FPipTB, a benzimidazole derivative, induces chondrosarcoma cell apoptosis via endoplasmic reticulum stress and apoptosis signal-regulating kinase 1

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Abstract

Chondrosarcoma is the second most common primary bone tumor and it responds poorly to both chemotherapy and radiation treatment. In this study, we investigated the anticancer effects of a new benzimidazole derivative, 2-(furanyl)-5-(piperidinyl)-(3,4,5-trimethoxybenzyl) benzimidazole (FPipTB) in human chondrosarcoma cells. FPipTB induced apoptosis in human chondrosarcoma cell lines (JJ012 and SW1353) but not in primary chondrocytes. Furthermore it triggered endoplasmic reticulum (ER) stress, which was characterized by changes in cytosolic calcium levels. Treatment of chondrosarcoma cells with FPipTB was associated with increased intracellular levels of ASK1, p38, p53 and Bax, followed by release of cytochrome c from mitochondria and activation of caspases. It is also known that ER stress activates apoptosis signal-regulating kinase 1 (ASK1), which mediates activation of JNK and p38 pathways. We also found that FPipTB induced p38 and p53 phosphorylation and upregulated Bax expression. To study the mechanism of Bax upregulation, we determined that Bax promoter activity was increased in FPipTB-treated cells, leading to an increase in intracellular levels of Bax. In addition, cell treated with Ca^{2+} chelator or p38 inhibitor showed reduced transcriptional activity. The results further suggest that FPipTB triggered ER stress, as indicated by changes in cytosolic calcium levels and activated the ASK1-MKK3/6-p38-p53-Bax pathway, causing chondrosarcoma cell death. Importantly, animal studies revealed a dramatic 40% reduction in tumor volume after 21 days of treatment. Thus, FPipTB may be a novel anticancer agent for the treatment of chondrosarcoma.

Key words: Chondrosarcoma; Benzimidazole; ER stress; ASK1

Introduction

Due to its resistance to both ionizing radiation and chemotherapy, chondrosarcoma is making the management of chondrosarcoma a complicated challenge [1]. Thus, the management of chondrosarcoma is a complicated challenge [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 novel and adequate therapies are needed [3].

The endoplasmic reticulum (ER) plays essential roles in multiple cellular processes including protein synthesis, folding, modification, and trafficking. A variety of toxic insults including disruption of cellular redox regulation, hypoxia, Ca^{2+} overload, and failure of protein synthesis, folding, transport, or degradation can impair ER function and result in ER stress [4-6]. There is increasing evidences that ER stress plays a crucial role in the regulation of apoptosis and it triggers several specific signaling pathways, including ER-associated protein degradation (ERAD) and the unfolded protein response (UPR) [7,8]. The ERAD system removes misfolded and unfolded proteins from the ER, and the UPR reduces accumulation of misfolded protein load in the ER by attenuating of protein synthesis. It has been reported that ER stress and calcium influx activate apoptosis signal-regulating kinase 1 (ASK1) [9-13]. ASK1 activation is required for Ca^{2+}-induced activation of p38 in synaptosomes [13] and in neuronal cell [14]. ASK1 is a member of the mitogen-activated protein (MAP) kinase kinase kinase family, which activates both the MKK4/7-JNK and MKK3/6-p38 MAP kinase pathways and constitutes a pivotal signaling pathway in various types of stress-induced cell death, including exposure to tumor necrosis factor, ER stress, Fas ligation, and H_2O_2 [9,10,15]. To regulate ASK1 activity, there are multiple steps, including dimerization, phosphorylation, and protein-protein interactions [10,15]. Phosphorylation of the Ser967 residue in ASK1 is required for formation of the ASK-14-3-3 complex, which maintains ASK1 inactive [16-18]. Following stimulation, phosphorylation of Thr845, which is essential for ASK1 activation, triggers the dissociate 14-3-3 from Ser967 [19], resulting in enhancement of ASK1 catalytic activity [16].

