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## Plumbagin Inhibits Osteoclastogenesis and Reduces Human Breast Cancer-induced Osteolytic Bone Metastasis in Mice through Suppression of RANKL Signaling

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### Abstract

Bone loss is one of the major complications of advanced cancers such as breast cancer, prostate cancer and multiple myeloma; agents that can suppress this bone loss have therapeutic potential. Extensive research within the last decade has revealed that RANKL, a member of the tumor necrosis factor superfamily, plays a major role in cancer-associated bone resorption, and thus is a therapeutic target. We investigated the potential of vitamin K3 analogue plumbagin (derived from Chitrak, an Ayurvedic medicinal plant), to modulate RANKL signaling, osteoclastogenesis and breast cancer-induced osteolysis. Plumbagin suppressed RANKL-induced NF- $\kappa$ B activation in mouse monocytes, an osteoclast precursor cell, through sequential inhibition of activation of I $\kappa$ B $\alpha$  kinase, I $\kappa$ B $\alpha$  phosphorylation and I $\kappa$ B $\alpha$  degradation. Plumbagin also suppressed differentiation of these cells into osteoclasts induced either by RANKL or by human breast cancer or human multiple myeloma cells. When examined for its ability to prevent human breast cancer-induced bone loss in animals, plumbagin (2 mg/kg body weight), when administered via the intraperitoneal route, significantly decreased osteolytic lesions resulting in preservation of bone volume in nude mice bearing human breast tumors. Overall, our results indicate that plumbagin, a vitamin K analogue, is a potent inhibitor of osteoclastogenesis induced by tumor cells and of breast cancer-induced osteolytic metastasis through suppression of RANKL signaling.

### Keywords

Plumbagin; NF- $\kappa$ B; RANKL; osteoclastogenesis

### Introduction

Bone metastasis is a common complication of advanced cancers such as breast cancer and prostate cancer. Depending on the site of primary tumor, metastasis to bone occurs in as many as 70% of patients with metastatic disease and often results in skeletal morbidity (1, 2). Sequelae of bone metastasis include hypercalcemia of malignancy, severe bone pain and debilitating skeletal morbidity (1, 2). Bone metastases are commonly characterized as

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Study design: BS, BOO and BBA. Study conduct: BS and BOO. Data analysis: BS and BOO. Data interpretation: BS and BOO. Drafting manuscript: BS and BOO. Approving final version of manuscript: BBA.

osteolytic, osteoblastic/osteosclerotic or mixed. While breast cancer is most often associated with osteolytic or mixed metastases, osteoblastic metastases are common in prostate cancer. Osteolytic lesions are due to a marked increase in osteoclast number with reduced osteoblastic activity. Parathyroid hormone-related protein (PTHrP) is known as a major player between tumor and bone cells and induces the osteolytic process in part through activation of the RANKL pathway (3). In contrast to osteolytic lesions, osteosclerotic metastases are defined by a dramatic increase in new bone formation, but they always possess a resorption component (4, 5).

Breast cancer cells produce factors that induce osteoclastogenesis, PTHrP and interleukins (IL)-1, -6 and -11, which act on bone-forming osteoblasts to increase production of an essential osteoclast stimulator, receptor activator of nuclear factor-kappaB (RANK) ligand (RANKL) (4, 6, 7). RANKL-induced osteoclastogenesis is mediated through the cell surface receptor RANK. The interaction of RANKL with RANK leads to recruitment of tumor necrosis factor (TNF) receptor-associated factor to cell surface receptor RANK and then activate NF- $\kappa$ B signaling pathways (8). Indeed, secretion of RANKL by various tumor cells induces osteoclastogenesis (9–12). Therefore, selective modulation of RANKL signaling pathways may have therapeutic potential for cancer-induced bone loss as well as bone-related diseases such as osteoporosis and osteoarthritis. A fully humanized monoclonal neutralizing antibody to RANKL, denosumab (also called Prolia [Amgen]), has been approved by the U.S. Food and Drug Administration for use in postmenopausal women with risk of osteoporosis.

Identification of novel targets for traditional medicine provides a reverse pharmacology or “bedside to bench” approach for novel drug discovery. We report here our investigation of the potential of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone; Figure 1A), derived from the root of an Ayurvedic plant, *Plumbago zeylanica* L. (also known as Chitrak), to modulate RANK signaling and bone loss. This compound has also been identified in *Juglans regia* (English walnut), *Juglans cinerea* (butternut and white walnut) and *Juglans nigra* (black walnut) (13). Plumbagin has been shown to exert antibacterial (14), antifungal (15), anti-atherosclerotic (16) and anticancer effects (17–19) through a variety of mechanisms, including induction of ROS (20); suppression of NF- $\kappa$ B (13), AKT/mTOR (21) and STAT3 (22); induction of p53 and JNK (23); activation of the NRF2-ARE pathway (24) and direct inhibition of histone acetyltransferase p300 (25). In animal studies it has been shown to be chemopreventive for colon cancer (26) and to exhibit antitumor activity in prostate cancer (27).

We investigated whether plumbagin can suppress the RANKL-induced NF- $\kappa$ B activation pathway and osteoclastogenesis. Whether this vitamin K analogue can affect osteoclast differentiation induced by tumor cells was also examined. Moreover, we investigated whether plumbagin could prevent human breast cancer-induced bone loss *in vivo*. The results show that plumbagin inhibited the RANKL-induced NF- $\kappa$ B activation pathway by inhibiting I $\kappa$ B $\alpha$  kinase (IKK); this effect correlated with suppression of osteoclastogenesis induced by RANKL or by breast cancer or multiple myeloma cells. Finally, plumbagin inhibited human breast cancer-induced osteolytic lesions in nude mice.

