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Differential Effect of Doxorubicin and Zoledronic Acid on Intraosseous versus Extraosseous Breast Tumor Growth *In vivo*

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Abstract

Purpose: Breast cancer patients with bone metastases are commonly treated with chemotherapeutic agents such as doxorubicin and zoledronic acid to control their bone disease. Sequential administration of doxorubicin followed by zoledronic acid has been shown to increase tumor cell apoptosis *in vitro*. We have therefore investigated the antitumor effects of clinically relevant doses of these drugs in a mouse model of breast cancer bone metastasis.

Experimental Design: MDA-MB-231/BO2 cells were injected via the tail vein into athymic mice. Tumor-induced osteolytic lesions were detected in all animals following X-ray analysis 18 days after tumor cell inoculation (day 18). Mice were administered saline, 100 μg/kg zoledronic acid, 2 mg/kg doxorubicin, doxorubicin and zoledronic acid simultaneously, or doxorubicin followed 24 h later by zoledronic acid. Doxorubicin-treated animals received a second injection on day 25. Tumor growth in the marrow cavity and on the outside surface of the bone was measured as well as tumor cell apoptosis and proliferation. The effects of treatments on bone were evaluated following X-ray and μCT analysis.

Results: Sequential treatment with doxorubicin followed by zoledronic acid caused decreased intraosseous tumor burden, which was accompanied by increased levels of tumor cell apoptosis and decreased levels of proliferation, whereas extraosseous parts of the same tumors were unaffected. Administration of zoledronic acid, alone or in combination with doxorubicin, resulted in significantly smaller tumor-induced osteolytic lesions compared with control or doxorubicin-treated animals.

Conclusions: This is the first study to show that sequential treatment with clinically relevant doses of doxorubicin, followed 24 h later by zoledronic acid, reduces intraosseous but not extraosseous growth of BO2 breast tumors. Our results suggest that breast cancer patients with metastatic bone disease may benefit from sequential treatment using doxorubicin and zoledronic acid.

Patients with advanced breast cancer frequently develop metastasis to bone, which are associated with tumor-driven bone loss caused by increased osteoclast activity (osteolysis; ref. 1). The current choice of treatment for cancer-induced bone disease are bone resorption inhibitors; however, these therapies are only palliative and do not provide a life-prolonging benefit to the patient. There is therefore a need to improve the treatments for cancers that metastasize to bone (e.g. by combining therapies that target both tumor cells and osteoclasts).

The third-generation nitrogen-containing bisphosphonate, zoledronic acid, is the only bisphosphonate licensed to treat cancer-induced bone disease from a variety of solid tumors and multiple myeloma (2). Zoledronic acid reduces osteoclastic bone resorption by inhibiting key enzymes of the mevalonate pathway responsible for post-translational modification of signaling GTPases, leading to loss of osteoclast function and, ultimately, apoptosis (3–6). The mevalonate pathway constitutes an important part of the metabolic process resulting in cholesterol synthesis, which is ubiquitous to all nucleated cells. Although its main target is likely to be the osteoclasts, zoledronic acid may also induce apoptosis in a variety of other cell types, including tumor cells. The concentration of bisphosphonates required to induce apoptotic cell death in tumor cells ranges from 5 to 20 μmol/L *in vitro* and is unlikely to be reached at extraosseous sites *in vivo* due to the high affinity of bisphosphonates to bone.

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There is increasing evidence from both *in vitro* and *in vivo* model systems for a role of nitrogen-containing bisphosphonates as potential antitumor agents (7–10). Zoledronic acid has been reported to reduce the tumor burden in bone from a variety of cancer types, including multiple myeloma (11), osteosarcoma (12), breast (13–15), prostate (16), and leukaemia/lymphoma (17). Interestingly, clinically relevant doses of zoledronic acid (100 μg/kg/mo) have no effect on breast cancer skeletal tumor growth in animals when administered alone (18), possibly accounting for the lack of life-prolonging effects seen in cancer patients following treatment with this drug.

Combining zoledronic acid with anticancer agents has the potential to significantly increase the potency of the anticancer drug, and zoledronic acid has been shown to synergistically increase cancer cell death when combined with a variety of anticancer agents *in vitro* [e.g. with dexamethasone in myeloma cells (19); paclitaxel, etoposide, cisplatinum, and irinotecan in lung cancer cells (20); and doxorubicin, paclitaxel, or tamoxifen in breast cancer cells (21, 22)]. Beneficial effects of combining zoledronic acid with anticancer agents are also reported from *in vivo* model systems. Kim et al. reported increased inhibition of growth of PC-3MM2 prostate cancer in bone, and a reduced incidence of lymph node metastasis, when combining zoledronic acid with imatinib mesylate or paclitaxel (23). In addition, Brubaker et al. show a significant inhibition of LuCap 23.1 prostate tumor growth in bone following combined treatment with zoledronic acid and docetaxel (24). In a mouse model of breast cancer, giving zoledronic acid in combination with UFT was reported to decrease the number of bone metastases (14). In these studies, very high doses of zoledronic acid were used, ranging from a single injection of 250 to 120 μg/kg twice daily, equivalent to a 15 mg i.v. dose given daily. By contrast, the current clinical dose of zoledronic acid approved for treatment of cancer patients with skeletal metastasis is 4 mg i.v. given every 3 to 4 weeks (18). It is therefore pertinent to test the effectiveness of combination therapy using clinically relevant doses of this bisphosphonate.