Benzimidazole derivatives provide useful precursors or subunits for the development of molecules of pharmaceutical or biological interest [20]. They are of widely interested because of their diverse biological activity and clinical applications. Substituted benzimidazole derivatives have different therapeutic applications, including anti-histamine [21], anti-ulcerative [22], anti-inflammatory [23], anti-oxidant [24], anti-HIV-1 [25], anti-bacterial, [26] and anti-cancer activities [27]. However, the roles of benzimidazole derivatives in chondrosarcoma remain largely undefined. In this study, we synthesized a new benzimidazole derivative 2-(furan-2-yl)-5-(piperidin-1-yl)-1-(3,4,5-trimethoxybenzyl) benzimidazole (FPipTB) and investigated its anticancer activity in human chondrosarcoma cells. Our data provide evidence in human chondrosarcoma cells, FPipTB decreased cells survival and tumor growth both *in vitro* and *in vivo*.

Materials and methods

Materials

2-(Furan-2-yl)-5-(piperidin-1-yl)-1-(3,4,5-trimethoxybenzyl)benzimidazole (FPipTB: Fig. 1A) was synthesized at the Graduate Institute of Pharmaceutical Chemistry, China Medical University (Taichung, Taiwan). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG, and rabbit polyclonal antibodies specific for ASK1, 14-3-3, p38, JNK, p53, cytochrome c, Bcl-2, Bcl-xl, Bax, Bak, caspase 3, and caspase 9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody specific for p-ASK1, p-MKK3/6, p-p38, p-JNK, and p-p53 were purchased from Cell Signaling (Danvers, MA, USA). All other chemicals were purchased 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 human chondrosarcoma cell line SW1353 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM/α-MEM which were supplemented with 10% fetal bovine serum (FBS) and maintained at 37℃ in a humidified atmosphere of 5% CO_2.

Primary cultures of human chondrocytes were isolated from articular cartilage as we previously described [28]. The cells were grown in plastic cell culture dishes in 95% air-5% CO_2 in DMEM supplied with 20 mM HEPES, 10% heat-inactivated FBS, 2 mM-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

MTT assay

Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treating with FPipTB for 2 days, cultures were washed with PBS. Then MTT (0.5 mg/ml) were added to each well and the mixture was incubated at 37℃ for 2 h. To dissolve formazan crystals, culture medium was then replaced with an equal volume of DMSO. After the mixture was shaken at room temperature for 10

min, absorbance of each well was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

Colony assay

To determine the long-term effects of FPipTB, cells (1000 per well) were treated with FPipTB at various concentrations for 3 hr simultaneously. After rinsing with fresh medium, cells were allowed to form colonies for 7 days before being stained with crystal violet (0.4 g/l). After washing three times with ddH_2O, acetic acid was added to a final concentration of 33% (v/v), and the absorbance was measured at 550 nm [29].

Quantification of apoptosis by flow cytometry

Apoptosis was assessed by using annexin V, a protein that binds to phosphatidylserine (PS) residues exposed on the cell surface of apoptotic cells, as previously described [30]. Cells were treated with vehicle or FPipTB for the indicated times, washed twice with PBS, and resuspended 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 the dark, and cells were immediately analyzed by FACScan and the Cellquest program (Becton Dickinson; Lincoln Park, NJ, USA) [29].

Quantitative assessment of apoptotic cells was also assessed by examining the cell cycle. Cells were collected by centrifugation and adjusted to 3×10^6 cells/ml. Pre-chilled ethanol was added to 0.5 ml of cell suspensions and the mixture was incubated at 4 ℃ for 30 min. Ethanol was then removed by centrifugation, and cellular DNA was stained with 100 μg/ml PI (in PBS containing 0.1% Triton-X 100, and 1 mM EDTA) in the presence of an equal volume of DNase-free RNase (200 μg/ml). After staining, cells were analyzed immediately with a FACScan and Cellquest program. The extent of apoptosis was determined by measuring the DNA content of cells below sub G_1 peak [31].

Determination of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the fluorometric probe JC-1 (Calbiochem, CA, USA), a positively charged mitochondria-specific fluorophore that indicates depolarization by a fluorescence emission shift from green (525 nm) to red (610 nm) [32]. Briefly, cells were plated in 6-well culture dishes, grown to confluence, and treated with vehicle or FPipTB. After incubation, cells were stained with JC-1 (5 μg/ml) for 15 min at 37℃. Then analyzed by FACScan using an argon laser (488 nm). Mitochondrial depolarization, which is specifically indicated by a decrease in the red/green fluorescence intensity ratio, was analyzed by Cellquest program.

