Zoledronic Acid Reduces Bone Loss and Tumor Growth in an Orthotopic Xenograft Model of Osteolytic Oral Squamous Cell Carcinoma

Chelsea K. Martin¹, Jillian L. Werbeck¹, Nanda K. Thudi¹, Lisa G. Lanigan¹, Tobie D. Wolfe¹, Ramiro E. Toribio², and Thomas J. Rosol¹

Abstract

Squamous cell carcinoma (SCC) is the most common form of oral cancer. Destruction and invasion of mandibular and maxillary bone frequently occurs and contributes to morbidity and mortality. We hypothesized that the bisphosphonate drug zoledronic acid (ZOL) would inhibit tumor-induced osteolysis and reduce tumor growth and invasion in a murine xenograft model of bone-invasive oral SCC (OSCC) derived from an osteolytic feline OSCC. Luciferase-expressing OSCC cells (SCCF2Luc) were injected into the perimaxillary subgingiva of nude mice, which were then treated with 100 μg/kg ZOL or vehicle. ZOL treatment reduced tumor growth and prevented loss of bone volume and surface area but had no effect on tumor invasion. Effects on bone were associated with reduced osteolysis and increased periosteal new bone formation. ZOL-mediated inhibition of tumor-induced osteolysis was characterized by reduced numbers of tartrate-resistant acid phosphatase-positive osteoclasts at the tumor-bone interface, where it was associated with osteoclast vacuolar degeneration. The ratio of eroded to total bone surface was not affected by treatment, arguing that ZOL-mediated inhibition of osteolysis was independent of effects on osteoclast activation or initiation of bone resorption. In summary, our results establish that ZOL can reduce OSCC-induced osteolysis and may be valuable as an adjuvant therapy in OSCC to preserve mandibular and maxillary bone volume and function.

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Introduction

Oral and oropharyngeal cancer is the eighth most common cancer worldwide (1). In 2009, the American Cancer Society estimated that 35,720 people in the United States would be diagnosed with cancer of the oral cavity and pharynx and 7,600 people would die (2). Approximately 90% of oral and oropharyngeal tumors are squamous cell carcinoma (OSCC; refs. 3, 4). Despite advances in cancer treatment, the 5-year disease-specific survival for OSCC has shown only moderate improvement over the past 30 years and is currently 61% for all stages combined (5). OSCC frequently invades bone (6) and is characterized by osteoclastic bone resorption (7). Bone invasion contributes to the clinical morbidity of OSCC patients and is associated with poorer prognosis (8, 9). Despite the frequency and clinical effect of bone invasion in OSCC, the mechanisms responsible for bone resorption and invasion are poorly understood.

We hypothesize that OSCC invasion into bone is facilitated by a vicious cycle of tumor growth and bone resorption, and inhibition of the vicious cycle by targeting osteoclastic bone resorption with zoledronic acid, a third generation bisphosphonate, will reduce bone loss and tumor growth. The vicious cycle theory was described in bone metastases of human breast cancer as a relationship between tumor-derived parathyroid hormone-related protein (PTHrP) and bone-derived transforming growth factor-β1 (TGF-β1; ref. 10). OSCC expresses several factors with the potential to stimulate osteolysis, including PTHrP (11–13), tumor necrosis factor-α (14), interleukin-6, and interleukin-11 (11). Regardless of the number and type of bone resorption agonists expressed by OSCC cells, the physical destruction of bone is accomplished by bone-resorbing osteoclasts.

Osteoclastic bone resorption is inhibited by bisphosphonates. Nitrogen-containing bisphosphonates, such as zoledronic acid (ZOL), interfere with the mevalonate pathway by inhibiting farnesyl pyrophosphate synthase (15). Inhibition of the mevalonate pathway leads to reduced prenylation of small GTP-binding proteins responsible for osteoclast function and survival (16) and leads to apoptosis (17). We hypothesize that inhibition of osteoclastic bone resorption with ZOL will not only reduce bone loss but will also inhibit OSCC xenograft growth and invasion by antagonizing the vicious cycle of osteolysis and tumor growth.