Doxorubicin is the first choice of chemotherapy for both early-stage and late-stage breast cancer. Doxorubicin is an anthracycline antibiotic that exerts its effects on cancer cells via two different mechanisms. Firstly, it acts as a DNA-intercalating agent whereby the drug wedges between the bases of the DNA, preventing synthesis and transcription (25). Secondly, doxorubicin inhibits the activity of topoisomerase type II leading to breaks in the DNA (26). Both of these mechanisms lead to disruption of DNA structure, ultimately leading to cell death.

Patients with late-stage breast cancer that has metastasized to bone may be treated with doxorubicin as a chemotherapeutic agent along with zoledronic acid to inhibit tumor-associated bone resorption, but the sequence in which the drugs are administered is not standardized and little is known regarding the optimal therapeutic regimen. Studies in our laboratory have shown that doxorubicin and zoledronic acid can synergistically increase apoptosis (21) and reduce invasion (27) in breast cancer cell lines *in vitro*. The synergistic effect was found to be sequence specific, and administration of doxorubicin 24 h before zoledronic acid was essential for synergy to be achieved. Using a mouse model of breast cancer growth in bone, we have now investigated whether clinically achievable doses of doxorubicin and zoledronic acid can act synergistically to induce anticancer effects *in vivo*. Our data show that sequential treatment with doxorubicin followed by zoledronic acid causes a substantial reduction of intraosseous breast tumor growth compared with the single agents.

Materials and Methods

In vitro *apoptosis assays.* MDA-MB-231/BO2-GFP cells (BO2 cells; ref. 28) were routinely cultured in RPMI 1640 with 10% FCS (both from Life Technologies/Invitrogen). Cells were treated with doxorubicin (1 nmol/L, 24 h), zoledronic acid (25 μmol/L, 1 h), or doxorubicin followed zoledronic acid. At 72 h, cells were stained with 8 μmol/L Hoechst 33341 (Sigma RBI) and 5 μmol/L propidium iodide (Molecular Probes) for 15 min at 37°C, and % apoptotic cells scored as described by Neville-Webbe et al. (20).

Bone tumors. Four-week-old female BALB/c *nu/nu* mice were used (Charles River Laboratories) and all studies were conducted in accordance with a code of practice established by the Experimental Review Board from the Laennec School of Medicine. Experiments were carried out in duplicate using two sets of $n = 5$ per experimental group. Tumor take rate was 80% for tumor burden experiments and 100% for survival experiments. Only animals with detectable bone tumors were entered into an experimental protocol.

BO2 cells (10^5) in 100 μL PBS were inoculated into the tail vein of anesthetized nude mice (28). Radiographs (MIN-R2000 film, Kodac) were taken with a cabinet X-ray system (MX-20, Faxitron X-Ray) and bone tumors were enumerated. The area of osteolytic lesions (mm^2) was measured by a computerized image analysis system (Visiolab 2000, Biocom). Tumor-induced osteolytic lesions were detected in all mice by day 18, and mice were randomized into groups of equal tumor burden. On day 18, animals were administered 100 μL saline s.c., 2 mg/kg doxorubicin (PharmaChemie) i.v., 100 μg/kg zoledronic acid ([1-hydroxy-2-(1H-imidazol-1-yl)ethylidene]bisphosphonic acid) supplied as the hydrated disodium salt by Novartis Pharma (s.c), doxorubicin and zoledronic acid simultaneously, and doxorubicin followed 24 h later by zoledronic acid (8 animals per group for tumor burden and 10 animals per group for survival). Doxorubicin-treated animals received a second injection of doxorubicin on day 25, and all animals were administered 400 mg/kg bromodeoxyuridine (BrdUrd; Sigma-Aldrich) 3 h before sacrifice (29). For survival experiments, survival was defined as time to moribund state or hind limb paralysis, at which point mice were sacrificed. The right hind legs were fixed and decalcified with 1% paraformaldehyde/0.5 mol/L EDTA in PBS, embedded in paraffin, and sectioned at 5 μm. The left hind legs were fixed in 10% formalin for 24 h and stored in 70% ethanol for subsequent μCT analysis.

Bone histology and measurement of tumor volume. Histologic sections (5 μm) of decalcified long bones were stained with Goldner's trichrome. Histologic analysis was done separately on tumor growing within the bone marrow cavity (intraosseous) and on tumor growing on the outside surface of the bone (extraosseous) separately. Tumor volume was measured by drawing round intraosseous and extraosseous tumors on four nonserial histologic sections per sample using Osteomeasure software (Osteometrics) and a computerized image analysis system.

