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PRECLINICAL STUDIES

Tetrahydrofurofuran-type lignans inhibit breast cancer-mediated bone destruction by blocking the vicious cycle between cancer cells, osteoblasts and osteoclasts

Ah Young Jun · Hyun-Jeong Kim · Kwang-Kyun Park · Kun Ho Son · Dong Hwa Lee · Mi-Hee Woo · Won-Yoon Chung

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Summary Breast cancer frequently spreads to bone. The interaction between bone metastases and microenvironment, referred as the "vicious cycle", increases both tumor burden and bone destruction. Therefore, inhibition at any point in this "vicious cycle" can reduce malignant osteolytic lesions in patients with advanced breast cancer. In this study, we evaluated whether tetrahydrofurofuran-type lignans derived from Magnoliae Flos, commonly used in traditional Asian medicine to treat inflammatory diseases, could block breast cancer-mediated bone loss. Aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin at noncytotoxic concentrations suppressed mRNA expression and secretion of osteolytic factor PTHrP in MDA-MB-231 metastatic human breast cancer cells. Fargesin inhibited TGF-β-stimulated cell viability, migration, and invasion and decreased TGF-β-induced PTHrP production in MDA-MB-231 cells. In addition, these lignans reduced RANKL/OPG ratio in PTHrP-treated hFOB1.19 human osteoblastic cells and inhibited RANKL-mediated osteoclast differentiation in mouse bone marrow macrophages. Aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin substantially reduced bone-resorbing activity of osteoclasts by inhibiting MMP-9 and cathepsin K activities. Furthermore, orally administered fargesin inhibited tumor growth and cancer-mediated bone destruction in mice with MDA-MB-231 cells injected into calvarial tissues. Aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin blocked initiation and progression of the "vicious cycle" between breast cancer metastases and bone microenvironment by inhibiting PTHrP production in breast cancer cells and osteoclastic bone resorption. Therefore, these tetrahydrofurofuran-type lignans have the potential to serve as beneficial agents to prevent and treat cancer-induced bone destruction in breast cancer patients.

Keywords Tetrahydrofurofuran-type lignans · Breast cancer · Cancer-induced bone destruction · PTHrP · RANKL/OPG · Osteoclast

A. Y. Jun · H.-J. Kim · K.-K. Park · W.-Y. Chung
Department of Applied Life Science, The Graduate School, Yonsei University, Seoul 120-749, Republic of Korea

A. Y. Jun · H.-J. Kim · K.-K. Park · W.-Y. Chung
Department of Oral Biology, Oral Cancer Research Institute, and Brain Korea 21 Project, Yonsei University College of Dentistry, Seoul 120-752, Republic of Korea

K. H. Son · D. H. Lee
Department of Life Science, Andong National University, Andong 760-740, Republic of Korea

M.-H. Woo
College of Pharmacy, Catholic University of Daegu, Gyeongsan 712-702, Republic of Korea

W.-Y. Chung (✉)
Department of Oral Biology, Yonsei University, College of Dentistry, 50 Yonsei-ro, Seodaemoon-Ku, Seoul 120-752, Republic of Korea
e-mail: wychung@yuhs.ac

Introduction

Bone is a highly specialized form of connective tissue that functions as an internal support system in all higher vertebrates. Bone is continuously destroyed and reformed through a balance of osteoclast-mediated bone resorption and osteoblast-mediated bone formation [1]. However, that balance is disturbed by hormonal changes such as estrogen deficiency, aging, and cancer metastasis to bone, with consequent bone loss [2]. Most cancer cells that metastasize to the skeleton stimulate the function of bone-degrading cells, the

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osteoclasts, causing the formation of malignant osteolytic lesions [3]. For breast cancer, bone is the most common site of metastasis. Advanced-stage breast cancer patients who develop bone metastases suffer increased morbidity and mortality, with enhanced bone pain, fractures, hypercalcemia of malignancy, spinal cord compression, and other nerve compression syndromes [4].

The propensity of breast cancer to metastasize to bone has been explained by Paget's "seed and soil" hypothesis: The "seeds" (tumor cells) require appropriate "soil" (the bone microenvironment) to establish skeletal metastasis [5]. Preclinical data support a model of interaction between tumor cells and the bone microenvironment, which creates a "vicious cycle" that accelerates both tumor growth and bone destruction. Tumor cells produce osteolytic factors, including parathyroid hormone-related protein (PTHrP) and several proinflammatory interleukins. These factors promote the expression of receptor activator of nuclear factor-κB ligand (RANKL) on the surface of osteoblasts, consequently causing excessive osteoclastic bone resorption. The outer structure of bone is primarily composed of a hard-mineralized matrix which stores abundant varieties of growth factors, including insulin-like growth factors, transforming growth factor (TGF)-β, fibroblast growth factors, and platelet-derived growth factors [6]. Bone resorption by osteoclasts results in the release of growth factors from the bone matrix, thereby further stimulating tumor growth and the expression of tumor-derived osteolytic factors. Therefore, inhibition at any point in the "vicious cycle" can reduce cancer-associated bone destruction in patients with advanced breast cancer.

Plant-derived compounds have long been recognized as potential preventive and therapeutic agents for human diseases [7]. Magnoliae Flos (Chinese name: Xin-yi), the flower buds of *Magnolia* spp., are commonly used in traditional Oriental medicine to treat nasal congestion with headache, sinusitis, and allergic rhinitis, and are listed in the Korean, Chinese, and Japanese Pharmacopoeias [8]. The primary bioactive components of Magnoliae Flos include volatile terpenoids and lignans, with antimicrobial, anti-angiogenic, anti-inflammatory, anti-diabetic, and anti-allergic effects [8, 9]. In particular, tetrahydrofuranoid lignans have been reported to be central marker/active compounds of Magnoliae Flos [9–13].

In the present study, we evaluated whether aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin, tetrahydrofurofuran-type lignans derived from Magnoliae Flos (Fig. 1), could possess significant potential as preventive and/or therapeutic agents for cancer-mediated bone destruction. We investigated the effects of these compounds on the production of PTHrP osteolytic factor in MDA-MB-231 human metastatic breast cancer cells, RANKL and osteoprotegerin (OPG) expression in PTHrP-stimulated osteoblastic cells, and RANKL-induced osteoclastogenesis and bone resorption in mouse bone marrow macrophages (BMMs). In addition, we determined the effect of fargesin on bone destruction by MDA-MB-231 cells locally injected over the calvarial tissues of mice.

Materials and methods

Reagents

Leibovitz's L-15 Medium (L-15), Dulbecco's Modified Eagle Medium Nutrient Mixture/F-12 (DMEM/F-12), Minimum Essential Medium Alpha (α-MEM), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Dulbecco's Phosphate Buffered Saline (PBS), antibiotic-antimycotic (100 x), Geneticin® Liquid (G418 Sulfate), penicillin-streptomycin, and 0.25 % trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY). Histopaque-1083, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). PTHrP(1–34) was purchased from Bachem (Torrance, CA) and recombinant mouse soluble RANKL (sRANKL) and macrophage-colony stimulating factor (M-CSF) were obtained from Koma Biotech (Seoul, Korea) and R&D Systems (Minneapolis, MN), respectively. All reagents used in this study were of analytical grade.