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We have designed a novel, bone-invasive, bioluminescent orthotopic xenograft nude mouse model of OSCC using PTHrP-expressing cells derived from a bone-invasive feline OSCC. As in humans, OSCC is the most commonly diagnosed tumor of the oral cavity in cats (18) and has a highly invasive, osteolytic phenotype (19) with similarities in clinical progression and pathology compared with human OSCC (20). The inhibitory activity of ZOL on OSCC growth and tumor-associated osteolysis was investigated using a combination of in vivo bioluminescent imaging (BLI), faxitron radiography, quantitative microcomputed tomography (microCT), and detailed maxillary histomorphometry. We found that ZOL may be an effective adjuvant treatment for preventing bone resorption associated with OSCC.

**Materials and Methods**

**Cells and reagents**

SCCF2 cells were originally derived in the authors’ laboratory in 2005 from a bone-invasive gingival SCC of a 7-year-old male castrated cat using methods previously described (21). The authors authenticated SCCF2 cells as feline malignant epithelial cells using karyotype analysis, cytokeratin immunocytochemistry, and electron microscopy. Formation of keratinizing SCC xenografts was confirmed in two prior passages (passages 56 and 77; passage 78 was used in this in vivo study). ZOL (Zometa, Novartis) was purchased from the USPHS’s Ortho Pharmaceutical Group. SCCF2 cells were originally derived in the authors’ laboratory in 2005 from a bone-invasive gingival SCC of a 7-year-old male castrated cat using methods previously described (21). The authors authenticated SCCF2 cells as feline malignant epithelial cells using karyotype analysis, cytokeratin immunocytochemistry, and electron microscopy. Formation of keratinizing SCC xenografts was confirmed in two prior passages (passages 56 and 77; passage 78 was used in this in vivo study). ZOL (Zometa, Novartis) was purchased from the USPHS’s Ortho Pharmaceutical Group. SCCF2 cells were originally derived in the authors’ laboratory in 2005 from a bone-invasive gingival SCC of a 7-year-old male castrated cat using methods previously described (21). The authors authenticated SCCF2 cells as feline malignant epithelial cells using karyotype analysis, cytokeratin immunocytochemistry, and electron microscopy. Formation of keratinizing SCC xenografts was confirmed in two prior passages (passages 56 and 77; passage 78 was used in this in vivo study). ZOL (Zometa, Novartis) was purchased from the USPHS’s Ortho Pharmaceutical Group.

**Transfection of SCCF2 cells with luciferase**

SCCF2 cells were stably transfected with a plasmid containing a luciferase–yellow fluorescent protein (YFP) fusion construct driven by a cytomegalovirus promoter and containing neomycin and ampicillin resistance cassettes [pCDNA3.1(+).yLuc-YFP, kindly provided by C. Contag at Stanford University] using Lipofectamine LTX and PLUS Reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase-expressing cells were selected with 0.6 mg/mL geneticin (Invitrogen), and luciferase expression was confirmed using BLI (Supplementary Materials and Methods).

**Animals and treatments**

All animals were 6-week-old male nu/nu mice (National Cancer Institute). Animal care procedures were approved by the Institutional Lab Animal Care and Use Committee using criteria based on both Animal Welfare Act and USPHS’s “Guide for the Care and Use of Laboratory Animals.” Sixty mice were assigned to one of four groups: non–tumor-bearing vehicle-treated mice, tumor-bearing vehicle-treated mice, non–tumor-bearing ZOL-treated mice, and tumor-bearing ZOL-treated mice.

General anesthesia of mice was induced and maintained with inhaled isofluorane. One million (1 × 10⁶) SCCF2Luc cells in 0.1 mL were injected through the maxillary gingiva into the perimyillary gingival submucosa using a 1-mL syringe and 26-gauge needle (BD). Non–tumor-bearing mice were similarly anesthetized and injected with cell-free PBS. This protocol reliably yields xenograft growth, which invades maxillary and premaxillary bone, and is associated with osteoclastic bone resorption. Treatment was initiated 7 days following injection of SCCF2Luc cells and consisted of twice weekly s.c. injections of ZOL at a dose of 100 μg/kg. Non–tumor-bearing mice received twice weekly injections of vehicle at the same volume. Two mice met early removal criteria and were removed from the study (one ZOL-treated, tumor-bearing mouse and one vehicle-treated, non–tumor-bearing mouse). Mice were euthanized by cervical dislocation during isofluorane anesthesia on the 28th day of treatment, and blood was collected for determination of plasma PTHrP and total calcium concentrations. Tissues were fixed in 10% neutral buffered formalin at 4°C for 48 hours, followed by storage in 70% ethanol at 4°C until histologic processing and evaluation.

