THE ROLE OF TURMERIC AS AN ADJUVANT THERAPEUTIC
FOR OSTEOLYTIC BREAST CANCER BONE METASTASES

A Thesis submitted to the University of Arizona College of Medicine -- Phoenix
in partial fulfillment of the requirements for the Degree of Doctor of Medicine

Ashley Leigh Lukefahr
Class of 2015

Mentor: Janet L Funk, MD, FACP
Acknowledgements

Thank you to Jen Frye, Minwha Lee, and Guadalupe Barrera for their assistance in the completion of this work. This work was supported by the National Institutes of Health-funded T32 Arizona Complementary and Alternative Medicine Research Training Program (ACAMTRP). The content is solely the responsibility of the authors and does not necessarily represent the official views of The University of Arizona or the National Institutes of Health.
Abstract

Zoledronic acid (ZA), the gold standard treatment for breast cancer-derived osteolytic bone lesions, induces apoptosis in mature osteoclasts. Curcumin, a plant-derived component of turmeric (Curcuma longa), inhibits osteoclast differentiation. This study aimed to determine the in vitro and in vivo effects of ZA and curcuminoids, alone and combined, on osteoclast differentiation and survival, breast cancer cell growth, breast cancer cell-induced osteolytic bone lesion area, and bone mineral density (BMD). Curcuminoids, but not ZA, inhibited osteoclast formation at doses that did not alter precursor viability, as assessed by osteoclastogenesis assays using murine RAW 264.7 cells. Combined curcuminoids and ZA did not differ from curcuminoids alone in their effects on osteoclast survival/formation. The half maximal inhibitory concentration (IC50) for ZA alone was 4 μM, while the IC50 for curcuminoids plus ZA was 6μM. Curcuminoids and ZA inhibit in vitro cell viability of human breast cancer-derived MDA-MB-231 cells, as assessed by MTT assays. The IC50 of ZA alone was projected to be 1.0677 x 10^4 μM, while the IC50 for curcuminoids alone (9.1 x 10^1 μM), was close to the IC50 for curcuminoids plus ZA (1.31 x 10^2 μM curcuminoids with 300 μM ZA). In vivo effects of ZA (2 μg/kg/d) and curcuminoids (25 mg/kg/d), alone and combined, on osteolytic bone lesions derived from inoculation with MDA-MB-231 cells were assessed. Radiographically-evident osteolytic bone lesion area did not differ between treatment groups, with a trend towards decreased osteolytic lesion area in mice treated with ZA. BMD in non-responders, without bone or pericardiac tumors, assessed by dual energy x-ray absorptiometry, was increased in mice administered ZA. Thus, for the first time, the combined in vitro effects of ZA and curcuminoids on osteoclast formation and survival were demonstrated, as well as the combined effects of ZA and curcuminoids on breast cancer-derived osteolytic bone lesions and BMD.
# Table of Contents

- **Introduction/Significance** .............................................................................................................. 1  
  - Background ..................................................................................................................................... 1  
  - Impact ............................................................................................................................................ 17  
  - Aims/Goals/Hypotheses ..................................................................................................................... 20

- **Research Materials and Methods** ................................................................................................... 22

- **Results** ........................................................................................................................................... 27

- **Discussion** ...................................................................................................................................... 56

- **Future Directions** .......................................................................................................................... 61

- **Conclusions** ................................................................................................................................... 62

- **References** ..................................................................................................................................... 63
List of Figures and Tables

INTRODUCTION/SIGNIFICANCE and RESEARCH MATERIALS AND METHODS

Figure 1: The essential steps in the process of tumor cell metastasis to bone.
Figure 2: Turmeric is obtained from the roots of the plant Curcuma longa.
Figure 3: Bone mineral density was determined in anesthetized mice at day 20 post-intracardiac injection using a PIXImus densitometer.

RESULTS

Figure 1: Zoledronic acid and curcuminoids inhibit in vitro cell viability of MDA-MB-231 cells, with differential effects when used in combination.
Figure 2: Zoledronic acid and curcuminoids dose-dependently inhibit the viability of RAW 264.7 cells.
Figure 3: Curcuminoids, but not zoledronic acid, inhibit osteoclast formation at doses that do not alter precursor viability.
Figure 4: Combined curcuminoids and zoledronic acid differ from zoledronic acid alone in their effects on osteoclast survival/formation.
Table 1: Assessment of treatment toxicity.
Figure 5: Zoledronic acid alone increases bone mineral density.
Figure 6: Responders demonstrated no differences in body weights throughout treatment.
Table 2: Incidence of radiographically-evident osteolytic bone lesions.
Figure 7: Radiographically-evident osteolytic bone lesion area did not differ between treatment groups.
Figure 8: Representative micrographs of osteolytic bone lesions.
INTRODUCTION/SIGNIFICANCE

BACKGROUND

Breast Cancer Bone Metastases – Clinical Perspective

At initial clinical presentation, 5% of all breast cancer patients exhibit primary distant metastases. Metastatic breast cancer is considered incurable (Ruiterkamp et al. 2011). In patients with secondary metastatic breast cancer, defined as recurrence by distant metastases, sites of metastases are bone (85%), liver (40-50%), pleuritis carcinomatosa (20%), lung (15-25%) and brain (6-16%). For these patients, median survival is 58 months after recurrence, with an overall 5-year survival of 44% (Ruiterkamp et al. 2011). Metastasis to distant sites is the main cause of mortality in breast cancer patients (Weigelt et al. 2005). At autopsy, approximately 70% of patients who die from breast cancer exhibit evidence of metastatic bone disease. These lytic bone lesions are associated with significant morbidity and poor patient prognosis (Coleman 2006). Based on the poor clinical outcome associated with breast cancer metastasis, it is important to develop novel methods that can prevent or ameliorate the metastasis of breast cancer cells. Given the predilection for breast cancer metastasis to bone, it is especially important to develop efficacious methods that can prevent breast cancer metastasis to bone.

In the United States, 40% of women diagnosed with breast cancer use natural products (Shen et al. 2002). In a recent article, Dr. Andrew Weil spoke to CNN regarding Steve Jobs’ decision to seek alternative cancer treatments (Weil): “I wish that all cancer patients could partner with integrative oncologists to guide them through the maze of confusing options, avoid fraudulent treatments and scams and make use of all safe and effective therapies to eliminate tumors, prevent recurrences and preserve quality of life.” Barriers to acceptance of alternative and complementary treatment on part of allopathic oncologists is in part due to lack of evidence of efficacy and, perhaps most importantly when considering botanical dietary supplements, absence of harm with respect to interference with standard treatments.

Bone Overview

The functions of the human skeletal system include structural support, protection of internal organs, reservoir of calcium and phosphate, and source of hematopoietic cells. Osseous tissue comprises living cells, including bone-forming osteoblasts and bone-resorbing
osteoclasts, embedded in an organic matrix that is mineralized. Bone can be classified as cortical (compact) or trabecular (spongy, cancellous). Cortical bone accounts for approximately 80% of total bone mass and is found primarily in the shafts of long bones. Approximately 70% of cortical bone is mineralized, with cortical bone serving a primarily structural/mechanical function. The outer surface of cortical bone is covered with periosteum; the periosteum comprises an outer fibrous layer composed of collagen fibers and fibroblasts and an inner cambium layer of flattened osteoprogenitor cells that can differentiate into osteoblasts. Endosteum comprises osteoprogenitor cells and connective tissue and lines the inner surface of bone. Thus both the periosteum and endosteum provide precursors for bone-forming osteoblasts. The bone matrix comprises organic matter such as type I collagen fibers composed of fibril bundles and embedded in a ground substance of proteoglycans and glycoproteins. The bone matrix also comprises inorganic matter of hydroxyapatite, an analogue of calcium phosphate. The association of hydroxyapatite with the collagen fibers accounts for the hardness of bone. Trabecular bone accounts for the remaining 20% of total bone mass and comprises a network of trabeculae filled with hemopoietic marrow, fat-containing marrow, or blood vessels (Berenson et al. 2006).

Bone remodeling describes the continuous process of destruction of existing bone by osteoclasts and the formation of new bone by osteoblasts, necessary to maintain bone strength and integrity. To maintain bone mass, the osteoblast-mediated bone formation and the osteoclast-mediated bone resorption are tightly coupled. In the activation phase, osteoclasts migrate to a specific site. During the resorption phase, osteoclasts erode bone in a process also known as osteolysis. In the reversal phase, the osteoclasts apoptose. In the final stage, the formation phase, osteoblasts deposit osteoid, which is subsequently mineralized (Berenson et al. 2006).

Osteoclast development and function is regulated by the osteoprotegerin (OPG)-receptor activator of nuclear factor NF-kappaB ligand (RANKL)-receptor activator of NF-kappaB (RANK) signal transduction pathway. RANKL and OPG are produced by bone marrow-derived stromal cells and osteoblasts. RANKL activates its cognate receptor RANK, allowing the pool of active osteoclasts to expand and increase their bone resorption activities. OPG, a soluble form
of RANK that acts as a decoy receptor, diminishes the pool of active osteoclasts and their bone resorption activity by neutralizing RANKL. The expression of RANKL and OPG is regulated by calciotropic cytokines, hormones, and drugs. In the proliferation phase of osteoclastic bone resorption, macrophage colony-stimulating factor (M-CSF) stimulates the proliferation of osteoclast precursor cells. In the differentiation phase, RANKL binds to RANK, inducing the differentiation of osteoclast precursors to pre-fusion osteoclasts. In the survival and fusion phase, RANKL on osteoblasts and stromal cells stimulates the formation of multinucleated mature osteoclasts in response to cytokines such as interleukins-1 and -6 (IL-1 and -6) and tumor necrosis factor beta (TNF-beta). In the activation phase, osteoclasts are activated to resorb bone by factors such as RANKL, IL-1, IL-6, and TNF-beta. Mature osteoclasts will undergo apoptosis in response to OPG (Berenson et al. 2006).

Metastasis of Breast Cancer Tumor Cells to Bone

The bone metastases of breast cancer are predominantly osteolytic, with mixed osteolytic and osteoblastic lesions and components of osteosclerosis often present. There are many hypotheses as to how and when tumor cells metastasize to bone, with anatomical factors, tumor cell phenotype, blood flow from the site of the primary tumor, inherent tumor cell motility, chemotactic stimuli, and properties of the metastatic site all appearing to play a role in this process. The essential steps in the process of metastasis to the bone can be summarized as follows: 1) a tumor cell detaches from the primary tumor site, 2) the tumor cell enters the vasculature (through attachment to the basement membrane, secretion of proteolytic enzymes to disrupt the basement membrane, and migration through the basement membrane) and systemic circulation, 3) the tumor cell survives the host immune response and physical forces in the circulation, 4) the tumor cell is arrested in a distant capillary bed, 5) the tumor cell leaves the capillary bed vasculature (through attachment to the epithelium, secretion of proteolytic enzymes to disrupt the basement membrane, and migration through the basement membrane), and 6) the tumor cell proliferates in the metastatic site (Figure 1; Guise and Mundy 1998). At various points throughout this process, the tumor cells must
Figure 1. The essential steps in the process of tumor cell metastasis to bone (Guise and Mundy 1998).
produce proteolytic enzymes, express or lose expression of cell adhesion molecules, and respond to chemoattractants. However, the propensity of breast cancer cells to metastasize specifically to bone has not been fully explained. Over 120 years ago Paget proposed the “seed and soil” hypothesis to explain the osteotropism of breast cancer cells; current thought is guided by the idea that the microenvironment in which the breast cancer tumor cells metastasize and thrive must be inherently conducive to the growth of the breast cancer tumor cell phenotype (Guise and Mundy 1998).

