Activation of p38 MAPK-regulated Bcl-xL signaling increases survival against zoledronic acid-induced apoptosis in osteoclast precursors

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ABSTRACT

The nitrogen-containing bisphosphonate zoledronic acid (ZA) induces apoptosis in osteoclasts and inhibits osteoclast-mediated bone resorption. It is widely used to treat osteoporosis. However, some patients are less responsive to ZA treatment, and the mechanisms of resistance are still unclear. Here, we identified that murine osteoclast precursors may develop resistance to ZA-induced apoptosis. These resistant cells survived the apoptotic effect of ZA following an increase in anti-apoptotic Bcl-xL. Pharmacologically inhibiting Bcl-xL facilitated ZA-induced apoptosis. Treatment with ZA activated p38 MAPK, increasing Bcl-xL expression and cell survival. Nuclear import of β-catenin regulated by p38 MAPK determined Bcl-xL mRNA expression and cell survival in response to ZA. ZA also inactivated glycogen synthase kinase (GSK)-3β, a negative upstream regulator of β-catenin, in a p38 MAPK-mediated manner. Synergistic pharmacological inhibition of p38 MAPK with ZA attenuated receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast differentiation and facilitated ZA-induced apoptosis. These results demonstrate that elevated Bcl-xL expression mediated by p38 MAPK regulated GSK-3β/β-catenin signaling is required for cell survival of ZA-induced apoptosis in both osteoclast precursors and osteoclasts. Finally, we demonstrated that inhibiting p38 MAPK-mediated pathway enhanced ZA effect on increasing the bone mineral density of ovariec-tomized mice. This result suggests that targeting these pathways may represent a potential therapeutic strategy.

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Introduction

Osteoporosis, characterized by low bone mineral density (BMD) and abnormal bone quality, leads to decreased bone strength and increased susceptibility to fractures [1]. Bisphosphonates, pyrophosphate analogues utilized as bone-specific anti-resorptive agents, are commonly used for the treatment of osteoporosis, generally by inhibiting osteoclasts [2]. However, poor compliance is often observed due to irritation of the upper gastrointestinal tract and a strict dosing schedule [3]. Therefore, new intravenous drugs such as zoledronic acid (ZA) have been developed that allow dosing at much longer intervals, improving adherence [4]. ZA inhibits the farnesyl diphosphate-mediated mevalonate pathway, thereby inhibiting osteoclast proliferation and inducing apoptotic cell death in osteoclasts [2,5]. Biopsy of human bone samples shows higher trabecular volume, higher numbers, and decreased separation after treatment with ZA [6]. A large international clinical trial demonstrated the efficacy of an annual intravenous (IV) infusion of ZA for the treatment of postmenopausal osteoporosis [7]. Patients treated with ZA showed significant improvements in BMD and bone metabolism markers. ZA treatment reduced the risk of vertebral fracture by 70% and of hip fracture by 41% over 3 years relative to placebo. However, some patients do not respond to bisphosphonate treatment [8–10].

Non-response to bisphosphonates was defined as a decrease in BMD greater than the least significant change at the 95% confidence level or occurrence of fractures [11]. New fractures in patients with good compliance to therapy for 12 months or more should be considered a sign of possible therapeutic failure [12]. The proportion of patients who experience a decrease in BMD after treatment with oral bisphosphonates varies from 8 to 41% among different body parts and drugs [9,13–16]. Non-response rates are similar in patients who receive annual IV ZA. A small case series showed that 15% (3/20) of patients had lower BMD in the spine and 40% (8/20) had lower BMD in the hip after one year of treatment with ZA [17]. A large long-term cohort study demonstrated that ZA non-responders had a higher risk of osteoporotic...
fracture than responders [18]. Furthermore, the long-term results of ZA treatment were not superior to the short-term results. The 3-year HORIZON-PFT (Health Outcomes and Reduced Incidence with Zoledronic Acid Once Yearly Pivotal Fracture Trial) study was extended to 6 years to investigate the long-term effects of ZA on BMD and fracture risk [19]. The report revealed small differences in bone density and markers in those who continued treatment to 6 years versus those who stopped after 3 years. This result suggests that the anti-resorptive effect is not proportional to duration of treatment. After a period of treatment, osteoclasts may develop resistance to ZA and escape drug-induced cell death.