Measurement of Ca^{2+} concentration

Approximately 2×10^5 JJ012 cells/well in 12-well plates were incubated with FPipTB to detect changes in Ca^{2+} levels. Cells were harvested, washed twice, re-suspended in Fluo 3/AM (3 μg/ml) at 37℃ for 30 min, and analyzed by FACScan and Cellquest [29].

Western blot analysis

Cellular lysates were prepared as we described [31]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against p-ASK1, ASK1, 14-3-3, p-p38, p38, p-JNK, JNK, p-MKK3/6, p-p53, p53, cytochrome c, Bcl-2, Bcl-xl, Bax, Bak, caspase 3, caspase 9, or caspase 12 (1:1000 dilution) for 1 h at room temperature. After washed three times, the blots were incubated with a peroxidase-conjugated donkey anti-rabbit secondary antibody (1:1000 dilution) for 1 h at room temperature. The signals were visualized by enhanced chemiluminescence with Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA).

Caspase activity assay

The assay is based on the ability of active enzyme to cleave chromophore from enzyme substrate LEHD-pNA (for caspase 9) or Ac-DEVD-pNA (for caspase 3). Cell lysates were

prepared and incubated with anti-caspase 9 and caspase 3. 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) for 2 h at 37 ℃. The release of *p*-nitroaniline was monitored at 405 nm. Results shown are the percent change in activity compared to untreated control.

shRNA transfection

shRNA against human ASK1 and control shRNA were purchased from Santa Cruz Biotechnology. Cells were transfected with shRNA (at a final concentration of 2 μg/ml) using Lipofectamine 2000 (Invitrogen Life, Carlsbad, CA, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using a Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) with minor modifications of the manufacturer's instructions. Briefly, ~2 × 10^6 cells were fixed with 1% formaldehyde and lysed in SDS lysis buffer. DNA in the cross-linked chromatin preparations was sheared to 200–1000 bp by sonication. Samples were precleared with 50% slurry of salmon sperm DNA and protein A-agarose slurry. After addition of antibodies and fresh protein A-agarose, the samples were incubated at 4 ℃ overnight. Normal mouse or rabbit IgG was used as a control. Precipitated chromatin complexes were eluted with 500 ml of elution buffer (1% SDS, 0.1M $NaHCO_3$) for 30 min and the protein-DNA cross-links were reversed with overnight incubation in 100 μM NaCl at 65 ℃. Immunoprecipitated Bax DNA (region -530 to -193) was analyzed by PCR using the following primers forward, 5′-TAATCCCAGCGCTTTGGAAG-3′; reverse, 5′-ACTGTCCAATGAGCATCTCCCGAT-3′.

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 (1×10^6 in 200 μl) were injected subcutaneously into the flanks of SCID mice and tumors were allowed to develop until they reached a size of approximately 100 mm^3 (~14 days). The mice were treated with vehicle or with 0.5 or 1.5 mg/kg (i.p.; total volume 200 μl) FPipTB every day for 21 days (10 mice/group). The volume of implanted tumors in the dorsal side of the mice was determined twice a week with a caliper and the formula $V = LW^2/2$, where V =is volume (mm^3), L is = largest diameter (mm), and W is = smallest diameter (mm). All mice were manipulated in accordance with Animal Care and Use Guidelines of the China Medical University (Taichung, Taiwan) under a protocol approved by the Institutional Animal Care and Use Committee.

To investigate the cell apoptotic effect of FPipTB in tumor tissues *in vivo*, paraffin-embedded tumor sections were prepared, mounted on silane-coated slides, deparaffinized in xylene, rehydrated, and washed in distilled water. Protein was removed by digesting the sections with 20 μg/ml proteinase K for 15 min. After washing, labeling was performed by covering the sections with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) reaction mixture at 37 ℃ for 60 min. The reaction was blocked in stop/wash buffer for 10 min. The TUNEL labeling was visualized using fluorescence microscopy. TUNEL staining was performed using the Apoptosis Detection kit (Trevigen, Gaithersburg, MD, USA).