The tumor cells of breast cancer bone metastases are found adjacent to osteoclasts that are actively resorbing bone. Breast cancer cells are capable of activating osteoclasts, and may do so via induction of osteoclast differentiation from hematopoietic precursors or activation of mature osteoclasts, with both processes likely invoked through soluble mediators or cell-to-cell contact (Guise and Mundy 1998). The mineralized bone matrix that is dissolved through the actions of bone-resorbing osteoclasts contains growth factors including insulin-like growth factors (IGFs), transforming growth factor alpha and beta (TGF-alpha and –beta), fibroblast growth factors (FGF-1 and -2), platelet-derived growth factors (PDGFs), and bone morphogenic proteins (BMPs). Most of these factors are released in their active form following the osteoclastic destruction of the bone matrix that occurs during the normal process of bone remodeling. The bone marrow includes the hematopoietic stem cell precursors that are capable of differentiating into any type of blood or immune cell, including osteoclasts. The differentiation process is supported by stromal cells, which are themselves capable of becoming osteoblasts (Akhtari et al. 2008).

In the bone microenvironment, breast cancer tumor cells overproduce parathyroid hormone-related peptide (PTHRP), which in turn activates osteoblasts to produce RANKL and downregulate OPG. This leads to osteoclast activation and osteolysis. The osteoclastic destruction of bone leads to the release of bone-derived growth factors such as transforming growth factor-beta (TGF-beta) and insulin-like growth factor 1 (IGF1), and a rise in extracellular levels of calcium (Ca^{2+}). TGF-beta and IGF1 both bind to receptors on the surface of the breast cancer tumor cells and activate autophosphorylation and signaling through the SMAD and mitogen-activated protein kinase (MAPK) pathways (Berenson et al. 2006). TGF-beta has been
shown to enhance the production of the osteolytic factor PTHrP in MDA-MB-231 cells (Guise and Mundy 1998). Extracellular calcium binds and activates a calcium pump. All of these actions serve to promote tumor-cell proliferation and PTHrP production. Therefore a “vicious cycle” exists in the bone-tumor microenvironment, whereby tumor cell growth and the osteolytic activity of osteoclasts are mutually stimulatory (Berenson et al. 2006).

Overview of Zoledronic Acid

For women with advanced breast cancer, bisphosphonates are currently a standard treatment for preventing the progression of skeletal metastases, and their use in primary prevention of bone metastases is also being studied. Bisphosphonates, in general, are analogues of endogenous pyrophosphate (H$_2$O$_3$P-O-PO$_3$H$_2$), with a carbon replacing the oxygen to transform the hydrolytically labile P-O-P phosphoanidride bond into a hydrolysis-resistant P-C-P phosphoether bond (Uludag 2002 and Santini et al. 2003). Regardless of attached side groups, this structure demonstrates a significant predilection for deposition in osseous tissue (>20% with parenteral administration) (Uludag 2002). Zoledronic acid displays a heterocyclic imidazole moiety attached via a two-carbon side chain to the P-C-P backbone (Chen et al. 2002).

Third generation bisphosphonates such as zoledronic acid are characterized by a cyclic side chain. The cyclic side chain of zoledronic acid contains nitrogen, placing it in the category of nitrogen-containing bisphosphonates. Zoledronic acid exhibits a reported antiresorptive potency of >10,000 versus the first generation bisphosphonate etidronate (Vepsäläinen JJ 2002). Zometa® (Novartis) is a zoledronic acid monohydrate with the chemical designation of (1-hydroxy-2-imidazol-1-yl-phosphonoethyl) phosphonic acid monohydrate and the molecular formula of C$_5$H$_{10}$N$_2$O$_7$P$_2$H$_2$O. Zometa® has a molecular weight of 290.11 and is an odorless, white crystalline material that is soluble at alkaline pH and sparingly soluble in water (Ibrahim et al. 2003).
Preclinical Evidence for the Molecular Mechanism of Action of Zoledronic Acid

Zoledronic acid inhibits the osteoclastic resorption of bone through the induction of osteoclast apoptosis. Osteoclasts internalize zoledronic acid through endocytosis. Zoledronic acid inhibits the mevalonate pathway farnesyl pyrophosphate synthase (FPPS), which synthesizes isoprenoid lipids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are required for the post-translational prenylation of small GTPases (e.g. Ras, Rho, Rab, Rac). These GTPases regulate osteoclast functions such as membrane ruffling and trafficking of intracellular vesicles. FPPS inhibition also leads to the formation of the mitochondrial toxin triphosphoric acid 1-adenosine-5’-yl ester 3-(3-methylbut-3-enyl) ester, which stimulates osteoclast apoptosis. By inhibiting osteoclast bone resorption, there is a reduction in the release of bone-derived growth factors, such as TGF-beta, that promote the growth of bone metastases. In turn, this reduces the secretion of tumor-derived growth factors, such as PTHrP and RANKL, thereby halting the “vicious cycle” of bone metastasis progression. Therefore, more recent clinical studies are investigating the use of zoledronic acid in the prevention of metastatic bone disease (Young and Coleman 2013).

In addition, preclinical studies suggest that zoledronic acid can alter bone formation as well as bone resorption. Rat osteoclasts cultured in vitro with zoledronic acid demonstrate increased apoptosis (similar to non-nitrogen-containing bisphosphonates), while human stem cell-derived osteoblasts cultured in vitro with zoledronic acid demonstrate decreased mineral deposition (Kellinsalmi et al. 2005).

Although the primary mechanism of action of zoledronic acid involves osteoclast apoptosis, the results of some clinical trials have pointed to an anti-tumor effect of zoledronic acid. Whether the mechanism of this effect involves the direct action of zoledronic acid on tumor cells, tumor cell sensitization to chemotherapy, antiangiogenic effects, or immunomodulatory effects remains to be seen. In vitro studies have demonstrated that zoledronic acid can inhibit tumor cell proliferation and invasion and induce tumor cell apoptosis, albeit at doses much higher than what can be achieved clinically (Young and Coleman 2013). Zoledronic acid has been reported to have a half-maximal inhibition (ICso) of cell viability of 1.5 x 10^1 μM in MDA-MB-231 cells. In addition, EDTA is incapable of reducing
cell viability (as assessed by MTS assay) of MDA-MB-231 cells at the same concentration that zoledronic acid is capable of significantly reducing cell viability (100 μM, 3 day treatment), indicating that the reduction in MDA-MB-231 cell viability observed with zoledronic acid is most likely not due to calcium chelating. In addition, 3 days of culture with zoledronic acid (50 μM) altered the nuclear morphology of MDA-MB-231 cells in a manner consistent with apoptosis: nuclei stained brightly and with separate globular structures under Hoechst 33285 staining. MDA-MB-231 cells incubated with zoledronic acid (100 μM) demonstrated PARP cleavage with appearance of the 30 kDa cleavage product that is a result of caspase 3 action (Senaratne et al. 2000).

Importantly, zoledronic acid, has demonstrated synergistic effects in combination with a variety of agents; for example, with imatinib mesylate in leukemia cells and lung cancer cells, with gemcitabine in prostate cancer cells, with dexamethasone in myeloma cells, and with doxorubicin in breast cancer cells and prostate cancer cells (Neville-Webbe et al. 2004).

**Preclinical In Vivo Evidence of the Effectiveness of Zoledronic Acid**

Several preclinical studies have examined the effectiveness of zoledronic acid in preventing and treating bone metastases of breast cancer in animal models that utilize the nude mouse MDA-MB-231 intracardiac injection model.

In one study, five week old inbred nude mice (Balb/c nu/nu) were inoculated with MDA-MB-231 cells, with one group receiving a treatment of 0.2 μg per mouse of zoledronic acid (subcutaneous; Novartis) every 2 days for a total of 10 doses, beginning 3 days prior to MDA-MB-231 injections, and were followed prospectively for 28 days post-MDA-MB-231 injections. Simultaneously, animals were implanted with pellets containing 0.25 mg of 17beta-estradiol. As compared to placebo control, those animals receiving zoledronic acid demonstrated a total tumor burden (total cumulative tumor area per animal, in bone and bone-associated soft tissue) that was significantly reduced (by 43%). In bone-associated soft tissue alone, tumor burden was reduced by 93%. On histomorphological analysis, zoledronic acid increased bone volume in tumor-bearing and non-tumor-bearing animals (Duivenvoorden et al. 2007).

In another study, four week old female Balb/c nu/nu mice were inoculated with a subclone of MDA-MB-231 cells selected for their *in vivo* capacity to metastasize to bone and
transfected for green fluorescent protein expression, with one group receiving zoledronic acid (0.3 μg per mouse per day, subq) as a treatment of established metastases from day 18 to 29 post-inoculation. The transfected subclones of MDA-MB-231 cells displayed a higher than normal predilection to bone, with osteolytic lesions higher in number and area than that typically observed with MDA-MB-231 cells. On analysis of radiography, zoledronic acid completely blocked the formation of new osteolytic lesions. On analysis of fluorescent tumor foci, zoledronic acid inhibited the expansion of existing tumor areas, and prevented the development of new tumor foci. Zoledronic acid did not affect the fluorescence intensity of tumor foci (Peyruchaud et al. 2001).

In another study, four week old female Balb/c nu/nu mice were inoculated with a subclone of MDA-MB-231 cells selected for their in vivo capacity to metastasize to bone and transfected for green fluorescent protein and luciferase expression. For preventive protocols, mice were administered single (100 μg/kg), daily (3 μg/kg), and weekly (20 μg/kg) doses of zoledronic acid. For treatment protocols, mice were administered single (100 μg/kg), daily (7 μg/kg), and weekly (50 μg/kg) doses of zoledronic acid. According to the authors, these doses are equivalent to the clinical dose of 4 mg every 3-4 weeks administered to breast cancer patients; however this is not the case by our calculations. Allometric scaling based on body surface area is an established method for determining the Human Equivalent Dose (HED) when moving from experimental animal models to dosing regimens for human clinical trials. For mice, to convert an animal dose in mg/kg to the HED in mg/kg, the animal dose must be divided by 12.3 (CDER 2005). Assuming that Zometa® is dosed at 4 mg every 3-4 weeks (minimum of every 28 days) to a 60 kg human, the equivalent mouse dose is 29.28 μg/kg/d. For preventive protocols, zoledronic acid was administered beginning one day prior to tumor cell inoculation, with the single dose administered on day -1. For treatment protocols, zoledronic acid was administered beginning on day 18 post-inoculation, with the single dose administered on day 18. Results of the treatment protocols suggest that the single dose of zoledronic acid resulted in osteolytic lesions that were 52% smaller than vehicle-treated controls. Daily dosing regimens resulted in lesions that were 66% smaller, while weekly dosing regimens resulted in lesions that were 50% smaller. Mice from all three groups in the treatment protocol displayed higher bone
mineral density versus vehicle-treated control mice. Mice from all three treatment groups also displayed higher bone volume to tissue volume ratios (BV/TV), indicating a prevention of bone loss, and lower tumor burden to soft tissue volume ratios (TB/STV), indicating decreased skeletal tumor burden. Results of the preventive protocols suggest that the single dose regimen did not alter the size of osteolytic lesions as compared to vehicle-treated controls; in contrast, daily and weekly dosing regimens resulted in osteolytic lesions that were 88% and 80% smaller, respectively. Bone mineral density and BV/TV ratios were higher in the daily and weekly dosing regimen groups, with TB/STV being significantly lower. Mice in the daily and weekly preventive dosing regimen groups displayed lower luciferase activity compared to vehicle-treated controls, indicating a smaller tumor burden with lower presence of inoculated tumor cells. It was noted that mice in the preventive group receiving weekly or single doses of zoledronic acid showed dense transverse lines on the tibial metaphysis (Daubiné et al. 2007).

Clinical Evidence of the Effectiveness of Zoledronic Acid

The American Society of Clinical Oncology Clinical Practice Guideline Update on the Role of Bone-Modifying Agents in Metastatic Breast Cancer recommends the use of bone-modifying therapies only in breast cancer patients with evidence of bone metastases. The recommended administration of zoledronic acid is 4 mg, administered over a period of at least 15 minutes, every three to four weeks. This recommendation is based on randomized control trial data indicating that skeletal related events are reduced by 39% in treated groups versus placebo, with delays in the time to first skeletal related event and reductions in pain scores (Van Poznak et al. 2011).