## Materials and Methods

### Reagents

Plumbagin (purity >97%) was purchased from Sigma-Aldrich (St. Louis, MO). A 10-mmol/L solution of plumbagin was prepared in dimethylsulfoxide, stored as small aliquots and then diluted as needed in cell culture medium. Dulbecco modified essential medium (DMEM)/F12, RPMI 1640, DMEM, 0.4% trypan blue vital stain and antibiotic-antimycotic

mixture were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Inc. (Lawrenceville, GA). Antibodies against I $\kappa$ B $\alpha$ , IKK $\alpha$  and IKK $\beta$  were obtained from Imgenex (San Diego, CA). Antibody against phospho-I $\kappa$ B $\alpha$  (Ser32/36) was purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates were purchased from Bio-Rad (Hercules, CA). Antibody against  $\beta$ -actin and the leukocyte acid phosphatase kit (387-A) for tartrate-resistant acid phosphatase (TRAP) staining were purchased from Sigma-Aldrich. Protein A/G-agarose beads were obtained from Pierce (Rockford, IL). [ $\gamma$ - $^{32}$ P]ATP was purchased from MP Biomedicals (Solon, OH).

### Cell lines

RAW 264.7, MDA-MB-231, MCF-7, MM.1S and U266 cells were obtained from American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in DMEM/F12 supplemented with 10% FBS and antibiotics. This cell line is a well-established osteoprogenitor cell system that has been shown to express RANK and differentiate into functional TRAP-positive osteoclasts when cultured with soluble RANKL (28). MDA-MB-231 and MCF-7 cells were cultured in DMEM, and MM.1S and U266 cells in RPMI 1640 with 10% FBS. The above-mentioned cell lines were procured more than 6 months ago and have not been tested recently for authentication in our laboratory.

### Electrophoretic mobility shift assays for NF- $\kappa$ B

Nuclear extracts were prepared as described previously (29).

### Western blot analysis

To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (30) and fractionated them by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence reagent (GE Healthcare).

### IKK assay

To determine the effect of plumbagin on RANKL-induced IKK activation, IKK assay was done by a method described previously (30).

### Osteoclast differentiation assay

RAW 264.7 cells were cultured in 24-well dishes at a density of  $5 \times 10^3$  cells per well and allowed to adhere overnight. The medium was then replaced, and the cells were treated with 5 nM RANKL for 5 days. All cell lines were subjected to TRAP staining using leukocyte acid phosphatase kit. For co-culture experiments with tumor cells, RAW 264.7 cells were seeded at  $5 \times 10^3$  per well and allowed to adhere overnight. The following day, U266 or MDA-MB-231 cells at  $1 \times 10^3$  per well, were added to the RAW 264.7 cells, treated with plumbagin and co-cultured for 5 days before subjected to TRAP staining.

### Bone resorption pit assay

The effect of plumbagin on bone resorption by pit formation assay was performed as described elsewhere (31).

### *In vivo* osteolytic bone metastasis assay

The estrogen-independent human breast cancer cell line MDA-MB-231 was cultured and resuspended in phosphate-buffered saline solution (PBS) to give a final concentration of  $1 \times$

$10^5/100 \mu\text{L}$ . 5-week-old female BALB/c *nu/nu* mice (Harlan, Indianapolis, IN) were inoculated with MDA-MB-231 cells ( $1 \times 10^5/100 \mu\text{L}$ ) direct into the left ventricle via a percutaneous approach. The mice were then randomly assigned to one of two groups, treated with vehicle (0.9% sodium chloride,  $n = 5$ ) or plumbagin (2 mg/kg body weight in vehicle,  $n = 9$ ) by intraperitoneal injection five times a week for 28 days and then sacrificed. Radiographs (Faxitron Radiographic inspection unit; Kodak, Rochester, NY) were obtained at baseline and just prior to sacrifice. Areas of osteolytic bone metastases of tibiae and femorae, recognized as well-circumscribed radiolucent lesions on radiographs, were quantitated using MetaMorph imaging software (Molecular Devices, Downingtown, PA). Radiographs were analyzed by investigators blinded to the composition of the groups in the experiment or the experimental protocol. Areal data for each group are presented as  $\text{mm}^2/\text{lesion}$  (mean  $\pm$  SEM).

### Quantitative micro-computed tomography (micro-CT) analysis

A  $\mu\text{CT}40$  scanner (SCANCO Medical AG, Bassersdorf, Switzerland) was used to acquire tomographic images (100 slices) of formalin-fixed tibiae using energy settings of 55 kV and 145 mA, integration time of 250 ms with 125 projections per  $180^\circ$ , at a  $12 \mu\text{m}$  voxel size (isotropic). Images were reconstructed using proprietary Scanco evaluation software. To obtain bone volume/total volume (BV/TV), trabecular number, trabecular thickness and trabecular connectivity density, contours were drawn within the cortices around the metaphyseal regions, such that the total volume included only trabecular bone at 0.2 mm below the growth plate and extending 0.12 mm. Bone volume included all bone tissue that had a material density greater than  $438.7 \text{ mgHA}/\text{cm}^3$ , thereby giving a measure of bone volume/total volume. The same threshold setting for the auto-contouring feature in the Scanco software was used for all bone samples. Cross-sectional images were exported in tiff format and imported into AMIRA 3-D graphics software (Mercury Computer Systems, Chelmsford, MA). Using a consistent threshold, AMIRA was used to generate 3-D renderings of the metaphyses. All micro-CT image acquisitions and analyses were performed by an individual blinded to the composition of the experimental groups.

### Statistical analysis

Data are presented as mean  $\pm$  SEM and analyzed using StatView software (version 5.0; SAS Institute, Inc.). Statistical significance of differences was assessed using the non-parametric Mann-Whitney U test. Values of  $p \leq 0.05$  were considered statistically significant.

## Results

The aim of this study was to investigate the effect of vitamin K3 analogue plumbagin on osteoclastogenesis induced by RANKL or by tumor cells and on cancer-induced bone loss in animals. We used the RAW 264.7 cell (murine macrophage) system, as it is a well-established model for osteoclastogenesis (32). Whether plumbagin could modulate osteoclastogenesis induced by tumor cells such as breast cancer and multiple myeloma was another focus of these studies. We also investigated the effects of plumbagin on cancer cell-mediated bone loss in athymic nude mice bearing human breast cancer.