Statistics

The values reported are means ± SEM. 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 significant.

Results

FPipTB induces cell apoptosis in human chondrosarcoma cells

To investigate the potential for FPipTB to induce cell death in human chondrosarcoma cells, we first examined the effect of FPipTB on cell survival in human chondrosarcoma cells using the MTT assay. Treatment of JJ012 and SW1353 cells with FPipTB induced cell death in a concentration-dependent manner (Fig. 1B). The IC_{50} values of FPipTB were 8 and 24.5 μM for JJ012 and SW1353 cells, respectively. FPipTB did not affect the viability of normal primary chondrocytes. The anticancer activities of FPipTB were further assessed with clonogenic assays (Fig. 1C), which correlated very well with previous *in vivo* assays of tumorigenicity in nude mice [33]. JJ012, SW1353 and primary chondrocytes formed clones in untreated control wells (Fig. 1C), addition of FPipTB led to a dose-dependent inhibition in clonogenicity in JJ012 and SW1353 cells but not primary chondrocytes (Fig. 1C). We then examined the effect of FPipTB on the induction of cell death in JJ012 and SW1353 cells using PI staining. Treating cells with FPipTB induced a concentration-dependent increase in cell death, resulting in an increase in the percentage of cells in the sub G1 phase (Fig. 2A-B). Next, we investigated whether FPipTB induced cell death through an apoptotic mechanism. Annexin V/PI double-labeling was used to detect PS externalization, a hallmark of the early phase of apoptosis. Compared to vehicle-treated cells, a high proportion of annexin V labeling was detected in cells treated with FPipTB (Fig. 2C-D). To determine whether FPipTB induced apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of Bcl-2 family proteins. Treatment of JJ012 cells with FPipTB induced an increase in Bax protein levels (Fig. 3A). In addition, FPipTB decreased the expression of Bcl-2, which led to an increase in the proapoptotic/antiapoptotic Bcl-2 ratio (Fig. 3A). FPipTB did not affect expression of Bak or Bcl-xl in human chondrosarcoma cells (Fig. 3A), but did increase cytochrome c expression in the cytosolic fraction (Fig. 3A). To further explore whether FPipTB-induced apoptosis is mediated by mitochondrial dysfunction, we determined the mitochondrial membrane potential with the mitochondria-sensitive dye JC-1 using flow cytometry. As shown in Fig. 3B, treatment of JJ012 cells with FPipTB for 48

h induced the loss of the mitochondrial membrane potential in a dose-dependent manner. Therefore, FPipTB-increased cell death is mediated by mitochondrial dysfunction in human chondrosarcoma cells. One of the hallmarks of the apoptotic process is the activation of cysteine proteases, which include both initiators and executors of cell death. Treatment with FPipTB increased expression and activity of the upstream protein caspase 9 in JJ012 cells (Fig. 3A&C). FPipTB also increased the expression and activation of caspase 3 in JJ012 cells (Fig. 3 A&D). Pretreatment of cells with the specific caspase 3 inhibitor (z-DEVD-FMK) or the specific caspase 9 inhibitor (z-LEHD-FMK) [34] reduced FPipTB-induced cell death, as shown by annexin V/PI double-labeling (Fig. 3E).

FPipTB-induced Ca^{2+} release and ER stress in chondrosarcoma cells

Depletion of luminal ER calcium stores is believed to promote ER stress and thus serves as a marker for ER stress. Therefore, we assessed the effect of FPipTB on the mobilization of Ca^{2+}. When JJ012 cells were treated with FPipTB, Ca^{2+} levels were significantly increased as compared with the vehicle-treated group (Fig. 4A). Moreover, pretreatment of cells with the Ca^{2+} chelator BAPTA-AM reduced FPipTB-increased apoptosis in human chondrosarcoma cells (Fig. 4B). Therefore, Ca^{2+} production is required for FPipTB-mediated cell death in human chondrosarcoma cells. Another major indicator of ER stress is an increase in expression of glucose-regulated protein 78/BiP (GRP78) and growth arrest and DNA damage-inducible gene 153 (GADD153) [35]. We found that FPipTB markedly increased the levels of GRP 78 and GADD 153, in a time-dependent manner (data not shown). Taken together, these results indicate that FPipTB-induced cell death is mediated by Ca^{2+} production and ER stress in chondrosarcoma cells.