One systematic review and meta-analysis of data revealed that the effects of zoledronic acid on fracture events were statistically significant, although the effects of zoledronic acid on breast cancer patient survival is unclear. Zoledronic acid reduces fracture events, with a relative risk of 0.66. Zoledronic acid appears to have a statistically significant effect on overall survival, with limited effect on disease-free survival and recurrence-free survival. Although patients with early-stage breast cancer demonstrated a decrease in risk of disease recurrence, patients with advanced breast cancer (bone metastases) who began zoledronic acid treatment after complete primary tumor resection demonstrated a statistically significant 35% increase in
the risk of disease recurrence. In addition, increases in the risk of bone pain, neutropenic fever, pyrexia, and rash are also seen with zoledronic acid therapy (Huang et al. 2012). Similarly, a Cochrane meta-analysis found that zoledronic acid reduced the risk of developing a skeletal related event, reduced skeletal morbidity rate (events per year), reduced the time to a skeletal related event, improved pain, and improved quality of life (Wong, Stockler, Pavlakis 2012).

Another systematic review and meta-analysis found that zoledronic acid reduced risk of fractures by 21% in breast cancer patients. The risk of death in these patients was reduced by 19%. The incidence rate of osteonecrosis of the jaw was found to be 0.52% (Valachis et al. 2013).

Some data indicates that zoledronic acid may be useful in preventing breast cancer bone metastases. A Phase II clinical trial by Aft et al. evaluated breast cancer patients with stage II-III newly diagnosed breast cancer who were randomly assigned to receive 1 year of neoadjuvant chemotherapy with or without the addition of 4 mg zoledronic acid every 3 weeks. Around 46% of patients were found to have disseminated tumor cells in the bone marrow at the time of diagnosis (28 of 58 in the chemotherapy alone group and 26 of 60 in the chemotherapy plus zoledronic acid group). After 3 months of therapy, fewer patients in the zoledronic acid group demonstrated detectable disseminated tumor cells in the bone marrow (17 of 56) versus the chemotherapy alone group (25 of 53; p=0.054). By 12 months, there was no significant difference in the number of patients with detectable disseminated tumor cells in the bone marrow between the group treated with zoledronic acid and the group treated with neoadjuvant chemotherapy alone. No significant difference was seen in disease-free survival rates at 12 and 24 months (Aft et al. 2010).

Another study by Solomayer et al. looked at bone marrow aspirates of 76 breast cancer patients (pT1-4, N1-2, M0) treated with adjuvant hormone and/or chemotherapy within 42 days of completing primary tumor resection and axillary lymph node dissection. Those who received additional zoledronic acid (3-4 mg every 4 weeks for 24 months) were found to have a larger decrease in the mean number of disseminated tumor cells and were more likely to have tumor cell-free bone marrow aspirates after 12 months of therapy as compared to those receiving hormone and/or chemotherapy alone (Solomayer et al. 2012).
Most recently, the final results of the AZURE trial (n=3360 women with stage II or III invasive breast cancer) suggest that zoledronic acid is effective in reducing the development of bone metastases regardless of reproductive status (premenopausal, perimenopausal, postmenopausal, or status unknown), but zoledronic acid only improves invasive disease-free survival in women who are at least 5 years post-menopausal. This is consistent with the current hypothesis that the effects of zoledronic acid are dependent on the hormonal milieu (Coleman et al. 2014).

Clinical Pharmacokinetics and Pharmacodynamics of Zoledronic Acid

Pharmacokinetic studies submitted to the FDA as part of the New Drug Application for Zometa® for treatment of patients with bone metastasis indicate a terminal half-life of at least 146 hours, with a distribution half-life of 14 minutes followed by an elimination half-life of 1.9 hours. In vitro studies indicated that 22% of Zometa® was protein bound. The use of Zometa® in patients with severe renal failure is not recommended, as Zometa® clearance appears to be a function of creatinine clearance (Ibrahim et al. 2003).

It has been reported that zoledronic acid has a terminal elimination half-life of approximately 7 days in humans, with the initially administered dose being retained in bone prior to being slowly released into circulation, resulting in low levels of circulating zoledronic acid in plasma that are short lasting (Caraglia et al. 2006). Within 24 hours after administration of zoledronic acid, plasma concentrations decline to 1% of peak plasma concentrations followed by a prolonged period of low plasma concentrations. This occurs independent of renal function status (Skerjanec et al. 2003). Another study confirmed that plasma levels of zoledronic acid decline in a multiphasic manner to less than 1% of peak systemic concentrations within 24 hours of dosing. Blood samples among patients receiving zoledronic acid demonstrated sustained, low levels of zoledronic acid 7 days after dosing in 2 out of 12 patients receiving 4 mg of zoledronic acid, 9/12 receiving 8 mg, and 12/12 receiving 16 mg. Measurable levels of zoledronic were observed 28 days after infusion in the blood of 1 out of 12 patients receiving 8 mg of zoledronic acid and 7/12 receiving 16 mg (Chen et al. 2002).

Zoledronic acid is primarily excreted through the kidneys, with 40% of the administered dose of zoledronic acid excreted unchanged within the first 24 hours post-dosing (Chen et al.}.
2002 and Skerjanec et al. 2003). Combined with the fact that the plasma concentration of zoledronic acid declines rapidly in the first 24 hours with a sustained, low concentration of zoledronic acid observed up to 28 days post-dosing, these data suggest that zoledronic acid is cleared by the kidneys with simultaneous uptake by osseous tissue followed by slow release during the process of bone remodeling. In dosing studies, the apparent volume of distribution in the central compartment is similar to the blood volume, suggesting that zoledronic acid has a limited ability to bind to the cellular constituents of blood. Due to the sustained, low release of zoledronic acid from bone to systemic circulation, it is estimated that the terminal half-life of zoledronic acid is actually greater than 6 months (~189 days) (Chen et al. 2002).

**Clinical Recommendations for the use of Bone-Modifying Therapies**

Zoledronic acid (Zometa®, Novartis) was approved by the U.S. Food and Drug Administration (FDA) on February 22, 2002 for the “treatment of patients with multiple myeloma and documented bone metastases from solid tumors, in conjunction with standard antineoplastic therapy,” with a recommended dosing regimen of 4 mg infused over 15 minutes every 3-4 weeks (Ibrahim et al. 2003). Denosumab (Xgeva®, Amgen) is a monoclonal antibody that targets RANKL that was approved by the FDA in 2010 “to help prevent skeletal-related events (SREs) in patients with cancer that has spread (metastasized) and damaged the bone” (FDA 2011). Pamidronate sodium (Aredia®, Novartis) was approved by the FDA in 1998 for the adjuvant treatment of osteolytic bone metastases of breast cancer with standard antineoplastic therapy (FDA 1998).

The American Society of Clinical Oncology (ASCO) recommends that only breast cancer patients with evidence of metastases be treated with bone-modifying therapies, with the recommended therapies being denosumab 120 mg subcutaneously every 4 weeks, pamidronate 90 mg intravenously over no less than 2 hours, or zoledronic acid 4 mg over no less than 15 minutes every 3-4 weeks. The ASCO finds insufficient evidence to recommend one therapeutic regimen over another. The ASCO recommends that patient serum creatinine levels be monitored prior to each dose, with no dosing changes required in patients with serum creatinine clearance of greater than 60 mg/min. The ASCO also recommends that patients
receive a dental examination and appropriate preventive dentistry prior to beginning bone-modifying therapies (Van Poznak et al. 2011).

**Turmeric Overview**

The word *turmeric* is derived from the Medieval Latin name *terramerita*, which became the French *terre merite*, meaning “deserved earth” or “meritorious earth.” In India, turmeric has been referred to by many names, each denoting a particular quality of the plant. These include: *Ranjani, Mangal prada, Krimighni, Mahaghnî, Anestha, Haridra, Varna-datri, Hemaragi, Bhadra, Pavitra, Hridayavilasini,* and *Shobhna.* There are approximately 55 words for turmeric in Sanskrit, where it was associated with both medicinal and religious uses. In English, turmeric has also been referred to as yellow root and Indian saffron. Turmeric has been used in all of the major traditional medical systems: *Ayurveda, Sidha, Unani,* and *Tibetan* (Ravindran et al. 2007).

Turmeric has at least 6000 years of documented use in traditional medicine and socioreligious practices. The earliest medicinal reference to turmeric is in the *Atharvaveda* text published approximately 6000 years before present, in which turmeric is indicated for the treatments of jaundice and leprosy. It is unknown whether this turmeric was *Curcuma longa* or another turmeric species. *Curcuma longa* has been used in Ayurvedic medicine, and is included in the sacred Ayurvedic texts *The C---- Samhita* and *The Sushruta Samhita* where it was referred to as *Haridra* (Ravindran et al. 2007).

Turmeric has been traditionally used as a dye of cloth materials, imparting a yellow color or red color, when alkaline, that fades with light. Turmeric is currently used in industry as a food additive for the coloring of cheese, spices, mustard, cereal products, pickles, potato flakes, soups, ice creams, yogurts, and more (Ravindran et al. 2007).

The dried rhizome of turmeric contains curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Figure 2; Simon et al. 1998). Cyclocurcumin was first successfully isolated by Kiuchi et al. (1993) as a racemic mixture in a chloroform extract. In support of the idea that the efficacy of turmeric depends on synergistic effects of these components, it was found that curcumin, demethoxycurcumin, bisdemethoxycurcumin, and cyclocurcumin alone had no effect on the larvae of *Toxocara canis.* However, in combination demethoxycurcumin
and bisdemethoxycurcumin demonstrated strong nematocidal activity, with no significant additive effects when curcumin or cyclocurcumin were added to this combination (Kiuchi et al. 1993).

**Turmeric and Bone**

Curcumin has demonstrated an ability to alter osteoblast proliferation and mineralization. *In vitro* work has demonstrated that curcumin dose-dependently decreases osteoblast viability through the processes of apoptosis at lower concentrations and necrosis at higher concentrations (Chan, Whu, Chang 2006). Cultured rat-derived osteoblast-like cells demonstrate decreased viability and mineralizing capabilities in a dose-dependent manner upon exposure to curcumin (Notoya et al. 2006).

Curcumin has also demonstrated the ability to alter osteoclast proliferation and lytic activity. *In vitro* work with human-derived preosteoclasts has demonstrated the ability of curcumin to dose-dependently inhibit RANKL-induced osteoclast differentiation and bone resorption (von Metzler et al. 2009). The increase in parathyroid hormone (PTH)-induced osteoclast-like cell formation in rat *in vitro* studies is inhibited with curcumin administration to culture (Yamaguchi et al. 2007).

Animal studies have further demonstrated the ability of curcumin to modulate bone structure. Rats administered oral curcumin for four weeks demonstrate increased trabecular width in femoral cancellous bone of the epiphyses and metaphyses (Folwarczna, Zych, Trzeciak 2010). Use of curcumin in the MDA-MB-231 bone metastasis model has demonstrated the ability of curcumin to decrease osteolytic lesion area through a reduction in osteoclast numbers at the bone-tumor interface without alteration of the tumor area found within bone (Wright et al. 2013).
Figure 2. Turmeric is obtained from the roots of the plant *Curcuma longa*. The dried rhizome of turmeric contains curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Singh 2007).
**In vitro Effects of Turmeric on Breast Cancer and MDA-MB-231 Cells**

Various studies have determined that curcumin is active against breast cancer cells through the downregulation of factors that include nuclear factor-kappa B (NF-kappaB), activator protein-1 (AP-1), cyclooxygenase-1 (COX-1), COX-2, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), cyclin E, interleukin-6 (IL-6), IL-11, transforming growth factor-beta (TGF-beta), matrix metalloproteinase-2 (MMP-2), MMP-9, and MMP-13 as well as the upregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1), p21, and p27. Work in MDA-MB-231/Her2 cells has demonstrated the ability of curcumin to repress cell proliferation, block cell migration, and arrest cells in the G1 phase of growth (Shehzad, Lee, Lee 2013). Microarray data analysis has demonstrated the ability of curcumin to inhibit the in vitro proliferation of MDA-MB-231 human invasive breast carcinoma cells through downregulation of elements in the epidermal growth factor (EGF) pathway, whose downregulation has been previously associated with impaired tumor growth (Cine et al. 2013).

