

# Zoledronic acid inhibits osteosarcoma growth in an orthotopic model

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## Abstract

Zoledronic acid (ZOL) has been shown to reduce osteolysis in bone metastasis. Its efficacy in osteosarcoma has not been convincingly proved in a clinically relevant model for the disease. *In vitro*, ZOL decreased osteosarcoma cell proliferation, mainly due to an increase in apoptosis in a dose-dependent fashion. There was a decrease in cell migration at  $\geq 10 \mu\text{mol/L}$  concentrations, but invasion was inhibited at a much lower dose of  $0.1 \mu\text{mol/L}$ . Reverse transcription-PCR showed that ZOL overall caused an increased expression of osteocalcin and decreased expression of alkaline phosphatase, osteopontin, osteonectin, and vascular endothelial growth factor, with no change in expression of osteoprotegerin. ZOL administration s.c. twice weekly at  $0.12 \text{ mg/kg}$  to SaOS-2 tumor-bearing mice resulted in primary tumor growth inhibition, reduction in lung metastases, and dramatic decrease in osteolysis. Furthermore, in the ZOL cohort, there was a clear reduction in the number of osteoclasts in bone exposed to tumor and a lower tumor vessel density. These data point to the adjuvant potential of ZOL in the management of osteosarcoma not only for its antiosteolytic properties but also for its ability to directly halt tumor cell growth and metastasis via its effects on viability, invasion, differentiation, and angiogenesis. [Mol Cancer Ther 2007;6(12):3263–70]

## Introduction

Osteosarcoma is a debilitating, if not fatal, high-grade malignant bone neoplasm that targets children and adolescents. Recently, the prognosis of these patients has improved substantially owing to the development of

various adjuvant chemotherapies. However, these chemotherapies are not fully effective, and as a result, 20% of all patients die due to metastasis of osteosarcoma cells to the lungs (1). As a consequence, various new osteosarcoma therapies are presently in clinical trials around the world.

Bisphosphonates are widely used to treat a variety of bone pathologies that revolve around excessive bone resorption and destruction (2). Third-generation bisphosphonates such as zoledronic acid (ZOL) show direct antitumor effects and synergistically augment the effects of anticancer agents in various cancer cell lines (3, 4). Investigators have recently reported the antiosteosarcoma effects of novel bisphosphonates *in vitro* (5–9) and *in vivo* (10, 11). However, the field is still in its infancy, and more thorough analyses of the pharmacodynamics due to ZOL in clinically relevant models need to be done.

Previously, we have shown that the rat osteosarcoma cell line UMR 106-01 expresses both receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and osteoprotegerin, the key regulators of osteoclastogenesis by Northern blot analysis (12). We also studied the expression of RANKL and osteoprotegerin in our *in vivo* UMR 106-01 osteosarcoma model and observed that both molecules are expressed in the tumor. Subsequently, we also showed that the tumor recruited osteoclasts to the resorption areas on the surface of trabecular and cortical bone adjacent to the tumor.<sup>3</sup> Thus, these observations indicate that osteosarcoma has the apparatus to regulate osteoclastogenesis.

When UMR 106-01 osteosarcoma cells were treated with pamidronate *in vitro*, we observed inhibition of proliferation, induction of apoptosis, and down-regulation of RANKL (12). We also showed a reduction in lung metastases in our *in vivo* osteosarcoma model when treated with pamidronate in a preliminary study.<sup>3</sup> *In situ* hybridization and tartrate-resistant acid phosphatase staining of the tumor tissue showed a significant reduction in RANKL expression and a decrease in the number of osteoclasts, respectively. The observation that pamidronate inhibited osteoclastogenesis paved the way for our current project.

We hypothesized that treatment with ZOL would lead to inhibition of bone resorption, leading to delay in the onset of skeletal complications, down-regulation of tumor growth, and development of metastases in mice injected with osteosarcoma cells. Our objectives were as follows: to treat SaOS-2 human osteosarcoma cells with ZOL and to compare the characteristics of the cells such as proliferation, apoptosis, migration, invasion, and the potential of the cells to induce osteoclastogenesis; to assess the pattern of tibial tumor formation and metastasis in mice injected with SaOS-2 cells following treatment with ZOL; to examine

Received 8/9/07; revised 10/8/07; accepted 10/15/07.

**Grant support:** The Australian Orthopaedics Association, the Victorian Orthopaedics Research Trust, and the Cancer Council Victoria.

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doi:10.1158/1535-7163.MCT-07-0546

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osteoclastogenesis and the expression of osteoprotegerin, alkaline phosphatase, osteopontin, osteonectin, and osteocalcin in treated and untreated SaOS-2 cells; and to evaluate whether ZOL has direct regulatory effects on angiogenesis in osteosarcoma by examining the expression of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor.

## Materials and Methods

### Cell Culture

The SaOS-2 cell line, derived from a primary osteosarcoma in a 11-year-old girl (13), was attained from the American Type Culture Collection and used within 20 passages. Cells were propagated in complete medium of  $\alpha$ -MEM plus 10% FCS. Single-donor human bone marrow-derived mononuclear cells were obtained from Cambrex and cultured in Eagle's MEM/pyruvate/glutamine plus 10% FCS. All cells were used at a seeding viability of >95% as determined by trypan blue dye exclusion.