The molecular mechanism of non-response or resistance to ZA in osteoporosis treatment is not clear. However, we can find some clues in the literature regarding ZA treatment for malignancy. The molecular mechanism of resistance to ZA has been reported for several types of malignancy, including breast cancer, prostate cancer and osteosarcoma [20–23]. The purpose of this study was to investigate the mechanism of resistance to ZA in osteoporosis. This in vitro study suggests that osteoclasts or precursors develop resistance after continuous exposure to ZA. The resistance is mediated by strong activation of the p38 mitogen-activated protein kinase (p38 MAPK)-dependent pathway. We also show that inhibiting this pathway reverses resistance and promotes ZA-induced cell death in osteoclasts as well as enhances ZA treatment of osteoporosis in mice.

Materials and methods

Reagents

ZA (dissolved in purified water), DAPI (4',6-diamidino-2-phenylindole), β-catenin inhibitor PNU-74654 and propidium iodide (PI) were purchased from Sigma-Aldrich (St Louis, MO, USA). p38 MAPK inhibitor SB203580 was obtained from Calbiochem (San Diego, CA). Bcl-xL inhibitor ABT-737 was purchased from SelleckChem (Houston, TX). Recombinant mouse receptor activator of nuclear factor-kappa B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF): PeproTech (Rocky Hill, NJ); anti-GSK-3β (phosphoY216 and Y279); Abcam (Cambridge, MA); antibodies against PARP, caspase-8, caspase-9, caspase-3, McI-1, Bcl-xL, Bcl-2, phospho-p38 MAPK at Thr180/Tyr182, p38 MAPK, β-catenin, phospho-AKT at Ser473, AKT, phospho-GSK-3β, for or, β-actin: Chemicon International (Temecula, CA); Alexa Fluor 488- and HRP-conjugated goat anti-rabbit: Invitrogen (Carlsbad, CA). For intracellular inhibition of p38 MAPK, a non-cytotoxic dose was used. For animal study, Zoledronic acid (Aclasta®, from Novatis, Basel, Switzerland) and SB203580 (from Calbiochem, San Diego, CA) were used. A combination of atletamine hypochloride, and zolazeepam hypochloride (Zoletil 50, Virbac, TX) was used for anesthesia.

Cell cultures

RAW264.7 mouse macrophage/osteoclast precursor cells were purchased from American Type Culture Collection (ATCC #TIB-71) and were grown on plastic in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO), 100 units of penicillin, and 100 μg/mL of streptomycin at 37 °C under 5% CO2. Cells were used at 3 to 5 passages.

To generate bone marrow-derived osteoclast precursors (BMOP), bone marrow cells were isolated from the long bones of 6-week old male C57BL/6J mice [24]. Cells collected from bone marrow were washed and resuspended in RPMI 1640 medium (Invitrogen Life Technologies, Gaithersburg, MD) with 10% FBS. Cells were induced with a medium containing 10 ng/mL recombinant mouse M-CSF for 6 days. The medium was replaced every 2 days.

Proliferation assay

To evaluate cell proliferation, extracellular reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) by NADH produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator was evaluated using colorimetry (Cell Counting Kit-8; Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Cells were cultured in 96-well tissue culture plates in DMEM medium with propofol. WST-8 reagent (5 μL/well) was added after 24, 48, and 72 h of culture. A microplate reader (SBS Max 340PC; Molecular Devices, Sunnyvale, CA) was used to measure absorbance at 450 nm, and the data were analyzed with Softmax Pro software (Molecular Devices).

Cell cycle and apoptosis assay

Cell cycle was analyzed using PI (Sigma-Aldrich), as described previously [25]. Cell death was determined by using PI-based staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Apo-Direct Apoptosis Detection Kit, eBioscience, USA) plus flow cytometry (FACS Calibur, BD Biosciences) with excitation at 488 nm and emission detected in the FL-1 and FL-2 channels. Samples were analyzed using CellQuest Pro 4.0.2 software (BD Biosciences), and quantification was performed using Flowjo software (Tree Star, Inc, Ashland, USA). Small cell debris was excluded by gating on a forward scatter plot. For PI staining, levels of cells were reported and gated as percentages of each phase of cell cycle. To observe nuclear condensation, DAPI (Sigma-Aldrich)-stained cells were observed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Western blot analysis