Curcumin dose-dependently decreases the in vitro invasive capabilities of MDA-MB-231 cells at doses below those that induce cell cytotoxicity (Mo et al. 2012). Further in vitro work indicates that MDA-MB-231 cells are capable of cellular uptake of curcumin, a response that is correlated with the ability of curcumin to dose-dependently inhibit MDA-MB-231 cell proliferation and activate cell apoptosis (Chang et al. 2012).

**Turmeric use in Clinical Trials**

The National Institutes of Health (NIH) lists 34 clinical trials that incorporate turmeric in their study design as of June 22, 2012. Of these, 11 are completed, 5 are active/not recruiting, 8 are recruiting, 1 is enrolling by invitation, 4 are not yet recruiting, 4 have an unknown status, and 1 has been withdrawn (ClinicalTrials.gov).

A Phase I clinical trial in the United Kingdom looking at 15 patients with advanced adenocarcinoma of the colon or rectum used a curcuminoid mixture containing 90% curcumin, 8% desmethoxycurcumin, and 2% bisdesmethoxycurcumin. Patients consumed the curcumin-containing supplement after 2 hours of fasting at doses of 450, 900, 1800, and 3600 mg for up to 4 months. No toxicity was reported with these dosing regimens, with two patients developing diarrhea (450 mg and 3600 mg), one patient experiencing nausea (900 mg), four
patients developing a rise in serum alkaline phosphatase levels, and three patients developing a rise in serum lactate dehydrogenase. The highest dose of 3600 mg was associated with an inhibition of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) induction in ex vivo blood sampled on day 1 and 29 post-dose versus immediately pre-dose. In the 3600 mg group, curcumin was detected in plasma samples 1 hour post-dose in 3/6 patients by HPLC (limit of detection 5 pmol/mL). All 6 patients demonstrated glucuronidated and sulfonated forms of curcumin and desmethoxycurcumin in plasma samples taken 0.5 and 1 hours post-dose. Patients in the 3600 mg group displayed curcumin and curcumin conjugates in 24-hour urine and fecal samples. No patients administered any dose of curcuminoids demonstrated changes in tumor markers and no improvements in radiologic progression of their disease (Sharma et al. 2004).

One study examined the effects of curcumin containing at least 95% curcuminoids: curcumin, bisdemethoxycurcumin, and demethoxycurcumin administered in single doses of 0.5, 1, 2, 4, 6, 8, 10, or 12 grams. Of the 24 patients, 7 experienced adverse effects in the 72 hours following curcumin administration: in the 1 g group 1 experienced diarrhea, in the 4 g group 1 experienced headache, in the 8 g group 1 experienced a rash and 1 experienced yellow stool, in the 10 g group 1 experienced headache and 1 experienced yellow stool, and in the 12 g group 1 experienced diarrhea. All adverse effects were toxicity grade 1 as measured by the National Cancer Institute Common Toxicity Criteria v.2.0. Curcumin could not be detected in the serum by HPLC (limit of detection 0.031 μg/mL), and 2 patients demonstrated detectable plasma levels of curcumin with 10 and 12 g doses (Lao et al. 2006).

The apparently low oral bioavailability of curcumin contradicts the systemic effects observed with curcumin treatment, such as the effects seen in inflammatory eye disorders. It is hypothesized that this may be due to the potency of the curcumin that does reach the general circulation as seen with high doses of administered curcumin and/or the effects of unmeasured or unrecognized metabolites of curcumin (Shureiqui and Baron 2011).
IMPACT

The work described in this current thesis, entitled “The Role of Turmeric as an Adjuvant Therapeutic for Osteolytic Breast Cancer Bone Metastases,” is applicable in many areas of breast cancer metastasis research, especially work investigating the lytic bone lesions that occur with breast cancer bone metastasis. The proposed project, therefore, serves to address the important problem of decreasing the ability of breast cancer cells to metastasize by altering the molecular signaling cascades involved in breast cancer cell invasion. In addition, the ability of turmeric to modify a model of breast cancer metastases will have been verified, as evidenced by the ability of the agent to modify the lytic bone lesions observed in an animal model of breast cancer bone metastasis. Scientific knowledge concerning a therapeutic agent that may ameliorate breast cancer cell metastasis will have increased, with the possibility that further research into clinical use of this agent will modify breast cancer progression and improve patient prognosis.

I believe the clinical implications of such research are widespread and significant: data would support safe use of turmeric in combination with traditional treatments; data would help convince the allopathic community of potential benefits of botanicals, of which turmeric is only one; and data may reveal novel treatments, with botanicals targeting biologic pathways for which no pharmaceuticals currently exist.
AIMS/GOALS/HYPOTHESES

Specific Aim 1: Investigate ability of turmeric in conjunction with zoledronic acid to modulate breast cancer cell growth.

Expected Results: As both agents in isolation appear to limit cell growth by stimulating apoptosis, I anticipate finding additive or more than additive effects in the combined treatment group for inhibition of cell proliferation as compared to treatment with either agent alone.

Specific Aim 2: Investigate ability of turmeric in conjunction with zoledronic acid to modulate osteoclast differentiation and growth

Expected Results: As the mechanism of action of zoledronic acid on osteoclast inhibition appears to be the direct induction of apoptosis in mature osteoclasts (Kellinsalmi et al. 2005) and the mechanism of action of curcuminoids on osteoclast inhibition appears to be a decline in osteoclast differentiation (Bharti, Takada, Aggarwal 2004), I anticipate finding additive or synergistic effects in the combined treatment group for inhibition of osteoclast number as compared to treatment with either agent alone.

Specific Aim 3: Determine whether turmeric in conjunction with zoledronic acid can decrease the osteolytic bone destruction in breast cancer cell bone metastases.

Expected Results: I anticipate finding additive or more than additive effects in the combined treatment group for the radiographic endpoint as compared to treatment with either agent alone. While zoledronic acid acts to stimulate apoptosis of mature osteoclasts (Young and Coleman 2013), curcumin dose-dependently inhibits RANKL-induced osteoclast differentiation (von Metzler et al. 2009). In addition, results of some clinical trials have pointed to an anti-tumor effect of zoledronic acid, while preclinical trials have demonstrated the ability of curcumin to repress breast cancer cell proliferation (Shehzad, Lee, Lee 2013 and Cine et al. 2013). Because these agents act via different mechanisms to limit the actions of osteoclasts and may both have direct effects on tumor cells, it is therefore hypothesized that the combination of turmeric and zoledronic agent will result in less bone destruction than is observed with either agent alone.
Specific Aim 4: Determine whether turmeric in conjunction with zoledronic acid can alter bone mineral density in breast cancer cell bone metastases.

Expected Results: As zoledronic acid has been shown to increase bone mineral density in experimental mouse models of bone disease (Croucher et al. 2003) and turmeric has been shown to preserve parameters of bone integrity, including bone mineral density, in experimental mouse models of bone disease (Wright et al. 2011; Hussan et al. 2012), I anticipate finding additive or synergistic effects in the combined treatment group for an increase in bone mineral density as compared to treatment with either agent alone.
RESEARCH MATERIALS AND METHODS

Chemicals
A curcuminoid-enriched turmeric product was utilized that comprises an 89.6% mixture of curcumin, demethoxycurcumin, and bis-demethoxycurcumin (Fisher Scientific #218580100, Lot A019754401). Chemical content of the curcuminoid product was assessed as previously described using an Agilent 1100 series high performance liquid chromatography 6 (HPLC) system (Agilent, Palo Alto, CA) (Funk et al., 2006). A curcuminoid dose of 25 mg/kg/d was chosen based on a prior study demonstrating significant reductions in osteolytic bone lesion area in mice administered curcuminoids (25 or 50 mg/kg/d) following inoculation with MDA-MB-231 cells (Wright et al. 2013).

The zoledronic acid obtained for these studies is anhydrous (Selleck Chemicals; Houston, TX), thus negating the need to adjust the dose to the hydrated form as Zometa® is formulated to contain 4.264 mg zoledronic acid monohydrate, corresponding to 4 mg zoledronic acid on an anhydrous basis (FDA, “Zometa”). Allometric scaling based on body surface area is an established method for determining the Human Equivalent Dose (HED) when moving from experimental animal models to dosing regimens for human clinical trials. For mice, to convert an animal dose in mg/kg to the HED in mg/kg, the animal dose must be divided by 12.3 (CDER 2005). Assuming that Zometa® is dosed at 4 mg every 3-4 weeks (minimum of every 28 days) to a 60 kg human, the equivalent mouse dose is 29.28 μg/kg/d. Based on previous data (Daubiné et al. 2007), and the need to demonstrate a sub-therapeutic effect of zoledronic acid for additive or synergistic effects to be observed, zoledronic acid doses of 1-2 μg/kg/d were chosen.

Animal procedures
Four-week old athymic nude mice (Harlan Laboratories) were housed in plastic cages with ad libitum access to water and autoclaved mouse chow. Anesthetized mice were administered a subclone of MDA-MB-231 cells (1 x 10^5 cells in 100 μL sterile PBS, 26 G needle), selected for their in vivo capacity to metastasize to bone, through an intracardiac injection into the left ventricle via a supine, percutaneous approach. Aspiration of pulsatile bright red blood prior to injection presumed correct placement in the left ventricle. Mice were randomly divided into
treatment groups (n=12) and administered vehicle (100 μL PBS IP, 100 μL DMSO SubQ, 5 d/wk), curcuminoids (25 mg/kg/d, 5 d/wk, IP), zoledronic acid (1 μg/kg/d, 5d/wk, SubQ for study to assess the safety of the proposed treatments and evaluate bone mineral density; 2 μg/kg/d, 5d/wk, SubQ for experimental study), or a double-injection of curcuminoids and zoledronic acid. Each group was prospectively followed for the development of osteolytic bone lesions at days 17 and 21 post-intracardiac injection. Radiographs were obtained utilizing a Digital Faxitron MS-20 with a digital camera at 4x magnification. Osteolytic bone lesion area was determined with investigator blinded to treatment using ImageJ software (Version 1.45 s, National Institutes of Health). Bone mineral density was determined in anesthetized mice at day 20 post-intracardiac injection using a PIXIImus densitometer (GE Lunar, Madison, WI) calibrated with a phantom of defined density using the manufacturer’s software (version 1.4x) (Figure 3). At termination, organ weights of the liver, kidneys, and spleen were determined to monitor for hepato-, renal- or immunotoxicity, respectively. The animal protocols for bone metastasis of tumor cells in mice were approved by the Institutional Animal Care and Use Committee at The University of Arizona in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell Cultures – MDA-MB-231 cells
Subconfluent human breast cancer MDA-MB-231 cells were plated at 5 x 10^4 cells/cm^2 and allowed to adhere overnight in incubation with fresh DMEM medium containing 10% fetal bovine serum and antibiotics (penicillin, streptomycin) at 37°C and 5% CO2 in a humidified atmosphere. The next morning, cells were incubated with curcuminoids (1-100 μM) for 28 hours, zoledronic acid (0.1-10,000 μM) for 24 hours, or a combined incubation involving a pre-incubation with curcuminoids for 4 hours followed by a co-culture of curcuminoids (1-100 μM) and zoledronic acid (300 μM) for an additional 24 hours. The 300 μM zoledronic acid dose was chosen based on the results of the zoledronic acid alone data, which indicated that this dose affected cell viability and could therefore be used to look for synergistic effects. The smallest volume of zoledronic acid-containing media that could be utilized to make a final concentration of 300 μM was added during the last 24 hours, and no media was refreshed, to minimize the change in concentration in curcuminoids that would result from the addition of zoledronic acid.
Figure 3. Bone mineral density was determined in anesthetized mice at day 20 post-intracardiac injection using a PIXImus densitometer.
Viable cell number was assessed by mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; ATCC), as per manufacturer’s protocol.