Microcomputed tomography imaging. Microcomputed tomography analyses were carried out using a Skyscan 1172 X-ray-computed microtomograph (Skyscan), imaged with an X-ray tube (voltage, 49 kV; current, 200 μA) and a 0.5 mm aluminum filter. Pixel size was 4.37 μm and scanning was initiated from the top of proximal tibia or distal femur. For each sample, 275 section images were reconstructed with NRecon software (version 1.4.3, Skyscan). After reconstruction, the volume of interest was designed by drawing interactively polygons on the two-dimensional acquisition images. For trabecular bone measurement, the volume of interest was composed only of cancellous bone, and the cortices were excluded. Trabecular bone volume fraction (BV/TV) was calculated covering 1 mm, starting from the lowest part of

the growth plate. BV/TV is the ratio of the volume of bone present (BV) to the volume of the cancellous space. For cortical bone measurement, the volume of interest was composed only of the cortices. Cortical volumes of tibia and femur were calculated covering 1.5 and 0.9 mm, respectively. Three-dimensional modeling and analysis of the bone were obtained with the CTAn (version 1.5.0.2, Skyscan) and CTvol (version 1.9.4.1, Skyscan) software.

Immunohistochemistry. Caspase-3 immunohistochemistry was done using rabbit polyclonal anti-active caspase-3 (AF835; 1:750) followed by a biotin-conjugated anti-rabbit secondary antibody (1:200; Vector Laboratories) as described by Marshman et al. (30). Immunohistochemistry for BrdUrd was carried out using mouse anti-human BrdUrd from DakoCytomation (1:175) and the secondary antibody was a biotin conjugated anti-mouse from Vector Labs (31). Two sections per sample were stained. Intraosseous and extraosseous tumors were assessed separately using a Leica BMRB upright microscope and scored for numbers of caspase-3-positive or BrdUrd-positive cells using OsteoMeasure software (Osteometrics).

Statistical analysis. Statistical analysis was by one-way ANOVA and post hoc analysis was by Mann-Whitney test for independent means. We have interpreted differences as being significant at $P \leq 0.05$. For Kaplan-Meier survival charts, statistical analysis is by one-tailed Mantel-Haenszel test and log-rank test for trend.

Results

Effects of doxorubicin and zoledronic acid on BO2 cells in vitro. MDA-MB-231/BO2 cells exhibit the unique property

