Novel Antiangiogenic Effects of the Bisphosphonate Compound Zoledronic Acid

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ABSTRACT

Bisphosphonate drugs inhibit osteoclastic bone resorption and are widely used to treat skeletal complications in patients with tumor-induced osteolysis. We now show that zoledronic acid, a new generation bisphosphonate with a heterocyclic imidazole substituent, is also a potent inhibitor of angiogenesis. In vitro, zoledronic acid inhibits proliferation of human endothelial cells stimulated with fetal calf serum, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (IC50 values 4.1, 4.2, and 6.9 μM, respectively), and modulates endothelial cell adhesion and migration. In cultured aortic rings and in the chicken egg chorioallantoic membrane assay, zoledronic acid reduces vessel sprouting. When administered systemically to mice, zoledronic acid potently inhibits the angiogenesis induced by subcutaneous implants impregnated with bFGF [ED50, 3 μg/kg (7.5 nmol/kg) s.c.]. These findings indicate that zoledronic acid has marked antiangiogenic properties that could augment its efficacy in the treatment of malignant bone disease and extend its potential clinical use to other diseases with an angiogenic component.

Bisphosphonates are widely used to inhibit osteoclastic activity in malignant and benign diseases involving excessive bone resorption such as tumor-induced osteolysis and osteoporosis (Fleisch, 1997; Berenson and Lipton, 1999). These compounds are characterized by two geminal phosphonic acid groups attached to a central carbon atom, which results in high binding to mineralized bone. Addition of various aliphatic or aromatic side chains to the carbon atom has generated a range of compounds that retain a similar affinity for bone and yet exhibit widely differing potencies as inhibitors of osteoclastic function (Fleisch, 1998). Although the geminal bisphosphonate moiety dictates the physicochemical properties of this class of compound, the overall pharmacological activity of each member is modulated by the other substituents. There is a growing body of literature indicating apoptotic and antitumor effects of nitrogen-containing bisphosphonates, at least in vitro (Senaratne et al., 2000; Shipman et al., 2000). However, in clinical trials in patients with breast cancer, conflicting data on the effect of the bisphosphonate clodronate have been reported on the development of bone and visceral metastases (Diel et al., 1998; Saarto et al., 2001).

Angiogenesis is a complex and tightly controlled physiological process in which new blood vessels are formed during normal growth and tissue repair. Apart from the high activity required for placentation, embryogenesis, wound healing, and endometrial repair after menstruation, angiogenesis is a relatively rare event in the normal healthy adult, and turnover of vascular endothelial cells is generally rather low. However, angiogenesis is a prominent pathological feature of many diseases; although much attention has focused on its essential role in tumor growth, it is also an important component of several other nonmalignant conditions such as rheumatoid arthritis, psoriasis, and transplant rejection (Folkman, 1995; Moulton et al., 1999). In this study, we have investigated the potential antiangiogenic properties both in vitro and in vivo of two bisphosphonates, the new generation compound zoledronic acid (Zometa) and an earlier one, pamidronate.

Materials and Methods

Test Compounds and Solutions. All studies were performed with the hydrated disodium salts of zoledronic acid, [1-hydroxy-2-(1H-imidazol-1-yl)ethylidene]bisphosphonic acid (ZOL), and pamidronate.
idronic acid, (3-amino-1-hydroxypropylidene)bisphosphonic acid (PAM), synthesized by Novartis. Stock solutions of each compound (10 mM) were prepared in 0.15 M NaCl solution containing 0.01 M phosphate buffer, pH 7.0, and then diluted in the relevant solvent for each experiment. The molecular weights of ZOL and PAM are 399.5 and 369, respectively.  

Cell Culture and Animals. Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell (Heidelberg, Germany) and cultivated in vitro according to the supplier’s recommendations. Normal human skin fibroblasts were provided by Prof. B. Nusgens (Laboratory of Connective Tissues Biology, University of Liège, Belgium) and cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS. Female mice (strain Tiffbm:MA) weighing 17 to 20 g were kept in groups of six animals per cage under standard conditions. Six mice were used per treatment group in each experiment, and all experiments were performed at least twice. Animal experiments were performed in accordance with the Swiss animal protection laws.