Cell Cultures – RAW 264.7 cells

The use of RAW 264.7 cells for osteoclastogenesis assays is well established (Collin-Osdoby et al. 2003). For cell viability studies, subconfluent murine macrophage RAW 264.7 cells were plated at 1.6 x 10^4 cells/well and allowed to adhere overnight in incubation with fresh DMEM medium containing 10% fetal bovine serum and antibiotics (penicillin, streptomycin) at 37°C and 5% CO2 in a humidified atmosphere. The next morning, cells were incubated with the following treatment regimens: zoledronic acid (0.1-1000 µM) during the last 24 hours of culture (without refreshing the media and using the smallest volume of zoledronic acid-containing media that could be utilized to make the final concentration), zoledronic acid (0.1-100 µM) for 72 hours of culture, and curcuminoids (0.1-300 µM) for 76 hours. Viable cell number was assessed by mitochondrial reduction of MTT (ATCC), as per manufacturer’s protocol.

For osteoclast assays, murine macrophage RAW 264.7 cells were plated at 2 x 10^4 cells/well and incubated overnight in fresh DMEM medium containing 10% fetal bovine serum and antibiotics (penicillin, streptomycin) at 37°C and 5% CO2 in a humidified atmosphere. The next morning, cells were incubated with their treatment regimens and RANKL (100 ng/mL) for 72 hours. Cells were incubated with the following treatment regimens: zoledronic acid (1-1000 µM) during the last 24 hours of culture (with 48 hours of exposure to RANKL only); zoledronic acid (0.1-30 µM) for 72 hours of culture; curcuminoids (0.1-10 µM) for 76 hours (including 4 hours of pre-treatment prior to RANKL administration); a co-culture of curcuminoids (0.1-3 µM) and zoledronic acid (0.1 µM) for 72 hours (with an additional 4 hours of curcuminoid pre-treatment prior to RANKL administration); and a co-culture of curcuminoids (0.1-3 µM) for 76 hours (including 4 hours of pre-treatment prior to RANKL administration) with the addition of zoledronic acid (30 µM) for the last 24 hours of culture. After the 4 hours of curcuminoid pre-treatment, new media containing fresh RANKL or fresh RANKL plus fresh curcuminoids was added to all wells. In the experiments where zoledronic acid was added during the last 24 hours of culture, new media containing fresh RANKL or fresh RANKL plus zoledronic acid was added to all wells. In the co-culture experiments where zoledronic acid was added during the
last 24 hours of culture with curcuminoids, new media containing fresh RANKL, fresh RANKL plus fresh curcuminoids, or fresh RANKL plus fresh curcuminoids plus zoledronic acid was added to all wells. The 0.1 and 30 μM zoledronic acid doses were chosen for the 72 hour and 24 hour experiments, respectively, based on the results of the MTT assay, which indicated that these doses did not affect cell viability. At the end of incubation, cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich) for osteoclast visualization. TRAP positive cells with three or more nuclei were counted in each well, with data expressed as percent of control wells.

**Statistical analyses**

All data were expressed as mean ± SEM. Differences between means were determined by one- or two-way ANOVA, as appropriate, with Student Newman-Keuls or Bonferroni post hoc test, respectively, using Instat software (Graphpad; San Diego, CA) or Prism software (Version 6.01 for Windows, Graphpad Software; La Jolla, CA). Half maximal inhibitory concentrations (IC$_{50}$) were calculated using a four-parameter sigmoidal model (Prism). Briefly, dose response regression curves were fitted to the equation $Y=\text{Bottom}+(\text{Top}-\text{Bottom})/(1+10^{(\text{LogIC}_{50}-x)\cdot\text{HillSlope}})$ with the bottom constrained to 0. Differences in regression curves were determined by the extra sum-of-squares F test.
RESULTS

MTT Assay: MDA-MB-231 cells

Zoledronic acid inhibited *in vitro* cell viability of MDA-MB-231 cells

The effects of zoledronic acid on *in vitro* cell viability of breast cancer-derived MDA-MB-231 cells were assessed using MTT reduction assays (Figure 1A). Viability of MDA-MB-231 cells was inhibited by zoledronic acid, with a minimum effective concentration of 1 μM zoledronic acid (93.2 ± 1.1% of control) and no clear dose-dependent relationship at the doses tested. A statistically significant increase in the viability of MDA-MB-231 cells was observed at 0.1 μM zoledronic acid (105.8 ± 2.6% of control), while statistically significant decreases in the viability of MDA-MB-231 cells were observed at 1, 10 (90.7 ± 0.8% of control), and 10000 (66.4 ± 0.8% of control) μM zoledronic acid (p<0.05, p<0.01, p<0.001, and p<0.001 respectively). No statistically significant changes in the viability of MDA-MB-231 cells were observed at 0.3 (101.7 ± 1.3% of control), 3 (99.3 ± 2.2% of control), and 30-3000 (30: 96.6 ± 0.8% of control, 100: 95.6 ± 1.1% of control, 300: 101.1 ± 1.3% of control, 1000: 101.1 ± 0.7% of control, 3000: 102.1 ± 0.8% of control) μM zoledronic acid (p>0.05). The half maximal inhibitory concentration for cell viability of MDA-MB-231 cells was projected to be 1.0677 x 10^4 μM zoledronic acid.

Curcuminoids inhibited *in vitro* cell viability of MDA-MB-231 cells

The effects of curcuminoids on *in vitro* cell viability of breast cancer-derived MDA-MB-231 cells were assessed using MTT reduction assays (Figure 1B). Viability of MDA-MB-231 cells was dose-dependently inhibited by curcuminoids, with a minimum effective concentration of 10 μM (94.8 ± 0.7% of control). Statistically significant decreases in the viability of MDA-MB-231 cells were observed at 10, 30 (76.4 ± 1.1% of control), and 100 (48.8 ± 0.8% of control) μM curcuminoids (p<0.05, p<0.001, and p<0.001, respectively). The half maximal inhibitory concentration for cell viability of MDA-MB-231 cells was 9.1 x 10^1 μM curcuminoids.
Figure 1

A

Percent of Control

IC_{50}=1.0677 \times 10^4 \mu M

Zoledronic Acid (\mu M)

B

Percent of Control

IC_{50}=9.1 \times 10^1 \mu M

Curcuminoids (\mu M)

C

Percent of Control

IC_{50}=1.31 \times 10^2 \mu M

Curcuminoids (\mu M) + 300 \mu M zoledronic acid
Zoledronic acid and cucruminoids inhibit *in vitro* cell viability of MDA-MB-231 cells, with differential effects when used in combination.

The effects of cucruminoids and zoledronic acid, alone and in combination, on *in vitro* cell viability of breast cancer-derived MDA-MB-231 cells were assessed by mitochondrial reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; ATCC), as per manufacturer’s protocol. All results are expressed as mean ± SEM, with statistical significance determined by ANOVA. *p<0.05, **p<0.01, ***p<0.001.

A. MTT assay of MDA-MB-231 cells exposed to zoledronic acid. The half maximal inhibitory concentration of zoledronic acid alone is projected to be 1.0677 x 10^4 μM zoledronic acid.

B. MTT assay of MDA-MB-231 cells exposed to curcuminoids. The half maximal inhibitory concentration of curcuminoids alone is 9.1 x 10^1 μM curcuminoids.

C. MTT assay of MDA-MB-231 cells exposed to combination of curcuminoids (1-100 μM) and zoledronic acid (300 μM). The half maximal inhibitory concentration is 1.31 x 10^2 μM curcuminoids with 300 μM zoledronic acid. The gray line indicates the MTT assay of curcuminoids alone, for comparison. The two curves generated by these data were statistically different by the extra sum-of-squares F test (p<0.0001).
Combined curcuminoids and zoledronic acid differ from zoledronic acid and curcuminoids alone in their effects on MDA-MB-231 cell viability

The combined effects of curcuminoids and zoledronic acid on *in vitro* cell viability of breast cancer-derived MDA-MB-231 cells were assessed using MTT reduction assays (Figure 1C). The 300 μM zoledronic acid dose was chosen because this was a dose that did not affect cell viability on its own and could therefore be used to look for synergistic effects. Viability of MDA-MB-231 cells was dose-dependently inhibited by the combination of curcuminoids and zoledronic acid, with a minimum effective concentration of 1 μM curcuminoids (93.5 ± 0.8% of control) when combined with 300 μM ZA (versus a minimum effective concentration of 10 μM for curcuminoids alone). Statistically significant inhibition in the viability of MDA-MB-231 cells was observed at 1, 3 (91.9 ± 1.2% of control), 10 (94.6 ± 1.5% of control), 30 (83.0 ± 0.8% of control), and 100 (56.4 ± 0.5% of control) μM curcuminoids in combination with 300 μM zoledronic acid (p<0.001, p<0.001, p<0.01, p<0.001, and p<0.001, respectively). The half maximal inhibitory concentration for cell viability of MDA-MB-231 cells in the combined treatment (IC<sub>50</sub>=1.31 x 10^2 μM curcuminoids with 300 μM zoledronic acid) was close to the half maximal inhibitory concentrations of curcuminoids alone (IC<sub>50</sub>=9.1 x 10^1 μM curcuminoids). The two curves generated by these data were statistically different by the extra sum-of-squares F test (p<0.0001), with the combination of curcuminoids and zoledronic acid differing from the curcuminoid alone treatment group.

**MTT Assay: undifferentiated RAW 264.7 cells**

**Zoledronic acid dose-dependently inhibits viability of RAW 264.7 cells**

Because zoledronic acid will also be added during the last 24 hours of culture in the osteoclastogenesis assays to elicit the effects of zoledronic acid on mature osteoclasts, the effects of zoledronic acid on the viability of undifferentiated cells when added for the last 24 hours of a 72 hour culture were assessed using MTT reduction assays (Figure 2A). Zoledronic acid dose dependently inhibited RAW 264.7 cell viability with a minimum effective concentration of 100 μM (76.808 ± 0.704% of control). Statistically significant decreases in the number of viable RAW 264.7 cells were observed at 100, 300 (70.503 ± 0.969% of control), and
Figure 2

(A) Plot showing the effect of Zoledronic Acid on cell viability over 24 hours. The IC$_{50}$ is $1.939 \times 10^3$ μM.

(B) Plot showing the effect of Zoledronic Acid on cell viability over 72 hours. The IC$_{50}$ is $1$ μM.

(C) Plot showing the effect of Curcuminoids on cell viability over 76 hours. The IC$_{50}$ is $1.6 \times 10^1$ μM.
Zoledronic acid and curcuminoids dose-dependently inhibit the viability of RAW 264.7 cells.

The effects of curcuminoids and zoledronic acid, alone and in combination, on *in vitro* cell viability of murine RAW 264.7 cells were assessed by mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; ATCC), as per manufacturer’s protocol. All results are expressed as mean ± SEM, with statistical significance determined by ANOVA. ***p<0.001.

A. MTT assay of RAW 264.7 cells exposed to zoledronic acid, 24 hours. Zoledronic acid dose dependently inhibited RAW 264.7 cell viability with a minimum effective concentration of 100 μM. Statistically significant decreases in the number of viable RAW 264.7 cells were observed at 100, 300, and 1000 μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for viable RAW 264.7 cells was projected to be 1.939 x 10^3 μM zoledronic acid.

B. MTT assay of RAW 264.7 cells exposed to zoledronic acid, 72 hours. Zoledronic acid dose-dependently inhibited RAW 264.7 cell viability with a minimum effective concentration of 1 μM. Statistically significant decreases in the number of viable RAW 264.7 cells were observed at 1, 3, 10, 30, and 100 μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for viable RAW 264.7 cells was 1 μM zoledronic acid.

C. Curcuminoids dose-dependently inhibited RAW 264.7 cell viability with a minimum effective concentration of 10 μM. Statistically significant decreases in the number of viable RAW 264.7 cells were observed at 10, 30, 100, and 300 μM curcuminoids (p<0.001). The half maximal inhibitory concentration for viable RAW 264.7 cells was 1.6 x 10^1 μM curcuminoids.
1000 (64.5 ± 1.5% of control) μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for viable RAW 264.7 cells was projected to be 1.939 x 10^3 μM zoledronic acid.