Harvested cells were lysed with a buffer containing 1% Triton X-100, 50 mM of Tris (pH 7.5), 10 mM of EDTA, 0.02% NaN3, and a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Mannheim, Germany). Following one cycle of freeze–thaw, cell lysates were centrifuged at 12,000 rpm at 4 °C for 20 min. Lysates were boiled in sample buffer for 5 min. Proteins were then subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA) using a semi-dry electroblotting system. After blocking with 5% skim milk in PBS, the membranes were incubated with a 1/1000 dilution of primary antibodies at 4 °C overnight. The membranes were then washed with 0.05% PBS-Tween 20 and incubated with a 1/5000 dilution of horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. After washing, the membranes were soaked in ECL solution (PerkinElmer Life Sciences Inc, Boston, MA) for 1 min and then exposed to film (BioMax; Eastman Kodak, Rochester, NY). ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, San Diego, CA) was used to separate the cytosolic and nuclear protein.

Immunostaining

To detect expression of proteins, we fixed, stained, and analyzed the cells as described elsewhere [26]. For confocal microscopy, cells were stained with anti-β-catenin, phospho-GSK-3β (Ser9), and phospho-p38 MAPK (Thr180/Tyr182) antibodies and then with Alexa 488-conjugated goat anti-rabbit IgG. DAPI (5 μg/mL) was used for nuclear staining. Cells were then visualized using a confocal laser-scanning microscope (BX51; Olympus, Tokyo, Japan).

Real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol and reverse-transcribed with the ReverTaqTM First Strand cDNA Synthesis kit (Fermentas, Vilnius,
Lithuania). cDNA was amplified with Taq polymerase (Fermentas). mRNA samples were analyzed with SYBR Green-based real-time quantitative RT-PCR (Applied Biosystems, Foster City, CA, USA) with β-actin as the reference gene in each reaction. The Bcl-xL and β-actin primers, designed using the primer 3 program (Steve Rozen and Helen Skatesky, Whitehead Institute for Biomedical Research, Cambridge, MA), were as follows: Bcl-xL, 5′-GCTGGGACACTTTTGTGAT-3′ (sense) and 5′-TGTC TGGCATCTCCGACTG-3′ (antisense) and β-actin, 5′-ACTGGCGCATCC TCTTCTC-3′ (sense) and 5′-TGCCACAGGATATCATACCC-3′ (antisense). Experiments were conducted in triplicate. The results, originally expressed as [2 − ((gene-of-interest of cycles) − (β-actin cycles))], are shown as a ratio to control.

Osteoclast formation assay

RAW264.7 cells were plated in triplicate at a density of 4 × 10⁴ cells with α-MEM medium (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10 ng/mL RANKL in 12-well tissue culture plates for 3 days. After RANKL treatment, cells were treated with 100 μM ZA (Sigma-Aldrich) with or without 25 μM SB203580 (Cayman Chemical, Ann Arbor, MI). To determine osteoclast differentiation, a leukocyte acid phosphatase kit (Sigma-Aldrich) was used to stain tartrate-resistant acid phosphatase (TRAP), an osteoclast marker, according to the manufacturer’s instructions. TRAP-positive multinucleated cells with 3 or more nuclei were scored as osteoclasts. The percentage of osteoclast differentiation in the RANKL-only group was defined as 100.

Animal preparation and grouping

Thrity-six-week-old wild-type C57BL/6 female mice weighing 25 to 30 g were used in this study. Each mouse was housed individually in our animal care center with a 12/12-hour light/dark cycle, and with free access to food and water. The mice were subjected either to bilateral ovariectomies (OVX) or to sham surgery. They were divided into 5 groups: 1) Shame control. 2) OVX only. 3) OVX and subcutaneous ZA administration (100 μg/kg) once a week. 4) OVX and subcutaneous ZA plus daily intraperitoneal SB203580 (1 mg/kg) administration. 5) OVX and SB203580 only. All pharmacological treatment started 1 week after OVX and was continued for 8 weeks. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of our institution.

Determination of bone mineral density

The BMD of the lumbar spine and proximal region of the right tibia in mice was measured 8 weeks after indicated treatment by using dual-energy X-ray absorptiometry (DXA) with an apparatus for small animals (Norland XR-600, CooperSurgical Inc., CT, USA). The mice were anesthetized by intraperitoneal injection of a combined anesthetic agent (10 mg/kg Zoletil) and the measurements were performed in a prone position.