Endothelial Cell Proliferation Assay. A proliferation assay based on incorporation of 5-bromo-2’-deoxyuridine (BrdU) was used to investigate the effect of ZOL and PAM on the functional response to angiogenic stimuli (Biotrak Cell Proliferation ELISA System V.2; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Subconfluent HUVEC were seeded at a density of 5 × 10⁴ cells/well into 96-well plates in endothelial cell growth medium containing 5% FCS (PromoCell number C-22110) or in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA; Invitrogen number 41965-039) containing 10% FCS, respectively. After 24 h, the growth medium was replaced by the corresponding serum-free medium supplemented with 1.5% FCS, 1.5% FCS and 10 ng/ml human basic fibroblast growth factor (bFGF), in the presence or absence of test compound. After 24 h of incubation, the BrdU ELISA was performed. Since bisphosphonates are strong calcium chelators, the experiments were repeated at least twice. Animal experiments were performed in accordance with the Swiss animal protection laws.

Apoptosis Evaluation. HUVEC were treated with different concentrations of test compound or with vehicle alone in complete medium. After 48 h, cells were stained with annexin V-fluorescein and counted. As a control for cation chelation, a flask of cells was also treated with 100 μM bisphosphonate and serum-free medium, and counted. After 48 h of incubation, the annexin V-fluorescent cells were analyzed by an in situ terminal deoxyuridine filter for fluorescein detection and a filter (Novo Nordisk A/S, Bagsvaerd, Denmark) using an excitation wavelength of 488 nm, a 515-nm bandpass filter, and an emission filter to detect fluorescein. A FACScalibur flow cytometer (BD Biosciences, Erembodegem, Belgium) was used to collect PI fluorescence through a 20x objective. Staining was performed as described previously (Bellahcène et al., 2000). HUVEC were treated with different concentrations of test compound or with vehicle alone in complete medium. After 48 h, cells were harvested and counted. The nuclei were harvested and counted. Then, 2 × 10⁵ living cells/well were incubated at 37°C for 2 h in the precoated wells. Attached cells were stained with crystal violet, and the incorporated dye was measured by reading absorbance at 560 nm.

Endothelial Cell Migration Assay. The cell migration assay was performed as described previously (Wood et al., 2000). Plates (24-well) were coated with 1.5% gelatin and fitted with circular fences to prevent cells from growing into the center of the well. Subconfluent HUVEC (10⁵ cells/well) were seeded into the outer area and incubated in 5% CO₂ at 37°C in endothelial cell growth medium containing 5% FCS. After removal of the fences at 24 h, the growth medium was replaced by serum-free endothelial cell basal medium containing 1.5% FCS supplemented with VEGF (10 ng/ml) or 5% FCS alone, in the presence or absence of test compound. To inhibit cell proliferation, 50 μg/ml fluorouracil (Roche Diagnostics, Basel, Switzerland) was added. Control experiments were performed without growth factor and with EDTA. After 48 to 60 h of incubation, the cells were fixed and stained with Diff-Quik (Dade Behring, Düsseldorf, Switzerland). The number of migrated cells was counted under a binocular microscope using the KS-400 software (Carl Zeiss, Jena, Germany).

Cell Chemotaxis Assay. HUVEC were treated with different concentrations of ZOL or with vehicle alone in complete medium. After 48 h, cells were harvested and counted. Then, 10⁵ living cells/well were placed in the top chamber of a modified Boyden chemotaxis chamber, and conditioned medium of UMR-106 cells was used as a chemoattractant in the bottom chamber. Cells that had traversed the filter after an overnight incubation at 37°C were stained and counted.

Aortic Ring Vessel Sprouting Assay. Rat aortic rings were prepared and embedded into a type I collagen gel as described previously (Montesano et al., 1983). These ex vivo organotypic cultures were treated with a single concentration of 50 μM bisphosphonate or EDTA. After 7 days of culture, the aortic rings were photographed under a microscope (25×), and the images were assessed visually.