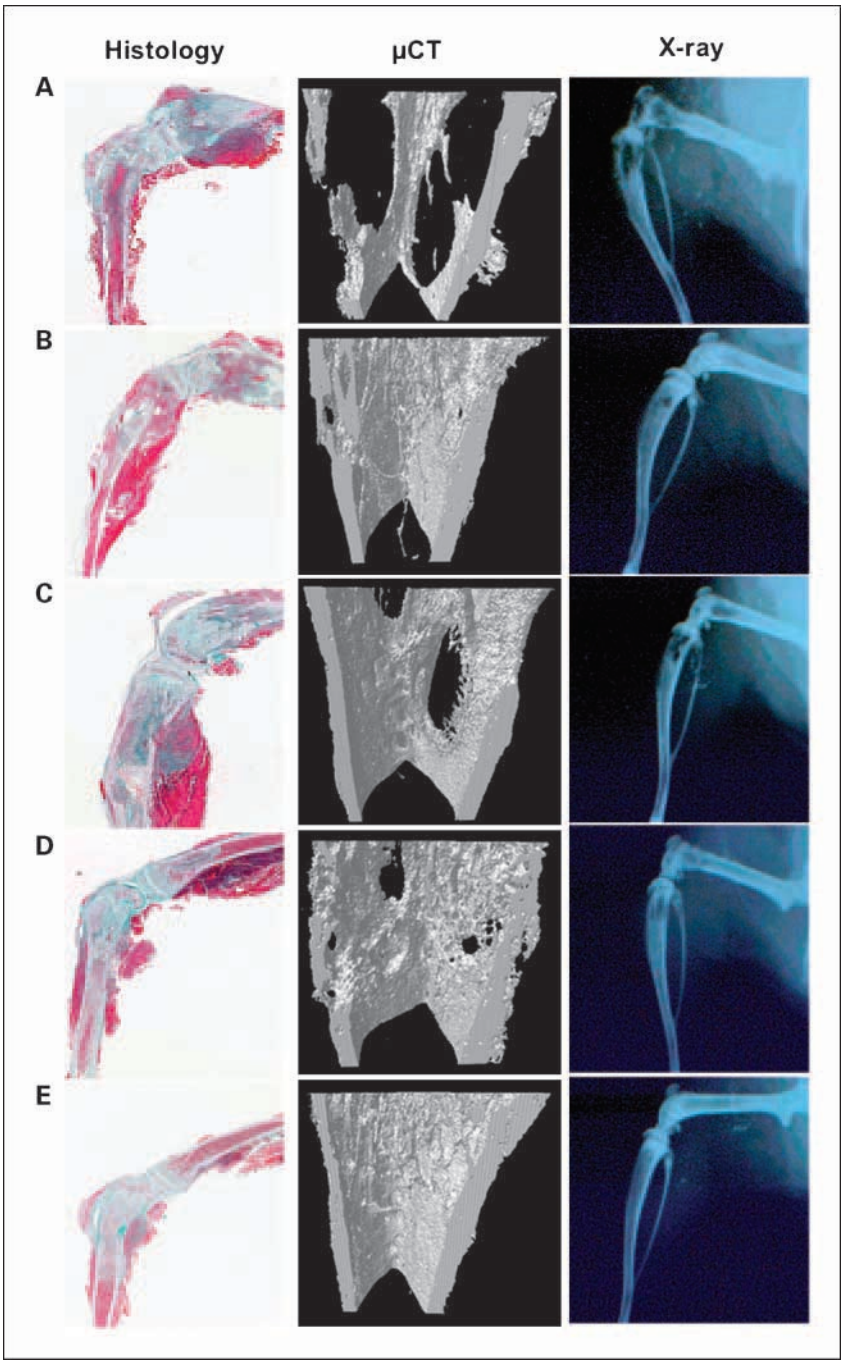

Fig. 1. Effects of zoledronic acid and doxorubicin, alone and in sequence or combination, on bone of BO2-bearing mice. Histomorphology following Goldner's trichrome staining; X-ray and μCT images from hind limbs of mice 32 d following inoculation with BO2 cells. Mice were treated with saline (control) (*A*), 100 μg/kg zoledronic acid (*B*), 2 mg/kg doxorubicin (*C*), doxorubicin and zoledronic acid simultaneously (*D*), or doxorubicin followed 24 h later by zoledronic acid (*E*).

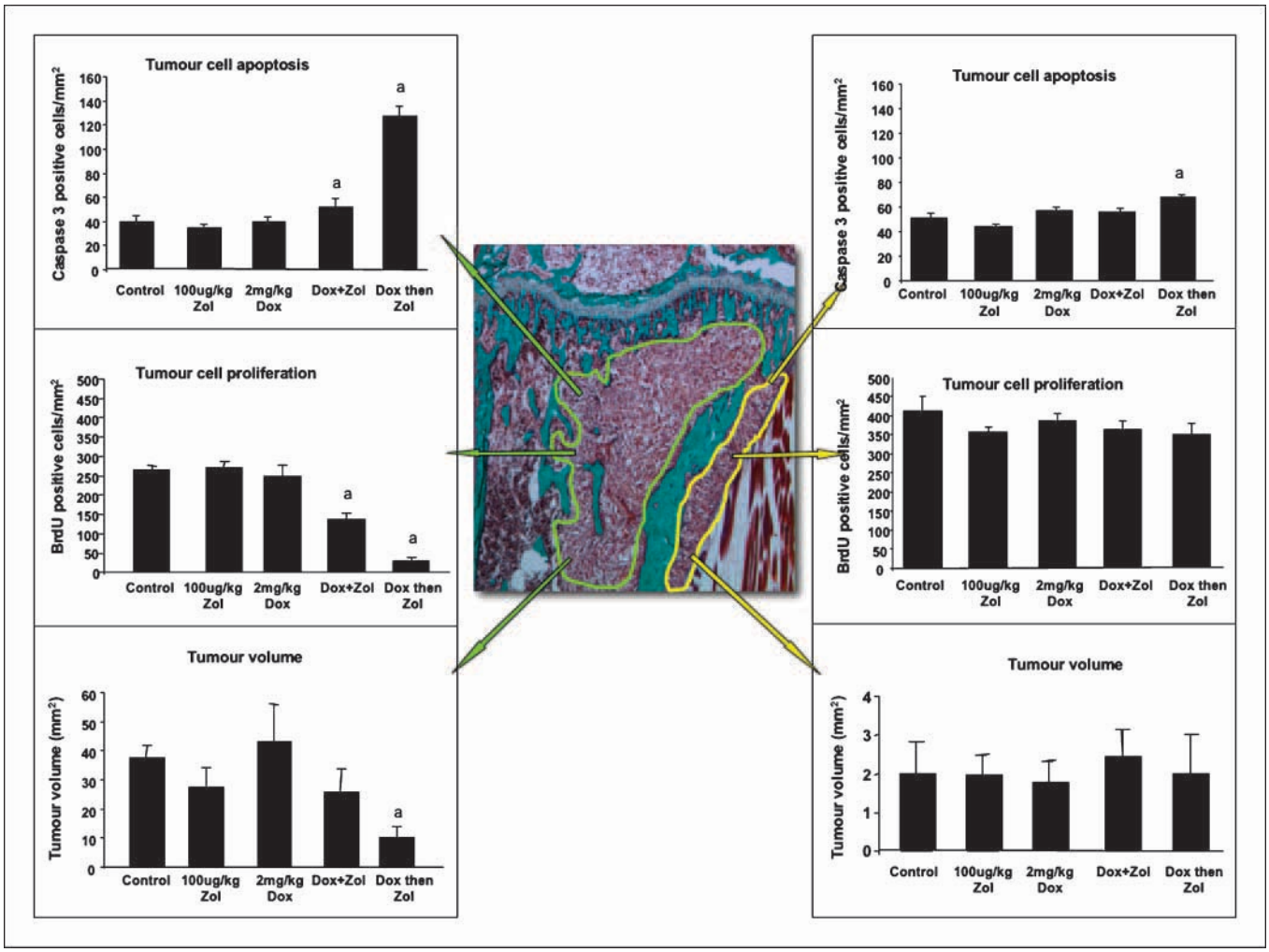

Fig. 2. Effects of zoledronic acid and doxorubicin, alone and in sequence or combination, on intraosseous and extraosseous tumor growth. Histograms representing tumor size, tumor cell proliferation, and tumor cell apoptosis in BO2 breast cancers growing intraosseously (*left panels*) and along the extraosseous bone surface (*right panels*). Mean ± SE. [a], $P < 0.05$, one-way ANOVA.

of homing to the bone after i.v. inoculation into the tail vein of female BALB/c nude mice resulting in osteolytic bone disease (28). Using this model of breast tumor growth in bone, we have investigated whether combined or sequential treatment of established tumors with clinically relevant doses of doxorubicin and zoledronic acid could inhibit breast cancer–induced bone disease compared with the single agents.

