Platycodin D Blocks Breast Cancer-Induced Bone Destruction by Inhibiting Osteoclastogenesis and the Growth of Breast Cancer Cells

Sun Kyoung Lee\textsuperscript{a,b} Kwang-Kyun Park\textsuperscript{a,b} Hyun-Jeong Kim\textsuperscript{a} Ki Rim Kim\textsuperscript{d} Eun Ji Kang\textsuperscript{a,b} Yu Li Kim\textsuperscript{a,b} Heein Yoon\textsuperscript{a,b} Yeong Shik Kim\textsuperscript{c} Won-Yoon Chung\textsuperscript{a,b}

\textsuperscript{a}Department of Oral Biology, Oral Cancer Research Institute, BK21 PLUS Project, Yonsei University College of Dentistry, Seoul, \textsuperscript{b}Department of Applied Life Science, The Graduate School, Yonsei University, Seoul, \textsuperscript{c}Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul, \textsuperscript{d}Department of Dental Hygiene, Kyungpook National University, Sangju, Republic of Korea

Key Words
Platycodin D • Breast cancer • Osteoclastogenesis • Bone destruction

Abstract
Background: Metastatic breast cancer cells are frequently associated with osteoclast-mediated bone resorption, resulting in severe bone destruction and increased mortality in patients. Platycodin D (PD) isolated from \textit{Platycodon grandiflorum} is a triterpenoid saponin with anti-cancer and anti-angiogenic potential. Methods: The \textit{in vivo} activity was determined in mice with the intratibial injection of human metastatic breast cancer cells. Osteoclast formation and activity were detected using tartrate-resistant acid phosphatase staining and calcium phosphate-coated plates. The expression of osteoclastogenesis-inducing molecules was detected by RT-PCR and western blotting in RANKL-treated bone marrow macrophages (BMMs). Cell viability and DNA synthesis were measured with MTT and BrdU incorporation assays. The induction of apoptosis was estimated using TUNEL staining and a caspase-3 activity assay. Results: The oral administration of PD inhibited MDA-MB-231 cell-induced osteolysis in an intratibial mouse model. PD treatment blocked RANKL-induced osteoclast formation by inhibiting the expression and nuclear translocation of NFATc1 and c-Fos in BMMs and consequently reduced osteoclast-mediated bone resorption. Furthermore, PD treatment induced apoptosis in osteoclasts and inhibited the growth of MDA-MB-231 cells. Conclusion: PD may block breast cancer-induced bone loss by suppressing the formation, activity, and survival of osteoclasts, as well as the growth of metastatic breast cancer cells.
Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide. Advanced breast cancer exhibits a high incidence of bone metastases at a frequency of approximately 70%, resulting in increased mortality [1, 2]. Metastatic breast cancer cells produce parathyroid hormone-related protein (PTHrP), interleukin (IL)-1β, IL-6 and IL-11, which directly or indirectly stimulate bone-resorbing osteoclast formation [3, 4]. Transforming growth factor (TGF)-β and insulin-like growth factors in the bone matrix are released when bone is resorbed by osteoclasts. These released growth factors disrupt the homeostatic balance of bone microenvironments by promoting the growth of cancer cells and the production of cancer cell-derived osteolytic factors [5, 6]. Recent studies have paid much attention to osteoclast-targeting agents with high potential for the treatment of cancer patients with bone metastases [7, 8]. In the clinic, anti-bone resorptive drugs blocking the differentiation and activation of osteoclasts are used to treat skeletal-related events by breast cancer metastases, and these agents have increased the quality of life of patients [9]. However, these treatments do not improve patient survival, likely because they are not effective in directly counteracting tumor cell expansion and cause adverse effects.