Chicken Egg Chorioallantoic Membrane Assay. Fertilized Lohman-selected White Leghorn eggs were incubated at 37°C in a humidified incubator. On the third day of development, the chorioallantoic membrane was detached from the shell by removal of 7 ml of albumen, and a rectangular window was cut in the egg shell. On day 8, two Silastic rings (inner diameter of 4 mm, height 500 μm, weight 30 mg) were placed on the surface of the chorioallantoic membrane (CAM). Bisphosphonates or EDTA were dissolved in sterile water to a final concentration of 1 mM and applied in 15-μl aliquots inside the rings. Vehicle alone (Millipore Corporation, Bedford, MA) was used as a negative control. CAMs were examined daily until day 10 and photographed in vivo under a microscope (60×). A minimum of 10 eggs was used for each treatment, and the experiments were repeated at least twice.

In Vivo Growth Factor Implant Model of Angiogenesis. The effect ZOL and PAM on growth-factor angiogenesis in vivo was investigated in mice in a growth factor implant assay (Wood et al., 2000). Sterile chambers made of perfluoro-alkoxy-Teflon (21 mm × 8 mm diameter, with 80 regularly spaced 0.8-mm perforations) were filled with 500 μl of 0.8% (w/v) agar containing 20 U/ml heparin (Novo Nordisk A/S, Bagsvaerd, Denmark) with or without growth
factor (bFGF, 0.3 μg/ml or VEGF, 2 μg/ml). The chamber was implanted aseptically under isoflurane anesthesia on the back of the animal. Five days after implantation, animals were anesthetized (3% isoflurane) and sacrificed with an overdose of pentobarbital (210 mg/kg i.p.). The chambers were recovered, the vascularized fibrous tissue around each implant was removed and weighed. The hemoglobin content of this tissue was determined colorimetrically with the Drabkin reagent kit (Sigma-Aldrich number 525), and converted to the equivalent blood volume using a calibration curve constructed with murine whole blood. ZOL (1, 10 and 100 μg/kg s.c.) and PAM (10, 100, and 1000 μg/kg s.c.) were dissolved in a 5% manniotol solution and administered once a day for 6 days starting 1 day before chamber implantation. The chambers were removed 24 h after the last dose, 5 days after implantation. Control animals received vehicle alone. Each experiment was performed on groups of six mice and repeated at least twice.

Statistics. All data are presented as mean ± SEM. They were analyzed by one-way analysis of variance followed by Dunnett’s test for comparison against a single control, or by the Bonferroni test for multiple comparisons. A p value of <0.05 was considered to be statistically significant. ED50 and IC50 values were calculated by sigmoid curve fitting.

Results

ZOL dose dependently inhibited HUVEC proliferation induced by FCS (Fig. 1A) and bFGF (Fig. 1B) with IC50 values of 4.1 ± 0.6 μM and 4.2 ± 0.4 μM, respectively. At a slightly higher concentration, it also had a similar effect on VEGF-induced proliferation (IC50, 6.9 ± 0.4 μM). By contrast, PAM at identical concentrations had no significant effect on cell proliferation. Parallel control experiments with EDTA indicated that cation chelation made only a minor contribution to the effect of ZOL. Because bisphosphonates have been reported to induce apoptosis in cancer cells (Senaratne et al., 2000; Shipman et al., 2000), we tested the hypothesis that ZOL could also induce endothelial cell apoptosis. As determined by the TUNEL assay, ZOL up to 30 μM had no effect on HUVEC DNA fragmentation, whereas 100 μM did induce a 4-fold increase in DNA fragmentation (p <0.05) (Fig. 1C). By contrast, 100 μM EDTA had no detectable effect. Analysis by flow cytometry of cells labeled with an annexin V-fluorescein conjugate confirmed that 48 h treatment with ZOL (1–30 μM) had no effect but 100 μM induced a 72% increase in apoptosis (p <0.05). In identical experiments with PAM or EDTA (1–100 μM), there was no significant effect on annexin V binding to HUVEC.

Since ZOL (100 μM) exerted a moderate effect on HUVEC viability, we wanted to confirm the presence of apoptotic cells by their morphology. To do so, we analyzed cellular DNA content of control and ZOL-treated HUVEC by laser scanning cytometry (Table 1). The DNA distribution pattern revealed a pronounced change in the cell cycle profile of treated versus control cells. Treatment with ZOL (100 μM) for 48 h produced a 72% increase in the number of cells in S phase, indicating a retardation of cell cycling. In a parallel experiment, 100 μM EDTA did not markedly alter the cell cycle profile; thus, cation chelation does not appear to contribute to the effect of ZOL. In addition, based on the DNA content analysis, ZOL increased the sub-G1 peak 3.7-fold in comparison to the control. On examination by fluorescence microscopy, this fraction consisted of cells with either fractional DNA content or nuclear fragmentation.