### Proliferation Assay

SaOS-2 cells were seeded at a density of 10,000 per well. Cells were treated with different concentrations of ZOL (0, 0.01, 0.1, 1, 10, and 100  $\mu$ mol/L; Novartis Australia) and plates incubated for 3 days. Cells were enumerated with trypan blue and a hemacytometer chamber. Nuclei were also enumerated with 4',6-diamidino-2-phenylindole and observed under a Nikon Eclipse TE-2000U microscope and SPOT Advanced software (SciTech) with a UV filter.

### Apoptosis Assay

SaOS-2 cells were seeded at a density of 10,000 per well. Cells were treated with different concentrations of ZOL (0, 0.01, 0.1, 1, 10, and 100  $\mu$ mol/L) and plates incubated for 3 days. Apoptotic cells were enumerated under high-power magnification after staining with the DEAD-End TUNEL assay kit (Promega) according to the manufacturer's instructions.

### Migration Assay

For the migration assay (14), chamber inserts with 8- $\mu$ m filter membranes (BD Biosciences) were used as supplied and all wells filled with complete growth medium. Cells were seeded in serum-free medium at a density of  $5 \times 10^4$  cells per insert inside the wells in duplicate, aliquoted with different concentrations of ZOL (0, 0.01, 0.1, 1, 10, and 100  $\mu$ mol/L), and incubated for 24 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. Cells that migrated through the pores toward serum-free medium containing 10  $\mu$ g/mL fibronectin (Sigma-Aldrich) onto the underside of the membranes were stained with Quick-Dip (Froline) and quantitated under high-power magnification.

### Invasion Assay

Twenty-four-well companion plates were filled with SaOS-2 complete medium. Modified Boyden chamber filter inserts were coated with 25% Matrigel (BD Biosciences). Inserts were placed into the medium-filled wells of the companion plates (14). Cells (50,000) were aliquoted to each insert. Cells were treated with different concentrations of ZOL (0, 0.01, 0.1, 1, 10, and 100  $\mu$ mol/L) and plates

incubated for 3 days. Cells were enumerated at  $\times 100$  magnification.

### Osteoclastogenesis Assay

Single-cell suspensions of SaOS cells were plated onto coverslips and placed in 24-well plates and incubated for 12 h. The wells were overlaid with single-cell suspensions of human bone marrow-derived mononuclear cells. Macrophage colony-stimulating factor (Sigma-Aldrich) was added to the cells at 50 ng/mL. Cells were treated with different concentrations of ZOL (0, 0.01, 0.1, 1, 10, and 100  $\mu$ mol/L) and incubated for 7 days. Osteoclasts were identified by tartrate-resistant acid phosphatase staining according to the manufacturer's (Sigma-Aldrich) instructions and quantified. Paraformaldehyde-fixed, paraffin-embedded sections were deparaffinized and stained for tartrate-resistant acid phosphatase and counterstained with hematoxylin.

### RNA Extraction and Reverse Transcription-PCR Evaluation of Common Bone Markers

mRNA from cultured cells (at 24 and 72 h posttreatment) and tumors was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen). Human mRNA reverse transcription-PCR primers were as follows: glyceraldehyde-3-phosphate dehydrogenase, forward ACCACCATGGAGAAGGCTGG and reverse CTCAGTGTAGCCCAGGATGC; alkaline phosphatase, forward CACTGAAATATGCCCTGGAGC and reverse CCTGATGTTATGCATGAGCTGG; VEGF, forward CCAGCACATAGGAGAGATGAGC and reverse CACCGCCTCGGCTGTGT-CACAT; osteocalcin, forward GAGAGCCCTCACATCCTCG and reverse AGACCGGGCCGTAGAAGC; pigment epithelium-derived factor, forward TGTCTCCAATTCGGCTACGATC and reverse CGAGTCAAACCTTGGTTACCCACTG; osteonectin, forward TGCCTGATGAGACAGAGGTGG and reverse GGATCTTCTTCACACGCAGC; osteopontin, forward TTGCTTTTGCCTCCTAGGCA and reverse GTGAAAACCTTCGGTTGCTGG; and osteoprotegerin, forward TGCTGTTCTACAAAGTTTACG and reverse CTTTGAGTGCTTTAGTGCGTG (from Sigma-Genosys). An Eppendorf MasterCycler was used with 40 cycles at  $T_{\text{ann}} = 64^\circ\text{C}$ ,  $T_{\text{ext}} = 72^\circ\text{C}$ , and  $T_{\text{den}} = 94^\circ\text{C}$ . The Expand Reverse Transcriptase-PCR and PCR Master (Roche Diagnostics) kits were used according to the manufacturer's instructions. A water control (no cDNA template) was always included in each series of PCRs. PCR products were run on a 1% agarose gel electrophoresis and sizes confirmed with a 100-bp ladder marker set (Invitrogen).