Statistical analysis

The results are expressed as means ± standard deviations (SDs). Groups were compared using Student’s 2-tailed unpaired t test or one-way ANOVA analysis followed by Dunnet’s post-hoc test when appropriate. Statistical significance was set at p < 0.05.

Results

ZA inhibits cell growth accompanying cell death and survival in osteoclast precursors

Previous studies showed the pro-apoptotic role of ZA in osteoclasts [2]. Mouse RAW264.7 cells, an osteoclast precursor cell line, were treated with ZA in various concentrations and evaluated at multiple time points. Analysis of cell proliferation at 48 or 72 h post-treatment demonstrated significant cell growth inhibition (p < 0.05) in cells treated with high-dose ZA (100 and 200 μM) (Fig. 1A). However, 200 μM ZA did not suppress growth more than 100 μM. By using DAPI-based nuclear staining (Fig. 1B), cell number decreased gradually in a time-dependent manner after treatment with ZA. Apoptotic cells, characterized by DNA condensation, could also be observed 48 h post-treatment. An unexpected result, observed 72 h post-treatment, was that some cells did not undergo apoptosis in response to ZA. PI staining followed by flow cytometry confirmed that some RAW264.7 cells underwent apoptosis (p < 0.05), as shown by PI signals in the sub-G1 phase, while some cells survived treatment with ZA (Fig. 1C). The evidence of apoptosis has also been confirmed by TUNEL staining followed flow cytometry (Fig. 1D). These results indicate that ZA treatment of osteoclast precursors caused mixed responses, including growth inhibition, cell apoptosis, and survival.

Inducible Bcl-xL mediates cell survival response in ZA-treated osteoclast precursors

Cell apoptosis and survival are regulated by a variety of proteins [27]. Therefore, we next checked the signaling of cell apoptosis and survival after ZA treatment. Protein expression was assessed by Western blot analysis in RAW264.7 cells treated with 100 μM ZA. The results demonstrated a time-kinetic cleavage of PARP, and caspase-3, indicating that apoptosis was induced by ZA (Fig. 2A). It is notable that an increase in Bcl-xL but not Bcl-2 or Mcl-1, which are anti-apoptotic proteins that influence cell survival [28], was identified in ZA-treated cells at 72 h post-treatment. To further test the role of increased Bcl-xL in cell survival, RAW264.7 cells and BMOP were pre-treated with a Bcl-xL inhibitor, ABT-737, prior to ZA stimulation. PI staining-based flow cytometry showed that combining ZA with ABT-737 facilitated cell apoptosis compared to ZA alone (p < 0.001, Fig. 2B). These findings suggest that inducible Bcl-xL expression plays an important role in cell survival after ZA treatment.

Activation of p38 MAPK positively regulates Bcl-xL expression and cell survival in ZA-treated osteoclast precursors

To investigate the upstream signaling pathways responsible for increased Bcl-xL levels, the survival role of p38 MAPK activation was next examined in response to ZA-induced apoptosis [21,29,30]. Western blot analysis showed the activation of p38 MAPK in ZA (100 μM)-treated RAW264.7 and BMOP cells, as revealed by protein phosphorylation at Thr180/Tyr182 (Fig. 3A). Inhibition of p38 MAPK with SB203580 effectively attenuated ZA (100 μM)-induced Bcl-xL upregulation in RAW264.7 cells at 48 h post-treatment (Fig. 3B). Inhibition of p38 MAPK followed by ZA treatment also facilitated cell apoptosis compared to ZA alone in either RAW264.7 cells or BMOP cells (p < 0.001, Fig. 3C). These results demonstrate that activation of p38 MAPK regulates inducible expression of Bcl-xL to determine cell survival in response to ZA treatment.