**BLI**

In vivo BLI was performed with the IVIS 100 system as previously described (20). Results were analyzed using LivingImage software, version 2.2 (Caliper Life Sciences). Mice were injected i.p. with 4.3 mg d-Luciferin in sterile PBS and imaged while under isofluorane anesthesia. A circular region of interest (ROI) surrounding the xenograft was used to determine the radiance (photons per second) for each xenograft. Peak ROI radiance was used for comparisons. ROI radiance was normalized by dividing radiance at days 7, 21, and 28 by the radiance at day 1.

**Faxitron radiography and microCT**

The mandible was removed from each skull, and the degree of bone loss was evaluated qualitatively using a Faxitron cabinet X-ray system (Hewlett-Packard) at 45 kVp for 3.5 minutes. Bone loss was evaluated quantitatively using microCT (Siemens Inveon Preclinical CT scanner and Inveon Research Workplace 3-Dimensional Image Software, Siemens AG). Image acquisition, ROI selection, and thresholding values are provided in Supplementary Materials and Methods. ROI bone surface area and bone volume were compared between treatment groups.

**Histopathology, tartrate-resistant acid phosphatase histochemistry, and histomorphometry**

The skulls were decalcified in 10% EDTA (pH 7.4) at 4°C for 14 days. Rostral skull with xenograft tumor was paraffin embedded, sectioned at 5 μm, and stained with H&E followed by microscopic evaluation. Invasiveness was determined by visually identifying tumor cells at the level of the periodontal ligament of the maxillary incisor and beneath nasal respiratory epithelium. Xenografts were evaluated histologically for qualitative evidence of necrosis and apoptosis in addition to overall tumor vascularity. Slides were scanned using the Aperio ScanScope slide scanner (Aperio). Two-dimensional tumor area was determined using morphometry tools in Imagescope software (Aperio). The degree of maxillary bone...
loss was determined by comparing maxillary bone area between the non–tumor-bearing side and the tumor-bearing side for vehicle- and ZOL-treated mice. Maxillary bone was classified as either preexisting (mature) bone or tumor-induced new bone (immature) based on collagen pattern (woven or lamellar), osteocyte density, and anatomic location.

Enzymatic histochemistry for tartrate-resistant acid phosphatase (TRAP, Sigma-Aldrich) was performed on tissue sections as previously described (22) and on murine calvarial bone cocultured with SCCF2 cells in vitro (Supplementary Materials and Methods). Bone histomorphometry was completed with the Imagescope software. The average percentage of perimeter of eroded bone, number of active osteoclasts, and average osteoclast length were compared between treated and untreated tumor-bearing mice. Sections with a minimum of 3 mm of direct tumor-bone interface were included (13 vehicle-treated mice and 12 ZOL-treated mice).

**PTHRP and calcium concentration assays**

SCCF2 secretion of PTHrP in vitro (Supplementary Materials and Methods) and mouse plasma concentrations of PTHrP were measured using a commercially available two-site immunoradiometric PTHrP (1–86) assay (Diagnostic Systems Laboratories, Inc.). Plasma calcium concentration was measured using a commercially available calcium assay kit (Bioassay Systems). The effect of SCCF2-derived PTHrP on MC3T3 cell (murine proosteoblast) expression of receptor activator of NF-κB (RANK) ligand (RANKL) was evaluated in vitro using a previously validated chicken anti-human PTHrP neutralizing antibody (refs. 23, 24; Supplementary Materials and Methods).