The potential effect of ZOL on endothelial cell adhesion was next investigated. As shown in Fig. 2A, ZOL induced a concentration-dependent, biphasic effect on the attachment of HUVEC to vitronectin. Pretreatment of cells with 1 and 3 μM ZOL increased attachment by up to 70%, whereas 30 and 100 μM ZOL reduced attachment by up to 76%. When the cells were pretreated with PAM or EDTA, no stimulation of adhesion was observed at lower concentrations, but higher concentrations inhibited attachment by up to 40% (Fig. 2A). Similar results
were obtained with the substrates fibronectin, osteopontin, and laminin (data not shown), indicating that the observed effects are not specific for a single substrate.

The potential effect of ZOL on HUVEC migration was also evaluated. At low concentrations (0.3–10 μM), ZOL stimulated HUVEC migration by 2- to 3-fold, the effect being greater at a low serum concentration (Fig. 2B). However, 30 μM ZOL completely inhibited migration regardless of the serum concentration. EDTA in the presence of 5% serum had no significant effect on migration.

The possibility that ZOL could inhibit angiogenesis was assessed using three different experimental models. The aortic ring assay is an ex vivo model for angiogenesis. Cultured aortic explants generate microvascular outgrowths (Fig. 3A). At a concentration of 50 μM, both PAM and ZOL completely inhibited the sprouting of capillaries (Fig. 3, C and D, respectively) as compared with the control (Fig. 3A) or EDTA-treated aortic rings (Fig. 3B). The CAM assay allows investigation of the ongoing angiogenic process in vivo (Fig. 3E). Twenty-four hours after application of 100 μM ZOL or PAM onto the CAM, a slight reduction in angiogenesis was ob-

**TABLE 1**

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<th>Treatment</th>
<th>Percentage of Cells in Each Phase of the Cell Cycle</th>
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<td>Control</td>
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<td>ZOL 100 μM</td>
<td>7.4</td>
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<td>EDTA 100 μM</td>
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Fig. 2. Effect of bisphosphonates on endothelial cell adhesion and migration. A, HUVEC were treated for 48 h with test compound in complete medium and harvested with EDTA-trypsin. Live cells (2 × 10⁴/well) were incubated (37°C, 2 h) in wells precoated with vitronectin (1 μg/ml). Attached cells were stained with crystal violet, and the incorporated dye was measured colorimetrically. Mean ± S.E.M. of four replicates from a representative experiment. B, Subconfluent HUVEC (10⁵/well) were seeded into the outer area of a plate well with a removable inner fence and then incubated for 24 h in growth medium containing 5% FCS. Fences were removed, and the medium was replaced by serum-free basal medium and the test solutions. To inhibit cell proliferation, 50 μg/ml fluorouracil was added. After 3 days, the cells were fixed and stained, and the migrated cells were counted. Mean ± S.E.M. of eight wells from two separate experiments. a, p < 0.05; b, p < 0.01, in Dunnett’s test versus control.

Fig. 3. Zoledronic acid inhibits angiogenesis in the rat aortic ring assay and in the chicken egg chorioallantoic membrane assay. Rat aortic rings were cultured ex vivo in collagen gels containing serum-free medium for 7 days with vehicle (A), 50 μM EDTA (B), 50 μM PAM (C), or 50 μM ZOL (D). Photographs are representative of rings from duplicate assays (magnification 25×). Chicken egg chorioallantoic membranes were treated on day 8 with 15 μl of vehicle (E), 1 mM EDTA (F), 1 mM PAM (G), or 1 mM ZOL (H) contained inside Silastic rings. CAMs were photographed (60×) in ovo after 24 h of treatment.
served with both compounds. At a high concentration of 1000 μM, a little vascularization was still present after PAM treatment (Fig. 3G), whereas no capillaries were observed in the CAM treated with ZOL (Fig. 3H). No inhibition of angiogenesis was observed on the CAM treated with 1000 μM EDTA (Fig. 3F).