Activation of β-catenin by p38 MAPK mediates Bcl-xL expression and cell survival in ZA-treated osteoclast precursors

To clarify how p38 MAPK controls Bcl-xL expression, upstream transcription factor β-catenin was investigated [31,32]. In the presence of the p38 MAPK inhibitor SB203580, RAW264.7 cells were treated with 100 μM ZA for 72 h. Immunostaining followed by fluorescence microscopy and Western blot analysis of nuclear proteins showed activation of β-catenin in ZA-treated cells (Figs. 4A & B), characterized by nuclear import. [33] Inhibiting p38 MAPK effectively abolished nuclear import of β-catenin, indicating that p38 MAPK regulates β-catenin activation. Real-time PCR demonstrated that inhibiting either β-catenin or p38 MAPK.
MAPK significantly \((p < 0.001)\) decreased ZA-induced Bcl-xL mRNA expression (Fig. 4C). Furthermore, treating RAW264.7 cells and BMOP with \(\beta\)-catenin inhibitor PNU-74654 also facilitated ZA-induced cell apoptosis \((p < 0.001, \text{Fig. 4D})\). These findings indicate possible regulation of \(\beta\)-catenin by p38 MAPK and also verify a \(\beta\)-catenin-mediated Bcl-xL signaling pathway for cell survival in ZA-treated osteoclast precursors.

**Fig. 2.** Inducible Bcl-xL mediates cell survival after ZA treatment. (A) RAW264.7 cells were treated with ZA (100 \(\mu\)M). Western blot analysis detected the expression of the proteins indicated. \(\beta\)-actin was used as an internal control. One representative data set with optical density of 3 individual experiments is shown as compared with the normalized control. (B) Pretreating RAW264.7 cells and BMOP with Bcl-xL inhibitor ABT-737 (0.05 \(\mu\)M) 0.5 h prior to ZA (100 \(\mu\)M) treatment for 48 h, the percentage of apoptotic cells was detected using propidium iodide staining followed by flow cytometry. The result is shown as the mean \(\pm\) SD of triplicate cultures obtained from 3 individual experiments. ***\(p < 0.001\) compared with untreated; ###\(p < 0.001\) compared with ZA.

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**Fig. 1.** ZA inhibits cell growth, followed by either cell death or survival. RAW264.7 cells were treated with ZA but without RANKL. (A) A WST-8 assay was used to detect cell proliferation. The data is shown as the mean \(\pm\) SD of triplicate cultures from 3 individual experiments as compared with the normalized control. \(*p < 0.05; **p < 0.001.\) (B) Immunostaining followed by fluorescence microscopy showed cell apoptosis. A representative image obtained from 3 individual experiments is shown. Scale bar = 50 \(\mu\)m. Propidium iodide (C) and TUNEL staining (D) followed by flow cytometry indicated the proportion of cells in the sub-G1 phase and apoptotic cells, separately. The result is shown as the mean \(\pm\) SD of triplicate cultures obtained from 3 individual experiments. **\(p < 0.001\) compared with untreated.
We next studied the involvement of GSK-3β inactivation by ZA in p38 MAPK-regulated β-catenin activation, since GSK-3β negatively regulates β-catenin [34] and is negatively regulated by p38 MAPK [35,36]. In ZA (100 μM)-treated RAW264.7 cells, phosphorylation of GSK-3β at serine residue Ser9, producing an inactive form, was increased in a time-dependent manner. By contrast, phosphorylation of GSK-3β at tyrosine residue Tyr216, which gives an active form, was time-dependently decreased (Fig. 5A). However, Akt, the regulator that most commonly phosphorylates GSK-3β at Ser9, was not activated by ZA treatment (data not shown). We therefore investigated the upstream role of p38 MAPK in GSK-3β inactivation, as seen in previous studies [35,36]. Immunostaining followed by fluorescence microscopy and Western blot analysis of nuclear proteins showed that inhibiting p38 MAPK decreased GSK-3β phosphorylation at Ser9 as well as nuclear import (Figs. 5B & C). After activation by ZA as shown in Fig. 3A, p38 MAPK also translocated into the nucleus, as did inactivated GSK-3β (Fig. 5D). These results indicate that ZA inactivates GSK-3β in a p38 MAPK-regulated manner.