Initial experiments were carried out *in vitro* to determine whether BO2 cells exhibited a synergistic apoptotic response following sequential administration of doxorubicin and zoledronic acid. Exposure of BO2 cells to 1 nmol/L doxorubicin for 24 h or 25 μmol/L zoledronic acid for 1 h did not induce significant levels of apoptosis at 72 h compared with control. Addition of doxorubicin to the cells 24 h before zoledronic acid resulted in a synergistic increase in the percentage of apoptotic cells compared with untreated control (4.58 ± 0.68 versus 0.32 ± 0.30%; $P = 0.006$), doxorubicin alone (0.50 ± 0.31%; $P = 0.007$), and zoledronic acid alone (0.49 ± 0.44%; $P = 0.009$; data not shown).

Effects of doxorubicin and zoledronic acid on osteolytic bone disease. To determine the effects of treatments on bone structure and integrity *in vivo*, comprehensive analysis of the hind limbs from all animals was carried out (Figs. 1 and 2; Table 1). Two weeks after treatment, there was a significant reduction in the area of osteolytic lesions in animals treated with zoledronic acid (1.65 ± 0.47 mm^2) compared with those treated with saline (8.59 ± 1.9 mm^2; $P = 0.0003$) or doxorubicin alone (6.64 ± 1.16 mm^2; $P = 0.0028$). Both simultaneous administration of doxorubicin and zoledronic acid and sequential treatment with doxorubicin then zoledronic acid resulted in significantly reduced osteolytic lesion area compared with control or doxorubicin alone. However, animals treated sequentially with doxorubicin followed by zoledronic acid exhibited significantly less area of osteolytic lesions in their hind limbs (1.24 ± 0.36 mm^2) compared with those treated simultaneously with doxorubicin and zoledronic acid (2.93 ± 0.75 mm^2; $P = 0.0181$).

Trabecular and cortical bone volumes were significantly increased in both the tibia and the femur of animals treated with a single dose of zoledronic acid, either alone, simultaneously, or sequentially with doxorubicin, compared with bones from animals treated with saline or doxorubicin alone

(Fig. 2; Table 1). The effects of zoledronic acid on bone integrity were not affected by combined or sequential doxorubicin treatment.

Sequential treatment with doxorubicin and zoledronic acid inhibits intraosseous tumor growth. The main aim of our study was to establish whether use of clinically relevant doses of doxorubicin and zoledronic acid exert anticancer effects against established breast tumors growing in bone. In contrast to previous studies, animals received a single dose of zoledronic acid of 100 μg/kg, equivalent to the 4 mg clinical dose administered to patients every 3 to 4 weeks. Thirty-two days following tumor cell inoculation, all tumors had expanded from the marrow cavity along the outside surface of the bone independent of the area of marrow space occupied by tumor cells (Fig. 2). We were therefore able to carry out a detailed investigation of the effects of drug treatments on intraosseous and extraosseous tumor areas separately.

As shown in Fig. 2 and Table 1, intraosseous tumor growth was significantly reduced in animals treated sequentially with doxorubicin followed by zoledronic acid compared with those treated with saline ($P = 0.0006$), doxorubicin alone ($P = 0.0028$), zoledronic acid alone ($P = 0.0192$), or doxorubicin and zoledronic acid simultaneously ($P = 0.0083$). There was no significant effect on tumor volume compared with that in the saline control group following treatment with the single agents or following simultaneous treatment with doxorubicin and zoledronic acid. These results show that, in agreement with the *in vitro* studies, sequential treatment of animals with established tumors using doxorubicin followed by zoledronic acid caused a substantial decrease in intraosseous breast tumor growth. In contrast, no significant differences in extraosseous tumor volume were observed between any of the treatment groups (Fig. 2). It is worth noting that these are not separate tumors initiated at different sites but rather a continuous tumor mass that has expanded from the bone marrow cavity where it was first established. Our data are in agreement with those reported by Van der Pluilm et al., who also showed differential effects on intraosseous but not total tumor volume following treatment with olpandronate (10). We next investigated the ability of the treatments to induce apoptosis and cell cycle arrest in the parts of the tumor growing inside the marrow cavity compared with the parts growing along the outside surface of the bone.