### In vivo Study

All animal experimentations were approved by the St. Vincent's Health Animal Ethics Committee. SaOS-2 orthotopic tumors were established and tissues analyzed as previously described (15). The primary tumors were not resected at any stage during the study to promote metastasis because this is a spontaneously metastasizing model. From week 1 post-inoculation, mice were injected twice weekly at a dose of 0.12 mg/kg s.c. in the midback dorsum with ZOL. Placebo groups received the same volume of normal saline.

### Statistical Analyses

Data were analyzed for statistical significance with Student's *t* test (two-tailed).  $P \leq 0.05$  was considered significant unless otherwise indicated.

## Results

### Cytotoxicity of ZOL on Cultured SaOS-2 Cells

ZOL reduced the proliferation of SaOS-2 cells *in vitro* in a dose-dependent manner (Fig. 1A). At 0.01, 0.1, 1, 10, and 100  $\mu\text{mol/L}$ , the inhibitions were 0%, 18%, 43%, 58%, and 76%, respectively. Results were similar for both the trypan blue and 4',6'-diamidino-2-phenylindole staining procedures. There was a corresponding increase in the degree of apoptosis in a dose-dependent manner (Fig. 1B). At 0.01, 0.1, 1, 10, and 100  $\mu\text{mol/L}$ , the degrees of induction were 0%, 1%, 6%, 13%, 15%, and 69% above baseline, respectively.

### Effect of ZOL on Migration and Invasion of SaOS-2 Cells in Culture

ZOL was unable to perturb cellular migration toward fibronectin in modified Boyden chambers at concentrations  $\leq 1 \mu\text{mol/L}$  (Fig. 1C). At 10 and 100  $\mu\text{mol/L}$ , the inhibitions were 75% and 100%, respectively. These results are only in a minor manner due to cell cytotoxicity because the degree of cell death after 24-h treatment with ZOL does not exceed 5% and 10% at 10 and 100  $\mu\text{mol/L}$  ZOL, respectively. ZOL was strikingly potent in its action against invasion of cells through Matrigel with a 4% inhibition occurring at 10 nmol/L and 100% inhibition at 100 nmol/L onward (Fig. 1D). These results are only partially due to cell cytotoxicity because at ZOL concentrations of 0.01 to 1  $\mu\text{mol/L}$ , the degree of cell death after 72-h treatment with ZOL does not exceed 50%, but invasion is completely inhibited even at 0.1  $\mu\text{mol/L}$ .

### ZOL Decreases Osteoclastogenesis of Human Bone Marrow-Derived Mononuclear Cells and Alters SaOS-2 Cell Morphology

ZOL reduced the maturation of osteoclastic cells from human bone marrow monocyte cells in a dose-dependent

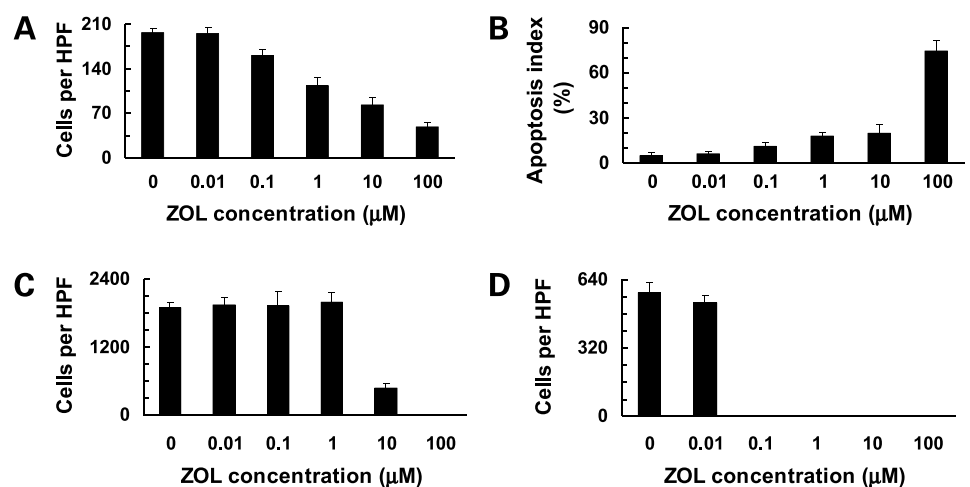
manner (Fig. 2A). At 0.01, 0.1, 1, 10, and 100  $\mu\text{mol/L}$ , the inhibitions were 1%, 6%, 13%, 15%, and 69%, respectively. SaOS-2 cells treated with 10  $\mu\text{mol/L}$  ZOL assumed a morphology (Fig. 2B) reminiscent of the phenomenon known as anoikis. Cell cytoplasm underwent significant shrinkage and the contacts between cell and plasticware were minimized, leading to cells assuming a rounded compact morphology.