### Plumbagin represses RANKL-induced NF- $\kappa$ B activation in RAW 264.7 cells

Binding of RANKL to its receptor RANK activates TNF receptor-associated factor 6, which is linked to activation of NF- $\kappa$ B. We investigated whether plumbagin modulates RANKL-induced NF- $\kappa$ B activation in monocytic RAW 264.7 cells. Cells were either pretreated with plumbagin for 4 hours or left untreated, then exposed to RANKL at indicated concentrations; nuclear extracts were prepared and NF- $\kappa$ B activation determined by electrophoretic mobility shift assay (EMSA). As shown in Figure 1B (left panel), RANKL

activated NF- $\kappa$ B; however, plumbagin suppressed RANKL-induced NF- $\kappa$ B activation in a dose-dependent manner. We also investigated the duration of incubation required for plumbagin to suppress RANKL-induced NF- $\kappa$ B activation. Cells were preincubated with 5  $\mu$ mol/L plumbagin for the indicated time period up to 4 hours and then exposed to RANKL. As shown by EMSA result in Figure 1B (right panel), RANKL-induced NF- $\kappa$ B was inhibited by plumbagin within 4 hours. Plumbagin by itself did not activate NF- $\kappa$ B.

### **Plumbagin abolished RANKL-induced phosphorylation and degradation of I $\kappa$ B $\alpha$**

Once cells are triggered with stimuli such as proinflammatory cytokines (i.e., TNF and IL-1), these stimuli activate the IKK complex, which phosphorylates I $\kappa$ B and thereby tags it for ubiquitination and degradation by the proteasome. The degradation of I $\kappa$ B thus allows NF- $\kappa$ B to translocate into the nucleus where it can act as a transcription factor.

To determine the effect of plumbagin on RANKL-induced I $\kappa$ B $\alpha$  degradation, we treated the cells with RANKL for different times in absence and presence of plumbagin; and then examined for NF- $\kappa$ B and I $\kappa$ B $\alpha$ . We found that plumbagin inhibited RANKL induced activation of NF- $\kappa$ B (Fig. 1C) as well as RANKL-induced I $\kappa$ B $\alpha$  degradation (Fig. 1D). RANKL induced I $\kappa$ B $\alpha$  degradation in control cells as early as 5 minutes after treatment, but in plumbagin-pretreated cells RANKL had no effect on I $\kappa$ B $\alpha$  degradation (Fig. 1D).

Next we investigated the effect of plumbagin on the phosphorylation of I $\kappa$ B $\alpha$ . To determine this, we used the proteasome inhibitor N-acetyl-leu-leu-norleucinal (ALLN) to block RANKL-induced I $\kappa$ B $\alpha$  degradation. Cells were either treated with plumbagin, ALLN or RANKL and then examined for phosphorylation of I $\kappa$ B $\alpha$ . Because of rapid phosphorylation and degradation of I $\kappa$ B $\alpha$ , no band was detected in cells treated with RANKL alone (Fig. 1E). Thus ALLN was required to see the phosphorylated band. Results clearly indicate that RANKL induces the phosphorylation of I $\kappa$ B $\alpha$  (sixth lane) and that plumbagin pretreatment inhibits the phosphorylation (eighth lane). (Fig. 1E).

### **Plumbagin suppresses IKK activation induced by RANKL**

Degradation of I $\kappa$ B is a tightly regulated event that is initiated upon specific phosphorylation by activated IKK. We examined the effect of plumbagin on RANKL-induced IKK activation using immune complex assays. The results indicated that RANKL activated IKK in a time-dependent manner and that plumbagin suppressed RANKL-induced activation of IKK (Fig. 1F, top panel). To check whether the apparent loss of IKK activity was due to downregulation of IKK protein expression, the levels of the IKK subunits IKK $\alpha$  and IKK $\beta$  were tested by western blot analysis. Neither RANKL nor plumbagin affected the expression of IKK $\alpha$  or IKK $\beta$  protein (Fig. 1E, middle and bottom panel).

### **Plumbagin inhibits RANKL-induced osteoclastogenesis**

Next we examined the effect of plumbagin on osteoclast differentiation induced by RANKL from osteoclast precursor murine monocyte RAW 246.7 cells. Cells were treated with 0.2, 0.5 or 1  $\mu$ mol/L of plumbagin in the presence of RANKL and allowed to differentiate into osteoclasts. As indicated in Figure 2A, RANKL induced formation of osteoclasts in the control cells at day 3. By contrast, the differentiation into osteoclasts induced by RANKL was significantly decreased in the presence of plumbagin. The reduction of osteoclast formation correlated with increasing concentration of plumbagin (Fig. 2B). As little as 0.2  $\mu$ mol/L plumbagin significantly suppressed RANKL-induced differentiation into osteoclasts. Under these conditions, the viability of cells was not significantly affected (data not shown).



### Plumbagin reduces RANKL-induced bone resorption

We also investigated whether the inhibition of RANKL-induced osteoclastogenesis by plumbagin leads to inhibition of bone resorption. RAW264.7 cells were seeded into calcium phosphate apatite-coated plates, treated with plumbagin along with RANKL and incubated for 5 days. After incubation, resorption pit formation was analyzed. RANKL induced pit formation (Fig. 2C, arrows), whereas plumbagin significantly inhibited RANKL-induced pit formation (Fig. 2C). These results suggest that plumbagin suppressed not only osteoclastogenesis but also bone resorption.

### Plumbagin acts at an early step of osteoclastogenesis induced by RANKL

To determine at which stage plumbagin inhibits osteoclastogenesis, the vitamin K analogue was added to osteoclast differentiation cultures beginning at day 0, 1 day later, 2 days later, 3 days later or 4 days later. Plumbagin inhibited osteoclastogenesis maximally when added from the beginning with RANKL treatment (Fig. 3A and 3B). The exposure of precursor cells to plumbagin at later stages (after 3 days) was less effective in preventing osteoclastogenesis (Fig. 3B, fifth column). Since the RAW 264.7 cells exposed to RANKL for 3 days have already committed to osteoclast differentiation, it is reasonable to suggest that plumbagin can block osteoclast differentiation but cannot reverse the differentiation process once cells have committed to the osteoclast.