**Statistical analysis**

Results were displayed as means ± SEM. Normality was determined with the Shapiro-Wilk test. Normally distributed, continuous data were analyzed using Student’s t test, and multiple group comparisons were made by one-way ANOVA followed by Bonferroni’s post hoc test. In the event the data were not normally distributed, a Wilcoxon rank-sum (Mann-Whitney) test was performed. Data with P values of <0.05 were considered statistically significant. When multiple comparisons were made within a data set (maxillary histomorphometry), the P value of 0.05 divided by the number of comparisons (7) was accepted as significant (P = 0.007). Categorical data (presence of invasion) were analyzed using Fisher’s exact test. All analyses were performed with STATA intercooled 10 software.

**Results**

**ZOL treatment reduced tumor growth**

Bioluminescent signal was detectable in all mice at all time points, with no initial difference between vehicle- and ZOL-treated groups. Tumor formation became visibly evident as mild facial swelling at 7 to 14 days after injection of tumor cells. Gross tumor formation occurred in all mice, which was located at the junction of maxillary and premaxillary bone on the lateral aspect of the skull.

At the 28th day of treatment, ZOL treatment reduced tumor radiance by 43% (P = 0.0097, two-tailed t test; Fig. 1). Histomorphometric evaluation of tumor sections revealed that ZOL treatment reduced tumor area (total area minus area of central necrosis) by 14% (P = 0.075, two-tailed t test; data not shown). The greater difference in tumor growth detected with BLI was attributed to the higher specificity of BLI for viable tumor cells with exclusion of necrotic areas and stromal elements, in addition to normalization to initial radiance measurements, which corrected for variation in the number of injected tumor cells.

**ZOL reduced loss of bone surface area and volume**

F Paxitron radiography revealed reduced bone loss and increased periosteal new bone formation in the region of xenograft growth in the ZOL-treated mice compared with vehicle-treated mice (Fig. 2A). Reconstructed, three-dimensional skull images (Fig. 2B) revealed that ZOL treatment reduced, but did not eliminate, loss of bone at the tumor-bone interface and was accompanied by extensive bone remodeling with new bone formation. Quantitative determination of bone volume and surface area was performed on a 2-mm-thick maxillary ROI (Fig. 2C). Xenograft growth in vehicle-treated mice reduced bone surface area by 14% (P < 0.0001, ANOVA) and bone volume by 19% (P < 0.0001, ANOVA; Fig. 3). There was no statistically significant loss of bone surface area and volume in ZOL-treated, tumor-bearing mice compared with non–tumor-bearing mice. ZOL treatment increased bone surface area and bone volume in tumor-bearing mice by 23% and 39%, respectively (P < 0.0001, ANOVA) compared with untreated tumor-bearing mice.

Figure 1. ZOL treatment reduced SCCF2Luc xenograft growth. At the 28th day of treatment, ZOL treatment reduced tumor radiance by 43% (P = 0.0097, two-tailed t test; Fig. 1). Histomorphometric evaluation of tumor sections revealed that ZOL treatment reduced tumor area (total area minus area of central necrosis) by 14% (P = 0.075, two-tailed t test; data not shown). The greater difference in tumor growth detected with BLI was attributed to the higher specificity of BLI for viable tumor cells with exclusion of necrotic areas and stromal elements, in addition to normalization to initial radiance measurements, which corrected for variation in the number of injected tumor cells.
**ZOL reduced microscopic evidence of maxillary bone loss and induced osteoclast degeneration but did not reduce invasion**

SCCF2Luc xenografts were composed of islands and trabeculae of malignant epithelial cells showing variable degrees of squamous differentiation and keratinization with regions of central necrosis. There was no difference in xenograft cellular morphology between vehicle- and ZOL-treated mice. SCCF2Luc xenografts were poorly vascularized; however, vessels seemed slightly more numerous and larger at the tumor-bone interface and were associated with larger aggregates of tumor cells. This pattern was observed in mice of both vehicle- and ZOL-treated groups.

