stress markers, including cytosolic-calcium level elevation and caspase 12 activation. Calcium chelator BAPTA-AM blocked FPTB-induced cell apoptosis in human chondrosarcoma cells. Together, these findings indicate that FPTB induced apoptotic cell death through ER stress in human chondrosarcoma cells.

GRP78, a 78 kDa glucose-regulated protein, is a major ER chaperone and plays a critical role in regulating ER. GRP78 upregulation is believed to increase the capacity to buffer stressful insults initiating from ER. Here, we demonstrate that FPTB increased GRP78 but not GRP94 expression by Western blot assay. Furthermore, the GRP78 siRNA antagonized the FPTB-mediated potentiation of cell apoptosis,

suggesting that GRP78 expression is an obligatory event in FPTB-induced cell death in these cells. A similar function has been reported that curcumin-induced apoptosis in human lung carcinoma cells through GRP78 up-regulation (Fettucciari *et al.,* 2006). In addition, hepatitis C virus induced cell apoptosis also through GRP78 up-regulation pathway (Pezzuto, 1997). Hsu et al., also reported that dehydrocostuslactone induced GRP78 (Bip) expression and cell apoptosis in liver cancer cells (Hsu *et al.,* 2009). On the other hand, FPTB also increased GADD153 expression. Therefore, GRP78/GADD153 may play pro-apoptotic role in FPTB-induced cell death. Calpains and caspases are two families of cysteine proteases that they are involved in regulating pathological cell death (Tan *et al.,* 2006). These proteases share several death-related substrates including caspases themselves, cytoskeletal proteins, Bax, and Bid (Fettucciari *et al.,* 2006). Calpain-mediated proteolysis proceeds in a limited manner but does not require a specific amino acid residue like that of 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 are not clear. In the present study, we found FPTB increased calpain I and II expression. Treatment of cells with FPTB 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 is possible that caspases 12 is downstream from calpain in mediating FPTB-induced chondrosarcoma cell apoptosis.

In conclusion, our data indicate that novel CA-4 derivative FPTB induced cell death in human chondrosarcoma cells. FPTB-induced apoptosis in chondrosarcoma cells through the ROS release leads to mitochondria dysfunction and involves caspase-9 and caspase-3-mediated mechanism. FPTB also induced cell death mediated by increasing ER stress, GPR78 activation, and Ca^{2+} release, which subsequently triggers calpain, caspase-12 and caspase-3 activity, resulting in apoptosis.

Conflict of interest

None of the authors of this study has a conflict of interest statement.

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Figure legends

Fig. 1 The effects of FPTB on cell viability and colony formation in human chondrosarcoma cells

(A) Chemical structure of FPTB. (B) JJ012 cells and primary chondrocytes were incubated with various concentrations of FPTB for 48 hr, and the cell viability was examined by SRB assay. Results are expressed as the mean ± S.E. of four independent experiments. *: $p < 0.05$ as compared with control group.

Fig. 2 FPTB induced the apoptosis of human chondrosarcoma cells.

(A) Cells were treated with vehicle or FPTB for 48 hr, the TUNEL positive cells were examined by flow cytometry. (B&C) Cells were treated with vehicle or FPTB for 48 hr, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control.

Fig. 3 FPTB induced mitochondrial dysfunction in human chondrosarcoma cells.

(A) Cells were incubated with FPTB for 48 hr, mitochondrial membrane potential was satiated with JC-1 and examined by flow cytometry (n=4). (B) JJ012 cells were incubated with FPTB (10 μM) for different time intervals, the levels of cytochrome c in mitochondria and cytosol were examined by Western blot analysis. (C) JJ012 cells were incubated with FPTB (10 μM) for different time intervals, the Bax, Bak, Bcl-2 and Bcl-XL expressions were examined by Western blot analysis. (D) JJ012 cells were pretreated for 30 min with catalase (10000 U/ml), NAC (4 μM), vitamin C (100 μM) and BAPTA-AM (10 μM) followed by stimulation with FPTB (10 μM) for 48 hr, the percentage of apoptotic cells and cells viability were the analyzed by flow cytometic analysis of Annexin v/ PI-stained cells. (Q1 = necrotic cells; Q2 = late apoptotic; Q3 = living cells; Q4 = early apoptotic). Results are

expressed as the mean ± S.E. of four independent experiments. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with FPTB-treated group.

Fig. 4 GRP78 and GADD153 expression are involved in FPTB-mediated cell apoptosis in human chondrosarcoma cells.

(A) JJ012 cells were incubated with FPTB (10 μM) for different time intervals, the GRP78, GRP94 and GADD 153 expression were examined by Western blot analysis. (B) JJ012 cells transiently transfected with GRP78-luciferase plasmids for 24 hr, before incubation with FPTB for 24 hr. Luciferase activity was measured, and the results were normalized to the b-galactosidase activity (n=5). (C) JJ012 cells were incubated with FPTB (10 μM) for 24 hr, the mRNA expression of GRP78 was examined by qPCR analysis. (D; upper panel) Cells were transfected with GRP78 or control siRNA for 24 hr, and GRP78 expression was examined by Western blot analysis. (D; lower panel) Cells were transfected with GRP78 or control siRNA for 24 hr, before incubation with or without FPTB for 24 hr, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (n=4). (Q1 = necrotic cells; Q2 = late apoptotic; Q3 = living cells; Q4 = early apoptotic). Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control.

Fig. 5 Calpain activation is involved in FPTB-mediated cell apoptosis in chondrosarcoma cells.

(A) JJ012 cells were incubated with FPTB (10 μM) for different time intervals, and calpain I and II expressions were examined by Western blot analysis. (B; upper panel) JJ012 cells were transfected with calpain I, calpain II or control siRNA, calpain I and calpain II expression was examined by Western blot analysis. (B; lower panel) JJ012 cells were transfected with calpain I, calpain II or control siRNA for 24 h, before incubation with FPTB

(10 μM) for 48 h, the percentage of apoptotic cells were then analyzed by flow cytometric analysis of TUNEL-stained cells (n=5). Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with FPTB-treated group.

Fig. 6 FPTB induced the activation of caspases in human chondrosarcoma cells.

(A) JJ012 cells were incubated with FPTB (10 μM) for different time intervals, the PARP, caspase-3, caspase-9 and caspase-12 expression were examined by Western blot analysis. (B&C) JJ012 cells were incubated with FPTB for 24 hr, and caspase-3 and caspase-9 activities were examined by caspase ELISA kit (n=5). (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 FPTB for 24 hr, the percentage of apoptotic cells were the analyzed by flow cytometric analysis of TUNEL-stained cells. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with FPTB-treated group.

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