### Plumbagin inhibits osteoclastogenesis induced by tumor cells

Bone loss is one of the most common complications in patients with breast cancer (33) or multiple myeloma (34). Whether this vitamin K3 analogue, plumbagin, also blocks tumor cell-induced osteoclastogenesis of RAW 264.7 cells was investigated. As shown in Figure 4, co-culture of RAW 264.7 cells with human multiple myeloma U266 (Fig. 4A) or MM.1S cells (Fig. 4B) induced osteoclast differentiation, and plumbagin suppressed this differentiation in a dose-dependent manner. We found, moreover, that human breast cancer cells, such as MDA-MB-231 (Fig. 4C) and MCF-7 (Fig. 4D), also induced formation of osteoclasts, and plumbagin suppressed this differentiation (Fig. 4C and 4D). These results indicate that osteoclastogenesis induced by tumor cells, is significantly suppressed by plumbagin.

### Plumbagin suppresses osteolysis in MDA-MB-231 breast cancer tumor-bearing mice

Breast cancer is one of the most common cancers affecting women in western countries, including the United States. In breast cancer patients, the frequency of bone metastasis is much higher than that of lung and liver metastases (1, 4). To determine whether plumbagin could suppress osteolytic bone metastasis, we injected nude mice with human breast cancer MDA-MB-231 cells, which are triple negative (negative for estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2), and treated them with plumbagin (2 mg/kg) or vehicle control for 4 weeks. To identify osteolytic bone metastasis, we analyzed osteolytic lesions by microradiography and micro-CT analysis. As seen in representative radiographs in Figure 5A, osteolytic bone metastasis and destruction of cortices (arrows) were observed in the MDA-MB-231 tumor-bearing control mice (left panel). In contrast, there was very little osteolysis in the plumbagin (2 mg/kg)-treated mice, and the cortices remained intact (left panel). Quantitative analysis of the radiographs measuring osteolytic lesion area and number confirmed this observation (Figs. 5B and 5C).

Due to extensive bone destruction, a decrease in residual trabecular/cancellous bone volume is considered characteristic of tumor-induced osteolytic bone disease. Micro-CT analyses of proximal tibiae (Fig. 6A) demonstrated that treatment with plumbagin resulted in a significant preservation of cancellous/trabecular bone volume (Fig. 6B), which was

accompanied by a significant increase in trabecular connectivity density in plumbagin-treated tumor-bearing mice compared to the vehicle-treated tumor-bearing mice (Fig. 6C). Although there was a small increase in trabecular thickness in plumbagin-treated mice, this was not statistically significant (data not shown).

## Discussion

Almost 90% of cancer-associated deaths are due to tumor metastasis. Some of the major organ sites of tumor metastasis include lung, liver, lymph node, brain and bone. Bone metastasis is commonly associated with prostate cancer, multiple myeloma and breast cancer. Breast cancer metastasis to bone leads to osteolytic lesions, and no treatment is available. Because RANKL, a member of the TNF superfamily, has been closely linked with bone loss, it is a novel therapeutic target. In the study presented here, we investigated the effect of plumbagin, an analogue of vitamin K derived from an Ayurvedic plant, for its potential to affect RANKL signaling, osteoclastogenesis and breast cancer-induced osteolytic lesions in mice.

We found that plumbagin exhibits numerous effects: first, it inhibited RANKL-mediated NF- $\kappa$ B activation; second, it inhibited RANKL-induced I $\kappa$ B $\alpha$  phosphorylation and degradation; third, it blocked activation of IKK; fourth, it suppressed RANKL-induced osteoclastogenesis; fifth, it abrogated osteoclastogenesis induced by breast cancer and multiple myeloma; and sixth, when given to animals, it suppressed human breast cancer-induced osteolytic lesions in animals. All these observations together suggest the potential of this compound in suppression of cancer-associated bone loss.

This is the first report to suggest that plumbagin can abrogate RANKL signaling. The RANKL/RANK interaction results in a cascade of intracellular events, including activation of NF- $\kappa$ B (28, 35). NF- $\kappa$ B signaling has been shown to play an important role in osteoclastogenesis (36). NF- $\kappa$ B p50<sup>-/-</sup> and p52<sup>-/-</sup> double knockout mice exhibit severe osteopetrosis caused by failure of osteoclast formation (37, 38). Both p50 and p52 expression are essential for RANK-expressing osteoclast precursors to differentiate into TRAP-positive osteoclasts in response to RANKL and other osteoclastogenic cytokines (39). IKK $\beta$ , a component of the NF- $\kappa$ B signaling pathway, plays an essential role in inflammation-induced bone loss and is required for osteoclastogenesis (40). IKK $\beta$ -deficient bone marrow cells do not differentiate to osteoclasts *in vitro* when stimulated by RANKL. These reports suggest that any drug that can block RANKL signaling would play an important role in suppression of osteoclast formation. In this study, we found that plumbagin indeed suppressed NF- $\kappa$ B activation by RANKL and this suppression correlated with inhibition of osteoclastogenesis.

Breast cancer, prostate cancer and multiple myeloma, all of which metastasize to bone frequently, express proteins that can migrate and anchor in bone, including chemokines (e.g., CXCR4) (41) and proteins that promote pericellular proteolysis and invasion (e.g., matrix metalloproteinases [MMPs]), angiogenesis (42) and osteoclastogenesis (1). Previous reports from our laboratory and others showed that plumbagin could suppress the expression of MMPs (for invasion) and vascular endothelial growth factor (VEGF; for angiogenesis) in various types of cancer cell lines, including human multiple myeloma (13, 22, 43). Aziz *et al.* reported that plumbagin was a potent inhibitor of prostate cancer cell invasion through suppression of cell invasion and metastasis marker protein MMP-9 and VEGF in prostate cancer cell lines and in athymic mice with ectopic implantation of hormone-refractory DU145 cells (27). These data are in agreement with our report that plumbagin can inhibit metastasis of breast cancer MDA-MB-231 cells to bone, thus leading to decrease of osteolysis in mice.