Isolation of the lignan compounds

The flower buds of *Magnolia biondii* were purchased from an Oriental Herb market (Seoul, Korea) and authenticated by Professor JH Lee (Gyeongsang National University, Jinju, Korea). A voucher specimen (YUCD-011) was deposited at Oral Cancer Research Institute of Yonsei University. The methanol extract (865 g) of the air-dried Magnolia flos (10.45 kg) was partitioned with chloroform and chloroform fraction (65 g) was chromatographically resolved on a silica gel eluting with a gradient of 100 % hexane to hexane-EtOAc (1:1) to afford 18 fractions. Fargesin, lirioresinol B dimethyl ether, and magnolin were isolated from fraction 5, 10, and 13 plus 14, as described previously [12]. Aschatin was purified by further chromatographic separation of fraction 8 on a silica gel column with hexane:$CHCl_3$:EtOAc (15:10:1) to give 4 subfractions. Subfraction 4 was dissolved in methanol and eluted with 60 % H_2O on RP-18 column. The chemical structures of these compounds were verified by comparison of the NMR data with the reported data [11, 12]. These compounds showed more than 97 % purity in high-performance liquid chromatography analysis (HPLC). HPLC chromatography was carried out RP-18 column (Waters YMC) with acetonitrile 62 % and distilled water:acetic acid (100:1) 38 % (flow rate, 1 mL/min; column temperature, 20 °C; detection, UV detector at 278 nm; Internal standard, propyl 4-hydroxy benzonate).

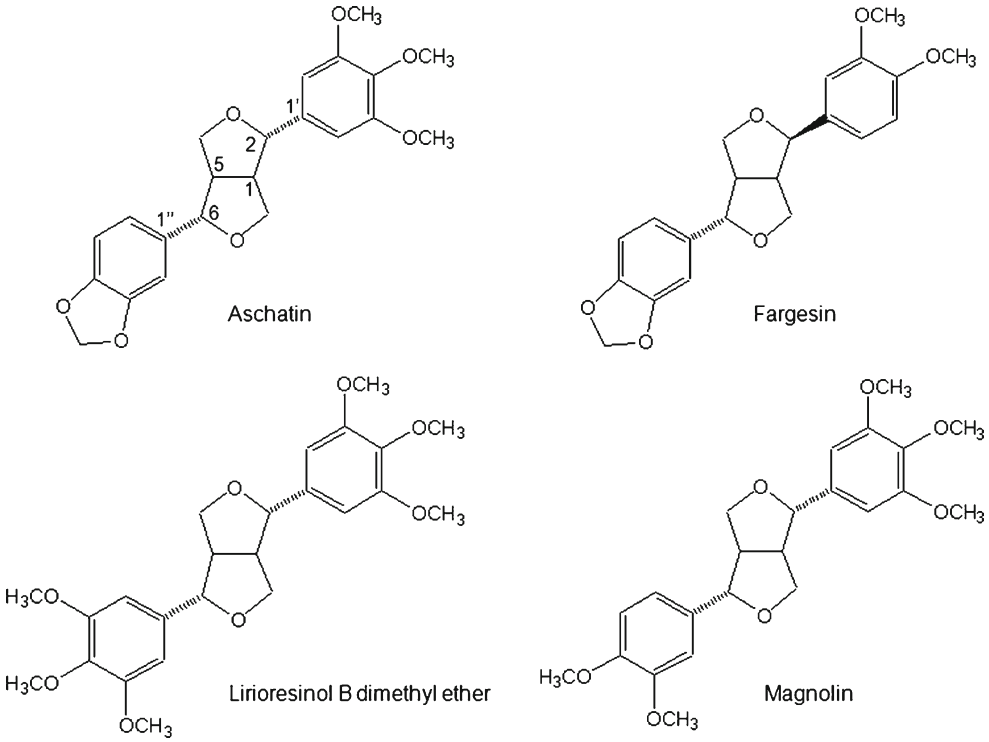


Fig. 1 Chemical structure of aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin

The lignans were dissolved in dimethyl sulfoxide (DMSO) and diluted with media just before use. The final concentration of DMSO in cell culture media was maintained below 0.1 %.

Animals

4-week-old male ICR mice (18±2 g) and 7-week-old female BALB/c athymic nude mice (20±2 g) were purchased from the Central Lab Animal (Seoul, Korea). All mice were provided unrestricted access to a standard chow diet (Orient, Seongnam, Korea) and tap water. They were maintained at a temperature of 22±2 °C with a relative humidity of 50±5 % and a 12 h light–dark cycle. The animal studies were conducted in accordance with the rules and regulations established by the Institutional Animal Ethics Committee of the Yonsei University College of Dentistry.

Cell lines and culture conditions

Human metastatic breast cancer MDA-MB-231 cells (Korean Cell Line Bank, Seoul, Korea) were maintained in L-15 medium containing 10 % FBS and 1 % antibiotic-antimycotic mixture, and cultured in DMEM containing 10 % FBS and 1 % antibiotic-antimycotic mixture in a humidified atmosphere of 5 % CO_2 at 37 °C. Human osteoblastic hFOB1.19 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/F12 containing 10 % FBS, G418 (0.3 mg/ml), and penicillin (100 U/ml)-streptomycin (100 μg/ml) in a humidified atmosphere of 5 % CO_2 at 34 °C. BMMs were isolated from the tibiae of 4-week-old ICR male mice. The ends of aseptically removed tibiae were trimmed off, and the marrow was flushed out using serum-free α-MEM. The bone marrow cells were centrifuged at 500 x *g* for 10 min. The cells were then resuspended with α-MEM and BMMs were purified by histopaque density gradient centrifugation. BMMs were cultured in α-MEM containing 10 % FBS, M-CSF (30 ng/ml), and 1 % antibiotic-antimycotic mixture in a humidified atmosphere of 5 % CO_2 at 37 °C.

MTT assay

MDA-MB-231 or hFOB1.19 cells (1×10^4 cells/well) were seeded into 96-well plates with DMEM or DMEM/F-12 containing 10 % FBS, respectively. MDA-MB-231 cells were cultured in the corresponding serum-free medium with various concentrations of lignan compounds for 24 h and 72 h, and hFOB1.19 cells were treated for 6 h and 24 h. MDA-MB-231 cells were also exposed to TGF-β (5 ng/ml) and/or fargesin at the indicated concentration for 24 h and 72 h. BMMs (5×10^4 cells/well) were cultured in α-MEM containing 10 % FBS, M-CSF (30 ng/ml), and lignan compounds at the indicated concentrations for 5 days with replacement of fresh medium every second day. MTT solution (5 mg/ml) was added to each well and the cells were incubated for an additional 4 h. The medium was then removed and DMSO was added to each well. The absorbance was determined

at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Cell viability was expressed as percentage of control.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

MDA-MB-231 cells were cultured in serum-free medium containing each lignan compound for 24 h to analyze PTHrP mRNA levels. hFOB1.19 osteoblastic cells were treated with lignans in the presence of PTHrP (10 nM) for 6 h to analyze RANKL and OPG mRNA levels. The cells were harvested and total RNA was isolated with TRIZOL Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with total RNA (2 μg), Oligo(dT)15 Primer, Recombinant RNasin® Ribonuclease Inhibitor, and M-MLV Reverse Transcriptase (Promega, Madison, WI). Using the recombinant Taq DNA polymerase kit (Takara, Shiga, Japan), PCR was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with cDNA (2–4 μg) and the following primers (10 pmol): PTHrP, forward 5′-AACTCGCCTCCAA CCTGCGC-3′, reverse 5′-CGCTCGGGACCTCCTCTG GT-3′; RANKL, forward 5′-ATCCCATCTGGTT CCCATAA-3′, reverse 5′-CCCTGACCAATACTTGGT GC-3′; OPG, forward 5′-GGGGACCACAATGAAC AAGTTG-3′, reverse 5′-AGCTTGCACCACTCC AAATCC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control, forward 5′-CCGCCTACTGCCCACTGCCACCAC-3′, reverse 5′-TCCATCCACTATGTCAGCAGGTCC-3′. Amplification consisted of 30 cycles with an annealing temperature of 62°C for PTHrP and 52°C for RANKL, OPG, and GAPDH. PCR products were electrophoresed on a 2 % SeaKem LE Agarose (Lonza, Basel, Switzerland) gel in Tris-Borate-EDTA buffer (pH 8.0) containing ethidium bromide. The PCR products were visualized on a UV transilluminator. The intensity of each band was quantitated by densitometric analysis using the TINA program (version 2.1) and normalized to that of GAPDH.