The effects of 72 hours of zoledronic acid treatment on in vitro cell viability of murine macrophage RAW 264.7 cells were assessed using MTT reduction assays. Zoledronic acid dose-dependently inhibited RAW 264.7 cell viability with a minimum effective concentration of 1 μM (79.4 ± 2.7% of control). Statistically significant decreases in the number of viable RAW 264.7 cells were observed at 1, 3 (37.6 ± 1.2% of control), 10 (37.5 ± 1.2% of control), 30 (21.2 ± 0.6% of control), and 100 (19.3 ± 0.1% of control) μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for viable RAW 264.7 cells was 1 μM zoledronic acid (Figure 2B).

**Curcuminoids dose-dependently inhibit viability of RAW 264.7 cells**

The effects of curcuminoids on in vitro viability of murine macrophage RAW 264.7 cells, which will be used in the 72 hour osteoclastogenesis cultures, were assessed after 76 hours of culture of undifferentiated cells using MTT reduction assays (Figure 2C). Curcuminoids dose-dependently inhibited RAW 264.7 cell viability with a minimum effective concentration of 10 μM (71.0 ± 6.4% of control). Statistically significant decreases in the number of viable RAW 264.7 cells were observed at 10, 30 (16.9 ± 0.1% of control), 100 (18.3 ± 0.6% of control), and 300 (19.3 ± 0.4% of control) μM curcuminoids (p<0.001). The half maximal inhibitory concentration for viable RAW 264.7 cells was 1.6 x 10^1 μM curcuminoids.

**Osteoclast formation assays**

**Zoledronic acid inhibited osteoclast formation at doses that also decreased precursor viability**

The effects of zoledronic acid on in vitro osteoclast formation were assessed using RAW 264.7 cells capable of expressing an osteoclast-like phenotype upon RANKL induction (Figure 3A). Formation of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by zoledronic acid (added at the start of RANKL-induced differentiation)
Figure 3

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Curcuminoids, but not zoledronic acid, inhibit osteoclast formation at doses that do not alter precursor viability.

The effects of zoledronic acid and curcuminoids on in vitro osteoclast growth were assessed using RAW 264.7 cells capable of expressing an osteoclast-like phenotype upon RANKL induction. All results are expressed as mean ± SEM, with statistical significance determined by ANOVA. *p<0.05, **p<0.01, ***p<0.001

A. The effects of zoledronic acid (0.1-30 μM) on osteoclast formation. Formation of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by zoledronic acid with a minimum effective concentration of 10μM. Statistically significant decreases in the numbers of cells with an osteoclast-like phenotype were observed at 10 and 30 μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to zoledronic acid was more (IC_{50}=4 μM zoledronic acid) than the half maximal inhibitory concentration for viable cell number as assessed by mitochondrial reduction of MTT in RAW 264.7 cells exposed to zoledronic acid (IC_{50}=1 μM zoledronic acid). The gray line indicates the corresponding MTT assay data, for comparison.

B. The effects of curcuminoids (0.1-10 μM) on osteoclast formation. Formation of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by curcuminoids with a minimum effective concentration of 1 μM. Statistically significant decreases in the numbers of cells with an osteoclast-like phenotype were observed at 1, 3, and 10 μM curcuminoids. The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to curcuminoids was less (IC_{50}=4 μM curcuminoids) than the half maximal inhibitory concentration for viable cell number as assessed by mitochondrial reduction of MTT in RAW 264.7 cells exposed to curcuminoids (IC_{50}=1.7 x 10^1 μM curcuminoids). The gray line indicates the corresponding MTT assay data, for comparison.

C. The combined effects of curcuminoids (0.1-3 μM) and zoledronic acid (0.1 μM) on osteoclast formation. Formation of cells with an osteoclast-like phenotype was not dose-dependently inhibited with the combined treatment of zoledronic acid and curcuminoids at the observed
doses (dose response up to 3 μM curcuminoids and 0.1 μM zoledronic acid). The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to the combination of curcuminoids and zoledronic acid was projected to be less (IC$_{50}$=3 μM curcuminoids) than the half maximal inhibitory concentration with curcuminoids (IC$_{50}$=4 μM curcuminoids) alone. These values were not statistically different by the extra sum-of-squares F test (p=0.8381). The gray line indicates the corresponding osteoclast data for curcuminoids alone, for comparison.
with a minimum effective concentration of 10 μM. Statistically significant decreases in the numbers of cells with an osteoclast-like phenotype were observed at 10 (20.0 ± 1.8% of control) and 30 (0.0 ± 0.0% of control) μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to zoledronic acid was more (IC₅₀=4 μM zoledronic acid) than the half maximal inhibitory concentration for viable cell number as assessed by mitochondrial reduction of MTT in RAW 264.7 cells exposed to zoledronic acid (IC₅₀=1 μM zoledronic acid). As these values were statistically different by the extra sum-of-squares F test (p=0.0403), the observed inhibitory effects of zoledronic acid on osteoclast formation may be secondary to changes in precursor cell growth and viability.

**Curcuminoids inhibited osteoclast formation, independent of effects on precursor cell growth**

The effects of curcuminoids on *in vitro* RANKL-stimulated osteoclast differentiation were assessed using RAW 264.7 cells capable of expressing an osteoclast-like phenotype upon RANKL induction (Figure 3B). Formation of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by curcuminoids with a minimum effective concentration of 1 μM (71.4 ± 3.3% of control). Statistically significant decreases in the numbers of cells with an osteoclast-like phenotype were observed at 1, 3 (62.5 ± 11.6% of control), and 10 (0.3 ± 0.2% of control) μM curcuminoids (p<0.05, p<0.01, and p<0.001, respectively). The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to curcuminoids was less (IC₅₀=4 μM curcuminoids) than the half maximal inhibitory concentration for viable cell number as assessed by mitochondrial reduction of MTT in RAW 264.7 cells exposed to curcuminoids (IC₅₀=1.7 x 10⁻¹ μM curcuminoids). These values were statistically different by the extra sum-of-squares F test (p<0.0001) and suggest that the observed inhibitory effects of curcuminoids on osteoclast formation are independent of changes in precursor cell growth.

**Combined curcuminoids and low dose zoledronic acid do not differ in their effects on osteoclast formation from curcuminoids alone**

The combined effects of curcuminoids and zoledronic acid on *in vitro* osteoclast differentiation were assessed using RAW 264.7 cells capable of expressing an osteoclast-like phenotype upon RANKL exposure (Figure 3C). A dose of 0.1 μM zoledronic acid was chosen, because this was a
dose that did not affect cell viability in MTT reduction assays. Formation of cells with an osteoclast-like phenotype was not dose-dependently inhibited with the combined treatment of zoledronic acid and curcuminoids at the observed doses (curcuminoid dose response up to 3 μM combined with 0.1 μM zoledronic acid). The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to the combination of curcuminoids and zoledronic acid was projected to be less (IC₅₀=3 μM curcuminoids) than the half maximal inhibitory concentration with curcuminoids (IC₅₀=4 μM curcuminoids) alone. However, these values were not statistically different by the extra sum-of-squares F test (p=0.8381), suggesting that the combined effects of curcuminoids and zoleodronic acid at the single dose tested are neither synergistic nor antagonistic at the doses tested.

**Osteoclast survival assays**

**Zoledronic acid inhibited mature osteoclast survival**

The effects of zoledronic acid on *in vitro* osteoclast survival were assessed using RAW 264.7 cells pretreated for 48 hours with RANKL to induce mature osteoclast formation (Figure 4A). Addition of zoledronic acid during the last 24 hours of culture (during which fresh RANKL or fresh RANKL plus zoleodronic acid was added to all wells) elicits the effects of zoleodronic acid on mature osteoclasts. Survival and further differentiation of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by zoleodronic acid with a minimum effective concentration (MEC) of 100 μM. Statistically significant decreases in the numbers of cells with an osteoclast-like phenotype were observed at 100 (2.1 ± 0.9% of control), 300 (0.0 ± 0.0% of control), and 1000 (0.0 ± 0.0% of control) μM zoleodronic acid (p<0.001). The half maximal inhibitory concentration for survival and continued differentiation of RANKL-stimulated cells with an osteoclast-like phenotype exposed to zoleodronic acid was less (IC₅₀=4.0 x 10^1 μM zoleodronic acid) than the half maximal concentration for decreased viability of undifferentiated precursor cells as assessed by mitochondrial reduction of MTT in
Combined curcuminoids and zoledronic acid differ from zoledronic acid alone in their effects on osteoclast survival/formation.

The effects of zoledronic acid and curcuminoids on in vitro osteoclast survival were assessed using RAW 264.7 cells capable of expressing an osteoclast-like phenotype upon RANKL induction. All results are expressed as mean ± SEM, with statistical significance determined by ANOVA. *p<0.05, ***p<0.001

A. The effects of zoledronic acid on in vitro osteoclast survival in RAW 264.7 cells pretreated for 48 hours with RANKL to induce mature osteoclast formation, followed by RANKL plus zoledronic acid (1-1000 μM) for the last 24 hours of culture. Growth of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by zoledronic acid with a minimum effective concentration (MEC) of 100 μM. Statistically significant decreases in the numbers of cells with osteoclast-like phenotype were observed at 100, 300, and 1000 μM zoledronic acid (p<0.001). The half maximal inhibitory concentration for survival and continued differentiation of RANKL-stimulated osteoclast-like phenotype exposed to zoledronic acid is 4.0 x 10^1 μM zoledronic acid. The gray line indicates the corresponding MTT assay data for zoledronic acid, for comparison.

B. The combined effects of curcuminoids and zoledronic acid on in vitro osteoclast growth and proliferation. Addition of zoledronic acid (30 μM, a dose which does not independently affect cell viability in MTT reduction assays) during the last 24 hours of culture elicits the effects of zoledronic acid on mature osteoclasts. Curcuminoids were added to the cells for the entire 72 hour incubation period, with an additional 4 hours of pre-treatment. Growth of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by the combination of curcuminoids and zoledronic acid with a minimum effective concentration (MEC) of 10 μM (p<0.05). Statistically significant increases and decreases in the numbers of cells with osteoclast-like phenotype were observed at 0.3 and 10 μM curcuminoids, respectively (p<0.05), when combined with low dose zoledronic acid. The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to the combination of curcuminoids and zoledronic acid is 6 μM curcuminoids plus 30 μM zoledronic
acid. The gray line indicates the corresponding osteoclast data for curcuminoids alone (see figure 3B), for comparison.
RAW 264.7 cells exposed to zoledronic acid during the last 24 hours of a 72 hour culture (projected to be IC\textsubscript{50} = 1.939 x 10\textsuperscript{3} μM zoledronic acid). These values were statistically different by the extra sum-of-squares F test (p<0.001) and suggest that the observed inhibitory effects of zoledronic acid on the survival and continued differentiation of mature osteoclast cultures cannot be attributed to inhibitory effects on precursor cell viability.

**Combined curcuminoids and zoledronic acid differ from zoledronic acid alone in their effects on osteoclast formation/survival**

The combined effects of curcuminoids and zoledronic acid on *in vitro* osteoclast formation and survival were assessed using RAW 264.7 cells capable of expressing an osteoclast-like phenotype upon RANKL exposure (Figure 4B). Addition of zoledronic acid (30 μM, a dose which does not independently affect cell viability in MTT reduction assays but approximates the IC\textsubscript{50} for inhibition of osteoclast survival) during the last 24 hours of culture elicits the effects of zoledronic acid on mature osteoclasts. Curcuminoids were added to the cells for the entire 72 hour incubation period, with an additional 4 hours of pre-treatment. Growth of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by the combination of curcuminoids and zoledronic acid with a minimum effective concentration (MEC) of 10 μM (p<0.05). Statistically significant increases and decreases in the numbers of cells with osteoclast-like phenotype were observed at 0.3 (178.3 ± 23.9% of control) and 10 (27.5 ± 8.3% of control) μM curcuminoids, respectively (p<0.05), when combined with low dose zoledronic acid. Because fresh RANKL or fresh RANKL plus curcuminoids and zoledronic acid was added to all wells for the last 24 hours, the conditions of these experiments were slightly different than those for the osteoclast formation assays, wherein cells were exposed to curcuminoids for a total of 76 hours with media refreshed only at the 4 hour time point when RANKL was added. With this caveat in mind, when one directly compares the effects of curcuminoids on osteoclast differentiation, the addition of zoledronic acid did not appear to alter its inhibitory effect. The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to the combination of curcuminoids and zoledronic acid was 6 μM curcuminoids plus 30 μM zoledronic acid. This IC\textsubscript{50} value was not statistically
different from the IC\textsubscript{50} for curcuminoids alone (see figure 3B) by the extra sum-of-squares F test (p=0.3286).