Platycodin D is a bioactive triterpenoid saponin that is isolated from the root of Platycodon grandiflorum (Companulaceae) and possesses various pharmacological activities, including anti-cancer, anti-angiogenic, anti-inflammatory, and anti-obesity effects [10-14]. In particular, many studies have reported the anti-cancer effects of platycodin D in breast cancer cells. Platycodin D induces apoptosis via mitochondrial-dependent and receptor-mediated pathways, the activation of reactive oxygen species-mediated apoptosis signal-regulating kinase 1, and the endoplasmic reticulum stress response in estrogen receptor-positive and less metastatic human breast cancer MCF-7 cells [15, 16]. Platycodin D reduces migration and growth via the suppression of epidermal growth factor receptor-mediated Akt and MAPK pathways and inhibits TGF-β-induced invasion through the blockade of Smad2/3 phosphorylation in highly metastatic MDA-MB-231 human breast cancer cells [17]. In addition, platycodin D enhances the anti-proliferative activities of doxorubicin on MCF-7 and MDA-MB-231 cells. However, the inhibitory effect of platycodin D for breast cancer-induced bone destruction remains unproved.

In the present study, we evaluated whether platycodin D could serve as a promising agent for preventing and treating breast cancer cell-induced bone loss by targeting both breast cancer cells and osteoclasts. We first investigated the inhibitory activity of platycodin D on breast cancer cell-induced bone destruction in mice that were intratibially injected with MDA-MB-231 human metastatic breast cancer cells. We further examined the effect of platycodin D on osteoclastogenesis and osteoclast-mediated bone resorption in bone marrow-derived macrophages and on the growth of MDA-MB-231 cells.

Materials and Methods

Materials and reagents

Platycodin D (PD) was provided by Professor Yeong Shik Kim [18]. Recombinant mouse receptor activator of nuclear factor kappa-B ligand (RANKL) was purchased from Koma Biotech (Seoul, Korea). Recombinant mouse macrophage-colony stimulating factor (M-CSF) was obtained from R&D System (Minneapolis, MN). Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium alpha (α-MEM), fetal bovine serum (FBS), antibiotics, phosphate-buffered saline (PBS), and Hank’s balanced salt solution (HBSS) were purchased from Gibco BRL (Grand Island, NY). Polyclonal anti-c-Fos antibody was obtained from Cell Signaling Technology (Danvers, MA). Polyclonal anti-β-Actin antibody, the staining kit for tartrate-resistant acid phosphatase (TRAP), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Histopaque-1083 were purchased from Sigma-Aldrich (St. Louis, MO).
Lee et al.: Platycodin D Inhibits Breast Cancer-Induced Bone Destruction

**Animals**

Three-week-old male ICR mice and 4-week-old female Balb/c nu/nu mice were purchased from Central Lab Animal (Seoul, Korea). The animal studies were conducted in accordance with the experimental protocols of the animal ethics committee of the Yonsei University College of Dentistry [19].

**Cell cultures**

The human breast cancer cell line MDA-MB-231 was obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM with 10% FBS and a 1% antibiotic-antimycotic mixture at 37°C. Mouse bone marrow-derived macrophages (BMMs) as osteoclast precursors were isolated from the tibiae of ICR mice [19] and cultured in α-MEM containing 10% FBS, M-CSF (30 ng/ml), and a 1% antibiotic–antimycotic mixture at 37°C. All of the cells were maintained in a humidified atmosphere of 5% CO₂.