PTHrP assay

MDA-MB-231 cells (2×10^4 cells/well) were seeded into 96-well plates with DMEM containing 10 % FBS and incubated to adhere overnight. The cells were then incubated in serum-free medium containing the indicated concentrations of lignan compounds and/or TGF-β (5 ng/ml) for 24 h and the culture media were collected. The collected culture media were centrifuged in a Centricon centrifugal filter device (Millipore, Bedford, MA) at 2,450 x *g* at 4 °C for 20 min. PTHrP levels in the culture media were quantified using a human PTHrP enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science, Wuhan, China) according to the manufacturer's instruction. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). PTHrP level was expressed as pg/mg protein.

Scratch-migration and Transwell invasion assays

MDA-MB-231 cells were allowed to grow to 90 % confluence in 6-well plates. The cell monolayer was scratched with the narrow end of a sterile micropipette tip. Detached cells were removed and the adherent cells were incubated in serum-free DMEM with and without various concentrations of TGF-β and/or fargesin in the presence of 0.5 μg/ml mitomycin C. Twenty-four hours later, the images of the scratched areas were captured using an inverted microscope (40x magnification).

Cell invasion assay was performed using a Transwell chamber (Corning Costar, Cambridge, MA) containing a polycarbonate membrane filter (6.5 mm diameter, 8 μm pore size). The bottom of the filter was covered with a layer of 0.1 % (w/v) gelatin. Matrigel (BD Biosciences, San Jose, CA), a mixture of basement membrane extracellular matrix proteins, was diluted with DMEM to a final concentration of 1 mg/ml and applied to the filter to coat the upper part of the filter. Suspensions of MDA-MB-231 cells (5×10^4 cells/0.1 ml) with the indicated concentrations of fargesin were added into the coated insert. The lower chamber contained 600 μl of culture media with 1 % FBS, fargesin, and TGF-β (5 ng/ml). Transwell plates were incubated for 24 h at 37 °C. The cells were fixed with 70 % methanol and the membranes were stained with hematoxylin. Non-invaded cells on the upper surface of the membrane were scraped with cotton swabs. Invaded cells on the bottom surface were mounted on slides and the images were captured using a light microscope (40x magnification).

Osteoclast differentiation and activity assay

BMMs (5×10^4 cells/well) were seeded into 96-well plates and cultured in α-MEM containing 10 % FBS, M-CSF (30 ng/ml), sRANKL (100 ng/ml), and each of the studied compounds at various concentrations for 5 days, replacing with fresh medium every second day. The cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using the Acid Phosphatase Leukocyte kit (Sigma-Aldrich). The number of TRAP-positive multinucleated (≥ 3 nuclei) cells, counted as the number of mature osteoclasts, was assessed using an inverted microscope (100x magnification).

The culture media were concentrated by centrifugation in a Centricon centrifugal filter device at 2,450 x g at 4 °C for 20 min. Protein concentration was determined using the Pierce BCA Protein Assay kit. Gelatin zymography and cathepsin K assay were performed as previously described

[14]. In gelatin zymography, clear zones against the blue background represented gelatinolytic activities of matrix metalloproteinase (MMP)-9. The level of cathepsin K was expressed as pg/mg protein.

Pit formation assay

BMMs (5×10^4 cells/well) were seeded onto BioCoat Osteologic MultiTest slides (BD Biosciences) and cultured in α-MEM containing 10 % FBS, M-CSF (30 ng/ml), and sRANKL (100 ng/ml) for 5 days to induce the formation of mature osteoclasts. BMMs were then treated with each lignan compound at the indicated concentrations for an additional 14 days. Culture media were changed every second day. The cells were lysed with 5 % sodium hypochlorite solution. The images of the resorbed pits were obtained under a light microscope (100x magnification).

A mouse model of breast cancer-associated bone destruction

The mice were randomly divided into 6 groups (7 mice per group): Control, MDA-MB-231 cell-inoculated, MDA-MB-231 cell-inoculated and fargesin (2 and 5 mg/kg body weight (BW))-treated, and MDA-MB-231 cell-inoculated and zoledronic acid (100 μg/kg BW)-treated groups. MDA-MB-231 cells (5×10^6 cells/0.1 ml PBS) were twice injected locally over mouse calvaria tissues at a one-week interval with a 1 ml syringe and a sterile 26-gauge needle. For 45 days after the first inoculation of the breast cancer cells, fargesin was orally administered to mice 5 times per week and zoledronic acid, as the positive control, was subcutaneously injected 3 times per week. Control mice received PBS and vehicle (PBS containing 0.1 % DMSO) instead of MDA-MB-231 cells and fargesin, respectively. The MDA-MB-231 cell-inoculated group was administered vehicle alone. Tumor volumes were measured every 3 days using an electronic digital caliper and calculated according to the following formula: $(a \times b^2)/2$, where "a" is the longer and "b" is the shorter dimension. On day 45, cardiac puncture was performed to extract the blood and the calvaria tissues were collected.

Calvaria tissues with tumor were fixed in 4 % neutral phosphate-buffered formalin at 4 °C. Formalin-fixed calvaria specimens were decalcified with 10 % EDTA solution (pH 5.5) at 40 °C for 5 days in the dark and then embedded in paraffin. Serial sections (2 μm thick) were mounted onto slides. The sections were deparaffinized in xylene, rehydrated, stained with hematoxylin and eosin (H&E), and then photographed under an Axio Imager microscope (Carl Zeiss, Jena, Germany). For immunohistochemical staining, the rehydrated sections were treated with 0.1 % Trypsin for 20 min at 37 °C for antigen retrieval, 3 % hydrogen peroxide for 15 min at room temperature to quench endogeneous peroxidase activity, and then goat serum for 30 min. The sections were incubated with a 1:50 dilution of primary antibodies against PTHrP (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for overnight. Then, the sections were treated with horseradish peroxidase (HRP)-conjugated secondary anti-mouse IgG antibodies (Sigma-Aldrich) diluted at 1:150 for 1 h. HRP activity was developed with the DAB system (Lab Vision, Fremont, CA). The sections were counterstained with hematoxylin, dehydrated, mounted, and examined under the microscopy (200x magnification).

Bone morphometric parameters of calvaria were determined using a SkyScan 6 1076 μCT System and CTAn software (SkyScan, Aartselaar, Belgium). Scans were taken with a source voltage of 100 kV and a source current of 140 μA. The resolution was set to 35 μm and the rotation step was 0.6°.

Blood samples were maintained at room temperature for 1 h, and then centrifuged at 1,910 x g for 20 min to obtain serum. Calcium levels in serum were detected with the QuantiChrom Calcium Assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instruction.

Statistical analysis

Data were expressed as mean±standard error (SE). Statistical analysis was performed by one-way analysis of variance (ANOVA) and Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

Effects of aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin on PTHrP in MDA-MB-231 human metastatic breast cancer cells

When MDA-MB-231 cells were treated with lignan compounds for 24 h and 72 h, aschatin, lirioresinol B dimethyl ether, and magnolin did not significantly alter cell viability at concentrations up to 100 μM, but treatment with fargesin decreased cell viability in a concentration- and time-dependent manner. The viability of MDA-MB-231 cells was inhibited by 26 % with treatment for 24 h and 51 % with treatment for 72 h with 100 μM fargesin (Fig. 2a).