Effects of simultaneous and sequential treatment with doxorubicin and zoledronic acid on tumor cell proliferation. Effects of treatment on tumor cell proliferation were assessed by analyzing the number of BrdUrd-positive cells in the intraosseous and extraosseous tumors from each treatment group following immunohistochemical staining. In the intraosseous parts of the tumors, both simultaneous and sequential administration of doxorubicin and zoledronic acid caused a significant reduction in the number of BrdUrd-positive cells compared with tumors in the control, doxorubicin, or zoledronic acid treatment groups (Fig. 2). Significantly fewer proliferating cells were observed in tumors from animals treated sequentially with doxorubicin followed by zoledronic acid compared with those treated simultaneously with doxorubicin and zoledronic acid. Numbers of BrdUrd-positive proliferating cells in the different treatment groups were $252.49 \pm 9.47/mm^2$ (control), $259.96 \pm 14.31/mm^2$ (zoledronic acid), $238.70 \pm 25.24/mm^2$ (doxorubicin), $131.52 \pm 12.69/mm^2$ (doxorubicin and zoledronic acid), and $30.35 \pm 6.23/mm^2$ (doxorubicin followed by zoledronic acid). These data indicate that administration of doxorubicin and zoledronic acid exerts antiproliferative effects on intraosseous BO2 tumors and that these effects can be significantly potentiated if zoledronic acid is administered 24 h after doxorubicin.

In the extraosseous parts of the tumors, no differences in numbers of BrdUrd-positive cells were observed between any of the treatment groups. It therefore appears that doses of doxorubicin and zoledronic acid that are sufficient to reduce tumor cell proliferation within the bone marrow cavity are not effective in the extraosseous environment. These data imply that the location of the tumor growth is a critical factor in susceptibility to drug treatment and also that different areas of a single tumor may exhibit differential response to anticancer therapies.

Effects of simultaneous and sequential treatment of doxorubicin and zoledronic acid on tumor cell apoptosis. As well as reducing the growth of intraosseous tumors through reduction of tumor cell proliferation, sequential treatment with doxorubicin then zoledronic acid may also increase apoptotic tumor cell death. The level of tumor cell apoptosis in each treatment group was assessed by counting numbers of cells expressing active caspase-3. The intraosseous parts of the tumors showed a 3-fold increase in tumor cell apoptosis following sequential treatment with doxorubicin then zoledronic acid compared with tumors

Table 1. Effects of administration of doxorubicin and zoledronic acid alone, simultaneously, or sequentially on bone morphology following BO2 tumor-induced bone disease

	Area of osteolytic lesions (mm^2)	Trabecular fraction (BV/TV%)		Cortical volume (mm^3)	
		Tibia	Femur	Tibia	Femur
Control	8.59 ± 1.90	6.95 ± 2.43	7.26 ± 7.77	1.02 ± 0.04	0.55 ± 0.22
Zoledronic acid	1.65 ± 0.48*	40.83 ± 13.45*	41.56 ± 8.22*	1.64 ± 0.19*	1.00 ± 0.15*
Doxorubicin	6.64 ± 1.16	15.11 ± 1.80	17.40 ± 6.44	1.34 ± 0.02	0.82 ± 0.15
Doxorubicin+ zoledronic acid	2.93 ± 0.75*	34.59 ± 5.50*	39.19 ± 5.18*	1.66 ± 0.24*	0.85 ± 0.07
Doxorubicin then zoledronic acid	1.24 ± 0.36*	38.53 ± 11.34*	40.93 ± 12.33*	1.75 ± 0.20*	0.88 ± 0.27

NOTE: Mean ± SE.
*$P < 0.05$, one-way ANOVA.