SCCF2 cells induced osteoclast formation and bone resorption *in vitro* (Supplementary Fig. S1), and SCCF2Luc xenografts were associated with marked osteolysis of premaxillary and maxillary bone (Fig. 4A). ZOL inhibited osteoclast formation and bone loss *in vitro* (Supplementary Fig. S1) and *in vivo* (Fig. 4B). Despite reduced bone loss, ZOL had no effect on tumor invasion (Fig. 4A and B). Tumor invasion around the maxillary incisor was observed in 13 of 15 vehicle-treated mice and 12 of 14 ZOL-treated mice. Tumor invasion into the nasal passage was observed in 12 of 15 vehicle-treated mice and in 10 of 14 ZOL-treated mice.

Nine of 14 tumor-bearing, ZOL-treated mice had foci of bone necrosis within xenografts, and six of these mice also showed necrosis of the maxillary incisor pulp cavity. Bone necrosis was often located in regions of tumor necrosis and only occurred in mice with pronounced tumor invasion. Bone and tooth necrosis was not observed on the non–tumor-bearing side of the maxilla and was not observed in ZOL-treated, non–tumor-bearing mice. No vehicle-treated mice had evidence of tooth necrosis, although 1 of 15

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**Figure 2.** ZOL inhibited SCCF2Luc xenograft-induced osteolysis. A, faxitron radiography of maxilla from three representative mice from each treatment group (arrowheads, xenograft margins; arrows, osteolysis; asterisks, new bone formation). ZOL reduced osteolysis and increased periosteal new bone formation compared with vehicle-treated mice. B, three-dimensional renderings of microCT data. Normal premaxillary bone (black arrow) and maxillary bone (white arrow) are indicated on the sham + vehicle mouse. SCCF2Luc xenografts in vehicle-treated mice were associated with marked loss of lateral premaxillary and maxillary bone revealing the root of the maxillary incisor (white arrows). ZOL reduced osteolysis of maxillary and premaxillary bone (minimal exposure of incisor root, black circle) and increased periosteal new bone formation. C, a 2-mm-thick ROI was selected (gray band), allowing bone surface area and volume to be calculated and compared between treatment groups.
vehicle-treated tumor-bearing mice had evidence of bone necrosis.

TRAP-positive osteoclasts were observed at the tumor-bone interface in treated and untreated mice; however, osteoclasts in ZOL-treated mice (Fig. 4D) had variable degrees of cytoplasmic vacuolar degeneration (numerous, variably sized, circular, clear cytoplasmic vacuoles) compared with those in vehicle-treated mice (Fig. 4C).

**Zolecronic acid reduced loss of maxillary bone area and resulted in fewer osteoclasts**

Maxillary bone area was determined using Imagescope software (Fig. 5A). There was no difference in total bone area on the non–tumor-bearing side of vehicle- and ZOL-treated mice. Xenograft growth was associated with a 48% reduction in total bone area (preexisting bone and new bone combined) compared with the non–tumor-bearing side ($P = 0.0002$, Wilcoxon rank-sum), whereas ZOL treatment prevented loss of total bone area. Xenograft growth was associated with a 64% reduction in preexisting bone ($P = 0.0003$, Wilcoxon rank-sum) in vehicle-treated mice compared with a 33% reduction in preexisting bone in ZOL-treated mice ($P = 0.0002$, two-tailed $t$ test). ZOL-treated mice retained 2-fold more total bone compared with vehicle-treated mice ($P = 0.0004$, Wilcoxon rank-sum), and 2-fold more preexisting bone compared with vehicle-treated mice ($P = 0.005$, Wilcoxon rank-sum). $P$ values of <0.007 were considered statistically significant (adjusted for multiple comparisons). The preservation of total bone in ZOL-treated mice, as detected by microCT and histomorphometry, was attributed to reduced loss of preexisting bone and increased new bone formation.

Activated osteoclasts at the xenograft-bone interface were detected using TRAP histochemistry. There was no statistically significant difference in the ratio of eroded to total bone surface between ZOL- and vehicle-treated mice (Fig. 5B); however, ZOL reduced the number of osteoclasts per millimeter of bone surface by 52% ($P = 0.0003$, two-tailed $t$ test). The average osteoclast length was increased in ZOL-treated mice by 39% ($P = 0.0001$, Wilcoxon rank-sum).