We also used an in vivo murine model in which angiogenesis is induced by subcutaneous implants impregnated with bFGF or VEGF (Wood et al., 2000). ZOL dose dependently inhibited the angiogenic response induced by bFGF, as measured by increases in blood content and weight of the tissue induced to grow around the implant, with ED₅₀ values of 3.1 and 2.9 μg/kg (7.8 and 7.2 nmol/kg), respectively (Fig. 4). By contrast, ZOL had a weaker effect on the angiogenic response induced by VEGF. The increase in tissue weight was dose dependently inhibited, but only the highest ZOL dose of 100 μg/kg produced a statistically significant 57% inhibition, to give approximate ED₅₀ values of 24 μg/kg for the wet weight parameter and >100 μg/kg for the blood content. PAM, administered at doses 10-fold higher than those used for ZOL, also produced a moderate, dose-dependent inhibition of the bFGF-induced angiogenic response, to give ED₅₀ values of 190 and 560 μg/kg for the blood and wet weight parameters, respectively (Table 2). However, the maximum efficacy at the highest PAM dose was still considerably less than that observed with a 10-fold lower dose of ZOL.

**Discussion**

In this study, we demonstrate for the first time that the new generation bisphosphonate, zoledronic acid, possesses significant antiangiogenic activity in several different in vitro and in vivo models. Bisphosphonates are widely used as inhibitors of bone resorption in a variety of human conditions including osteoporosis and malignant osteolytic diseases. The precise mechanism of action of bisphosphonates is not yet fully clarified. At the cellular level, the compounds inhibit the formation of mature, multinucleated osteoclasts from their mononuclear precursors (Boonekamp et al., 1986). Internalization of bisphosphonates by actively resorbing osteoclasts disrupts the cytoskeleton and vesicular trafficking (Zimolo et al., 1995), leading to a cessation of bone resorption and the induction of apoptosis (Hughes et al., 1995). At the molecular level, nitrogen-containing bisphosphonates block the mevalonate biosynthetic pathway (Luckman et al., 1998), thereby reducing the synthesis of farnesyl diphosphate and geranylgeranyl diphosphate, essential substrates for the prenylation of small GTP-binding proteins such as Rab, Rac, Ras, and Rho. These proteins are also important components in signaling pathways involved in endothelial migration. Our demonstration that ZOL inhibits angiogenesis provides an additional potential mechanism for the anti-resorptive activity of bisphosphonates. Indeed, osteoclastic bone resorption requires efficient vascularization by the hemangiogenic endothelial cells. Recently, it was shown that two bone matrix proteins involved in bone resorption, osteopontin and bone sialoprotein, stimulate angiogenesis (Bellahcène et al., 2000).

Long-term cancer trials with bisphosphonates such as ZOL indicate that, in addition to reducing bone resorption, some of these drugs may also influence tumor burden and even prolong survival in some patient subgroups (Berenson et al., 1998; Diel et al., 1998; Hortobagyi et al., 1998). The mechanism of action responsible for these antitumor properties of bisphosphonates is not yet established and clearly warrants further investigation. Recent in vitro data indicate that the more potent nitrogen-containing bisphosphonates can inhibit the proliferation of human tumor cell lines and induce their apoptosis (Senaratne et al., 2000). Angiogenesis, which is essential for tumor growth, could also be a potential mechanism by which bisphosphonates affect tumors but this possibility has received little attention. Our results now demonstrate potent, antiangiogenic properties of the bisphosphate ZOL. Initial experiments investigated the effect of ZOL on the proliferation, migration, and adhesion of HUVEC in vitro. Cell proliferation induced by serum, bFGF,
or VEGF was dose dependently inhibited by ZOL (3–30 μM), whereas Pam, another less potent bisphosphonate, had no significant effect at identical concentrations. Parallel control experiments with EDTA indicated that divalent cation chelation made only a minor contribution to the antiproliferative effect of ZOL. At even higher concentrations, ZOL induced apoptosis of the endothelial cells.