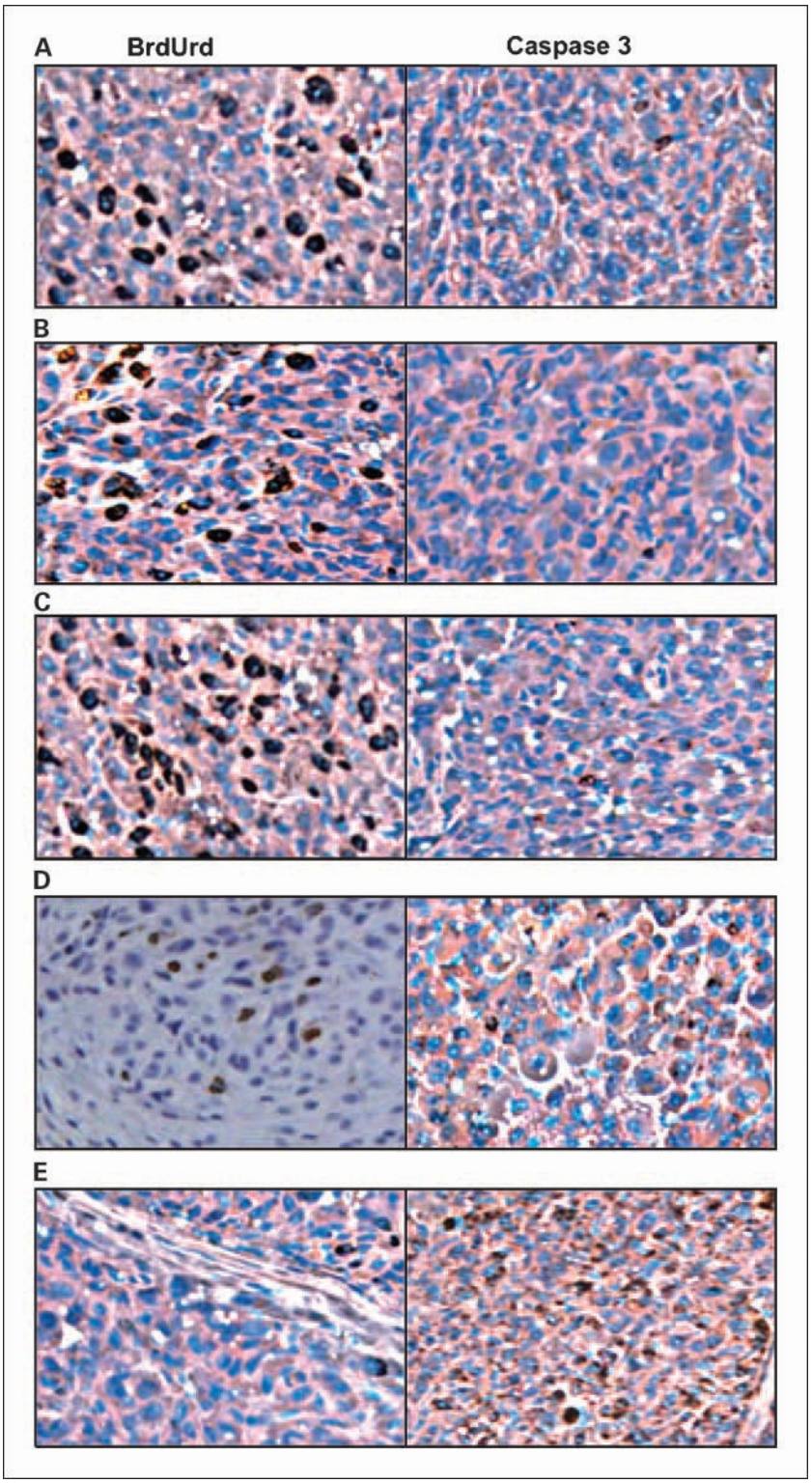

Fig. 3. Effects of zoledronic acid and doxorubicin, alone and in sequence or combination, on intraosseous tumor cell apoptosis and proliferation. Photomicrographs of intraosseous BO2 tumors following immunohistochemistry for active caspase-3 or BrdUrd. Mice were treated with saline (control; *A*), 100 μg/kg zoledronic acid (*B*), 2 mg/kg doxorubicin (*C*), doxorubicin and zoledronic acid simultaneously (*D*), or doxorubicin followed 24 h later by zoledronic acid (*E*).

from the control group (127.37 ± 7.76 versus 39.6 ± 5.46; $P = 0.0092$; Fig. 3). The level of apoptosis was also increased in the simultaneous doxorubicin and zoledronic acid treatment group compared with control (52.46 ± 6.16; $P = 0.0424$), but these levels were significantly lower than those recorded in the sequential treatment group ($P = 0.0185$). Treatment with doxorubicin or zoledronic acid alone did not induce caspase-3 activation.

The effects of the drugs were limited to the intraosseous parts of the tumors, with the exception of a small but significant increase in caspase-3-positive tumor cells following sequential treatment with doxorubicin followed by zoledronic acid

compared with control (67.65 ± 2.4 versus 50.93 ± 3.97/mm^2; $P = 0.0376$). No significant differences in numbers of caspase-3-positive cells were seen between any of the other treatment groups (Fig. 2). These results indicate that administration of clinically relevant doses of doxorubicin with zoledronic acid exert potent antitumor effects compared with either drug alone; furthermore, sequential administration with doxorubicin given 24 h before zoledronic acid appears to be superior to simultaneous administration of the two drugs.

Effects of simultaneous and sequential treatment of doxorubicin and zoledronic acid on survival of mice bearing BO2 tumors. A survival experiment was carried out to determine whether reduction of intraosseous tumor burden and/or inhibition of osteolytic bone disease following treatment with doxorubicin and zoledronic acid would affect survival of tumor-bearing mice. Ten animals were included per treatment group as described in Materials and Methods and were monitored twice weekly until they became moribund and/or experienced hind limb paralysis at which point they were sacrificed. Kaplan-Meier survival curves are presented in Fig. 4. Sequential treatment with doxorubicin followed 24 h later by zoledronic acid resulted in mice surviving significantly longer than any other treatment group ($P < 0.0001$). Median survival for mice treated sequentially with doxorubicin followed by zoledronic acid was 103 days compared with 82 days for those treated simultaneously with doxorubicin and zoledronic acid, 78 days for animals treated with zoledronic acid, 67 days for animals treated with doxorubicin, and 60 days for animals treated with saline (control). Interestingly, administration of zoledronic acid either alone or at the same time as doxorubicin resulted in significantly increased survival compared with treatment with saline or doxorubicin.

Discussion

It has been clearly shown that several different bisphosphonates can reduce tumor-induced osteolytic bone disease in animal models of a variety of cancers that metastasize to bone, including breast (8, 10, 32, 33), bladder (34, 35), and prostate cancer (16, 36–38) as well as multiple myeloma (11, 39, 40).