Inhibiting p38 MAPK facilitates ZA-induced apoptosis and inhibition of RANKL-induced differentiation in osteoclasts

Our studies indicate a strategy for cell survival in response to pro-apoptotic stimulation by ZA. To confirm that this pathway benefits cell survival, we next examined the role of p38 MAPK in ZA-induced cell apoptosis and inhibition of RANKL-induced differentiation in osteoclasts [37]. RAW264.7 cells were pre-treated with RANKL, followed by treatment with ZA and p38 MAPK inhibitor SB203580. Compared with ZA alone, inhibiting p38 MAPK conferred a synergistic effect on ZA-induced cell apoptosis (p < 0.001, Fig. 6A) during RANKL-induced differentiation in osteoclasts [38]. TRAP staining followed by microscopic observation showed osteoclast differentiation in RAW264.7 cells treated with ZA for 6 days. Notably, inhibiting p38 MAPK facilitated ZA-induced inhibition of RANKL-induced differentiation (Fig. 6B). These findings imply that targeting
p38 MAPK facilitates ZA-induced apoptosis and inhibits differentiation in osteoclasts.

SB203580 enhances ZA treatment effect on increasing bone density in osteoporotic mice

We further confirmed the therapeutic role of combination ZA with p38 MAPK inhibitor, SB203580 in a mouse model of osteoporosis by using DXA. The mice were ovariectomized and treated with ZA plus SB203580 or not. We found that the mice treated with ZA and SB203580 for 8 weeks showed significantly higher BMD in femur than those treated with ZA only (Fig. 6C). Treating with SB203580 alone didn’t produce any therapeutic effect. Regarding lumbar spine, mice treated with ZA plus SB203580 and ZA alone both increased BMD. However, there was no significant difference between two groups.

Discussion

The most significant finding of this study is that osteoclast precursors develop a survival advantage against the pro-apoptotic effects of ZA via p38 MAPK-regulated GSK-3β inactivation, followed by β-catenin-mediated Bcl-xL expression (Fig. 6D). Blocking this pathway may enhance ZA treatment of osteoporosis or osteoclast-associated bone disorders in our osteoclast differentiation cell model and osteoporosis animal model.

The HORIZON trial showed that ZA reduces fracture risk [4]. However, this trial recruited many patients who had previously suffered vertebral fractures. This high-risk population might show a greater reduction in fracture risk after treatment than patients without previous osteoporotic fractures. Some patients still showed lower BMD despite treatment with ZA or other bisphosphonates [17,18]. In addition, long-term ZA administration (6 years) showed little benefit compared to 3 years [19]. This result suggests that resistance to ZA may occur in some situations, as seen in our study.

Although ineffectiveness of ZA treatment or its mechanism of action has seldom been reported for osteoporosis, ZA resistance in cancer therapy is well documented [20–23]. These resistant signaling pathways involve p38 MAPK, heat-shock protein 27, or farnesyl diphosphate synthase. In the present study, although most osteoclast precursors underwent DNA fragmentation and cell death, some cells did develop a survival response after ZA treatment. Increased duration of treatment or drug concentration did not induce cell death more effectively. This finding is consistent with previous clinical results [19].

We found that Bcl-xl was induced, mediating cell survival responses after ZA treatment, but Bcl-2 and Mcl-1 were not. Bcl-xl, a member of the Bcl-2 family of proteins, is a transmembrane molecule in mitochondria that works at the level of the intrinsic apoptotic pathway to act as a pro-survival protein by inhibiting the release of mitochondrial contents for caspase activation. Bcl-xl has been reported to promote survival in several cellular models, such as macrophages [39], leukemic cells [40], and hepatoma cells [41]. Our model demonstrated abundant Bcl-xl expression in surviving cells after ZA treatment. We also found that the Bcl-2/Bcl-xl inhibitor ABT-737 increased the lethality of ZA to osteoclast precursors. ABT-737 has been reported to sensitize hematopoietic malignant solid tumors to chemotherapy and radital therapy [42] and to overcome the drug resistance of leukemic cells [43]. Further studies of ABT-737 in combined therapy for osteoporosis would be worthwhile.
Out results reveal that p38 MAPK also plays an important role in the cell survival response of osteoclast precursors. p38 MAPK was reported to have dual roles in both cell survival and death pathways [44]. In osteoclasts, p38 MAPK mediates survival and differentiation but not bone resorption [37,45]. Inhibiting p38 MAPK prevents inflammatory bone destruction in animal models of arthritis [46,47]. We found that osteoclast precursors also express p38 MAPK. This result is consistent with a previous report that p38 MAPK in osteoclast precursors was involved in the early steps of osteoclast formation [48]. We showed that p38 MAPK was expressed in osteoclast precursors, acting in a pro-survival role after ZA treatment. The mechanism of p38 MAPK induction by ZA is still unknown, but may involve reactive oxygen species [49] or mitogen-activated protein kinase kinase [50]. Therapies targeting p38 MAPK may be beneficial against destruction of bone in inflammatory arthritis [46,47] as well as in ZA-resistant prostate cancer or other malignancies [21,29,30]. Our results suggest that this strategy may also work for treatment of osteoporosis. Inhibiting p38 MAPK with SB203580 lowered Bcl-xL expression and facilitated ZA-induced cell death in both RAW 264.7 cells and BMOPs. These findings strongly indicate that combined therapy with ZA and SB203580 would be more effective than ZA alone. Further studies are needed to verify the clinical relevance of this finding.

