The novel benzimidazole derivative, MPTB, induces cell apoptosis in human chondrosarcoma cells

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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 benzimidazole derivative (5-methyl-2(pyridine-3-yl)-1-(3,4,5-trimethoxybenzyl)benzimidazole; MPTB) in human chondrosarcoma cells. MPTB induced cell apoptosis in two human chondrosarcoma cell lines, JJ012 and SW1353 but not in primary chondrocytes. MPTB induced upregulation of Bax and Bak and dysfunction of mitochondria in chondrosarcoma. MPTB triggered endoplasmic reticulum (ER) stress, as indicated by changes in cytosol calcium levels, and increased glucose regulated protein (GRP) expression. MPTB also increased calpain expression. Transfection of cells with GRP78 or calpain siRNA reduced MPTB-mediated cell apoptosis in JJ012 cells. Importantly, animal studies have revealed a dramatic 44% reduction in tumor volume after 21 days of treatment. This study demonstrates novel anticancer activity of MPTB against human chondrosarcoma cells and in murine tumor models.

Key words: Benzimidazole; Chondrosarcoma; ER; GRP; Calpain

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 [1; 2]. 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 [3]. 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 [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; 6; 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, GRP 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 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].

Several studies have revealed that a variety of therapeutic drugs are derived from benzimidazole analogues [20]. The benzimidazole structure also occurs in naturally, being part of the vitamin B_{12} molecule. Although the effects of benzimidazole derivatives on tumor apoptosis have been studied in various cancers [21; 22; 23], the cytotoxic activity of benzimidazole derivatives in chondrosarcoma remains largely undefined. Based on these considerations, we synthesized the new benzimidazole derivative (5-methyl-2(pyridine-3-yl)-1-(3,4,5-trimethoxybenzyl)benzimidazole; MPTB) (Fig. 1A) and investigated its anticancer activity in human chondrosarcoma cells. Our data indicate that MPTB reduces survival and tumor growth of human chondrosarcoma cells *in vitro* and *in vivo.*

Materials and Methods

Materials

Benzimidazole derivative (MPTB) 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 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). The GRP78 and GRP94 luciferase plasmids were provided from Dr. Kazutoshi Mori (Kyoto University, Kyoto, Japan) [24]. 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 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℃ 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).

Sulforhodamine B (SRB) assay

Cell viability by MPTB was measured with the SRB assay. MPTB was added in a range of concentrations for 48 hr. 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 mins 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.

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 MPTB for indicated time intervals. After treatment, cells were washed twice with PBS (pH 7.4) 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 mins 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 assessed by 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 the cells and incubated at 4 °C for 30 min. Ethanol was then removed by centrifugation and DNA of the cells was stained with Propidium iodide (PI) [100 μg/ml PI, 0.1% Triton-X, 1 mM EDTA in PBS] in the presence of an equal volume of DNase-free RNase (200 μg/ml) and analysed immediately by a FACScan and the Cellquest program (Becton Dickinson; Lincoln Park, NJ, USA). The extent of apoptosis was determined by measuring DNA content of the cells below the Sub G_1 peak.

DAPI staining

4'-6-diamidino-2-phenylindole (DAPI), a DNA-binding fluorescent dye, was used to determine whether the mechanism of growth inhibition after MPTB treatment is through apoptosis. After treatment with MPTB 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.

Colony assay

To determine the long-term effects of MPTB, cells (1,000 per well) were treated with MPTB at various concentrations for 3 hrs 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.

Detection of Ca^{2+} concentrations

JJ012 cells were seeded at approximately 2×10^5 cells/well in 12-well plates and incubated with MPTB 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.

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).

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), 10mM dithiothreitol, 1mM EDTA, 10% glycerol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.4) for 2 h at 37 ℃. 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.

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.

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 ng 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 sec and 60°C for 60 sec. 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).

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 MPTB 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 MPTB 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.

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 MPTB 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 $V = (LW_2)\ \pi/6$: where V, volume (mm_3); 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.

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.