PTHrP has been described as a key osteolytic factor in breast cancer-induced bone destruction [15]. In RT-PCR analysis and ELISA assay, treatment with aschatin, fargesin, lirioresinol B dimethyl ether, or magnolin at noncytotoxic doses decreased both mRNA expression (Fig. 2b) and levels secreted into culture media (Fig. 2c) of PTHrP in MDA-MB-231 cells. In particular, treatment with fargesin substantially reduced PTHrP mRNA expression and levels of secretion at concentrations lower than those required for the other components.

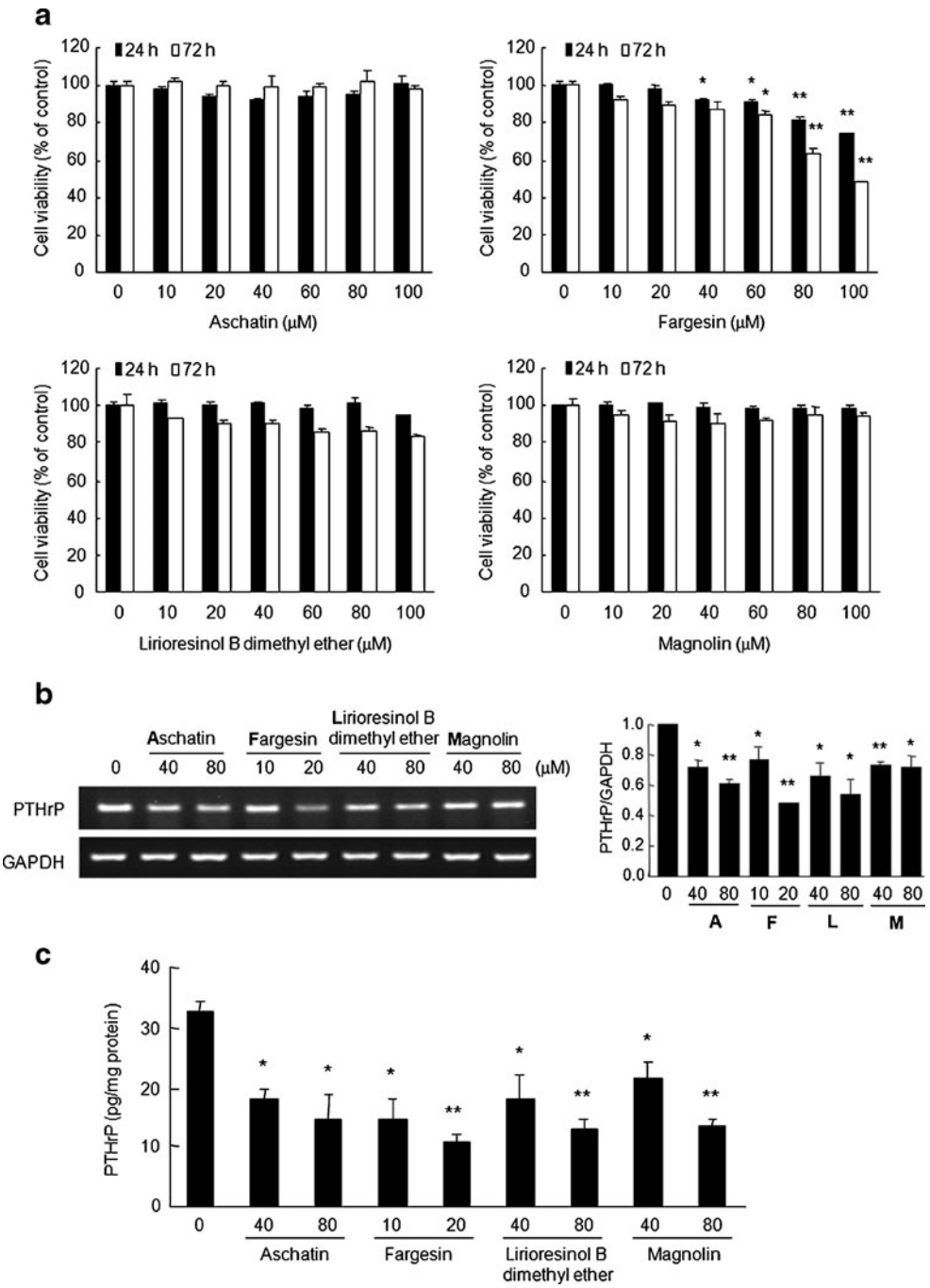


Fig. 2 Effects of lignan compounds on PTHrP production in MDA-MB-231 human metastatic breast cancer cells. **a** MDA-MB-231 cells were treated with serum-free DMEM containing lignan compounds at the indicated concentrations for 24 h and 72 h, respectively. Cell viability was determined by an MTT assay. **b** The cells were incubated in serum-free media with lignan compounds for 24 h. The cells were lysed and total RNA was subjected to RT-PCR for PTHrP. Graph indicate the relative intensity of PTHrP compared to that of GAPDH. **c** The culture medium was collected after MDA-MB-231 cells were incubated in serum-free DMEM with lignan compounds for 24 h. The PTHrP level was measured with a commercially available ELISA kit. Data are expressed as mean ± SE of three independent experiments in triplicate. *$P<0.05$, **$P<0.005$ versus serum-free medium-treated cells

We also examined the effects of fargesin on TGF-β-stimulated MDA-MB-231 metastatic breast cancer cells. TGF-β, a very abundant growth factor in bone, enhances colonization, growth, and PTHrP production of bone metastases [16]. Treatment with fargesin inhibited TGF-β-induced viability (Fig. 3a), migration (Fig. 3b), invasion (Fig. 3c), and PTHrP secretion (Fig. 3d) in MDA-MB-231 cells in a dose-dependent manner. These results indicate that aschatin, lirioresinol B dimethyl ether, and magnolin as well as fargesin can block PTHrP production of metastases in bone microenvironment.

Effects of lignan compounds on RANKL and OPG in hFOB1.19 human osteoblastic cells

In hFOB1.19 human osteoblastic cells exposed to each lignan compound for 6 h or 24 h, fargesin treatment at 100 μM significantly decreased cell viability by 32 % and