To further explore how p38 MAPK regulates Bcl-xL, we identified a transcription factor, β-catenin, functioning upstream of Bcl-xL. The influence of β-catenin-induced Bcl-xL expression on cell survival has been well documented [31,32]. Our results show that osteoclast precursors also feature this cascade, regulated by p38 MAPK. The synthetic compound PNU-74654 is a β-catenin inhibitor that functions by blocking the interaction of β-catenin and T cell factor [51]. The biological effect of PNU-74654 is under further investigation for anti-cancer therapy. We found that inhibition of β-catenin by PNU-74654 also decreased downstream Bcl-xL mRNA expression and promoted the death of osteoclast precursors after ZA treatment. To our knowledge, this is the first evidence of a potential therapeutic effect of PNU-74654 in osteoporosis.

We also found that p38 MAPK induced nuclear import of β-catenin and GSK-3β, which is known to act upstream of β-catenin. Inhibitory phosphorylation of GSK-3β has been reported to increase levels of β-catenin and Bcl-xL [34]. Here, we found that inhibitory phosphorylation of GSK-3β was induced time-dependently after ZA treatment of osteoclast precursors. This Akt-independent process was consistent with previous reports that reported direct phosphorylation of GSK-3β by p38 MAPK in several types of cell [35,36].

RANKL is the most important known signal for osteoclast survival, differentiation, and functioning. In the presence of RANKL, osteoclasts are more resistant to bisphosphonate-induced apoptosis and retain their bone absorptive ability [52,53]. This phenomenon may explain the ineffectiveness of some bisphosphonates against inflammation-related bone loss [54]. However, we found that inhibiting p38 MAPK led to sensitization of osteoclast precursors to ZA treatment, even in
the presence of RANKL. Combined ZA and SB203580 therapy not only increased the apoptosis of osteoclast precursors but also diminished osteoclast differentiation.

We finally confirmed the therapeutic role of combination ZA with p38 MAPK inhibitor, SB203580 in an ovariectomized mouse model of osteoporosis. Both ZA alone and ZA plus SB203580 rescued bone loss after O VX. With SB203580, ZA worked better to increase the BMD of femur. This result verifies our finding of in vitro study that blocking p38 MAPK pathway may enhance ZA treatment of osteoporosis. In the literature, the BMD changes of extremities are usually more sensitive than those of lumbar spine [55]. This might be the reason that our results showed that the treatment effect of ZA plus SB203580 was superior to ZA alone only in femur but not in lumbar spine. Further studies should be conducted to claim the role of combined therapy of ZA and SB203580 more clearly.

Bisphosphonates were reported to stop bone loss by not only inhibiting osteoclasts but also influencing directly the function of osteoblastic lineage [56]. Bisphosphonates were shown to prevent apoptosis of osteoblasts and osteocytes in vitro and in vivo [57,58]. However, some other studies showed that bisphosphonates decreased proliferation and inhibited differentiation of osteoblasts [59,60]. It was thought that this apparent contradiction was due to drug dosage [56]. The complex interaction of bisphosphonates, apoptosis and bone cells requires further investigation.

In conclusion, our study demonstrates that osteoclast precursors develop a cell survival response against ZA treatment via p38 MAPK-regulated GSK-3β inactivation, followed by β-catenin-mediated Bcl-xL expression. Targeting this pathway along with ZA may serve as an advanced combination therapy to treat osteoporosis or osteoclast-associated bone disorders.

Conflict of interest

Dr CF Lin and Prof. IM Jou are supported by grants from the National Science Council, Taiwan. There is no other financial support or conflict of interest to disclose.

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