**Plasma PTHrP was increased in tumor-bearing mice**

SCCF2 cells secreted PTHrP in vitro (Fig. 6B), and SCCF2Luc xenograft growth resulted in elevated plasma PTHrP in vivo (Fig. 6A). Tumor-bearing mice ($n = 12$) had a 2.7-fold increase ($P = 0.037$, ANOVA) in plasma PTHrP (0.65 pmol/L versus 2.42 pmol/L) compared with non–tumor-bearing control mice ($n = 5$). ZOL treatment resulted in a nonstatistically significant, 20% reduction in plasma PTHrP in tumor-bearing mice. Elevated plasma PTHrP in tumor-bearing mice was not associated with hypercalcemia (Fig. 6A). SCCF2 cells secreted PTHrP into conditioned medium, and addition of bone-conditioned medium to SCCF2 cultures increased SCCF2 secretion of PTHrP (Fig. 6B). SCCF2conditioned medium stimulated expression of RANKL in MC3T3 cells (a murine preosteoblast cell line; Supplementary Fig. S2) but did not have a significant effect on MC3T3 expression of osteoprotegerin (data not shown). Neutralization of SCCF2-derived PTHrP in conditioned medium with a neutralizing antibody inhibited the expression of RANKL in MC3T3 cells (Supplementary Fig. S2).

**Discussion**

There are few preclinical, in vivo models that recapitulate the bone invasive behavior of OSCC. In vivo OSCC studies have been reported using two osteolytic OSCC cell lines: BHY, derived from a human gingival OSCC (25), and SCCVII, derived from a C(3)H/HeN mouse OSCC from ventral mouth (26, 27). Other cell lines showing variable bone invasion in vivo include UMScC1 (28), UMScC11A, UMScC11B (29), BICR31, BICR56 (30), and HSC3 (12). We have designed a novel, bone-invasive, bioluminescent orthotopic xenograft nude mouse model of OSCC using SCCF2Luc cells derived from SCCF2 cells.
from a feline OSCC. SCCF2Luc cells reliably develop into invasive xenografts without requiring coinjection of fibroblasts (required for BHY cells; ref. 25) or Matrigel (required for UMSCC11A cells; ref. 29). Tumor progression in the SCCF2Luc model are typical of spontaneous bone-invasive OSCC, showing rapid tumor growth with bone invasion characterized by osteoclastic bone resorption.

ZOL treatment reduced tumor growth and bone loss in the SCCF2Luc xenograft model and was associated with a reduced number of activated osteoclasts at the tumor-bone interface. These results are similar to those reported by Cui and colleagues (2005), who showed that a third generation bisphosphonate (YM529, minodronic acid) reduced osteoclast number and tumor growth in the SCCVII syngeneic

**Figure 4.** ZOL reduced SCCF2Luc-associated osteolysis and induced vacuolar degeneration of osteoclasts but did not reduce tumor invasion. Photomicrographs of H&E-stained tissue sections. A, SCCF2Luc xenografts were associated with marked osteolysis of premaxillary and maxillary bone (white asterisks) along the infiltrating tumor front (black arrows; bar, 500 μm). Higher magnification from A (box), illustrating islands of squamous carcinoma cells (arrows) infiltrating the remaining bone (white asterisk; bar, 100 μm). B, treatment with ZOL reduced resorption of premaxillary and maxillary bone (black asterisks; bar, 500 μm). Despite reduced bone resorption, higher magnification (box) revealed islands of squamous carcinoma cells infiltrating the bone and the periodontal ligament (black arrows; bar, 100 μm). TRAP-positive osteoclasts were observed at the tumor-bone interface of bone in vehicle-treated (C) and ZOL-treated (D) mice. Osteoclasts were multinucleated (small black arrows) and, in ZOL-treated mice (D), were characterized by vacuolar degeneration (white arrows). Islands of OSCC are indicated by "T." Bar, 50 μm.
mouse model of bone invasive OSCC (26). In contrast to our study, Cui and colleagues injected SCCVII cells percutaneously into the masseter muscle adjacent to the mandible rather than orthotopically into the oral submucosa, and they did not quantify the effects of YM29 treatment on tumor-induced bone loss. The addition of complete histomorphometric characterization of bone loss in our study not only corroborated the subjective and objective findings of the radiographic and microCT examination but showed the effect of ZOL treatment on preexisting bone independent of periosteal new bone formation.