ASK1 signaling pathway was involved in FPipTB-mediated apoptosis in chondrosarcoma cells

Because ER stress and calcium induce ASK1 activation, we assessed the activation of ASK1 after FPipTB treatment. ASK1 activation, as indicated by phosphorylation at the

activation loop Thr845 and dephosphorylation at Ser967, was assessed by immunoblot analysis. Treatment of JJ012 cells with FPipTB significantly increased phosphorylation at Thr845 concomitant reduction of phosphorylation at Ser967 (Fig. 5A). Dissociation of ASK1 from 14-3-3 (an inhibitory protein) was previously reported to lead to ASK1 activation [36]. Therefore, we used coimmunoprecipitation to determine whether FPipTB-induced ASK1 dephosphorylation was accompanied by dissociation of the ASK1-14-3-3 complex. As shown in Fig. 5B, FPipTB rapidly induced ASK1 dissociation from 14-3-3. To further investigate whether FPipTB induced apoptosis through the ASK1 pathway, JJ012 cells were transfected with ASK1 shRNA for 24 h. ASK1 shRNA inhibited ASK1 expression and inhibited FPipTB-induced cell death (Fig. 5C). Thus, ASK1 activation is involved in FPipTB-induced cell apoptosis. Furthermore, pretreatment of cells with BAPTA-AM reduced FPipTB-induced ASK1 phosphorylation of Thr845 and dephosphorylation of Ser967 (Fig. 5D), indicating that Ca^{2+} production and ER stress are upstream events of ASK1 activation during FPipTB-induced cell death.

ASK1-MKK3/6-p38-p53 signaling pathways are mediated FPipTB-induced cell death

ASK1 belongs to the MAPKKK family and activates the p38 and JNK pathways via MKK3/6 and MKK4/7, respectively [37,38]. As shown in Fig. 6A, treatment of JJ012 cells with FPipTB resulted in time-dependent phosphorylation of MKK3/6, p38 but not JNK. Pretreatment of cells with p38 inhibitor (SB203580) or transfection of cells with p38 mutant antagonized the FPipTB-induced cells death (Fig. 6B). Taken together, these findings suggest that the ASK1-MKK3/6-p38 pathway is involved in FPipTB-induced cell death of chondrosarcoma. P53 is a transcription factor that plays a key role in the regulation of cell viability downstream of MAPK. Activation of p53 entails phosphorylation of its serine residues, primarily Ser15 [39,40]. Fig. 6C shows that FPipTB caused an increase in p53 phosphorylation at Ser15 in a time-dependent manner. The FPipTB-induced increase in p53 phosphorylation was attenuated by BAPTA-AM and SB203580 (Fig. 6D). These results support a causal role for the ASK1-MKK3/6-p38 signaling cascade in FPipTB-induced p53

activation. P53 has been shown to induce apoptosis by causing mitochondrial dysfunction via transactivation of Bax expression [41]. To confirm that FPipTB-induced cell death was mediated by Bax, we used ChIP assay show that Bax promoter could recruit p53 in the presence of FPipTB and this phenomenon was blocked by BAPTA-AM and SB203580 (Fig. 6E). These data demonstrate that the transcriptional activity of p53 is necessary for the Bax promoter and ASK1-MKK3/6-p38 pathway is involved in it.

FPipTB inhibits tumor growth in the mouse xenograft model of JJ012 cells

On the basis of the FPipTB-induced apoptotic effect exhibited *in vitro*, we decided to determine whether FPipTB possessed antitumor activities *in vivo*. We established xenografts of JJ012 cells in SCID mice; as tumors reached 100 mm^3 in size, the mice were divided into three groups and treated with either vehicle or FPipTB. FPipTB induced a dose-dependent inhibition of tumor growth (Fig. 7A&B). Moreover, in these two animal models, body weights were not significantly affected by FPipTB (Fig. 7C). In addition, an increase of TUNEL-positive cells was observed in tumors of the FPipTB-treated mice when compared with tumors taken from vehicle-treated mice (Fig. 7D). Finally, *ex vivo* analysis of tumors excised from mice showed significantly increased Bax, Bcl-2, p-MKK3/6, p-p38, and p-p53 expression in the FPipTB-treated group compared with that in the control group, as shown by Western blot (Fig. 7E). Taken together, these results suggest that FPipTB inhibits tumor growth by inducing JJ012 cell apoptosis *in vivo*.