Results

MPTB induces cell apoptosis in human chondrosarcoma cells

To investigate the cytotoxicity of benzimidazole derivative in human chondrosarocoma cells, we synthesized novel benzimidazole derivative (MPTB) and examined the effects on cell survival. Treatment of JJ012 and SW1353 cells with MPTB induced cell death in a concentration-dependent manner, as assessed by SRB assay (Fig. 1B&C) but not primary chondrocytes (data not shown). The IC_{50} values of MPTB were 7.1 and 7.5 μM for JJ012 and SW1353 cells, respectively. The anticancer activities of MPTB 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. 1D; upper panel). However, upon addition of MPTB, a dose-dependent inhibition in clonogenicity was observed; quantitative data are shown in the lower panel of Figure 1D. We next investigated whether MPTB 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 MPTB (Fig. 2A&B). We then investigated the effect of MPTB-induced apoptosis by using the cell cycle assay. Compared with vehicle-treated JJ012 cells, treatment of cells with MPTB increased sub G1 phase of cell cycle (Fig. 2B). In addition, stimulation of JJ012 cells with MPTB also significantly increased the condensation of chromatin by DAPI staining using immunofluorescence microscopy (Fig. 2C). These data indicate that MPTB induces cell apoptosis in human chondrosarcoma cells.

MPTB causes mitochondrial dysfunction

To explore whether MPTB-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 MPTB induced marked changes in ΔΨm as evident from the disappearance of red fluorescence or the increase of green fluorescence (Fig. 3C). Some cells were devoid of red fluorescence, which is an indication of the loss of ΔΨm and the severity of cell damage (Fig. 3C). Next we determined the mitochondrial membrane potential with JC-1, using flow cytometry. As shown in Fig. 3A&B, treatment of JJ012 cells with MPTB reduced red fluorescence (P2 region) and increased green fluorescence (P3 region). These results indicated that MPTB reduces mitochondrial membrane potential in human chondrosarcoma cells. To further determine whether MPTB induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of cytochrome c and Bcl-2 family proteins. As shown in Fig. 3C, MPTB a greatly increased the cytosolic cytochrome c compared with the control group. On the other hand, treatment of JJ012 cells with MPTB induced Bax and Bak protein levels (Fig. 3D). In addition, MPTB did not affect the expression of Bcl-XL and Bcl-2, which led to an increase in the proapoptotic/antiapoptotic Bcl-2 ratio (Fig. 3D). These data suggest that MPTB induced cell apoptosis through mitochondrial dysfunction.

MPTB induces 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 MPTB on Ca^{2+} mobilization. When JJ012 cells were treated with MPTB, Ca^{2+} levels were significantly increased as compared with the vehicle-treated group. The results demonstrated that MPTB promoted Ca^{2+} productions in a time-dependent manner (Fig. 4A). In contrast, pretreatment of cells with the Ca^{2+} chelator BAPTA-AM reduced MPTB-induced cell apoptosis (Fig. 4B). Thus, increased Ca^{2+} flux is involved in MPTB-mediated cell death in human chondrosarcoma cells.

MPTB increases GRP expression and calpain activity

GRP is a major ER chaperone that plays a critical role in regulating ER homeostasis [24]. MPTB markedly increased the levels of GRP78 and GRP94, in a time-dependent manner (Fig. 5A). Stimulation of cells with MPTB also increased GRP78 and GRP94 mRNA expression and luciferase activity (Fig. 5B&C). To further investigate whether MPTB induced cell apoptosis through GRP activation, cell were transfected with GRP78 siRNA, which specifically inhibited GRP78 expression (Fig. 5D; upper panel), but also reduced MPTB-induced cell apoptosis (Fig. 5D; lower panel). We next determined whether the calpain activity is induced by MPTB in chondrosarcoma cells. Transfection of cells with calpain I and II siRNA reduced calpain I and II expression, respectively (Fig. 5E; upper panel) and markedly reduced MPTB-mediated cell apoptosis (Fig. 5E; lower panel). Thus, our data suggest that GRP and calpain activation are involved in MPTB-mediated cell deaths.