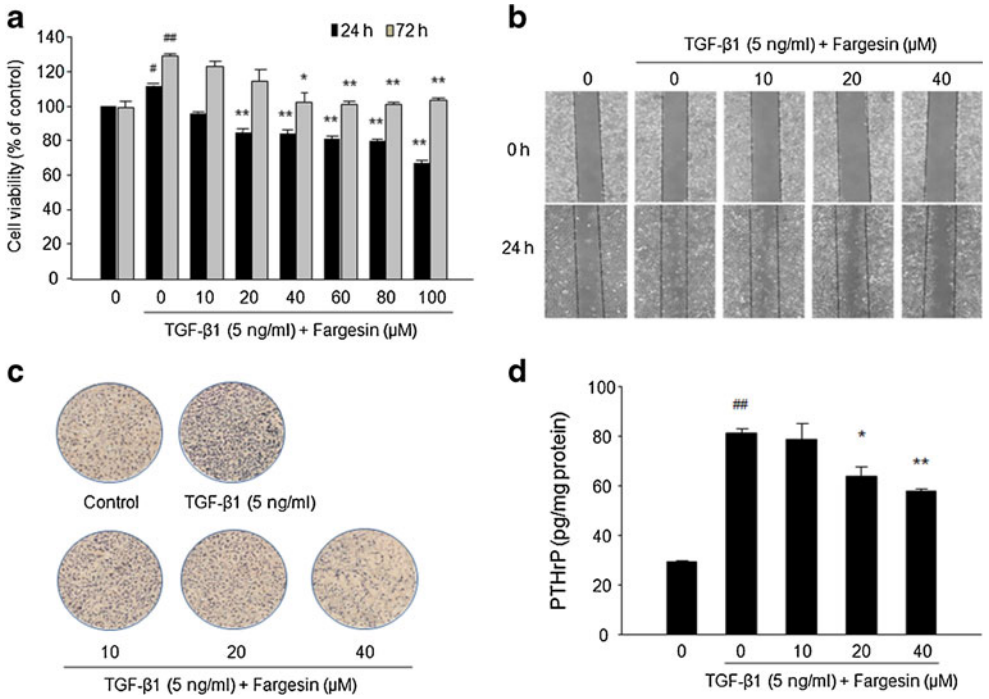


Fig. 3 Effects of fargesin on invasive potential and PTHrP production of TGF-β-stimulated MDA-MB-231 breast cancer cells. **a** MDA-MB-231 cells were exposed to TGF-β (5 ng/ml) and/or fargesin at indicated concentrations for 24 h and 72 h, respectively. Cell viability was determined by an MTT assay. **b** In 6-well plates with MDA-MB-231 cells grown to 90 % confluence, one artificial wound edge per well was scratched into monolayers with the narrow end of a sterile micropipette tip. The cells were incubated in serum-free DMEM with or without the various concentrations of fargesin and/or TGF-β for 24 h. The scratched areas were photographed (40x magnification). **c** Cell invasion assay was performed using a Transwell chamber containing a polycarbonate membrane filter (6.5 mm diameter, 8 μm pore size), coated with 0.1 % (w/v) gelatin and Matrigel, as described in Materials and methods. MDA-MB-231 cell suspensions with the indicated concentrations of fargesin were added into the coated insert and the lower chamber contained 600 μl of culture media with 1 % FBS, fargesin, and TGF-β (5 ng/ml). Transwell plates were incubated for 24 h. After hematoxylin staining, the membranes with invaded cells were mounted on slides and images were captured for each membrane (40x magnification). **d** MDA-MB-231 cells were incubated in serum-free medium containing the indicated concentrations of fargesin and/or TGF-β (5 ng/ml) for 24 h and the culture media were collected. PTHrP levels in the culture media were quantified using a human PTHrP ELISA kit. Data are expressed as mean ± SE of three independent experiments in triplicate. $^{\#}P<0.05$, $^{\#\#}P<0.005$ versus serum-free medium-treated cells, $^{*}P<0.05$, $^{**}P<0.005$ versus TGF-β-treated cells

36 %, respectively, but the other lignan compounds tested did not show remarkable cytotoxicity (Fig. 4a). PTHrP stimulation upregulated RANKL mRNA expression and downregulated OPG mRNA expression, resulting in an increased RANKL/OPG ratio in osteoblastic cells. However, treatment with lignan compounds dose-dependently blocked these effects of PTHrP, reducing the RANKL/OPG ratio of PTHrP-treated hFOB1.19 cells (Fig. 4b).

Effects of lignan compounds on osteoclast formation and activity

Osteoclast formation and activation by RANKL is a very important component of the "vicious cycle" of breast cancer-induced osteolysis [17]. Fargesin, lirioresinol B dimethyl ether, and magnolin at concentrations of 20 μM began to show cytotoxicity in BMMs treated for 5 days (Fig. 5a). Aschatin, fargesin, lirioresinol B dimethyl ether, or magnolin at noncytotoxic concentrations inhibited the formation of TRAP-positive multinucleated cells (osteoclasts) in BMMs stimulated with RANKL for 5 days in a dose-dependent manner. Fargesin (IC_{50}=4.33 μM) showed more potent inhibition than the other components (IC_{50}=7.43 μM for aschatin, 8.88 μM for lirioresinol B dimethyl ether, > 10 μM for magnolin) (Fig. 5b). The levels of MMP-9 (Fig. 5c) and cathepsin K (Fig. 5d) in culture media also decreased following treatment with lignan compounds. Furthermore, the formation of resorption pits in calcium phosphate-coated plates was noticeably blocked when BMMs were treated with RANKL for 5 days to induce mature osteoclast formation and then further incubated with different concentrations of lignan compounds in the presence of RANKL for 14 days (Fig. 5e). These results suggest that aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin can inhibit the

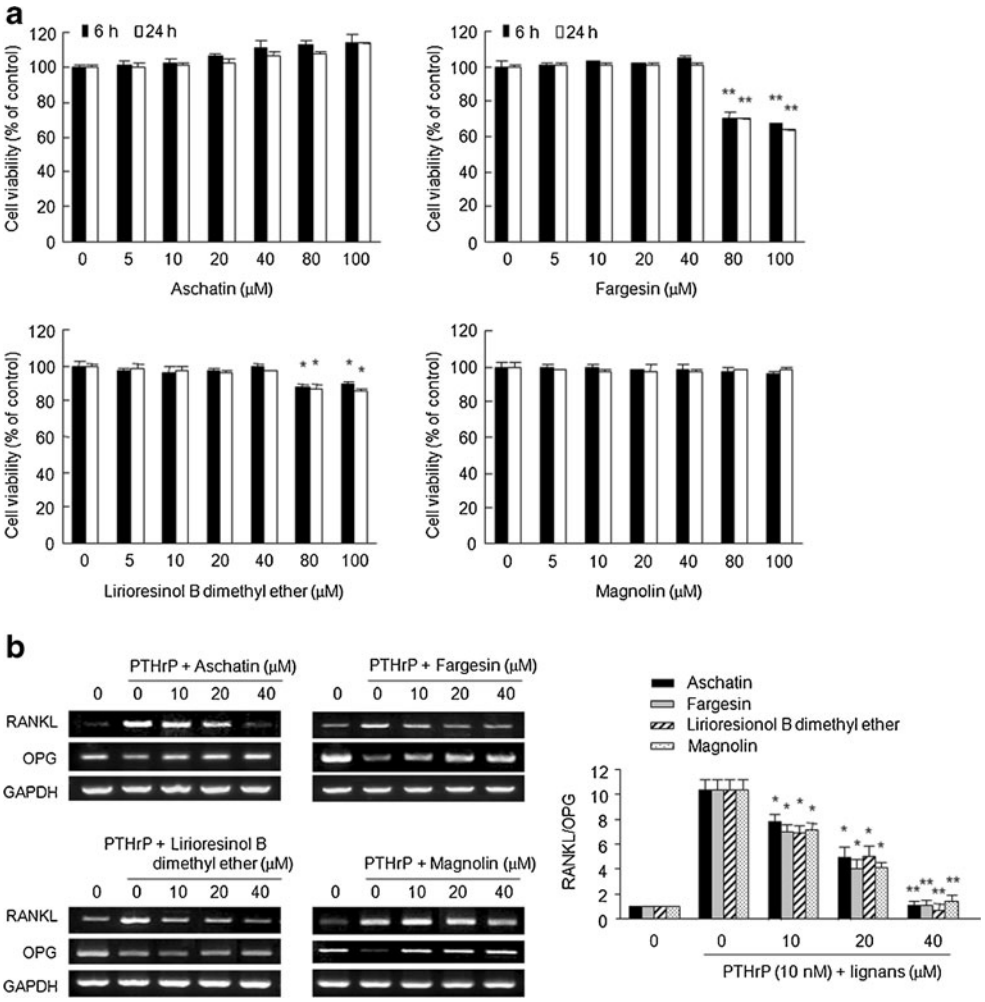


Fig. 4 Effects of lignan compounds on RANKL and OPG mRNA expression in hFOB1.19 human osteoblastic cells. **a** hFOB1.19 cells were treated with lignan compounds at the indicated concentrations for 6 h and 24 h, respectively. Cell viability was determined by an MTT assay. Data are expressed as mean ± SE of three independent experiments in triplicate. *$P<0.05$, **$P<0.01$ versus serum-free medium-treated cells. **b** hFOB1.19 cells were exposed to lignan compounds in the presence of PTHrP (10 nM) for 6 h. RANKL and OPG mRNA levels were analyzed by RT-PCR. Photographs are representative of three independent experiments. The ratio of RANKL to OPG was determined after band intensities of RANKL and OPG were normalized to that of GAPDH. Data are expressed as mean ± SE of three independent experiments. *$P<0.05$, **$P<0.001$ versus PTHrP-treated cells

formation of resorption pits by reducing RANKL-induced osteoclastogenesis and osteoclast-secreted levels of MMP-9 and cathepsin K.