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 [42]. The development of novel therapeutic agents targeting the malignant behavior of chondrosarcoma cells is important to improve the prognosis. Benzimidazole derivatives have been demonstrated to possess the effects of anti-bacterial, anti-fungal, and anti-viral [25,26,43]. It also has been reported that benzimidazole derivatives induced anti-mitotic and anti-cancer effects in many human cancer cells [44]. However, the anti-cancer effects of benzimidazole derivatives on chondrosarcoma cells are mostly unknown. Here, we synthesized a new benzimidazole derivative 2-(furan-2-yl)-5-(piperidin-1-yl)-1-(3,4,5-trimethoxybenzyl)benzimidazole and examined its anticancer effect in human chondrosarcoma cells. We found a new benzimidazole derivative FPipTB induced cell death in human chondrosarcoma cell lines but not primary chondrocytes. In this study, we identified FPipTB as a potential lead base on anticancer activity in human chondrosarcoma cells with good pharmacological properties. Our results revealed that FPipTB induced a significant concentration-dependent induction of chondrosarcoma apoptosis. We also showed that the ER stress-ASK1-MKK3/6-p38-p53-Bax signaling pathway is involved in FPipTB-mediated cell death.

ER is the primary site for protein synthesis, folding, and trafficking [45]. Under a variety of stressful conditions, the accumulation of unfolded or misfolded proteins in the ER results in the onset of ER stress [45]. Elevation of cytosolic calcium levels or depletion of ER calcium stores represents typical responses of cells to various stimuli. Our study found that FPipTB induced a number of ER stress markers, including cytosolic-calcium level elevation, GRP 78, and GADD 153 activation. In addition, treatment of cells with calcium chelator BAPTA-AM blocked FPipTB-induced cell apoptosis. Together, these findings indicate that FPipTB triggered ER stress, as indicated by changes in cytosol calcium levels is a critical mediator in FPipTB induced cell apoptosis.

ER stress can cause apoptotic cell death via a variety of mechanism, among which is the activation of stress kinase. ASK1 is a ubiquitously expressed MAPKKK that is activated by various stressors such as oxidative stress, calcium overload, TNF, Fas ligand, lipopolysaccharide, and ER stress and selectively activates the JNK and p38 MAPK pathways [9,37,46]. The activity of ASK1 is regulated by phosphorylation at different sites. Phosphorylation at Ser967 is essential for ASK1 association with 14-3-3 protein, which attenuates ASK1 activity. ER stress induces dephosphorylation of Ser967 and phosphorylation of Thr845 in the activation loop of ASK1, and both are correlated with ASK1 activity and ASK1-dependent apoptosis [36]. Here, we found that FPipTB enhanced dephosphorylation of ASK1 at Ser967 and phosphorylation at Thr845. Furthermore, ASK1 shRNA inhibited the FPipTB-induced cell death. Therefore, the ASK1 activation is required for FPipTB-induced cell apoptosis of chondrosarcoma. In the present study, we demonstrated that BAPTA-AM antagonized the FPipTB-induced ASK1 dephosphorylation at Ser967 and phosphorylation at Thr845. Therefore, ASK1 may function as a downstream signaling molecule of ER stress and Ca^{2+} after FPipTB treatment. We further examined the selectivity of FPipTB in chondrosarcoma cells and primary chondrocytes. We found that FPiPTB did not affect the ASK-1 phosphorylation in primary chondrocytes (data not shown). It appears that ASK-1 has greater resistant to apoptosis compounds and that FPiPTB shows greater anticancer potential in human chondrosarcoma cells. Recent studies have shown that ASK1 plays a critical role in apoptosis by stimulating the downstream MKK3/6-p38 signaling pathway [47,48] and ASK1 also is an upstream molecule of JNK and p38, which have been shown to be involved in the regulation of cell cycle and cell death [49]. In this study, we found that FPipTB increased the MKK3/6 and p38 but not JNK phosphorylation. Pretreatment of cells with p38 inhibitor and mutant antagonized the FPipTB-induced cell apoptosis. P53 has been described as having an important role in promoting cell apoptosis by regulating transcription of proapoptotic gene. Here, we noted that BAPTA-AM and SB203580 prevented FPipTB-induced p53 phosphorylation. Thus, it is plausible that FPipTB activates the ASK1-MKK3/6-p38 cascade to cause p53 phosphorylation and subsequent cell