MPTB increases caspase 3, 7, 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. MPTB increased the expression and activation of caspase-3/7 in JJ012 cells (Fig. 6A&B). Pretreatment of cells with the specific caspase-3 inhibitor z-DEVD-FMK reduced the MPTB-induced cell death (Fig. 6D). Notably, MPTB also increased cleaved-PARP expression (Fig. 6A). Upstream caspase-9 activities increased significantly upon treatment with MPTB in JJ012 cells (Fig. 6A&C). Pretreatment of cells with caspase-9 inhibitor z-LEHD-FMK reduced MPTB-mediated cell apoptosis (Fig. 6D). It has been reported that calpains promote caspase-12 activation during ER stress-induced apoptosis [18], we found that MPTB increased cleaved-caspase-12 in chondrosarcoma cells (Fig. 6A).

MPTB inhibits tumor growth in the mouse xenograft model

To determine whether MPTB 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 MPTB (0.5 or 1.5 mg/kg/day). MPTB dose-dependently inhibited of tumor growth (Fig. 7B). The average tumor volume in mice treated with MPTB 1.5 mg/kg/day was statistically significantly lower than the average tumor volume of vehicle-treated controls (Fig. 7A). Moreover, in these two animal models, body weights were not significantly affected by MPTB (Fig. 7C). *Ex vivo* analysis by Western blot of excised tumors showed significant increases in Bax, Bak, GRP78, GRP94, calpain I and calpain II expression in the MPTB-treated groups compared with tumors from controls (Fig. 7D). These results suggest that MPTB inhibits tumor growth by inducing JJ012 cell apoptosis *in vivo*.

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 [30]. Novel therapeutic agents targeting the malignant behavior of chondrosarcoma cells are needed to improve the prognosis. It has previously been reported that benzimidazole derivatives induce antimitotic and antitumor effects in many human cancer cell lines [21; 22; 23]. However, the antitumor effects of benzimidazole derivatives on chondrosarcoma cells are mostly unknown. In this study, we synthesized a new benzimidazole derivative and examined its anticancer effects in human chondrosarcoma cells. We found that the novel benzimidazole derivative MPTB induced cell apoptosis in human chondrosarcoma cells but not primary chondrocytes. In this study, we identified MPTB as a potential lead base on anti-tumor activity in human chondrosarcoma cells with good pharmacological properties.

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 [31]. The other is the release of apoptotic proteins including cytochrome *c* from the intermembrane space of mitochondria into the cytosol [32]. Here, we also found that MPTB 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 [33]. On the other hand, MPTB treatment results in a significant increase of Bax and Bak but not Bcl-XL and Bcl-2 expression, suggesting that changes in the ratio of proapoptotic and anti-apoptotic Bcl-2 family proteins might contribute to apoptosis-promotion activity of MPTB. In agreement of these observations, we noted that the mitochondrial dysfunction may be involved in MPTB-induced cell apoptosis of human chondrosarcoma cells.

ER is the primary site for protein synthesis, folding, and trafficking [34]. Under a variety of stressful conditions, the accumulation of unfolded or misfolded proteins in the ER results in the onset of ER stress [34]. Elevation of cytosolic calcium levels or depletion of ER calcium stores represent typical responses of cells to various stimuli. Our study found that MPTB induces a number of ER stress markers, including elevated cytosolic calcium levels and activation of caspase 12. The calcium chelator BAPTA-AM blocked MPTB-induced cell apoptosis in human chondrosarcoma cells. These findings indicate that MPTB induces apoptotic cell death through stimulation of ER stress in human chondrosarcoma cells.

GRP upregulation is believed to increase the capacity to buffer against stressful insults initiating from the ER [35]. Notably, we demonstrated in this study that MPTB increased GRP expression. Stimulation of cells with MPTB also increased GRP78 and GRP94 mRNA expression and promoter activity. These results indicate that MPTB increases GRP transcription activity. Furthermore, the GRP78 siRNA antagonized MPTB-mediated potentiation of cell apoptosis, suggesting that GRP78 expression is an obligatory event in MPTB-induced cell death in these cells. 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 proapototic proteins Bax and Bid [37]. 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 MPTB increased calpain I and II expression. 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 MPTB-induced chondrosarcoma cell apoptosis.

In conclusion, our data indicate that the novel benzimidazole derivative MPTB

induces cell death in human chondrosarcoma cells *in vitro* and *in vivo*. MPTB-induced cell death is mediated by increasing ER stress, GPR 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.