Effect of fargesin on in vivo breast cancer-mediated bone destruction

We investigated the inhibitory effects of fargesin on tumor growth and bone destruction in BALB/c nude mice with metastatic human breast cancer MDA-MB-231 cells xenografted onto the calvaria. Oral administration of fargesin for 45 days inhibited tumor growth in a dose-related manner. On day 45, fargesin treatment at doses of 2 and 5 mg/kg inhibited tumor growth by 19 % and 68 %, respectively, and subcutaneously injected zoledronic acid at 100 μg/kg inhibited tumor growth by 40 % (Fig. 6a). The reconstructed 3D images derived from μCT data (Fig. 6b) and histological examination (Fig. 6c) supported the findings that bone resorption and invasion in the calvaria of MDA-MB-231 cell-injected mice were substantially inhibited by oral administration of fargesin or subcutaneous injection of zoledronic acid. PTHrP expression in tumor tissues was markedly reduced by fargesin treatment (Fig. 6d). Compared with control mice, inoculation of MDA-MB-231 cells decreased percent bone volume (BV/TV, %) and bone surface density (BS/TV, 1/mm) in calvaria and increased serum calcium levels. However, fargesin or zoledronic acid treatment protected against breast cancer-induced bone destruction and allowed these parameters to remain normal (Fig. 6e). These results suggest that orally administered fargesin may have in vivo potential as a preventive/therapeutic agent against breast cancer-mediated osteolysis.

Discussion

Bone metastases are a serious complication in patients with breast, prostate, kidney, thyroid, and lung cancers and portend a poor outcome, with a median survival of less than 6 months,. Up to 70 % of patients with advanced breast cancer develop bone metastasis, which causes osteolytic lesions [18]. The colonized breast cancer cells in bone rapidly

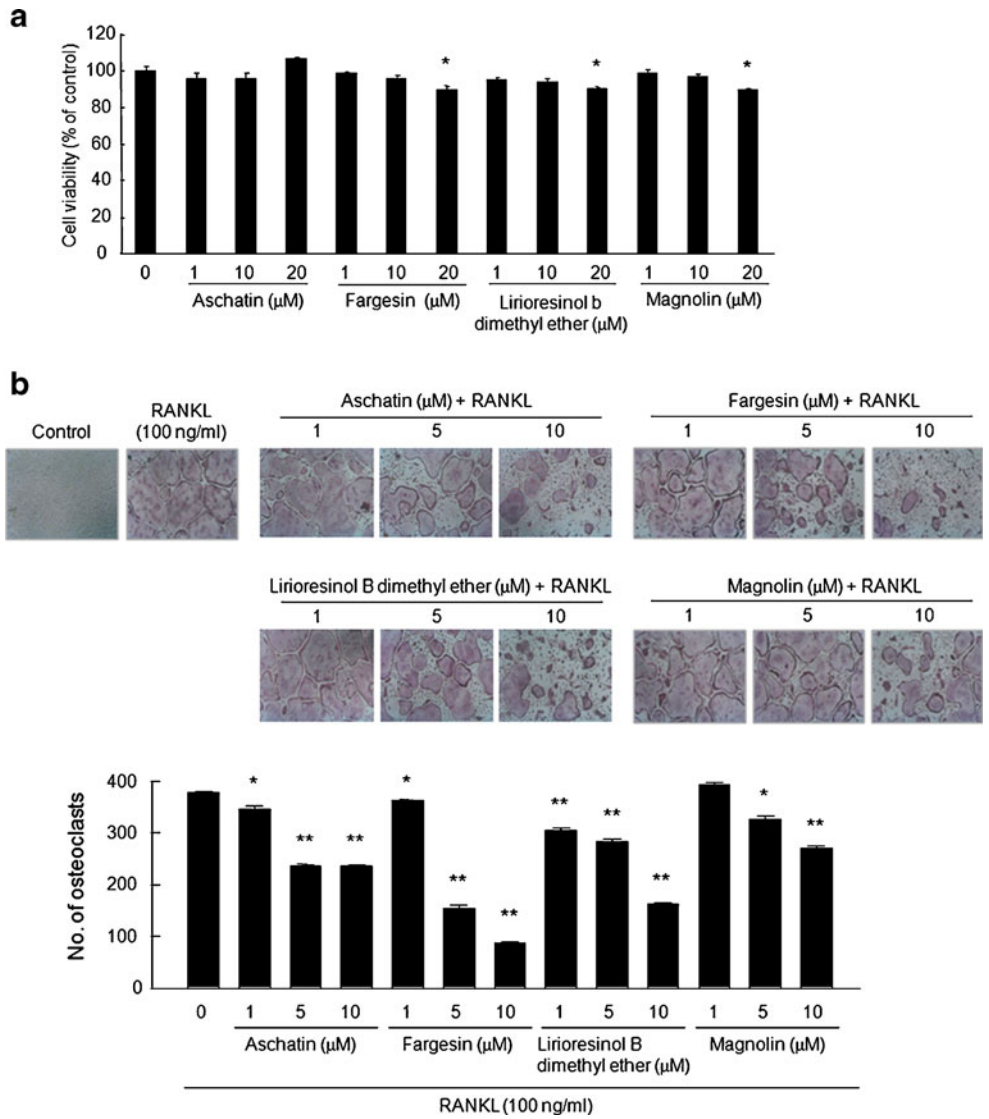


Fig. 5 Effects of lignan compounds on RANKL-induced osteoclast formation and bone resorption. **a** BMMs were treated with lignan compounds at indicated concentrations in the presence of M-CSF (30 ng/ml) for 5 days. Cell viability was determined by an MTT assay. *$P<0.05$ versus M-CSF-treated BMMs **b** BMMs were cultured in α-MEM containing 10 % FBS, M-CSF (30 ng/ml), and RANKL (100 ng/ml) with lignan compounds at indicated concentrations. Five days later, the cells were stained for TRAP and the culture media were collected. TRAP-positive multinucleated (≥ 3 nuclei) cells were counted as mature osteoclasts (100x magnification). **c** The collected media (60 μg protein) were electrophoresed in an 8 % SDS-polyacrylamide gel containing 0.2 % (w/v) gelatin and incubated at 37 °C for 24 h. Gelatinolytic activity was detected as clear bands in a blue background. **d** Cathepsin K levels in the collected media were detected with a SensiZyme Cathepsin K Activity Assay kit. **e** BMMs were cultured in α-MEM containing 10 % FBS, M-CSF (30 ng/ml), and RANKL (100 ng/ml) on BD BioCoat Osteologic MultiTest slides for 5 days. Then, the cells were treated with lignan compounds at indicated concentrations for an additional 14 days. The cells were lysed and pit areas were observed under phase contrast microscopy (100x magnification). Data are expressed as mean ± SE of three independent experiments in triplicate. $^{\#}P<0.001$ versus M-CSF-treated BMMs *$P<0.05$, ** $P<0.0001$ versus RANKL-treated BMMs

proliferate through their interaction with the bone microenvironment and finally lead to devastating bone destruction [19]. Currently, bisphosphonates, inhibitors of bone resorption for the prevention and treatment of osteoporosis, are used clinically to treat cancer patients with bone metastases. However, long-term bisphosphonate therapy at high doses has been closely associated with significant risks, including nephrotoxicity, electrolyte abnormalities, and osteonecrosis of the jaw [20–22]. On the other hand, OPG or monoclonal antibodies against RANKL have also been considered as promising therapeutic agents to treat the destructive bone metastasis of breast cancer. However, these protein therapeutics are associated with certain shortcomings such as difficulty of manufacture in large quantities, cost, immunogenic potential, and need for parenteral administration [6, 23, 24]. Therefore, it is essential to find novel agents with anti-bone-resorptive and anti-