In the study reported here, we show that plumbagin suppressed the osteoclastogenesis induced by breast cancer and by multiple myeloma cells. Both breast cancer and multiple myeloma are known to produce RANKL (12, 34) and thus exhibit constitutive NF- $\kappa$ B activation (44, 45). This NF- $\kappa$ B activation can cause induction of osteoclastogenesis via expression of RANKL. In breast cancer, osteolytic lesions occur in 80% of patients with stage IV metastatic disease (46). These lesions have increased osteoclast activity and net bone destruction (47). Our results suggest that plumbagin may have potential in the treatment of cancer-induced bone lesions.

The most widely used agents for prevention of bone loss include synthetic calcitonin, bisphosphonate drugs, raloxifene and teriparatide (synthetic parathyroid hormone). The bisphosphonates, potent inhibitors of osteoclast formation and activity, are the current standard of care and are the most used drugs for treatment of cancer-induced osteolytic diseases. Several reports have indicated, however, that bisphosphonates increase the risk of severe osteonecrosis of the jaw in cancer patients (48), with devastating consequences for affected patients (49). It is prudent, therefore, to develop safer antiresorptive strategies to combat lytic and mixed bone lesions in patients with metastatic cancer in the skeleton.

Plumbagin, a naphthoquinone derived from Ayurvedic medicine, has been used routinely in traditional medicine and is quite safe. Numerous reports indicate that it exhibits anticancer activities against a variety of tumors through activation of multiple cell signaling pathways (13, 21, 22, 27, 43, 50). Compared to bisphosphonates or denosumab, plumbagin is very inexpensive and has minimal side effects. Our data provide the rationale for further animal and human studies with plumbagin for breast cancer-associated bone loss.

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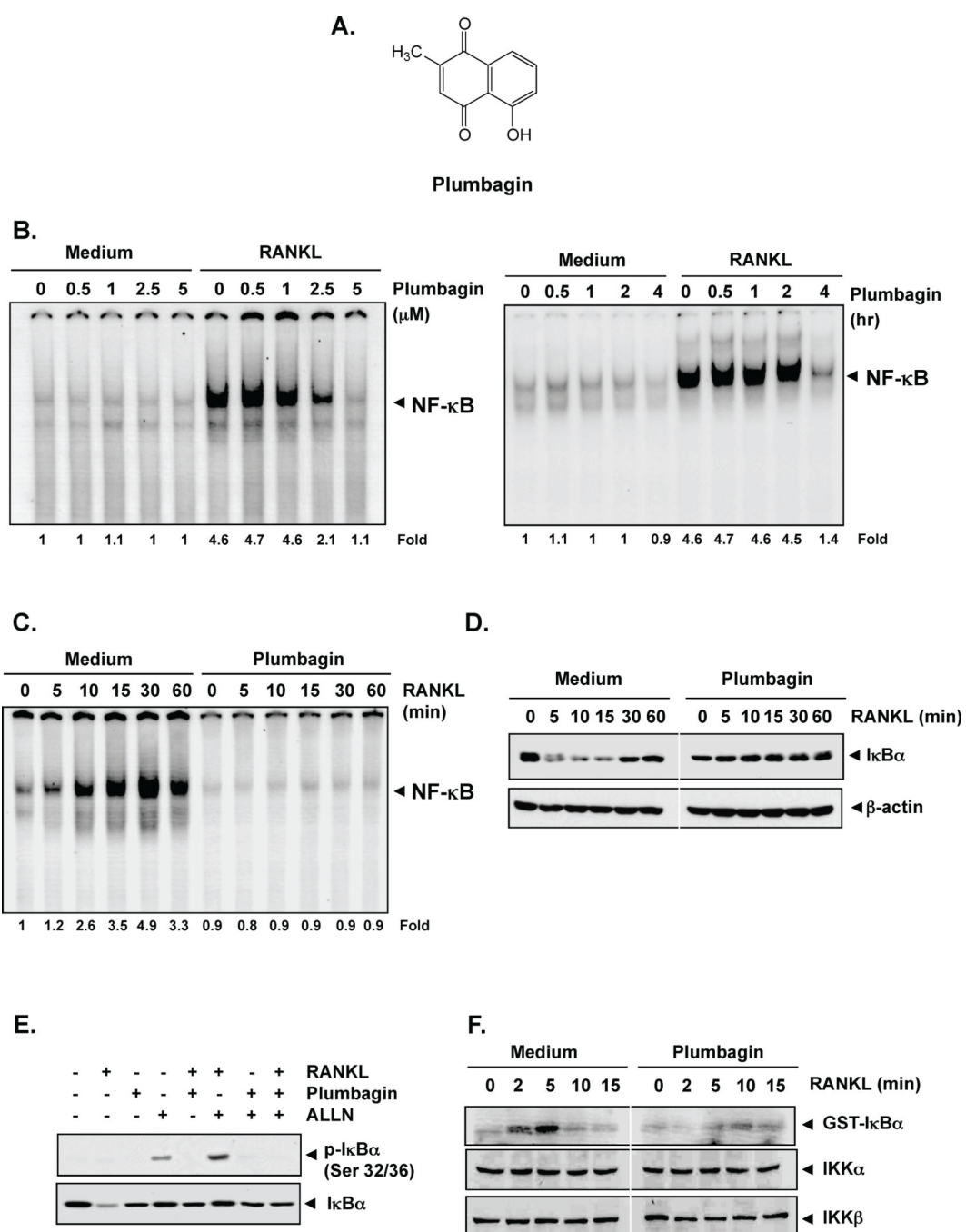
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**Figure 1. RANKL induces NF-κB activation and plumbagin abolishes RANKL-induced NF-κB activation**

(A) Chemical structure of plumbagin.