### ZOL Alters mRNA Levels of Several Markers in SaOS-2 Cells

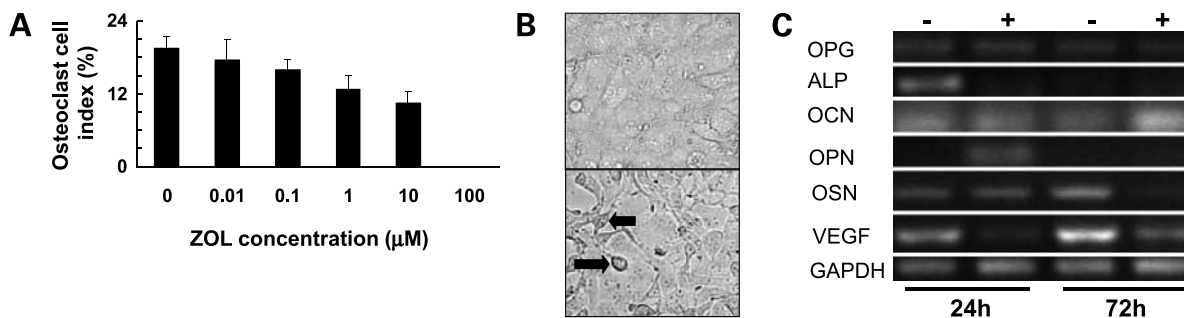
Figure 2C reveals results for reverse transcription-PCR analysis of RNA levels in samples treated with 10  $\mu\text{mol/L}$  ZOL after 24 and 72 h of treatment. There was a positive signal for osteopontin in ZOL-treated cells compared with untreated cells at 24 h but the level reached baseline at 72 h. The decrease was marked for alkaline phosphatase, which resulted in a sharp decrease to zero in signal intensity at 24 h when cells were treated with ZOL and did not recover at 72 h. There was an  $\sim 3$ -fold decrease in VEGF mRNA signal when cells were exposed to ZOL at both time points. There were no differences in osteoprotegerin and osteonectin signals as a result of ZOL treatment for 24 h, but levels dropped substantially at 72 h for osteonectin. Osteocalcin mRNA increased at 72 h.

### ZOL Reduces Primary and Secondary Osteosarcoma Growth in Mice

ZOL caused a significant decrease in tumor growth in SaOS-2-bearing mice. At 3 weeks, the ZOL-treated mice had a mean tumor volume of  $48 \pm 6 \text{ mm}^3$  and a mean tumor mass of  $40 \pm 46 \text{ mg}$  (Fig. 3A and B). In contrast, placebo mice had a mean tumor mass of  $147 \pm 43 \text{ mg}$  and a mean tumor volume of  $68 \pm 7 \text{ mm}^3$  (both  $P < 0.01$ ). At 5 weeks, the ZOL-treated mice had a mean tumor mass of  $330 \pm 60 \text{ mg}$  and a mean tumor volume of  $70 \pm 11 \text{ mm}^3$  (Fig. 3A and B). In contrast, placebo mice had a mean tumor mass of  $765 \pm 132 \text{ mg}$  and a mean tumor volume of  $195 \pm 21 \text{ mm}^3$  (both  $P < 0.01$ ). The number of macrometastases at 5 weeks decreased significantly from  $4.3 \pm 1.0$  to  $1.3 \pm 1.2$  in the ZOL-administered mouse cohort ( $P < 0.001$ ; Fig. 3C).



**Figure 1.** *In vitro* activity of ZOL against SaOS-2 human osteosarcoma cells. **A**, effect of varying concentrations of ZOL on cell proliferation. **B**, effect of varying concentrations of ZOL on apoptotic cell death. **C**, effect of varying concentrations of ZOL on cell migration. **D**, effect of varying concentrations of ZOL on cell invasion.



**Figure 2.** Effect of ZOL on osteoclastogenesis, cell morphology, and gene expression. **A**, effect of varying concentrations of ZOL on osteoclastogenesis in a coculture of SaOS-2 cells and human bone marrow – derived mononuclear cells in the presence of 50 ng/mL macrophage colony-stimulating factor. **B**, morphology of cells without (*top*) or with (*bottom*) 10 μmol/L ZOL. Magnification, ×200. **C**, mRNA analysis of cells without or with 10 μmol/L ZOL at 24 and 72 h.

At 3 weeks, the placebo group had tumors at the primary site that were palpable in contrast to ZOL-treated animals with some tumors unnoticeable (Fig. 4A and B). This difference was more exaggerated at 5 weeks when the placebo cohort showed significantly large tumors whereas the ZOL-treated mice showed modest tumor sizes (Fig. 4C and D). X-ray of tumor-containing hind-limbs of mice

revealed the ability of ZOL to reduce osteolysis due to SaOS-2 tumor growth at 3 weeks and more so at 5 weeks (Fig. 4E–H). The slight beneficial difference of ZOL at 3 weeks was amplified at 5 weeks. There were no visible signs of toxicity due to ZOL administration in mice as evidenced by no difference in the body weights (data not shown) between the cohorts and lack of overt signs of distress such as diarrhea, hunching, hyperactivity, or hypoactivity.