There are several possible mechanisms for reduced osteoclastic bone resorption in the ZOL-treated mice. ZOL inhibits osteoclastic bone resorption by inducing osteoclast apoptosis through impaired prenylation of small GTPases as a result of farnesyl transferase inhibition (31), accumulation of unprenylated small GTPases in their active state with inappropriate activation of downstream signaling pathways, and the induction of an ATP analogue, which induces direct apoptosis (32). Additionally, ZOL has also been shown to increase the in vitro expression of the osteoclast inhibitor osteoprotegerin in human osteoblasts (33). Our study revealed that osteoclasts in ZOL-treated mice continued to form and maintained their ability to resorb bone but existed in reduced numbers and showed evidence of vacuolar degeneration. The mechanism of the vacuolar degeneration is not known; however, a study by Coxon and colleagues showed that targeted inhibition of the small GTPase Rab resulted in reduced osteoclast function accompanied by dome-shaped cell morphology and large intracellular vacuoles, which was attributed to disruption of Rab-dependent intracellular membrane trafficking in osteoclasts (34). In our study, accumulation of numerous cytoplasmic vacuoles may have contributed to the increased osteoclast diameter (length) observed in the ZOL-treated mice.

ZOL significantly reduced, but did not completely inhibit, bone resorption in this model of bone-invasive OSCC. Interestingly, tumor-bearing mice continued to lose preexisting bone during ZOL therapy, albeit at a reduced level, and showed increased periosteal new bone formation. This may be explained by differential affinity of ZOL for different bone surfaces. ZOL is reported to be incorporated more readily into bone, as it is being formed by osteoblasts and into bone exposed by osteoclast activity compared with quiescent bone surfaces (31). Periosteal new bone formation would be relatively spared from bone resorption because of the higher levels of ZOL expected to be incorporated into the matrix as it is formed (31). ZOL treatment did not totally eliminate the presence of osteoclasts; therefore, it seems that osteoclasts in the tumor-bone microenvironment are at least partially protected from the proapoptotic effects of ZOL. Interestingly, a recent in vitro study revealed that a human OSCC cell line was capable of reducing osteoclast apoptosis through down-regulation of the proapoptotic factor Bim in osteoclasts (35).

ZOL has been shown to inhibit tumor growth in other models through direct antineoplastic mechanisms, which include inhibition of cancer cell adhesion, invasion, viability,

![Figure 5. ZOL-mediated reduction in bone loss was characterized by reduced loss of preexisting bone and reduced number of bone-resorbing osteoclasts at the tumorbone interface. A, SCCF2Luc xenografts were associated with a 48% reduction in total bone area (preexisting bone and new bone combined) compared with the non-tumor-bearing side (\( \ast, P = 0.0002 \)). ZOL prevented loss of total bone area. Vehicle-treated mice lost 64% of preexisting bone area (\( \ast, P = 0.0003 \)) compared with 33% in ZOL-treated mice (\( \ast, P = 0.0002 \)). ZOL-treated mice retained 2-fold more total bone compared with vehicle-treated mice (\( \ast, P = 0.0004 \); Wilcoxon rank-sum) and 2-fold more preexisting bone compared with vehicle-treated mice (\( \ast, P = 0.005 \)). B, histomorphometric evaluation of TRAP-stained sections revealed no difference in the percentage of eroded bone between ZOL- and vehicle-treated mice; however, ZOL reduced the number of osteoclasts per millimeter by 52% (\( \ast, P = 0.0003 \)). The average osteoclast length was increased in the ZOL-treated mice by 39% (\( \ast, P = 0.0001 \)).](image-url)
It is likely that ZOL-mediated inhibition of osteoclastic bone resorption contributed to reduced xenograft growth in this model. Culture of SCCF2 cells in bone-conditioned medium stimulated PTHrP secretion in vitro, and SCCF2-derived PTHrP stimulated murine preosteoblast expression of RANKL. ZOL showed in vitro and in vivo inhibition of SCCF2-associated bone resorption. Interestingly, ZOL treatment reduced the average plasma PTHrP concentration in tumor-bearing mice by 20%, although statistical significance was not reached. Taken together, the in vitro and in vivo data support the role of tumor-derived PTHrP in a vicious cycle of OSCC growth and osteoclastic bone resorption. Bone-derived factors that may have stimulated SCCF2 expression of PTHrP include TGF-β, fibroblast growth factor, insulin-like growth factors IGF-I and IGF-II, platelet-derived growth factor, and bone morphogenic proteins (42). By reducing osteoclastic bone resorption, ZOL has the potential to reduce the liberation of stored growth factors, making a less favorable environment for the growth of tumor cells (33).