**Animal model for breast cancer-induced bone destruction**

MDA-MB-231 cells (1 × 10⁶ cells/0.1 ml HBSS) were inoculated into the right tibia of 5-week-old female Balb/c nu/nu mice (6 mice per group). The control mice were injected with HBSS instead of cancer cells. Platycodin D was dissolved in PBS (vehicle). Twenty-four hours later, platycodin D at 0, 1 or 2 mg/kg body weight (BW) was administered five days per week by oral gavage for 44 days. Control and MDA-MB-231 cell-injected mice without platycodin D received the vehicle alone. The tibiae were analyzed by micro-computed tomography (µCT) using a SkyScan 1076 system (SkyScan, Aartselaar, Belgium) as described previously [20]. The right hind limbs were imaged with an X-ray source voltage of 100 kV, a current of 100 mA, and a 0.5-mm aluminum filter. The scanning angular rotation was 360°, and the angular increment was 0.5°. Two-dimensional (2D) images were generated using NRecon software (SkyScan). After generating the 2D images, three-dimensional (3D) microstructural images were reconstructed using the Rapidform 2006 software (INUS Technology, Seoul, Korea). For the quantitative analysis of bone destruction, resident software (CTAn, SkyScan) was used and calculated the following parameters within the volume of interest: bone volume over total volume (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), and structure model index (SMI). For the histological analysis of the bone, the hind limbs were fixed in 10% neutral-buffered formalin solution and decalcified in 10% EDTA (w/v, pH 7.4). Paraffin-embedded longitudinal serial sections (5 µm) were cut from the femur and tibia and stained using Goldner’s trichrome method according to manufacturer’s protocol (Electron Microscopy Science, Hatfield, PA) for the detection of bone (green) and tumor (purple gray) [19]. The tumor areas were measured with ImageJ software (ImageJ software, Bethesda, MD) and calculated as the percentage of the total tumor area per field.

**Osteoclast formation assay**

BMMs (1 × 10⁴ cells) were cultured in media containing M-CSF (30 ng/ml), RANKL (100 ng/ml), and platycodin D (1-5 µM) for 5 days. In another experiment, BMMs (1 × 10⁴ cells) were cultured in media with M-CSF and RANKL for 5 days and exposed to platycodin D (5 µM) from the indicated days to day 5. The cultures were fed every 2 days with fresh medium. The cells were fixed with fixative solution for 30 sec at room temperature, and enzyme histochemistry for TRAP was performed with a commercial kit (Sigma Aldrich). Multinuclear cells (≥ 3 nuclei) were counted as osteoclasts.

**Pit formation assay**

Osteoclast activity assay substrate (OAAS) mineral-coated 24-well plates (Osteogenic Core Technology, Cheonan-si, Korea) were used for the pit formation assay as described previously [19]. Briefly, BMMs (5 × 10⁴ cells) were seeded onto OAAS plates and cultured in α-MEM containing 10% FBS, M-CSF (30 ng/ml), and RANKL (100 ng/ml) for 5 days. BMMs were then treated with platycodin D at the indicated concentrations for an additional 10 days. The cells were lysed with 5% sodium hypochlorite solution. The images of the resorbed pits were obtained with light microscopy. The resorbed pit areas were measured using ImageJ software.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

The total RNA was extracted from the BMMs (1 × 10⁴ cells) that were treated with M-CSF (30 ng/ml), RANKL (100 ng/ml), and platycodin D (1-5 µM) for 48 h using Trizol (Invitrogen, Carlsbad, CA). Single-
stranded cDNA was transcribed from the RNA (2 µg) using Promega's reverse transcription system (Madison, WI). A polymerase chain reaction was carried out in a reaction mixture containing cDNA (2 µg), MgCl₂ (25 mM), dNTPs (10 mM), primers (1 µM), and Taq polymerase (1 unit) (Takara, Shiga, Japan) with the following primers: NFATc1, forward 5′-TGGCTCTCTCAGGTGTCGTC-3′; reverse 5′-CGTCTTCCACCTCCAGTG-3′; c-Fos, forward 5′-ATGATGTCTCGGCTTTAAGCGG-3′; reverse 5′-CAGTCTGGCTGATAAAGGAAGCG-3′; GAPDH, forward 5′-GATGTCGATGACCTGACTC-3′; reverse 5′-AGGGTCTCAATGGCAACT-3′. The amplification consisted of 30 cycles with an annealing temperature of 52°C for NFATc1, c-Fos and GAPDH. The PCR products were electrophoresed, and the detected band was analyzed with the TINA program (version 2.10e, Raytest, Straubenhardt, Germany).