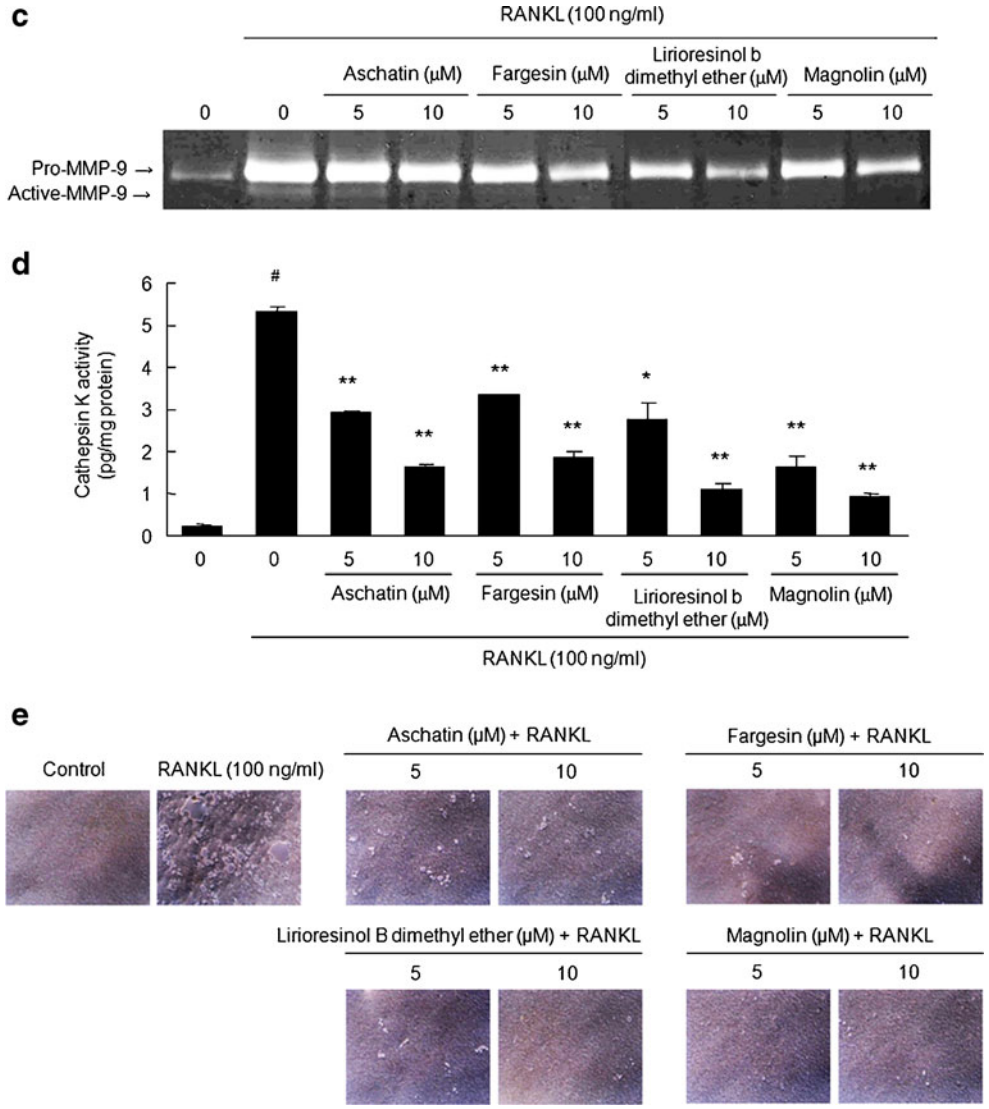
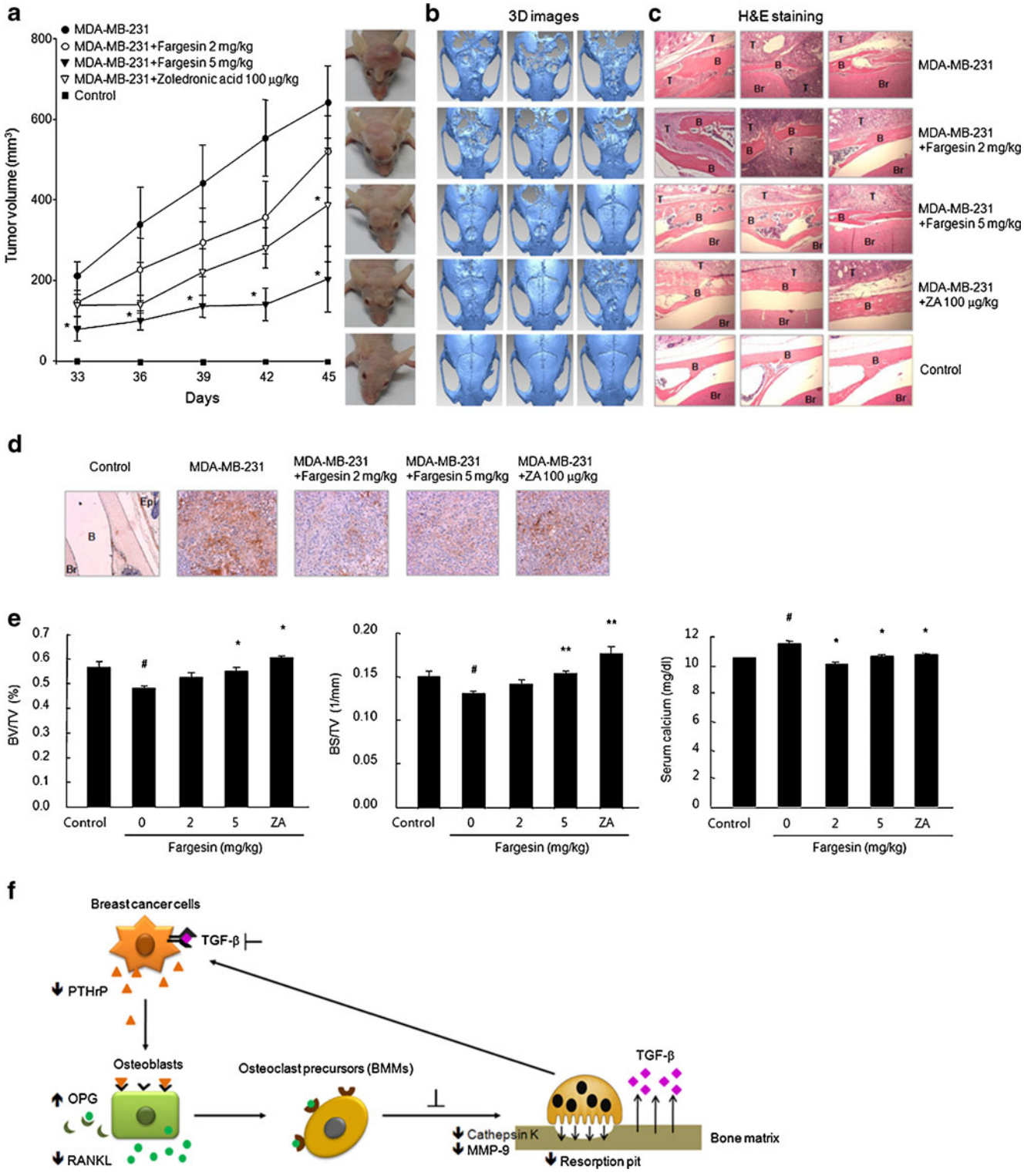


Fig. 5 (continued)

cancer activities for the prevention and treatment of cancer-mediated bone diseases in patients. In particular, small molecules derived from medicinal plants may be premier candidates. In this study, we evaluated whether aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin, tetrahydrofurofuran-type lignans, could inhibit breast cancer-mediated bone destruction, and we focused on interactions between breast cancer metastases and the bone microenvironment.