Animal Experiments

**Tolerability of treatments in mice without bone metastases**

In a separate experiment to evaluate the safety of the proposed treatments, 4-week old athymic nude mice were administered vehicle (100 µL PBS IP, 100 µL DMSO SubQ, n=6), curcuminoids (25 mg/kg/d, n=7), zoledronic acid (1 µg/kg/d, n=9), or a combination of curcuminoids/zoledronic acid (n=6) 5x/week for twenty-one days. There were no statistically significant overt toxic effects on mice, as assessed by total body weights or organ weights normalized to body weight (liver, spleen, or kidneys) of athymic mice, analyzed by one-way ANOVA with Bonferroni post-hoc test versus vehicle controls (Table 1).

**Effects of treatments on BMD in mice without bone metastases**

In the separate experiment to evaluate the safety of the proposed treatments, the effects of curcuminoids and zoledronic acid administration on bone mineral density (BMD) in mice without bone metastases were assessed on day 20 post-intracardiac injection in athymic nude mice administered vehicle, curcuminoids (25 mg/kg/d), zoledronic acid (1 µg/kg/d), or a combination (Figure 5). In mice administered zoledronic acid alone, BMD of the total femur (0.061 ± 0.00094 g/cm\textsuperscript{2}, n=18, p<0.01), distal femur (0.071 ± 0.0016 g/cm\textsuperscript{2}, n=18, p<0.001), total tibia (0.049 ± 0.0010 g/cm\textsuperscript{2}, n=18, p<0.001), and proximal tibia (0.063 ± 0.0019 g/cm\textsuperscript{2}, n=18, p<0.001) were statistically different from vehicle control BMD of the total femur (0.056 ± 0.0010 g/cm\textsuperscript{2}, n=12), distal femur (0.058 ± 0.0014 g/cm\textsuperscript{2}, n=12), total tibia (0.044 ± 0.00070 g/cm\textsuperscript{2}, n=12), and proximal tibia (0.049 ± 0.0015 g/cm\textsuperscript{2}, n=12) as assessed by one-way ANOVA with Bonferroni post-hoc test. The zoledronic acid-induced increase in BMD appeared to be abrogated by curcuminoids, as BMD in mice treated with both agents (or by curcuminoids alone) was no different than control. The BMD in mice administered the combination of zoledronic acid and curcuminoids was as follows: total femur (0.057 ± 0.0015 g/cm\textsuperscript{2}, n=12), distal femur (0.064 ± 0.0023 g/cm\textsuperscript{2}, n=12), total tibia (0.046 ± 0.0011 g/cm\textsuperscript{2}, n=12), and proximal tibia (0.054 ± 0.0021 g/cm\textsuperscript{2}, n=12). The BMD in mice administered curcuminoids distal femur (0.064 ± 0.0023 g/cm\textsuperscript{2}, n=12), total tibia (0.046 ± 0.0011 g/cm\textsuperscript{2}, n=12), and
Table 1. Assessment of treatment toxicity

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Control</th>
<th>Curcuminoids</th>
<th>Zoledronic Acid</th>
<th>Curcuminoids+Zoledronic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Body Weight</strong></td>
<td>20.7 ± 0.61</td>
<td>19.57 ± 0.61</td>
<td>21.67 ± 0.82</td>
<td>18.50 ± 0.67</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Liver (g/g BW)</strong></td>
<td>5.48 ± 0.10</td>
<td>5.31 ± 0.17</td>
<td>5.23 ± 0.14</td>
<td>5.35 ± 0.20</td>
</tr>
<tr>
<td><strong>Spleen (g/g BW)</strong></td>
<td>0.56 ± 0.04</td>
<td>0.65 ± 0.08</td>
<td>0.47 ± 0.02</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td><strong>Kidneys (g/g BW)</strong></td>
<td>0.71 ± 0.02</td>
<td>0.72 ± 0.02</td>
<td>0.65 ± 0.01</td>
<td>0.73 ± 0.02</td>
</tr>
</tbody>
</table>

Four-week old athymic nude mice non-responders without bone or pericardiac tumors were administered vehicle (100 μL PBS IP, 100 μL DMSO SubQ, n=6, n=12 for kidneys), curcuminoids (25 mg/kg/d, n=7, n=14 for kidneys), zoledronic acid (1 μg/kg/d, n=9, n=18 for kidneys), or a combination of curcuminoids and zoledronic acid (n=6, n=12 for kidneys) 5x/week for 21 days. All results are expressed as mean ± SEM, with statistical significance determined by one-way ANOVA with Bonferroni post-hoc test (p<0.05) versus vehicle control.
Figure 5

A

B

C

D

Vehicle Control  Curcuminoids  ZA  Curc + ZA

Vehicle Control  Curcuminoids  ZA  Curc + ZA

Vehicle Control  Curcuminoids  ZA  Curc + ZA

Vehicle Control  Curcuminoids  ZA  Curc + ZA
Figure 5

**Zoledronic acid alone increases bone mineral density.**

Four-week old athymic nude mice non-responders without bone or pericardiac tumors were administered vehicle (100 μL PBS IP, 100 μL DMSO SubQ, n=12), curcuminoids (25mg/kg/d, n=14), zoledronic acid (1 μg/kg/d, n=18), or a combination of curcuminoids and zoledronic acid (n=12) 5x/week for 21 days. All results are expressed as mean ± SEM, with statistical significance determined by ANOVA. **p<0.01, ***p<0.001 versus vehicle control.

A: Total Femur.
B: Distal Femur.
C: Total Tibia.
D: Proximal Tibia.
proximal tibia (0.054 ± 0.0021 g/cm², n=12). The BMD in mice administered curcuminoids alone was as follows: total femur (0.052 ± 0.00092 g/cm², n=14), distal femur (0.053 ± 0.0013 g/cm², n=14), total tibia (0.043 ± 0.00050 g/cm², n=14), and proximal tibia (0.048 ± 0.0011 g/cm², n=14).

Effects of treatments on body weights in mice with bone metastases
Four-week old athymic mice administered vehicle (100 μL PBS IP, 100 μL DMSO SubQ, n=8), curcuminoids(25 mg/kg/d, n=9), zoledronic acid (2 μg/kg/d, n=10), or the combined curcuminoids/zoledronic acid (n=9) 5x/week for 21 days following intracardiac injection of MDA-MB-231 cells to induce the development of bone metastases demonstrated no statistically significant differences in total body weights, as analyzed by two-way ANOVA (Figure 6).

Effects of treatment on radiographically-evident osteolytic bone lesion area in mice with bone metastases
The incidence of radiographically-evident osteolytic bone lesions in four-week old athymic nude mice administered vehicle (PBS IP, DMSO SubQ), curcuminoids (25 mg/kg/d), zoledronic acid (2 μg/kg/d), or a combination of curcuminoids/zoledronic acid, following intracardiac injection of breast cancer-derived MDA-MB-231 cells, did not differ greatly between groups (Table 2). The effects of curcuminoids and zoledronic acid administration on radiographically-evident osteolytic bone lesion area were assessed in four-week old athymic nude mice, following intracardiac injection of breast cancer-derived MDA-MB-231 cells (Figure 7). At day 17 post-intracardiac injection, while lytic lesion area tended to be lower in all zoledronic acid-treated mice, lytic lesion area in vehicle controls (3.419±0.762 mm², n=8) was not statistically different from curcuminoids alone (4.203±0.872 mm², n=9), zoledronic acid alone (1.537±0.412 mm², n=10), or the combined curcuminoids/zoledronic acid (1.599±0.525 mm², n=9). At day 21 post-intracardiac injection, while lytic lesion area again tended to be lower in all zoledronic acid-treated mice, lytic lesion area in vehicle controls (9.284±1.908 mm², n=8) was not statistically different from curcuminoids alone (9.830±1.674mm², n=9), zoledronic acid alone (4.543±1.283 mm², n=10), or the combined curcuminoids/zoledronic acid (5.342±1.715mm², n=9).
Figure 6

![Graph showing weight changes over days for different treatment groups.](image)

- Control
- 25mg/kg Curcuminoids
- 2ug/kg Zoledronic Acid
- 25mg/kg Curc + 2ug/kg ZA
Figure 6

Responders demonstrated no differences in body weights throughout treatment.

The effects of curcuminoids and zoledronic acid administration on total body weight was assessed in four-week old athymic nude mice, following intracardiac injection of breast cancer-derived MDA-MB-231 cells. Data is expressed as mean±SEM. Twenty-one days of vehicle (PBS IP, DMSO SubQ), curcuminoids (25 mg/kg/d), zoledronic acid (2 μg/kg/d), or the combined curcuminoids/zoledronic acid demonstrated no statistically significant effect on total body weights of athymic mice, as analyzed by two-way ANOVA. 
<table>
<thead>
<tr>
<th>Incidence</th>
<th>Vehicle Control</th>
<th>Curcuminoids</th>
<th>Zoledronic Acid</th>
<th>Curcuminoids+ Zoledronic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>8/12 (67%)</td>
<td>9/11 (82%)</td>
<td>10/11 (91%)</td>
<td>9/11 (82%)</td>
</tr>
</tbody>
</table>

Incidence of radiographically-evident osteolytic bone lesions 21 days post-intracardiac injection of MDA-MB-231 cells in 4 week-old athymic nude mice administered vehicle (PBS IP, DMSO SubQ), curcuminoids (25 mg/kg/d), zoledronic acid (2 μg/kg/d), or the combined curcuminoids/zoledronic acid 5x/week for 21 days.
Figure 7

- Vehicle Control
- 25 mg/kg/d Curcuminoids
- 2 ug/kg/d Zoledronic Acid
- Curc + ZA
**Figure 7**

**Radiographically-evident osteolytic bone lesion area did not differ between treatment groups.**

The effects of curcuminoids and zoledronic acid administration on radiographically-evident osteolytic bone lesions were assessed in four-week old athymic nude mice, following intracardiac injection of breast cancer-derived MDA-MB-231 cells. Data is expressed as mean±SEM. At day 17 post-intracardiac injection, lytic lesion area in vehicle controls (3.419±0.762 mm², n=8) was not statistically different from curcuminoids alone (4.203±0.872 mm², n=9), zoledronic acid alone (1.537±0.412 mm², n=10), or the combined curcuminoids plus zoledronic acid (1.599±0.525 mm², n=9). At day 21 post-intracardiac injection, lytic lesion area in vehicle controls (9.284±1.908 mm², n=8) was not statistically different from curcuminoids alone (9.830±1.674 mm², n=9), zoledronic acid alone (4.543±1.283 mm², n=10), or the combined curcuminoids/zoledronic acid (5.342±1.715 mm², n=9).
Data was assessed by two-way ANOVA. Representative radiographs of osteolytic bone lesions are demonstrated in Figure 8.
Figure 8

Vehicl  Cur  ZA  Cur+Z
Figure 8

**Representative micrographs of osteolytic bone lesions.**

Four-week old athymic nude mice were inoculated with MDA-MB-231 human breast cancer cells in the left cardiac ventricle and administered vehicle (100 uL PBS IP, 100 uL DMSO SubQ; n=8), curcuminoids (25 mg/kg/d, n=9), zoledronic acid (2 μg/kg/d, n=10), or curcuminoids plus zoledronic acid (n=9) 5x/week for 21 days. Mice were followed for the development of radiographically evident osteolytic bone lesions. Representative x-rays are presented, with arrows indicating sites of osteolytic bone lesions. For data interpretation, see figure 7.
DISCUSSION

The effects of curcuminoids and zoledronic acid on in vitro cell viability of breast cancer-derived MDA-MB-231 cells, as assessed using MTT reduction assays, indicate that zoledronic acid and curcuminoids are both capable of inhibiting MDA-MB-231 cell viability. Interestingly, while anti-proliferative effects of zoledronic acid on breast cancer cells have previously been documented, curcuminoids are 100-fold more potent than zoledronic acid in inhibiting the viability of MDA-MB-231 cells. In combination, the addition of zoledronic acid at 300 μM did not alter the anti-proliferative effects of the curcuminoids. However, possible synergistic effects of these two agents cannot be ruled out before testing a higher dose of zoledronic acid since the anti-proliferative half-maximal inhibition (IC₅₀) for zoledronic acid was 30-fold higher than the dose tested here.