Western blot analysis
BMMs (1 × 10⁶ cells) were treated with M-CSF (30 ng/ml), RANKL (100 ng/ml), and platycodin D (1-5 µM) for 48 h. The nuclear/cytosol fractionation was performed using a nuclear/cytosol fractionation kit (BioVision, Mountain View, CA) according to the manufacturer’s manual. The protein concentration was measured with a BCA kit (Pierce, Rockford, IL). Equal amounts of protein (40 µg) were loaded onto a sodium dodecyl sulfate–polyacrylamide gel and electrophoresed. The blots were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) and then incubated with the primary antibodies for NFATc1, Lamin B, and β-actin (1:1000) in 3% skim milk and c-Fos (1:1000) in 5% bovine serum albumin in TBS-T for 24 h at 4°C. After washing, the blots were incubated for 1 h with a secondary antibody coupled to horseradish peroxidase (1:2000) and visualized with the ECL kit. The detected band was visualized with the TINA program.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay
BMMs (1 × 10⁶ cells) in Lab-Tek chamber slides were cultured in α-MEM containing 10% FBS, M-CSF (30 ng/ml), and RANKL (100 ng/ml) for 5 days. The differentiated osteoclasts were then treated with platycodin D at the indicated concentrations for 24 h. MDA-MB-231 cells (1 × 10⁶ cells) were seeded onto Lab-Tek chamber slides (Nunc, Rochester, NY) and cultured in serum-free media with platycodin D (1–20 µM) for 24 h. For the detection of apoptosis in MDA-MB-231 cells and osteoclasts, TUNEL staining was performed with the In Situ Cell Death Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instruction. TUNEL-positive cells were viewed at excitation 488 nm/emission 512 nm by fluorescence at a magnification of ×200. The percent of TUNEL-positive nuclei was calculated as the number of TUNEL-labeled nuclei per the total number of cells.

Caspase-3 activity assay
MDA-MB-231 cells (1 × 10⁶ cells) were plated onto 100 mm culture dishes and incubated with platycodin D (1–20 µM) in serum-free DMEM for 24 h. BMMs (1 × 10⁶ cells) in 60-mm culture dishes were cultured in α-MEM containing 10% FBS, M-CSF (30 ng/ml), and RANKL (100 ng/ml) for 5 days. The differentiated osteoclasts were then treated with platycodin D at the indicated concentrations for 24 h. The activity of caspase-3 in MDA-MB-231 cells and osteoclasts was measured using the active caspase-3 enzyme-linked immunosorbent assay (ELISA) kit (R&D system) according to the manufacturer’s protocol.

MTT assay
MDA-MB-231 cells and BMMs were seeded onto 96-well culture plates at a density of 1 × 10⁴ cells/well. MDA-MB-231 cells were cultured in serum-free media with platycodin D (1–20 µM) for 24 h. BMMs were cultured in 10% FBS/α-MEM in the presence of M-CSF (30 ng/ml) and platycodin D (1-10 µM) for 5 days. The cultures were fed every 2 days with fresh medium. The cells were exposed to 20 µM of an MTT solution (5 mg/ml) and incubated at 37°C for 4 h. The medium was removed, and the cells were lysed with 200 µL of DMSO. The absorbance was determined at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA).

5-bromo-2'-deoxyuridine (BrdU) incorporation assay
To determine the extent of cellular proliferation, the incorporation of BrdU was quantified using a BrdU labeling and detection kit (Roche Diagnostics). Briefly, MDA-MB-231 cells (1 × 10⁴ cells) were cultured with platycodin D (1–20 µM) for 24 h in 96-well microtiter plates and 10 µM BrdU was then added to each
well. The cells were continuously cultured under the same conditions for 2 h, during which time BrdU was incorporated into freshly synthesized DNA. Following the fixation of the cells, cellular DNA was partially digested by nuclease treatment. The incorporated BrdU was quantified by the peroxidase-labeled antibody to BrdU. The absorbance at 450 nm, which is directly correlated with the level of BrdU incorporated into cellular DNA, was measured using a microplate reader.

**Statistical analysis**

Statistical analysis was performed with SPSS statistical software (IBM, Endicott, NY). The data are expressed as mean ± standard error (SE). Statistical analysis was performed by a one-way ANOVA and Student’s *t*-test to express the difference between the two groups.