(B) Left, RAW 264.7 cells ( $1 \times 10^6$  cells) were incubated with or without the indicated concentrations of plumbagin for 4 hours, treated with RANKL (10 nmol/L) for a further 30 minutes and then tested for NF-κB activation by EMSA. Right, RAW 264.7 cells ( $1 \times 10^6$  cells) were incubated with plumbagin (5 μmol/L) for the indicated times, treated with RANKL (10 nmol/L) for a further 30 minutes and assayed for NF-κB activation by EMSA. Fold value is based on the value for medium (control), arbitrarily set at 1.

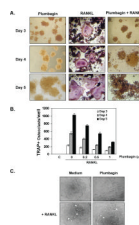
(C) RAW 264.7 cells ( $1 \times 10^6$  cells) were incubated with plumbagin ( $5 \mu\text{mol/L}$ ) for 4 hours and then treated with RANKL ( $10 \text{ nmol/L}$ ) for the indicated times and assayed for NF- $\kappa\text{B}$  activation by EMSA. Fold value is based on the value for medium (control), arbitrarily set at 1.

(D) Cells ( $1 \times 10^6$  cells) were incubated with plumbagin ( $5 \mu\text{mol/L}$ ) for 4 hours and then treated with RANKL ( $10 \text{ nmol/L}$ ) for the indicated times. Cytoplasmic extracts were prepared, fractionated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed with anti-I $\kappa\text{B}\alpha$ .

(E) RAW 264.7 cells ( $1 \times 10^6$  cells) were pretreated with plumbagin ( $5 \mu\text{mol/L}$ ) for 4 hours, incubated with ALLN ( $50 \mu\text{g/mL}$  for 30 minutes) and then treated with RANKL ( $10 \text{ nmol/L}$ ) for 15 minutes. Cytoplasmic extracts were prepared, fractionated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed using either anti-phospho-I $\kappa\text{B}\alpha$  (upper panel) or anti-I $\kappa\text{B}\alpha$  (lower panel).

(F) RAW 264.7 cells ( $3 \times 10^6$  cells) were pretreated with plumbagin ( $5 \mu\text{mol/L}$ ) for 4 hours and then incubated with RANKL ( $10 \text{ nmol/L}$ ) for the indicated times. Whole-cell extracts were immunoprecipitated using antibody against IKK $\alpha$  and analyzed by an immune complex kinase assay using recombinant GST-I $\kappa\text{B}\alpha$  as described in Materials and Methods. To examine the effect of plumbagin on the level of IKK proteins, whole-cell extracts were fractionated by 10% SDS-PAGE and examined by western blot analysis using anti-IKK $\alpha$  (middle panel) and anti-IKK $\beta$  (bottom panel) antibodies.