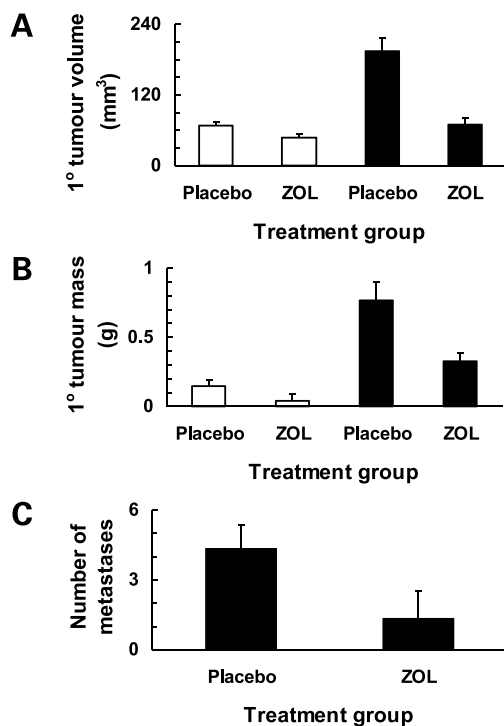
Histology confirmed the X-ray data that ZOL was beneficial in reduction of osteolysis at the site of tumor cell inoculation. There was markedly greater bone cortex degradation in the untreated group and this was exacerbated at 5 weeks (Fig. 4I–L). There was also a greater destruction of the growth plate cartilage layer of the tibiae in untreated mice, with tumors attempting to traverse this layer and causing massive lysis at the proximal end of the bone (data not shown). Confirming the results attained with counting macrometastases, histologic examination of representative lung sections revealed numerous secondary growth pockets within the lung parenchyma in untreated mice at both 3 and 5 weeks (Fig. 4M and N). Metastases were hard to visualize, if not nonexistent, in ZOL-treated lungs (Fig. 4O and P).

### ZOL Reduces Osteoclastogenesis and Vascular Density *In vivo*

Tumor sections stained for tartrate-resistant acid phosphatase revealed a significant ( $P < 0.001$ ) reduction in osteoclast generation at the growing tumor site as a result of ZOL treatment (Fig. 5). Figure 5A shows that the ZOL group exhibited only 6% of osteoclasts in comparison with the placebo cohort of mice. CD31 staining of tumor sections revealed a significant ( $P < 0.001$ ) reduction in CD31-stained cells in the neoplastic tissue when animals were on the ZOL regimen (Fig. 6). There was an almost 75% decrease in vessel density when animals were given ZOL (Fig. 6A).

### Discussion

ZOL has been used clinically for the treatment of several bone-related pathologies, including cancer. In one study, at baseline, all the patients with painful bone metastases from