**Results**

**Platycodin D inhibited bone destruction as induced by MDA-MB-231 breast cancer cells in mice**

We first examined the inhibitory activity of platycodin D on the generation of osteolytic lesions in mice that were intratibially injected with MDA-MB-231 human metastatic breast cancer cells. Radiographic and µCT-derived 3D images showed that the inoculated MDA-MB-231 cells in the tibia caused severe bone destruction but that treatment with platycodin D significantly inhibited the production of these lesions. Goldner’s trichrome staining also indicated that tibial bone marrow was filled with cancer cells, and bone was noticeably resorbed in the mice that were treated with vehicle alone. However, tumor growth in bone marrow and bone loss decreased in mice with the oral administration of platycodin D (ED₅₀ = 1.55 mg/kg) (Fig. 1A). While the bone morphometric parameters BV/TV, Tb.Th, and Tb.N decreased and Tb.Sp and SMI increased in the mice that were injected with MDA-MB-231 cells, treatment with platycodin D at 2 mg/kg significantly blocked cancer cell-induced alterations in these parameters (Fig. 1B).

**Platycodin D reduced RANKL-induced osteoclast formation and activity**

Breast cancer-induced bone destruction is caused by osteoclasts, which are directly or indirectly activated by breast cancer-secreted osteolytic factors [21]. When BMMs as osteoclast precursors were exposed to platycodin D for 5 days, the cell viability increased by 31% and 79% at 1 and 3 µM but decreased by 21% and 86% at 5 and 10 µM, respectively (Fig. 2A). RANKL stimulation in the presence of M-CSF for 5 days caused the formation of TRAP-positive osteoclasts in mouse BMMs, but treatment with platycodin D at these non-cytotoxic concentrations dramatically inhibited RANKL-induced osteoclast formation (Fig. 2B). The inhibitory effect of platycodin D on the activity of mature osteoclasts was determined by measuring the area of resorbed pits on the calcium phosphate-coated plates. Platycodin D significantly reduced the formation of resorption pits (Fig. 2C).

**Platycodin D blocked the activation of key transcription factors, leading to RANKL-induced osteoclastogenesis**

Osteoclastogenesis is a complicated procedure that includes many steps, such as the commitment, differentiation and activation of immature osteoclasts [22]. To further examine which step of osteoclastogenesis could be blocked by platycodin D, BMMs were exposed to 5 µM platycodin D on different days in the presence of RANKL and M-CSF. Osteoclast formation was inhibited by 86% in the BMMs that were exposed to platycodin D immediately and 1 day after the RANKL treatment. The addition of platycodin D at day 2, 3, and 4 inhibited osteoclast formation by 50%, 44%, and 43%, respectively (Fig. 3A). To explore the molecular mechanisms underlying the anti-osteoclastogenic activity of platycodin D, we examined the expression of c-Fos and NFATc1, which play critical roles in osteoclastogenesis [23], in the BMMs that were treated with RANKL and platycodin D for 2 days. The mRNA expressions of c-Fos and NFATc1 were up-regulated in RANKL-stimulated BMMs, but platycodin D
treatment inhibited the RANKL-induced mRNA expression of these transcription factors in a dose-dependent manner (Fig. 3B). Moreover, RANKL stimulation remarkably increased the protein levels of c-Fos and NFATc1 in the cytosol and nucleus, but treatment with platycodin D inhibited these RANKL-induced protein levels in the nucleus. Platycodin D at 5 µM induced the accumulation of c-Fos in the cytosol (Fig. 3C).

**Platycodin D induced the apoptosis of mature osteoclasts**

Next, we investigated whether platycodin D could induce apoptosis in mature osteoclasts. Platycodin D treatment noticeably increased the number of mature osteoclasts with TUNEL-stained nuclei in a dose-dependent manner (Fig. 4A). In addition, the activity of caspase-3, which is a member of the cysteine-aspartic acid protease family and the major executor of proteolysis in cell apoptosis [24], significantly increased by 2-fold and 9-fold in RANKL-induced osteoclasts treated with platycodin D at 3 and 5 µM, respectively (Fig. 4B).