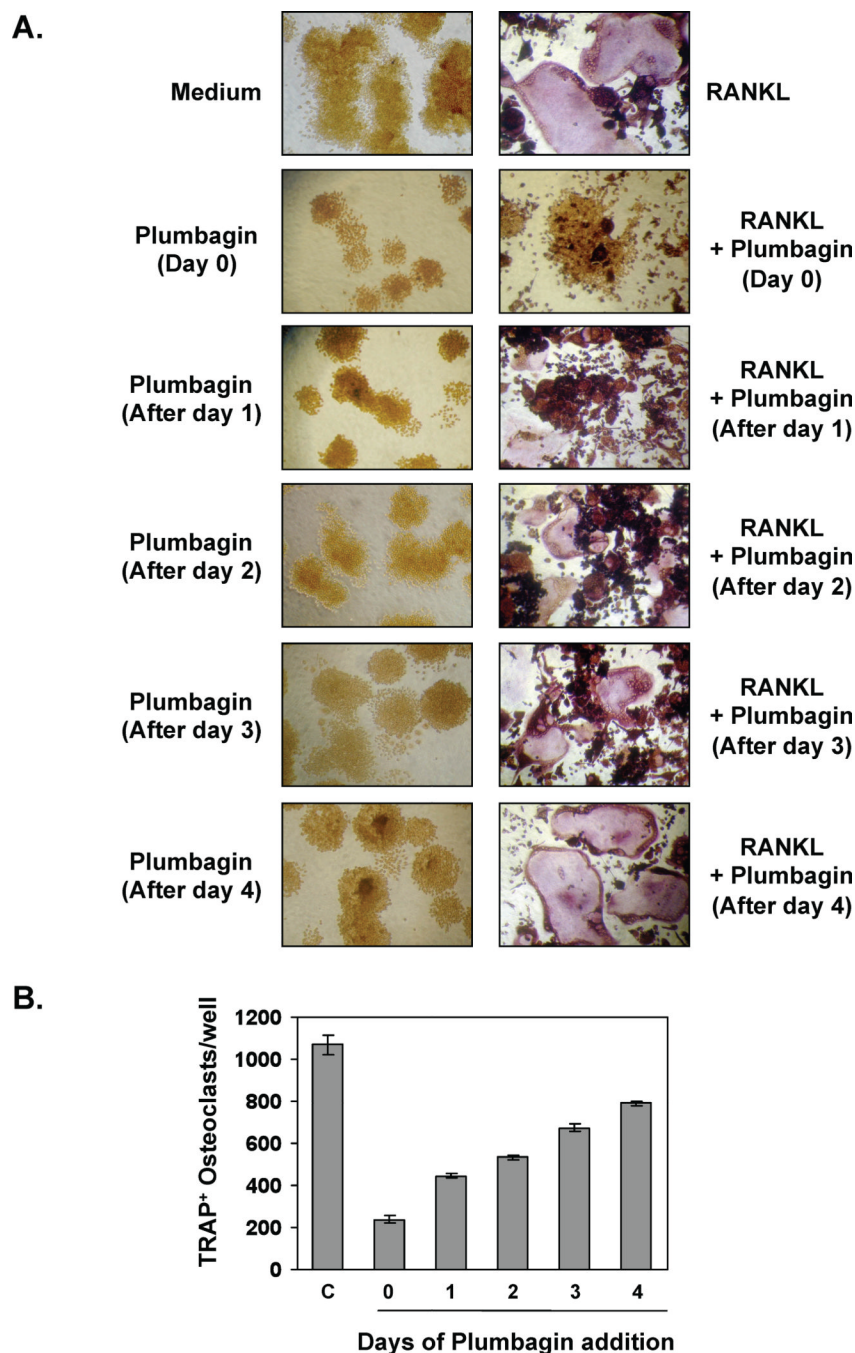


**Figure 2. Plumbagin suppresses RANKL-induced osteoclastogenesis**

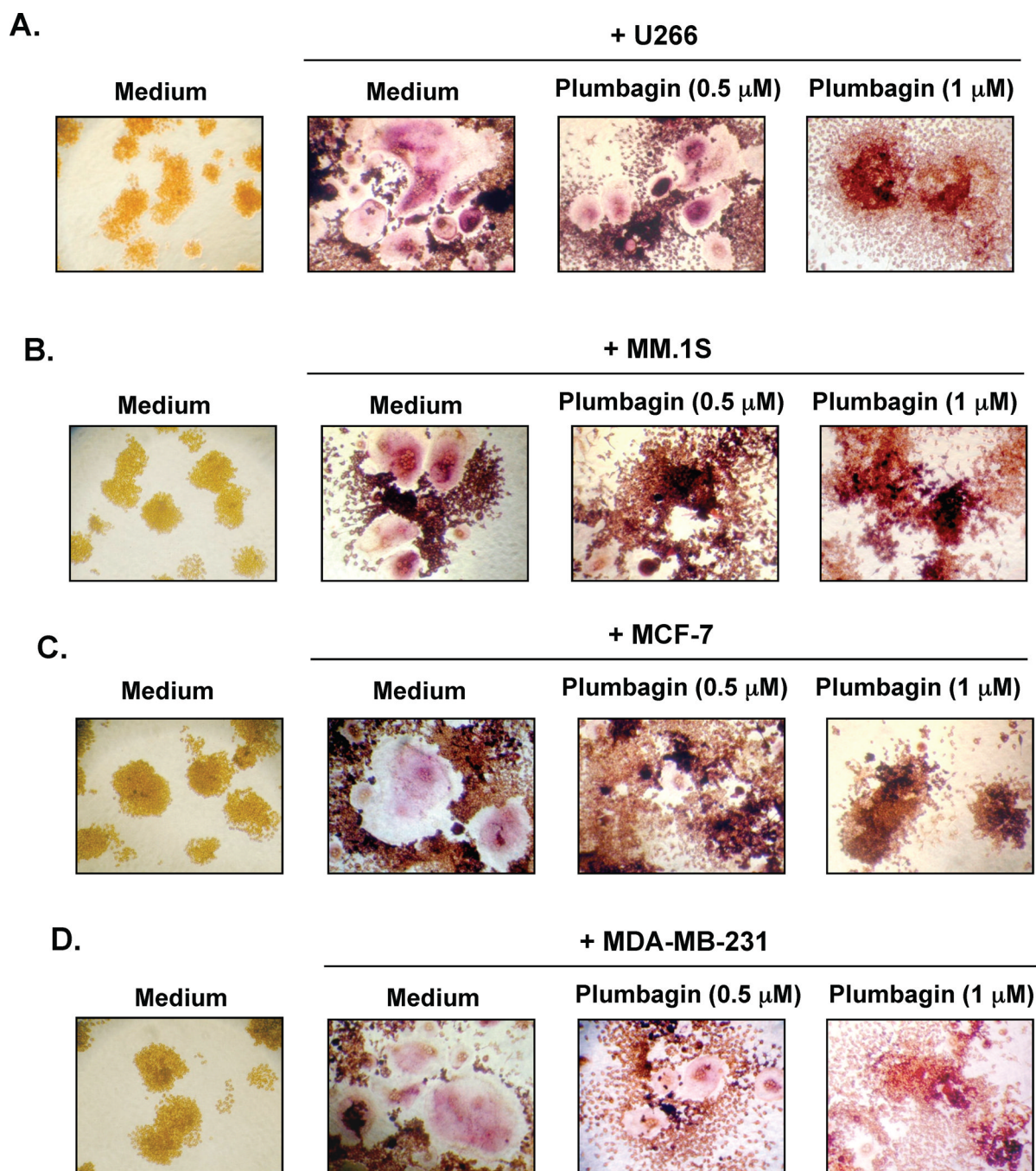
(A) RAW 264.7 cells ( $5 \times 10^3$  cells) were incubated with either medium or RANKL (5 nmol/L) or RANKL and plumbagin (1  $\mu$ mol/L) for 3, 4 or 5 days and then stained for TRAP expression to examine osteoclast formation. TRAP-positive cells were photographed (original magnification,  $\times 100$ ).

(B) Cells ( $5 \times 10^3$  cells) were incubated with either medium or RANKL (5 nmol/L) along with the indicated concentration of plumbagin for 3, 4 or 5 days and then stained for TRAP expression. Multinucleated osteoclasts were counted. Cells exposed to medium alone were used as a control (C).

(C) RAW264.7 cells ( $1 \times 10^4$  cells) were seeded into calcium phosphate apatite-coated plates, treated with plumbagin (1  $\mu$ mol /L) for 4 hours and then with RANKL (5 nmol/L). After 5 days of incubation, cells were subjected to lysis and images were obtained under light microscopy. Arrows, pit formation.

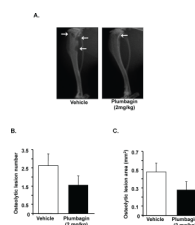


**Figure 3. Inhibition of RANKL-induced osteoclastogenesis by plumbagin is an early event**  
 (A) RAW 264.7 cells ( $5 \times 10^3$  cells) were incubated with RANKL (5 nmol/L) and then plumbagin (1  $\mu$ mol/L) was added either on day 0, after one day, after two days, after three days or after four days period. At the end of five days, cells were stained for TRAP expression.  
 (B) Multinucleated osteoclasts (i.e., those containing three nuclei) were counted. Cells untreated to plumbagin but exposed to RANKL, is indicated as a control (C).