Acknowledgments

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Figure legends

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

(A) Chemical structure of MPTB. JJ012 (B) and SW1353 (C) cells were incubated with various concentrations of MPTB for 48 hr, and the cell viability was examined by SRB assay (n=5). (D) For the colony-forming assay, the clonogenic assay was performed as described under *Materials and Methods*. The quantitative data are shown in the lower panel. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with controls.

Fig. 2 MPTB induced apoptosis of human chondrosarcoma cells.

(A) JJ012 cells were treated with vehicle or MPTB for 48 hr. The percentage of apoptotic cells was analyzed by flow cytometry of Annexin V/PI double staining (n=4). (B) Cells were treated with vehicle or MPTB for 48 hr. The cell cycle analysis (PI staining) were examined by flow cytometry (n=4). (C) JJ012 cells were treated with vehicle or MPTB (10 μM) for 48 hr. The cells were stained with DAPI. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with controls.

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

(A&B) Cells were incubated with MPTB for 48 hr, mitochondrial membrane potential was satiated with JC-1 and examined by flow cytometry (n=4). (C) JJ012 cells were incubated with MPTB for 48 hr, and JC-1 staining was performed as described under *Materials and Methods.* Note that MPTB induced marked changes in $\Delta\Psi m$ as evident from the disappearance of red fluorescence or the increase of green fluorescence. (D) JJ012 cells were incubated with MPTB for different time intervals, the levels of cytochrome c, Bax, Bak, Bcl-2 and Bcl-XL expressions were examined by Western blot analysis. Results are expressed as the mean ± S.E. *, $p < 0.05$

compared with controls.

Fig. 4 MPTB induced Ca^{+2} release in chondrosarcoma cells.

(A) JJ012 cells were incubated with MPTB (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 MPTB (10 μM) for 24 hr. The percentage of apoptotic cells were the analyzed by flow cytometry of PI-stained cells. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with controls; #, $p < 0.05$ compared with the MPTB-treated group.

Fig. 5 GRP and calpain activation are involved in MPTB-mediated cell apoptosis in human chondrosarcoma cells.

(A) JJ012 cells were incubated with MPTB (10 μM) for different time intervals. GRP78 and GRP94 expression was examined by Western blot analysis. (B&C) JJ012 cells were incubated with MPTB for 24 hr, the mRNA expression and luciferase activity of GRP was examined by qPCR analysis and luciferase assay. Cells were transfected with GRP78 or control siRNA for 24 hr. GRP78 expression was examined by Western blot analysis (D; upper panel). Cells were transfected with GRP78 or control siRNA for 24 hr, before incubation with or without MPTB for 24 hr. The percentage of apoptotic cells was also analyzed by flow cytometry of PI staining (D; lower panel). Cells were transfected with calpain I, calpain II or control siRNA for 24 hr. Calpain I and calpain II expression was examined by Western blot analysis (E; upper panel). Cells were transfected with calpain I, calpain II or control siRNA for 24 hr, before incubation with or without MPTB for 24 hr. The percentage of apoptotic cells was also analyzed by flow cytometry of PI staining (E; lower panel). Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with controls. #, $p < 0.05$ compared with the MPTB-treated

group

Fig. 6 MPTB induces the activation of caspases in human chondrosarcoma cells.

(A) JJ012 cells were incubated with MPTB (10 μM) for different time intervals. Levels of PARP, caspase-3, caspase-7 caspase-9 and caspase-12 expression were examined by Western blot analysis. JJ012 cells were incubated with MPTB for 24 hr. Caspase-3/7 (B) and caspase-9 (C) activities were examined by caspase ELISA kit. (D) Cells were pretreated for 30 mins with z-DEVD-FMK (caspase 3 inhibitor) or z-LEHD-FMK (caspase 9 inhibitor), followed by stimulation with MPTB for 24 hr. The percentage of apoptotic cells was the analyzed by flow cytometry of PI-stained cells. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with controls; #, $p < 0.05$ compared with the MPTB-treated group.

Fig. 7 MPTB inhibits tumor growth in SCID mice.

(A&B) Mice were injected s.c. with JJ012 tumor cells. After the tumors reached 100 mm^3 in size, MPTB (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 Bax, Bak, GRP78, GRP94, calpain I and calpain II expression in tumors with and without MPTB treatment.

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