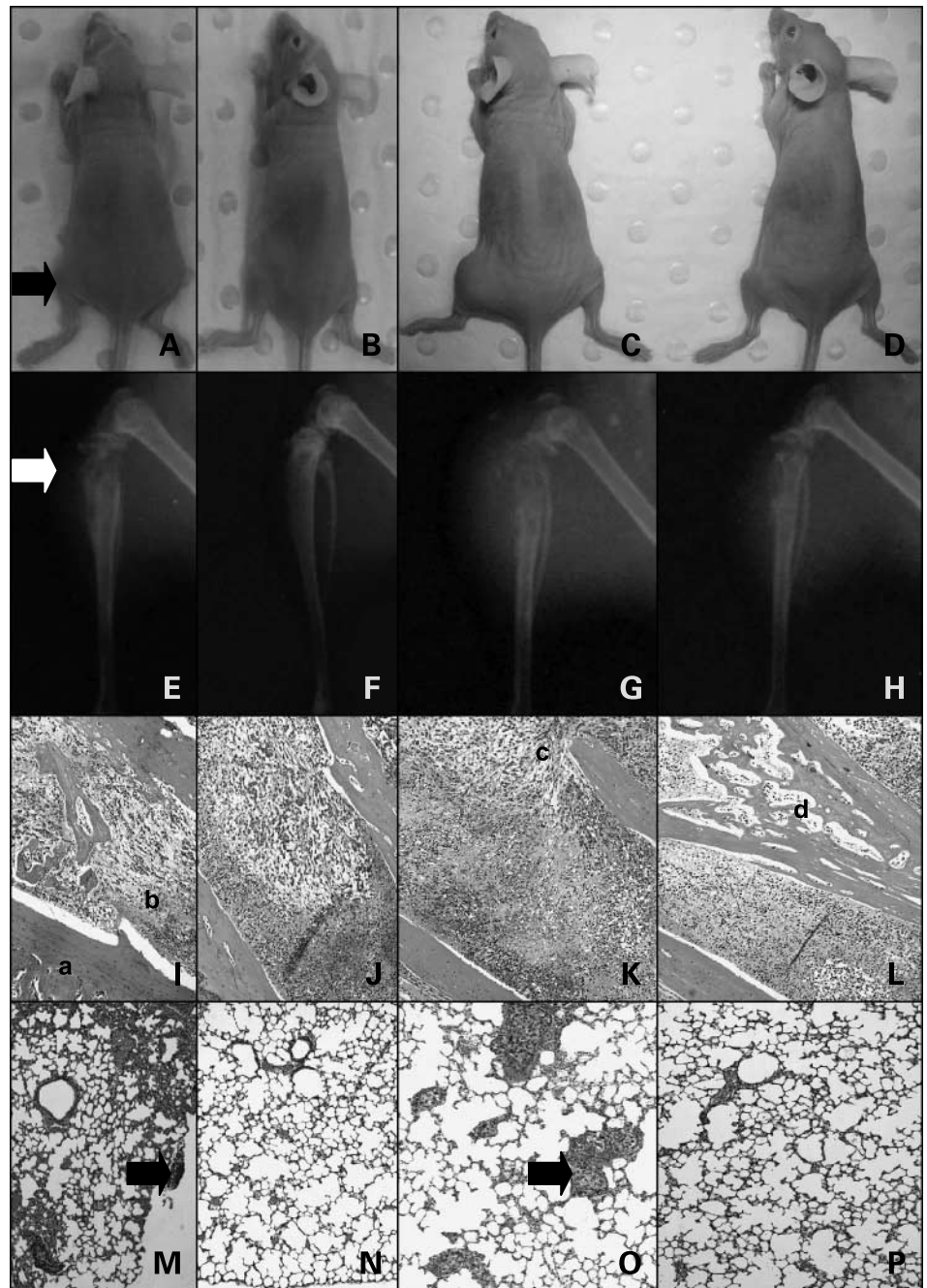


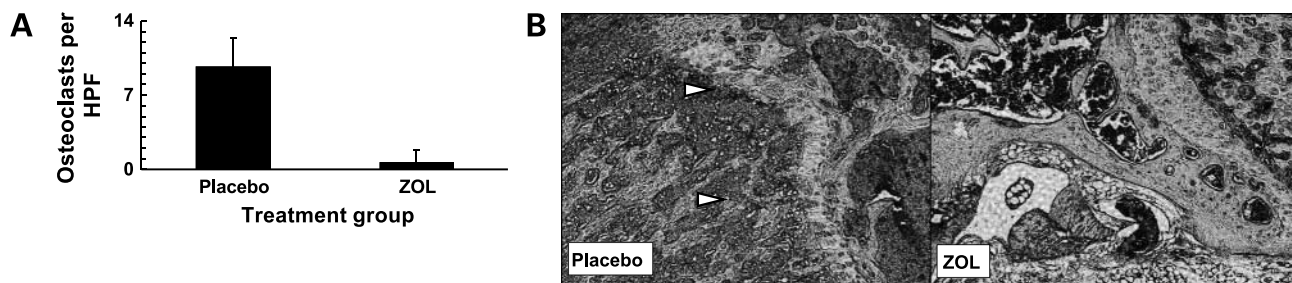
**Figure 3.** Efficacy of ZOL in orthotopic and spontaneously metastasizing osteosarcoma model using SaOS-2 cells. From week 1 post-inoculation, mice were injected with ZOL twice weekly at a dose of 0.12 mg/kg s.c. and placebo groups received the same volume of normal saline. **A**, primary tumor volumes of mice at 3 wks (*white columns*) and 5 wks (*black columns*) in the placebo or ZOL-treated groups ( $n = 6$ ). **B**, primary tumor mass of mice at 3 wks (*white columns*) and 5 wks (*black columns*) in the placebo or ZOL-treated groups ( $n = 6$ ). **C**, number of macrometastases in the lungs (nodules per lung) of mice in the placebo or ZOL-treated groups at 5 wks.

primary breast or prostate experienced a decrease in pain at rest and on movement after treatment with ZOL (16). This has followed some convincing data proving the beneficial effects of using ZOL for reducing bone destruction as a result of cancer spread from primary sites such as the breast in animal studies (17). The safety of prolonged use of ZOL in cancer patients, however, is an ongoing debate between the proponents (18, 19) and the opponents (20, 21) who refer to the tendency of this agent to cause osteonecrosis of the jaw in patients, although this is yet to be convincingly proved.

For osteosarcoma, studies examining the efficacy due to ZOL have only been centered around murine models using rodent osteosarcoma cells injected i.v. (11) or transplanted intraosseously (10). Both these models form pulmonary metastasis, resembling the clinical scenario. However, these murine cells do not allow a close examination of the effects of ZOL on human osteosarcoma cells. Whereas a handful of studies have looked at the effects of ZOL on human osteosarcoma cells *in vitro* (5, 6, 8, 9), there is no study done *in vivo*. The main reason for this discrepancy is that there was no clinically relevant model available for testing

**Figure 4.** Effect of ZOL on osteolysis and micrometastasis. **A**, representative mouse from the placebo group at 3 wks after tumor inoculation. *Arrow*, the left leg in which cells were injected. **B**, representative mouse from the ZOL group at 3 wks after tumor inoculation. **C**, representative mouse from the placebo group at 5 wks after tumor inoculation. **D**, representative mouse from the ZOL group at 5 wks after tumor inoculation. **E**, representative X-ray of the limb of a mouse in the placebo group at 3 wks after tumor inoculation. *Arrow*, site of primary tumor growth. **F**, representative X-ray of the limb of a mouse in the ZOL group at 3 wks after tumor inoculation. **G**, representative X-ray of the limb of a mouse in the placebo group at 5 wks after tumor inoculation. **H**, representative X-ray of the limb of a mouse in the ZOL group at 5 wks after tumor inoculation. **I**, representative H&E section of a limb of a mouse in the placebo group at 3 wks after tumor inoculation. *a*, intact bone cortex; *b*, tumor. Magnification,  $\times 100$ . **J**, representative H&E section of a limb of a mouse in the ZOL group at 3 wks after tumor inoculation. **K**, representative H&E section of a limb of a mouse in the placebo group at 5 wks after tumor inoculation. *c*, degraded bone cortex with tumor leakage into soft tissue. **L**, representative H&E section of a limb of a mouse in the ZOL group at 5 wks after tumor inoculation. *d*, region of bony destruction. **M**, representative H&E section of the lungs of a mouse in the placebo group at 3 wks after tumor inoculation. *Arrow*, a single micrometastasis. Magnification,  $\times 100$ . **N**, representative H&E section of the lungs of a mouse in the ZOL group at 3 wks after tumor inoculation. **O**, representative H&E section of the lungs of a mouse in the placebo group at 5 wks after tumor inoculation. *Arrow*, a single but larger micrometastasis than at 3 wks. **P**, representative H&E section of the lungs of a mouse in the ZOL group at 5 wks after tumor inoculation.