**Figure 4. Plumbagin blocks osteoclastogenesis induced by tumor cells**

RAW 264.7 cells were incubated in the presence of U266 (A), MM.1S (B), MCF-7 (C) or MDA-MB-231 cells (D) for 24 hours, exposed to plumbagin (1  $\mu$ mol/L) for 5 days and finally stained for TRAP expression. Multinucleated osteoclasts (i.e., those containing three nuclei) in co-cultures were counted.



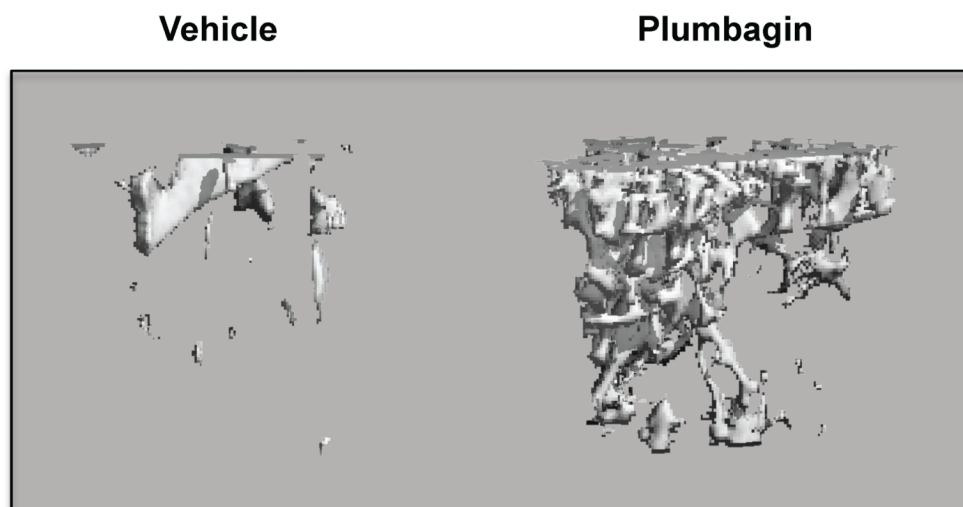
**Figure 5. Plumbagin decreases breast cancer (MDA-MB-231)-induced bone loss in mice**

Nude mice were injected with human breast cancer MDA-MB-231 cells and then treated with 2 mg/kg of plumbagin (n=9; five times a week) or vehicle control (n=5); they underwent weekly radiographic imaging.

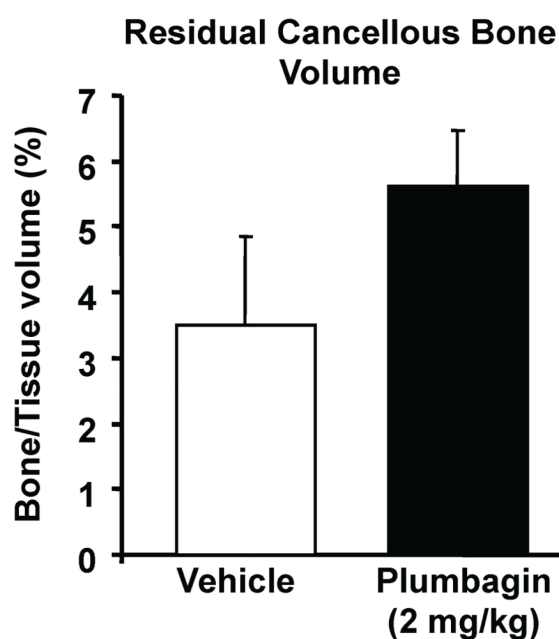
(A) Representative radiographs of mice treated with vehicle (left) or plumbagin (right). Arrows, osteolytic bone lesions caused by injection of MDA-MB-231 cells; treatment with plumbagin reduced these osteolytic bone lesions.

(B and C) The effect of plumbagin on the number and area of osteolytic lesions in human MDA-MB-231 breast cancer-bearing mice. Quantitation of discrete lytic lesions using MetaMorph, a computerized image acquisition and quantitation software indicates that treatment of tumor-bearing mice with plumbagin reduced the number (B) as well as average area (C) of osteolytic lesions in mice bearing MDA-MB-231 tumors compared to vehicle-treated tumor-bearing mice.

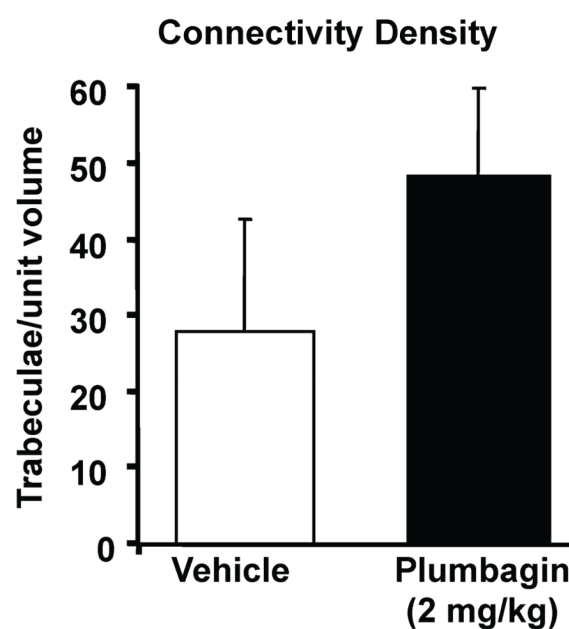
A.



B.



C.



**Figure 6. Plumbagin reduces osteolysis and preserves trabecular/cancellous bone in human MDA-MB-231 breast cancer-bearing mice**

(A) Three-dimensional computer reconstructions of residual bone by micro-CT showing significant bone loss in proximal tibia of a vehicle-treated tumor-bearing mouse and inhibition of bone loss in a tumor-bearing mouse treated with plumbagin. Each computer rendering is from the mouse with the median BV/TV in that group.

(B and C) Graphical representation of micro-CT data. Tumor-bearing mice treated with plumbagin exhibit greater trabecular/cancellous bone volume (B) and significantly greater relative trabecular connectivity density (C) than tumor-bearing mice treated with vehicle.