death.

Mitochondrial dysfunction has been implicated as being a key mechanism in apoptosis in various cell death paradigms [50]. 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 [51]. The other is the release of apoptotic proteins including cytochrome c from the inter-membrane space of mitochondria into the cytosol [52]. Here, we also found that FPipTB 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 [53]. On the other hand, FPipTB treatment results in a significant increase of Bax expression, and decrease of Bcl-2, suggesting that changes in the ratio of proapoptotic and anti-apoptotic Bcl-2 family proteins might contribute to apoptosis-promotion activity of FPipTB. Bax-binding motifs are present within the putative promoter regions of p53. The expression of Bax has been shown to be directly induced by p53 at a transcriptional level [54]. Further characterization of the transcriptional response to FPipTB in chondrosarcoma cells demonstrated that Bax induction was p53-dependent, and then this phenomenon was blocked by BAPTA-AM and SB203580. Taken together, these results indicate that p53-dependent transcriptional induction of Bax play important roles in the sensitivity of cancer cells to apoptosis by FPipTB.

In conclusion, our data indicate that the novel benzimidazole derivative FPipTB induces cell death in human chondrosarcoma cells *in vitro* and *in vivo*. We showed that FPipTB induced ER stress as indicated by changes in cytosol calcium. In addition, FPipTB increased cell death by activating of ER stress, ASK1, MKK3/6, p38, and p53 and resulted in the transactivation of Bax expression. Thus, FPipTB is a promising chemotherapeutic agent worthy of further development for treatment of human chondrosarcoma cells.

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Conflicts of interest

The authors state no conflict of interest.

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Figures and legends

Figure 1. The effect of FPipTB on cell viability and colony formation in human chondrosarcoma cells. (A) Chemical structure of FPipTB. (B) JJ012, SW1353 and primary chondrocytes were incubated with various concentrations of FPipTB for 48 h, and the cell viability was examined by MTT assay. (C) For the colony-forming assay, the clonogenic assay was performed as described in Materials and Methods. The quantitative data are shown in the lower panel. Results are expressed as the means ± S.E.M. of four independent experiments. *, $p<0.05$ as compared with control group.

Figure 2. FPipTB induced apoptosis of human chondrosarcoma cells. (A-B) Cells were treated with vehicle or FPipTB for 48 h, the percentage of apoptotic cells was analyzed by flow cytometric analysis of PI-stained cells. (C-D) Cells were treated with vehicle or FPipTB for 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining. Results are expressed as the means ± S.E.M. of four independent experiments. *, $p<0.05$ as compared with control group.

Figure 3. FPipTB induced mitochondrial dysfunction in human chondrosarcoma cells. (A) JJ012 cells were incubated with FPipTB (10 μM) for different time intervals, the cytosolic cytochrome c, Bax, Bak, Bcl-2, Bcl-xl, caspase 3 and caspase 9 expression were examined by Western blot analysis. (B) JJ012 cells were incubated with various concentration of FPipTB for 48 h, the mitochondrial membrane potential was examined by flow cytometry (n=4). Caspase 3 (C) and caspase 9 (D) activities were examined by caspase ELISA kit (n=5). (E) Cells were pretreated for 30 min with caspase 3 inhibitor (20 μM) and caspase 9 inhibitor (100 μM) followed by stimulation with FPipTB (10 μM) for 48 h, the percentage of apoptotic cells were the analyzed by flow cytometric analysis of annexin V/PI-stained cells. Results are expressed as the means ± S.E.M. *, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with FPipTB-treated group.