**Figure 5.** Effect of ZOL on *in vivo* osteoclastogenesis at the primary lesion site. **A**, number of osteoclasts observed at primary lesion site at 4 wks posttreatment. *HPF*, high-power field. **B**, microphotograph depicting osteoclasts stained for tartrate-resistant acid phosphatase activity (white arrowheads).

molecules such as ZOL (22). To address this issue, we recently developed and characterized a new model for osteosarcoma using the SaOS-2 cell line in a manner that closely mimics the clinical presentation and progression of the disease (14).

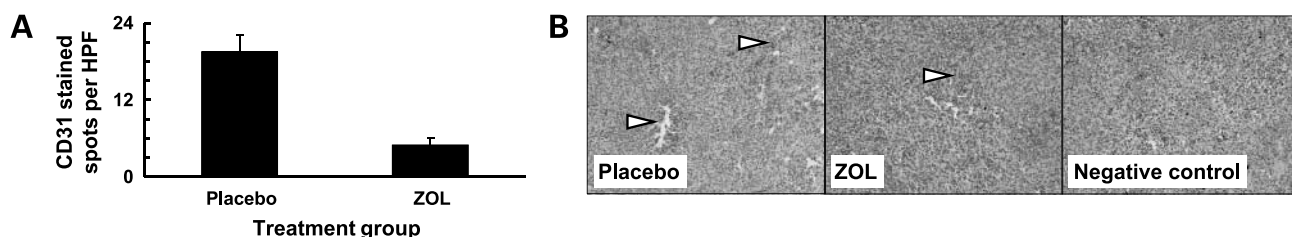
ZOL reduced the proliferation of SaOS-2 cells in a dose-dependent manner with a maximum of 76% at 100  $\mu\text{mol/L}$ . There was a corresponding increase in the degree of apoptosis in a dose-dependent manner with a maximum of 69% at 100  $\mu\text{mol/L}$ , suggesting that the cell loss was due mostly to apoptosis and very little to cell cycle stasis, as shown by other researchers (6, 23). However, Ory et al. (11) have shown that in mouse POS-1 osteosarcoma cells and rat OSRGA osteosarcoma cells, most of the growth-inhibitory activity is attributed to cell cycle arrest rather than apoptosis induction. Thus, the effect, although always growth inhibitory, may depend on the cell line used. An early study using ZOL with a human osteosarcoma cell panel showed that death via apoptosis was actually not reliant on caspase activation but more on a form of apoptotic death involving cell detachment referred to as anoikis (5). This study seminally showed that ZOL, in fact, was capable of direct cytostatic effects on osteosarcoma cells, albeit *in vitro*.

ZOL was unable to perturb cellular migration toward fibronectin in modified Boyden chambers at concentrations <10  $\mu\text{mol/L}$ . At the highest dose of 100  $\mu\text{mol/L}$ , the inhibition was complete at 100%. Kubista et al. (6) have shown that at 50  $\mu\text{mol/L}$  ZOL concentration, human osteosarcoma cells were inhibited from migrating in a

scratch assay, although whether SaOS-2 cells were tested in their study is unclear. Our results have relevance to metastasis because a decrease in the ability of osteosarcoma cells to migrate *in vivo* from the primary site will lead to a decreased secondary tumor burden in the lungs.

However, ZOL was potent in its action against invasion of cells through Matrigel, with a 4% inhibition occurring at even the surprisingly low dose of 10 nmol/L and 100% complete inhibition at 100 nmol/L onward. The effect of ZOL on the invasive properties of osteosarcoma cells has not been reported in the literature, so these findings shed important light on the ability of this agent to drastically perturb the ability of the osteosarcoma cells to invade. This, akin to the findings with migration study (above), and even more so because of the lower doses that were active in the invasion study, directly implicates ZOL as an agent that has the capacity of curbing the metastasis of osteosarcoma from its primary site, exclusively from its effects on limiting the actual size of the primary tumor. The doses evaluated in this study cover those used clinically (11). We would like to add that the inhibitory effects on migration and invasion are not entirely dependent on the ability of ZOL to decrease proliferation because the trends for the latter two assays do not directly correlate with that of the proliferation assay.