**Platycodin D inhibited the growth of MDA-MB-231 breast cancer cells**

We also examined whether platycodin D could reduce proliferation and induce apoptosis in MDA-MB-231 human metastatic breast cancer cells. Platycodin D treatment for 24 h...
Lee et al.: Platycodin D Inhibits Breast Cancer-Induced Bone Destruction

Cellular Physiology and Biochemistry

Decreased the viability of MDA-MB-231 cells in a dose-dependent manner, and its IC50 value was 17.8 µM (Fig. 5A). A BrdU incorporation assay indicated that treatment with 10 or 20 M platycodin D for 24 h inhibited DNA synthesis in the proliferating MDA-MB-231 cells by 12% or 51%, respectively (Fig. 5B). Treatment with platycodin D at 10 and 20 µM considerably promoted the activity of caspase-3 (Fig. 5C) and cell number with TUNEL-stained nuclei by 8% and 21% (Fig. 5D), respectively, in MDA-MB-231 cells.

Discussion

Breast cancer can be treated effectively in its early stage. However, the survival rates of patients with bone metastatic breast cancer are significantly decreased. Numerous...
studies are in progress to develop new agents to inhibit bone metastasis and the resulting bone loss in patients with breast cancer [26]. Bioactive components in medicinal plants are being considered as a promising research materials [27, 28]. Platycodin D, with diverse pharmacological activities, including anti-inflammatory, anti-obesity and anti-nociceptive effects [29-31], inhibits growth and induces cell death in several cancer cells [32-35]. In this study to verify the effect of platycodin D as a protective and therapeutic agent for breast cancer-associated bone diseases, orally administered platycodin D significantly suppressed osteolysis and tumor growth in the tibial bone marrow of MDA-MB-231 human metastatic

Fig. 3. Platycodin D inhibits a multistep process of RANKL-induced osteoclastogenesis. (A) The BMMs were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 5 days. Platycodin D (5 µM) was added at the indicated time periods after the treatments of M-CSF and RANKL. Differentiated osteoclasts were detected by TRAP staining, and the images were obtained under light microscopy (magnification, ×100). TRAP-positive multinucleated (>3 nuclei) osteoclasts were counted. (B, C) The BMMs were incubated with M-CSF (30 ng/ml), RANKL (100 ng/ml) and platycodin D at the indicated concentrations for 2 days. The mRNA expressions of c-Fos and NFATc1 were analyzed by RT-PCR (B). The protein levels of cytosolic and nuclear c-Fos and NFATc1 were detected by western blotting (C). The detected band was densitometrically analyzed with the TINA program. The data are expressed as the means ± SE, *p<0.05, **p<0.01 vs. control mice *p<0.05, **p<0.01, ***p<0.0001 vs. RANKL-alone-treated cells.
**Fig. 4.** Platycodin D induces apoptosis in mature osteoclasts. The BMMs were incubated in media with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 5 days. The differentiated osteoclasts were treated with platycodin D at the indicated concentrations for 24 h. The induction of apoptosis in osteoclasts was analyzed by (A) TUNEL staining and (B) caspase-3 activity assay. The images of TUNEL-positive cells were obtained under fluorescence microscopy (magnification, ×200). The percentage of TUNEL-positive cells was estimated as described in the Materials and Methods. The data are expressed as the means ± SE, *p<0.001, **p<0.0001 vs. RANKL-alone-treated cells.

**Fig. 5.** Platycodin D inhibits the growth of MDA-MB-231 cells. The MDA-MB-231 cells were incubated for 24 h in the presence of platycodin D at the indicated concentrations. (A) The cell viability and (B) the amount of newly synthesized DNA were examined using the MTT assay and BrdU incorporation assay, respectively. The induction of apoptosis in MDA-MB-231 cells was detected by (C) the caspase-3 activity assay and (D) TUNEL staining. Images of TUNEL-positive cells were obtained under fluorescence microscopy (magnification, ×200). The percent of TUNEL-positive nuclei was estimated as described in the Materials and Methods. The data are expressed as the means ± SE, *p<0.05, **p<0.01 vs. cancer cells without platycodin D.
breast cancer cell-inoculated mice as evidenced by radiographic images, the 3D reconstruction of \( \mu \)CT data, Goldner's trichrome staining, and bone morphometric parameters.