Figure 4. FPipTB-induced Ca^{2+} release and ER stress in chondrosarcoma cells. (A) JJ012 cells were incubated with various concentration of FPipTB for 2 h, Ca^{2+} flux was examined by flow cytometry (n=4). (B) JJ012 cells were pretreated for 30 min with BAPTA-AM (10 μM) followed by stimulation with FPipTB (10 μM) for 48 h, the percentage of apoptotic cells were the analyzed by flow cytometric analysis of annexin V/PI-stained cells. Results are expressed as the means ± S.E.M. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with FPipTB-treated group.

Figure 5. ASK1 signaling pathway is involved in the FPipTB-induced cell apoptosis in chondrosarcoma cells. (A) JJ012 cells were incubated with FPipTB (10 μM) for different time intervals, the phosphoryaltion of ASK Ser967 and Thr845 was examine by Western blot analysis. (B) JJ012 cells were incubated with FPipTB (10 μM) for different time intervals and then immuoprecipitated with the anti-ASK1 antibody. The immunoprecipitated complex was then subjected to immunoblotting with an anti-14-3-3 antibody. IP: Immunoprecipitation. IB: Immunoblotting. (C) Cells were transfected with ASK1 or control shRNA for 24 h, the ASK1 expression was examined by Western blot analysis (upper panel). Cells were transfected with ASK1 or control shRNA for 24 h followed by stimulation with FPipTB (10 μM) for 48 h, the percentage of apoptotic cells was the analyzed by flow cytometric analysis of PI-stained cells (lower panel). (D) JJ012 cells were pretreated for 30 min with BAPTA-AM (10 μM) followed by stimulation with FPipTB (10 μM) for 180 min, the ASK1 phosphorylation was examined by Western blot analysis. Results are expressed as the means ± S.E.M. of four independent experiments. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with FPipTB-treated group.

Figure 6. ASK1-MKK3/6-p38-p53 signaling pathway is involved in FPipTB-induced cell death. (A) JJ012 cells were incubated with FPipTB (10 μM) for different time intervals, the p-MKK3/6, p-p38, and p-JNK expressions were examined by Western blot analysis. (B) JJ012 cells were pretreated for 30 min with SB203580 (10 μM) or transfection with p38

mutant followed by stimulation with FPipTB (10 μM) for 48 h, the percentage of apoptotic cells was the analyzed by flow cytometric analysis of annexin V/PI-stained cells. (C) JJ012 cells were incubated with FPipTB (10 μM) for different time intervals, the p-p53 expression was examined by Western blot analysis. (D) JJ012 cells were pretreated for 30 min with BAPTA-AM (10 μM) and SB203580 (10 μM) followed by stimulation with FPipTB (10 μM) for 120 min, the p-p53 phosphorylation was examined by Western blot analysis. (E) ChIP assay done on JJ012 cells were pretreated for 30 min with BAPTA-AM (10 μM) and SB203580 (10 μM) followed by stimulation with FPipTB (10 μM) for 120 min, and then sonicated and chromatin fragments were immunoprecipitated with anti-p53 antibody. Bax promoter contains p53 binding site. The binding of p53 on Bax promoter was analyzed by PCR and electrophoresed in agarose gels. Input, amplification of 1% of the total material used for immunoprecipitation. Results are expressed as the means ± S.E.M. of four independent experiments. *, $p < 0.05$ compared with control. $^{\#}$, $p < 0.05$ compared with FPipTB-treated group.

Figure 7. Effects of FPipTB on tumorigenicity and *in vivo* growth of xenografts in SCID mice. (A-B) The JJ012 cells (1×10^6) were injected subcutaneously into the 5-week-old SCID mice. After the tumors reached 100 mm^3 in size, the animals treated with an intraperitoneal injection of FPipTB (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) TUNEL assay in tissues from chondrosarcoma cells xenografts. Apoptosis happened only in approximately 1% of tumor tissue in the control group, while FPipTB treated tumors show marked green staining of fragmented nuclei, indicative of apoptosis. (E) Western blot analysis determined levels of Bax, Bcl-2, p-p38, p-p53, and p-MKK3/6 expression in tumor with and without treatment.