OSCC expression of PTHrP influences in vitro tumor cell proliferation, migration, and invasiveness (43) and participates in the regulation of osteoclastogenesis by increasing stromal cell expression of RANKL (12). A small subset of OSCC patients experienced hypercalcemia with increased serum PTHrP concentration (44). Plasma PTHrP concentrations were increased in mice bearing SCCF2Luc xenografts. Although there was no evidence of a humoral role of PTHrP in this model (lack of hypercalcemia), it is likely that tumor-derived PTHrP in the xenograft-bone microenvironment functioned in a paracrine manner to stimulate osteoclastic bone resorption by increasing RANKL expression in osteoblasts.

Osteonecrosis of the jaw (ONJ) is occasionally observed in cancer patients receiving aminobisphosphonate therapy (estimated incidence of 5–10%; ref. 45) and is characterized by exposure of mandibular or maxillary bone, which fails to heal over a period of 6 to 8 weeks (46). In this study, ZOL was well tolerated in both the tumor-bearing and non–tumor-bearing mice, and there was no evidence of ONJ. The presence of tumor-associated necrotic bone within xenografts of ZOL-treated mice was attributed to the retention of bone undergoing necrosis as a result of tumor infiltration. Bone necrosis would be expected in the vehicle-treated mice as well; however, the robust osteoclastic response in vehicle-treated mice would have removed necrotic bone as it developed. The cause of tooth necrosis in ZOL-treated mice was not evident but seemed as an extension of bone necrosis and occurred in mice that showed tumor infiltration near the base of the tooth root. It is possible that OSCC patients with existing bone disease of the mandible or maxilla may be at increased risk for the development of ONJ; however, the potential benefits of protecting bone volume and function in cases of nonresectable, bone-invasive cancer may outweigh the risks in some patients.

ZOL inhibited OSCC-induced osteolysis and reduced tumor growth in this xenograft mouse model; however, the presence of invasion was unchanged. The results of this experiment suggest that ZOL would be valuable as an adjunct and angiogenesis (36, 37). For example, the antiangiogenic activity of bisphosphonates is associated with reduced circulating levels of vascular endothelial growth factor in humans with metastatic bone disease (38) and in cats with OSCC (39). There was no histologic evidence of an antiangiogenic effect of ZOL treatment in this study. Although a proapoptotic activity of ZOL in tumor cells has been shown in human cell lines (40, 41), evaluation of ZOL-treated SCCF2Luc tumors did not reveal microscopic evidence of increased necrosis or apoptosis. The complex pharmacokinetics of ZOL in the bone microenvironment pose significant challenges to in vitro studies attempting to model the exposure of tumor cells to clinically relevant concentrations of ZOL and make comparisons of in vitro and in vivo cytotoxicity difficult.
therapy with the purpose of maintaining mandibular and maxillary bone volume and function, particularly if combined with antineoplastic agents capable of reducing tumor invasion. We have provided evidence supporting the role of OSCC-derived PTHrP in a vicious cycle of tumor growth and bone resorption during OSCC progression. Further studies are required to characterize the factors released from bone, which stimulate OSCC growth and PTHrP secretion, and to determine if ZOL may provide an additive or synergistic antitumor effect when combined with other therapeutic modalities for the treatment of bone invasive OSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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