Breast cancer cells metastasize to the bone and secrete various osteolytic factors, thereby modifying the bone microenvironment. Most osteolytic factors stimulate osteoclast differentiation by promoting osteoblastic/stromal RANKL expression, causing excessive bone resorption and consequently decreasing the survival rates of breast cancer patients [21]. Therefore, bisphosphonates (anti-bone resorptive agents) and denosumab (a monoclonal antibody against RANKL), which were primarily developed as therapeutic agents for osteoporosis, have been used in therapy for patients with bone metastatic breast cancer [9, 36]. Platycodin D noticeably suppressed the number of multinucleated osteoclasts in the BMMS that were treated with RANKL and the osteoclast-mediated formation of the resorption pit. Moreover, platycodin D showed a potent inhibition of the multistep process of RANKL-induced osteoclastogenesis by blocking the fusion between mononuclear osteoclasts and the acquisition of the bone-resorbing activity of mature osteoclasts (days 2-4), as well as the growth of BMMS as osteoclast precursors and their differentiation into mononuclear osteoclasts (days 0-1). The differentiation and cell-cell fusion of osteoclast precursors are mainly triggered by the binding of RANKL to RANK on the osteoclast precursor and amplified by downstream signaling molecules [37-39]. In particular, NFATc1 is a master regulator of osteoclastogenesis that promotes the expression of osteoclast-specific genes in cooperation with c-Fos [40-42]. Platycodin D treatment reduced the levels of nuclear NFATc1 and c-Fos by inhibiting the expression and/or nuclear translocation of these transcription factors in RANKL-treated BMMS. Furthermore, platycodin D induced apoptosis in the mature osteoclasts that were differentiated with RANKL stimulation for 5 days, supported by the increased number of cells with TUNEL positive nuclei and up-regulated caspase-3 activity. These results indicate that the in vivo inhibitory activity of platycodin D on breast cancer-mediated bone destruction can be attributed to inducing the apoptosis of mature osteoclasts as well as blocking the differentiation and activation of osteoclasts.

Next, we investigated the effect of platycodin D on the growth of MDA-MB-231, human metastatic breast cancer cells, which are among the major elements in the vicious cycle of breast cancer bone metastasis. Cell growth is determined by cell proliferation and cell death [43]. Our data showed that platycodin D inhibited the cell viability and DNA synthesis in proliferating cells and increased apoptosis as represented by TUNEL-positive nuclei and caspase-3 activity in MDA-MB-231 cells. Apoptosis occurs via two different pathways: the extrinsic pathway via death receptor or the intrinsic pathway dependent on mitochondria. Although these pathways were triggered by various signal molecules, they finally converged on the activation of caspase-3, and caspase-3 lead to cell death by the cleavage of substrates, including poly (ADP-ribose) polymerase; pro-caspases -6, -7, and -9; and \( \beta \)-catenin [24, 44-47].

Collectively, platycodin D inhibits breast cancer-mediated bone destruction by blocking osteoclast formation and function and by inducing the apoptosis of both mature osteoclasts and MDA-MB-231 cells. The ideal treatment for breast cancer patients should aim at inhibiting breast cancer cell growth and preventing bone resorption. Therefore, platycodin D with anti-cancer and anti-bone resorptive activity may possess preventive and therapeutic benefits for breast cancer-induced bone destruction.

**Acknowledgements**

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011578)" Rural Development Administration, Republic of Korea.
Disclosure Statement

The authors declare no conflict of interest.

References

Lee et al.: Platycodin D Inhibits Breast Cancer-Induced Bone Destruction