ZOL reduced the SaOS-2 cell-influenced maturation of osteoclastic cells from human bone marrow-derived mononuclear cells in the presence of macrophage colony-stimulating factor in a dose-dependent manner with a maximum inhibition of 100% at 100  $\mu\text{mol/L}$ . The osteoclast cells were in direct contact with SaOS-2 cells in a modified



**Figure 6.** Effect of ZOL on tumor vessel density. **A**, number of CD31 stained spots observed at primary lesion site at 4 wks posttreatment. **B**, microphotograph depicting immunostained cells signifying vessels (white arrowheads).

coculture system, which allowed cross-talk at both the cellular and, more importantly, molecular levels. These results are inconclusive because the decrease in osteoclastogenesis may directly be a result of the cytotoxic effects of ZOL on SaOS-2 cells, depriving the human bone marrow-derived mononuclear cells of a source of differentiation factors required for osteoclast maturation.

mRNA analysis of untreated and ZOL-treated SaOS-2 cells revealed some interesting results. There was no change in the levels of osteoprotegerin mRNA when SaOS-2 cells were treated with ZOL. This is similar to our earlier findings with pamidronate and the rat UMR 106-01 osteosarcoma cells (12). Osteoprotegerin is known to act as a decoy, binding to the RANK protein and thereby limiting the proper functioning of the RANKL (24). There was a decrease in osteonectin mRNA signal at 72 h as a result of ZOL treatment, implicating an antimetastatic effect. Osteonectin is part of the package of matricellular proteins that carry out critical functions in desmoplastic responses of tumors, allowing them to dissociate from their primary sites and metastasize (25).

There was an increase in osteocalcin signal when cells were exposed to ZOL. Likewise, there was an increase in osteopontin signal from zero at 24 h but which decreased to baseline at 72 h. A dramatic decrease in alkaline phosphatase mRNA was also noted. These three molecules form the major constituents of a panel of bone markers typically used for assessing the maturation status of osteoblastic cells, with alkaline phosphatase being an early, osteopontin a mid-phase, and osteocalcin a late osteoblast phase marker (26). Thus, it is apparent that ZOL stimulates the differentiation of SaOS-2 cells, and this constitutes yet another mechanism by which ZOL may lead to reduced malignancy of osteosarcoma.

There was an ~3-fold decrease in VEGF mRNA signal when cells were exposed to ZOL. The fact that VEGF is arguably the most potent proangiogenic endogenously produced molecule makes the down-regulation by ZOL, shown for the first time in this study, an important finding. Finally, there was no positive signal for pigment epithelium-derived factor, the most potent angiogenesis inhibitor produced endogenously, and previously shown to possess antiosteosarcoma activity (27, 28) in either untreated or treated cells (data not shown). Thus, the effect of ZOL on SaOS-2 growth *in vitro* and *in vivo* is independent of pigment epithelium-derived factor function.

ZOL caused a significant decrease in tumor growth in SaOS-2-bearing mice both at 3 and 5 weeks postseeding. ZOL reduced osteolysis at 3 weeks and more so at 5 weeks due to SaOS-2 tumor growth. There was a greater destruction of the growth plate cartilage layer of the tibiae in untreated mice, with tumors attempting to traverse this layer and cause massive lysis at the proximal tibial region. These results mirror those of the two previous studies (10, 11) using mouse osteosarcoma cells in that primary tumor growth, bone lysis, and lung metastasis are minimized when ZOL is used in nonorthotopic models. We also observed a reduced capacity for osteoclastogenesis

at the lesion site in mice given ZOL. As suggested by our *in vitro* results, this could be very well due to the direct cytotoxic action of ZOL on osteosarcoma cells, thereby depriving the bone marrow cells of factors stimulating osteoclast differentiation.

In the present study, at 5 weeks, the number of macro-metastases decreased significantly from 4.3 to 1.3 in the ZOL-administered mouse cohort. Tissue sections revealed numerous secondary growth pockets within the lung parenchyma in untreated mice at both 3 and 5 weeks, whereas micrometastases were hard to visualize in ZOL-treated lungs. Thus, ZOL may not only control local tumor growth and concomitant osteolysis but even lead to a decrease in the number of metastases established in the lungs, the site of usually fatal secondary lesions in osteosarcoma.

Metastasis may be curbed directly via the action of ZOL on cell migration and invasion, probably driven by reduced expression of osteonectin, or indirectly by the ability of ZOL to halt aggressive proliferation of the SaOS-2 cells in the tibial site. In addition, the prodifferentiation and VEGF down-regulation effected by ZOL could be additional factors bolstering its overall efficacy. Indeed, vessel density of tumors in the ZOL group was significantly lower than in the placebo group. In reality, both these mechanisms may be at play *in vivo* because *in vitro* data strongly point to both being important.

In total, these data point to the adjuvant potential of ZOL in the management of osteosarcoma not only for its antiosteolytic properties but also for its ability to directly halt tumor cell growth and metastasis, thus acting via various mechanisms conducive to reduced malignancy of osteosarcoma.

#### Acknowledgments

We thank Drs. Sonia Yip and Michael Copeman (Novartis Pharmaceuticals Australia Pty Limited) for the supply of zoledronic acid, Dr. Anne Nadesapillai for administrative assistance, and Karla Contreras for technical assistance.

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