It is important to note that the MDA-MB-231 cells used in this study are a subclone that have been cultured to target bone for optimal development of osteolytic bone lesions. Therefore this limits the ability to compare the results of the in vitro experiments described here to published data obtained using other MDA-MB-231 clones. For example, the study by Senaratne et al. that looked at zoledronic acid and MDA-MB-231 cells found an IC₅₀ of cell viability of 1.5 x 10^1 μM, while our current study found a half maximal inhibitory concentration for cell viability of MDA-MB-231 cells that was projected to be 1.0677 x 10^4 μM zoledronic acid (Senaratne et al. 2000). It is possible that this difference can be attributed to the fact that our MDA-MB-231 cells are a subclone of MBD-MB-231 cells distributed by ATCC.

To our knowledge, this paper is the first to describe in vitro assays with zoledronic acid and RAW 264.7 cells stimulated with RANKL to express an osteoclast-like phenotype. Zoledronic acid was found to dose-dependently inhibit osteoclast formation, but only at doses that also decreased precursor viability. The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to zoledronic acid was more (IC₅₀=4 μM zoledronic acid) than the half maximal inhibitory concentration for viable cell number as assessed by mitochondrial reduction of MTT in RAW 264.7 cells exposed to zoledronic acid (IC₅₀=1 μM zoledronic acid). Thus, it is likely that the observed inhibitory effects of zoledronic
acid on osteoclast formation are secondary to changes in precursor cell growth and do not reflect a specific inhibitory effect on osteoclast differentiation.

In contrast, the results of the in vitro assays with curcuminoids and RAW 264.7 cells stimulated with RANKL to express an osteoclast-like phenotype indicate that the observed inhibitory effects of curcuminoids on osteoclast formation are independent of changes in precursor cell growth. Thus, curcuminoids inhibit OC differentiation while zoledronic acid does not. In combination with a single, low dose of zoledronic acid, no differences in the effects on osteoclast formation were observed. Formation of cells with an osteoclast-like phenotype was not dose-dependently inhibited with the combined treatment of zoledronic acid and curcuminoids at the observed doses (dose response up to 3 μM curcuminoids and 0.1 μM zoledronic acid). The half maximal inhibitory concentration for formation of cells with an osteoclast-like phenotype exposed to the combination of curcuminoids and zoledronic acid was projected to be less (IC₅₀=3 μM curcuminoids) than the half maximal inhibitory concentration with curcuminoids (IC₅₀=4 μM curcuminoids) alone. These values were not statistically different by the extra sum-of-squares F test (p=0.8381) and suggest that the combined effects of curcuminoids and zoledronic acid at the single dose tested are neither synergistic nor antagonistic.

The experiments wherein RANKL and zoledronic acid were added to the final 24 hours of culture of RAW 264.7 cells pretreated with 48 hours of RANKL to induce mature osteoclast formation were aimed at elucidating the effects of zoledronic acid on mature osteoclast survival. Consistent with the well described ability of bisphosphonates to induce osteoclast apoptosis the results of these studies indicate that the survival of cells with an osteoclast-like phenotype secondary to RANKL exposure was dose-dependently inhibited by zoledronic acid (MEC = 100 μM). The half maximal inhibitory concentration for survival and continued differentiation of RANKL-stimulated osteoclast-like phenotype exposed to zoledronic acid was less (IC₅₀=4.0 x 10⁻¹ μM zoledronic acid) than the half maximal concentration for decreased viability of undifferentiated precursor cells as assessed by mitochondrial reduction of MTT in RAW 264.7 cells exposed to zoledronic acid during the last 24 hours of a 72 hour culture (projected to be IC₅₀=1.939 x 10⁻³ μM zoledronic acid). These values were statistically different
by the extra sum-of-squares F test \( p<0.001 \), suggesting that the observed inhibitory effects of zoledronic acid on the survival and continued differentiation of mature osteoclast cultures cannot be attributed to inhibitory effects on precursor cell viability. These results are consistent with the known mechanism of action of zoledronic acid, involving the direct induction of apoptosis in mature osteoclasts (Kellinsalmi et al. 2005).

The experiment examining the combined effects of curcuminoids on osteoclast formation (treatment precedes initial stimulation with RANKL) with the effects of zoledronic acid on mature osteoclast survival (zoledronic acid added only for last 24 hours of culture) is a set-up that takes advantage of the mechanisms by which the individual compounds target osteoclasts. The mechanism of action of zoledronic acid on osteoclast inhibition appears to be the direct induction of apoptosis in mature osteoclasts (Kellinsalmi et al. 2005), while the mechanism of action of curcuminoids on osteoclast inhibition appears to be a decline in osteoclast differentiation (Bharti, Takada, Aggarwal 2004). Growth of cells with an osteoclast-like phenotype subsequent to RANKL exposure was dose-dependently inhibited by the combination of curcuminoids and zoledronic acid with a minimum effective concentration (MEC) of 10 \( \mu \)M \( p<0.05 \). However, the IC\textsubscript{50} for curcuminoid inhibition of osteoclast differentiation was not altered by the addition of zoledronic acid for the final 24 hours of culture. This suggests a lack of synergistic or additive effect of these two treatments. However, it must be noted that only a single zoledronic acid dose was tested and that this dose in isolation also had no effect on osteoclast survival. Thus, additional experiments using pro-apoptotic doses of zoledronic acid will be required to further test the postulate that combined treatment with these two agents may have added benefits.

Because curcuminoids and zoledronic acid blocked osteoclasts by different mechanisms in our \textit{in vitro} experiments, inhibiting differentiation and survival, respectively, we anticipated seeing additive or synergistic effects on osteoclast-driven osteolytic breast cancer bone metastases \textit{in vivo}. In addition, the inhibitory effects of each agent on the proliferation of MDA-MB-231 cells \textit{in vitro} lent further support to the hypothesis that the combination of these two agents would yield greater benefits \textit{in vivo} in blocking bone metastases in an MDA-MB-231 bone metastases model.
Our *in vivo* studies did document that combined treatment with clinically relevant doses of these two agents was well tolerated and without evidence of toxicities. However, although there was a trend towards a decrease in osteolytic bone lesion area with zoledronic acid treatment (which did not reach statistical significance), the addition of curcuminoids did not enhance this effect. No statistically significant changes between the group administered curcuminoids and the vehicle control were observed. Furthermore, no statistically significant changes between the zoledronic acid and zoledronic acid plus curcuminoids groups were observed. These results do not necessarily negate or refute the hypothesis, as these results are particularly notable since the absence of curcuminoid effects on bone metastases differs from prior studies that established the effects of curcuminoids on osteolytic bone lesion area (Wright *et al.* 2013). These prior studies by Wright *et al.* examining the effects of curcuminoids on osteolytic bone lesion area in mice inoculated with MDA-MB-231 cells found that curcuminoids dosed at 25 and 50 mg/kg/d for 21 days reduced lytic lesion area by 57 and 51%, respectively (Wright *et al.* 2013). It is important to note that curcuminoids in the Wright *et al.* experiment were administered every other day for 21 days versus the 5 times per week regimen used in this study. It is possible that this difference in dosing regimen contributed to the low efficacy seen in the current study.

A prior *in vivo* study by Daubiné *et al.* (2007) indicated that zoledronic acid is effective in preventing metastases of breast cancer in animal models that utilize the nude mouse MDA-MB-231 intracardiac injection model. Daily (3 μg/kg), and weekly (20 μg/kg) dosing regimens resulted in osteolytic lesions that were 88% and 80% smaller, respectively. Based on this data, we chose a lower submaximal dose (2 μg/kg/d) in order to examine the combined effects of zoledronic acid and curcuminoids. While this dose of zoledronic acid did tend to decrease lytic lesion area by half, a statistically significant effect of zoledronic acid did not occur in these studies. Again, it is important to note that our study used a subclone of MDA-MB-231 cells, while the Daubiné *et al.* (2007) study utilized a subclone transfected for green fluorescent protein and luciferase expression. In addition, the Daubiné *et al.* (2007) study administered zoledronic acid beginning on day -1 relative to intracardiac injection. Thus, it is possible that the different dosing scheme tested here and/or the difference in MDA-MB-231 subclone used
accounted for the less dramatic effect of zoledronic acid in blocking osteolytic bone lesions in the current study. At the same time, it is notable that the addition of the non-traditional agent, curcuminoids, to zoledronic acid did not alter its tendency to limit the progression of bone metastases, suggesting that the clinical use of this dietary supplement may at the very least “do not harm” in women with breast cancer. At the same time, however, the fact that the addition of curcuminoids to zoledronic acid in animals without bone metastases seemed to abrogate the ability of zoledronic acid to enhance BMD at these pharmacologic doses does sound a cautionary note.
FUTURE DIRECTIONS

The MTT assay of MDA-MB-231 cells combining zoledronic acid and curcuminoids should be performed with increasing doses of both curcuminoids and zoledronic acid.

The MTT assay of RAW 264.7 cells combining zoledronic acid and curcuminoids should be performed.

Further osteoclastogenesis assays should be performed. For the osteoclast formation assays, further studies with increasing doses of both curcuminoids and zoledronic acid should be performed. For the osteoclast survival assays, further studies with increasing doses of both curcuminoids and zoledronic acid should be performed. In addition, an experiment involving exposure of RANKL-treated RAW 264.7 cells to curcuminoids for 72 hours (with an additional 4 hour pre-treatment prior to the addition of RANKL) with media being refreshed for the last 24 hours would allow direct comparison with the combined curcuminoids plus zoledronic acid experiment assessing the effects of zoledronic acid on osteoclast survival.

The in vivo animal studies described here should be repeated with varying doses of curcuminoids and zoledronic acid to determine the most efficacious dosing regimens to use for the in vivo experiments described in this paper.

Future experiments to assess more specific and sensitive parameters of osteolytic bone lesion area, including bone histomorphologic parameters and the differential and combined effects of zoledronic acid and curcuminoids on osteoblasts, would lead to better descriptions of the combined effects of zoledronic acid and curcuminoids. This may also uncover more subtle interactions between zoledronic acid and curcuminoids that may not be seen on the macroscopic level.
CONCLUSIONS

Curcuminoids, but not zoledronic acid, the gold standard treatment for breast cancer derived osteolytic bone metastases, inhibit osteoclast formation at doses that do not alter precursor viability, as assessed by osteoclastogenesis assays using the murine RAW 264.7 cell line, which develops an osteoclast-like phenotype subsequent to RANKL exposure. Zoledronic acid and curcuminoids both inhibit in vitro cell viability of human breast cancer cell-derived MDA-MB-231 cells, with differential effects in combination, as assessed by MTT mitochondrial reduction assays. The 100-fold higher IC50 for curcuminoid inhibition of MBD-MB-231 viability as compared to zoledronic acid alone was not altered by the addition of zoledronic acid. In vivo studies examining the effects of combined treatment with these two agents demonstrated good tolerability and the absence of any adverse effects when non-traditional curcuminoids were added to the gold standard treatment of zoledronic acid. However, no additive or synergistic effects in blocking the progression of bone metastases were noted - an outcome that may be due to the lack of effect of curcuminoids demonstrated here in the bone metastases model. To our knowledge, this study is the first to assess the combined effects of zoledronic acid and curcuminoids on breast cancer-derived osteolytic bone lesions and BMD. Clearly, given the well described, complementary in vitro effects of these agents, as documented here and by others, further in vivo studies testing additional dosing strategies are warranted.
REFERENCES


35. Singh S. From exotic spice to modern drug? Cell. 2007; 130: 765-768.