ZOL also exerted a concentration-dependent, biphasic effect on the adhesion and migration of HUVEC in vitro. ZOL concentrations of 1 and 3 μM increased cell adhesion but inhibited it at 30 and 100 μM. Similarly, cell migration was stimulated by 0.3 to 10 μM ZOL, whereas 30 μM completely inhibited it. These findings suggest that ZOL could interfere with cytoskeletal function in endothelial cells. Other bisphosphonates have been reported to inhibit the binding of osteoclasts and tumor cells to both mineralized and nonmineralized extracellular matrices in vitro (Boissier et al., 1997; Colucci et al., 1998). It has been proposed that bisphosphonates could interfere with integrin receptors, but a study with the bisphosphonate ibandronate (10−6 M, 24 h) found no evidence of altered integrin expression by breast cancer cells (Boissier et al., 1997). Nonetheless, in a set of preliminary experiments, we have observed that ZOL induced a biphasic modulation of the cell surface expression of αvβ3 integrin in HUVEC; i.e., increased expression at low ZOL concentrations and significantly decreased expression at higher concentrations (Bonjean et al., 2002). Consistent with these findings, a similar biphasic response has been reported for the interaction between soluble recombinant αvβ3 integrin and a cyclic RGD peptide with antiangiogenic properties (Legler et al., 2001).

The observation that ZOL affects adhesion, migration, and proliferation in HUVEC prompted us to investigate the possibility that it could also inhibit angiogenesis. In cultured aortic rings and in the in vivo CAM assay, ZOL inhibited the ongoing angiogenic process. Moreover, in an in vivo model of growth factor-mediated angiogenesis, ZOL dose dependently inhibited the angiogenic response induced by bFGF with an ED50 value of approximately 3 μg/kg (7.5 nmol/kg).

In our in vivo data are the first to demonstrate that ZOL affects angiogenesis occurring in a nonmineralized tissue. Interestingly, for VEGF-induced angiogenesis, a ZOL dose of 100 μg/kg was required to achieve any significant inhibition. This different sensitivity between bFGF- and VEGF-induced angiogenesis to ZOL inhibition may indicate a relatively specific effect on the bFGF signaling pathway as opposed to a general cytostatic or cytotoxic effect of ZOL on the angiogenic process per se.

The pharmacokinetic behavior of bisphosphonates is characterized by their short plasma half-life, accumulation in bone, and low exposure of visceral tissues (Pleisch, 1997, 1998; Berenson and Lipton, 1999). It is unlikely that the low doses of ZOL used in the in vivo experiment could produce the sustained micromolar concentrations needed in vitro to affect endothelial cell proliferation and adhesion. An alternative explanation is that the high local concentration of ZOL in bone nodules as yet unidentified signaling molecules or precursor cells released from the bone marrow that are essential for angiogenesis in distant tissues. Evidence already exists that endothelial progenitor cells originating in the bone marrow are required for physiological and pathological neovascularization (Asahara et al., 1999; Aernout et al., 2002). More research is obviously required to explore this hypothesis and to elucidate the mechanism by which ZOL exerts such a potent effect on bFGF-induced angiogenesis in vivo.

These novel antiangiogenic properties of ZOL suggest that it may potentially be a useful antiangiogenic therapy for diseases in which bFGF-mediated angiogenic effects play an underlying role in the pathogenesis, such as the growth of some solid tumors and various inflammatory diseases such as rheumatoid arthritis. Consistent with the antiangiogenic effects reported here, ZOL inhibits prostate revascularization in castrated rats treated with testosterone (Boissier et al., 2002). It has also been reported that ZOL can decrease tumor burden and increase survival in a murine model of established myeloma (Croucher et al., 2001), reduce tumor burden and serum paraprotein levels in a SCID mouse model of human myeloma (Yaccoby et al., 2002), reduce tumor expansion in a murine model of breast cancer metastases (Peyruchaud et al., 2001), and have disease-modifying as well as antinoceptive effects in a rodent model of bone cancer (Walker et al., 2002). Moreover, ZOL has been shown to be effective in reducing the skeletal complications of bone metastases in patients with breast cancer and osteolytic lesions of multiple myeloma (Rosen et al., 2001). In addition, ZOL is effective in animal models of arthritis, exerting a partial chondroprotective effect (Podworny et al., 1999; Muehleman et al., 2002). In summary, our findings demonstrate that ZOL has potent antiangiogenic properties that could contribute to its efficacy in the treatment of malignant bone diseases and extend its potential clinical use to other cancers and diseases with an angiogenic component.

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