Bone metastases have been shown to cause bone lesions by releasing a variety of bone-resorbing factors [6]. Among those factors, PTHrP is considered a major inducer of local osteolysis and consequential hypercalcemia of malignancy in advanced stages of breast cancer through its role in triggering the "vicious cycle" [25]. In fact, PTHrP expression is detected in 92 % of patients with skeletal metastases from breast cancer, whereas it is expressed in about 50 % of

Fig. 6 Effect of fargesin on tumor growth and bone destruction in mice with MDA-MB-231 cells inoculated into calvarial tissues. MDA-MB-231 cells were subcutaneously injected over the mouse calvaria twice at a one-week interval. Fargesin (2 and 5 mg/kg) was orally administered 5 times per week and zoledronic acid (ZA, 100 μg/kg) was subcutaneously injected 3 times per week for 45 days after the first inoculation of the breast cancer cells. On day 45, the blood and calvarial tissues were collected. **a** Tumor volumes were measured every 3 days using an electronic digital caliper and calculated according to the following formula: $(a \times b^2)/2$, where "a" is the longer and "b" is the shorter dimension. **b** 3D microarchitectural images of calvaria were reconstructed with micro-CT data and **c** calvarial tissues were stained with hematoxylin and eosin. **d** PTHrP expression in tumor tissues were investigated by immunohistochemical staining as described in Materials and methods (200x magnification). **e** Percent bone volume (BV/TV, %) and bone surface density (BS/TV, 1/mm) were analyzed with μCT and serum calcium levels were detected with a commercially available assay kit. Data are expressed as mean±SE. $^{\#}P<0.05$ versus control mice. $^{*}P<0.05$, $^{**}P<0.005$ versus MDA-MB-231 cell-injected mice. **f** A mechanism underlying the effects of tetrahydrofurofuran lignans on the vicious cycle of breast cancer-induced osteolysis



primary breast cancers and in only 17 % of nonskeletal metastases [26]. In addition, administration of anti-PTHrP antibody was found to suppress osteolytic bone metastasis in mice injected with MDA-MB-231 human breast cancer cells [27]. Recent studies also suggest a multifunctional role for PTHrP in cancer progression [28]. Thus, detection of natural small molecules that target tumor-derived PTHrP could be a promising strategy to prevent or attenuate breast cancer-associated bone destruction. Our data indicated that aschatin, fargesin, lirioresinol B dimethyl ether, and

magnolin at noncytotoxic concentrations decreased mRNA expression and secretion of PTHrP in MDA-MB-231 cells. Of these, fargesin showed more potent inhibition compared to the other compounds. PTHrP expression in breast cancer cells has been reported to be elevated mainly by TGF-β [15, 29, 30]. TGF-β is one of the growth factors which are stored in bone and is released from the bone matrix as a consequence of cancer-mediated osteolysis as well as from metastases themselves, increasingly promoting the progression of metastatic cancer cells in bone [31]. In our study, fargesin suppressed TGF-β-induced cell viability, migration, invasion, and PTHrP production. These results suggest that tetrahydrofurofuran lignans, including aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin, can prevent initiation and amplification of breast cancer-associated bone destruction by targeting PTHrP in bone metastases.

Tumor-derived osteolytic factors, including PTHrP, stimulate osteoblastic expression of RANKL, with decreased expression of OPG [6, 22]. The RANKL expressed by osteoblasts binds to RANK on the surface of osteoclast precursors and causes them to differentiate to mature osteoclasts, subsequently resulting in bone resorption. RANKL can also bind to OPG, its decoy receptor, and this binding inhibits the differentiation of osteoclasts. The increased RANKL/OPG ratio in osteoblasts has been recognized as a prerequisite in virtually occurrence of cancer-induced bone destruction [16, 32]. Hence, inhibition of osteoblastic RANKL and/or induction of OPG might be additional rational approaches to the prevention or treatment of cancer-mediated bone diseases and the resultant hypercalcemia of malignancy. Our results showed that treatment with lignan compounds at noncytotoxic concentrations inhibited PTHrP-induced elevation of the RANKL/OPG ratio in hFOB1.19 osteoblastic cells.

The bone-resorbing activity of osteoclasts is a very important factor in breast cancer-mediated bone destruction. Mature osteoclasts dissolve the inorganic mineral component and the organic matrix in bones by secreting acids and several proteolytic enzymes, primarily MMP-9 and cathepsin K, causing the release of growth factors necessary for the survival and proliferation of the breast cancer cells [1, 33, 34]. Therefore, the progression of metastatic bone diseases may be blocked through inhibition of osteoclast-mediated bone resorption. Our study indicated that aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin at noncytotoxic concentrations inhibited osteoclastogenesis and reduced the secreted levels of MMP-9 and cathepsin K in RANKL-treated BMMs. Moreover, osteoclast-mediated pit formation was decreased in calcium phosphate-coated plates treated with lignan compounds. These results demonstrated that lignan compounds have anti-bone-resorptive activity through the inhibition of osteoclast function as well as RANKL-induced osteoclast formation.

Fargesin, with a piperonyl type of phenyl ring and 2*R* stereochemistry, showed more potent inhibition than the other compounds in the study of PTHrP expression in metastatic breast cancer cells and RANKL-induced osteoclast formation in BMMS. To further estimate the potential of tetrahydrofurofuran-type lignans as novel therapeutic agents against cancer-mediated bone destruction, we administered fargesin to mice with MDA-MB-231 cells injected into the calvarial tissues. Oral administration of fargesin inhibited tumor growth and PTHrP expression in tumor tissues. Moreover, administration of fargesin considerably reduced bone destruction and serum calcium level in mice injected with MDA-MB-231 cells. Fargesin treatment showed better efficacy for the inhibitions of tumor growth and PTHrP expression than treatment with zoledronic acid. Although bone destruction was more greatly reduced in mice treated with zoledronic acid, fargesin treatment led to recovery of bone morphometric parameters and serum calcium levels in MDA-MB-231 cell-injected mice to almost the levels of control mice.

Taken together, aschatin, fargesin, lirioresinol B dimethyl ether, and magnolin, with tetrahydrofurofuran structure in common, interrupted each stage of the vicious cycle between breast cancer metastases and the bone microenvironment (Fig. 6f). These lignan compounds at noncytotoxic concentrations reduced PTHrP production in human metastatic breast cancer cells, inhibited the PTHrP-induced increase in RANKL/OPG ratio in human osteoblastic cells, and prevented RANKL-induced differentiation of osteoclast precursors and subsequent bone resorption. In particular, fargesin, as a representative of tetrahydrofurofuran lignans, demonstrated a beneficial effect in vivo, with the advantage of oral dosing. Therefore, these lignans, particularly fargesin, may be promising candidates for the development of therapeutic agents for patients with bone metastases.

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Declaration of interests The authors declare that they have no conflict of interest.

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