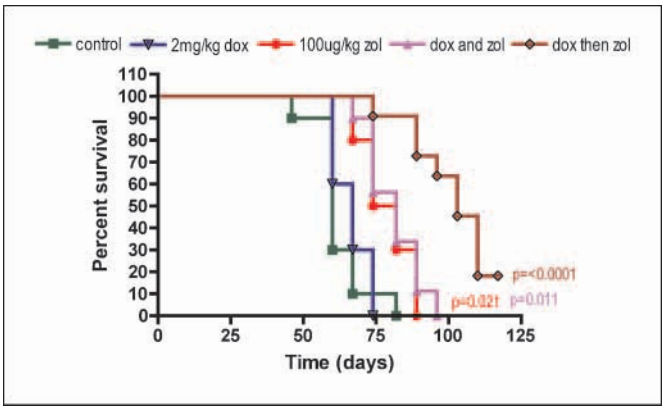

Fig. 4. Effects of zoledronic acid and doxorubicin, alone and in sequence or combination, on survival of mice bearing BO2 tumors. Kaplan-Meier survival curves representing percent survival of mice bearing BO2 tumors in their hind limbs. Statistical analysis is by one-tailed Mantel-Haenszel test and log-rank test for trend.

Inhibiting osteoclast-mediated bone resorption leads to a decrease in the release of tumor-promoting growth factors from bone; thus, it has been postulated that this is the mechanism by which bisphosphonates delay the further progression of bone metastasis. In all of these studies, however, high doses of bisphosphonates were used: Croucher et al. used 120 μg/kg zoledronic acid twice weekly for 12 weeks to prevent the occurrence of osteolytic bone disease in a mouse model of multiple myeloma (11). Corey et al. used 200 μg/kg zoledronic acid twice weekly from 4 to 9 weeks to reduce osteolytic lesions caused by metastatic prostate cancer cells in mice (16). Repeated dosing with zoledronic acid in this study was shown to reduce the volume of established intraosseous prostate tumors. Zoledronic acid has also been shown to reduce osteolytic lesions and intraosseous tumor growth in the BO2 model of breast cancer induced bone disease. However, to reduce growth of established tumors, a dose of 120 μg/kg zoledronic acid daily for 12 consecutive days was used (28). In the current study, we have investigated the effect of a clinically relevant dose of zoledronic acid (100 μg/kg) on tumor-induced osteolytic lesions and BO2 tumor growth in bone. A single dose of 100 μg/kg research grade zoledronic acid (disodium salt, 4.75 hydrate) is equivalent to 4 mg every 3 to 4 weeks, the dose currently administered to breast cancer patients with bone metastasis. In our study, 100 μg/kg zoledronic acid significantly reduced osteolytic lesion area but did not affect tumor growth, induce tumor cell apoptosis, or inhibit tumor cell proliferation either within the bone marrow cavity or on the external surface of the bone. These findings are in accordance with Daubiné et al. (18) who also reported that a single dose of 100 μg/kg zoledronic acid did not reduce intraosseous BO2 tumor growth. In this study, however, an accumulative dose of 100 μg/kg, where mice were administered zoledronic acid daily or weekly, significantly reduced intraosseous tumor burden. Thus, it appears that although a single clinical dose of zoledronic acid is sufficient to prevent cancer-induced bone disease, multiple doses of zoledronic acid are required to induce anticancer effects *in vivo*. These data indicate that zoledronic acid acts directly on breast cancer cells as opposed to an indirect effect through inhibition of bone resorption.

Combining bisphosphonates with cytotoxic drugs *in vivo* has been shown previously to synergistically increase the anticancer effects compared with either drug alone. In a prostate model of LuCap 23.1 cells growing in the tibia, combined administration of zoledronic acid and docetaxel resulted in a significant reduction in tumor growth within the bone. Mice were injected with 100 μg/kg zoledronic acid twice weekly for 7 weeks (24), which is equivalent to 32 mg/mo (8 times) the accumulative dose given to patients with cancer-induced bone disease, probably accounting for the reduction in tumor volume observed. Using the MDA-231F9AD/Luc breast carcinoma model, combining ibandronate with doxorubicin has been reported to be more effective at suppressing both bone and adrenal metastases compared with ibandronate or doxorubicin alone (9). This combination was only effective when the ibandronate and doxorubicin were administered before the tumors were established (in a preventive setting). No antitumor effects were reported against established tumors growing in the bone or adrenal glands following combination treatment with ibandronate and doxorubicin (9). Our data are the first to show a synergistic therapeutic effect of combining a

bisphosphonate with doxorubicin to significantly reduce established breast cancer tumor burden within the bone marrow cavity. In contrast, clinically relevant doses of doxorubicin (2 mg/kg) or zoledronic acid (100 μg/kg) used alone are not cytotoxic for BO2 breast cancer cells growing in the bone. Simultaneous administration of doxorubicin and zoledronic acid increases the cytotoxicity of these drugs to intraosseous BO2 tumors, stimulating tumor cell apoptosis and decreasing tumor cell proliferation. However, as reported by Yoneda et al., who combined ibandronate and doxorubicin (9), addition of zoledronic acid simultaneously with doxorubicin did not alter the area of bone occupied by tumor compared with control or doxorubicin or zoledronic acid alone treatment groups. The antitumor effects observed following sequential treatment (doxorubicin administered 24 h before zoledronic acid) were substantially more potent than those seen in the doxorubicin and zoledronic acid (simultaneous) treatment group, indicating that doxorubicin sensitizes the tumor cells to subsequent exposure to zoledronic acid.

Many groups have shown antitumor effects of bisphosphonates in bone (reviewed in ref. 7), and it has often been speculated that these effects may be limited to the bone microenvironment due to the high local concentration of bisphosphonate in bone relative to other organs and plasma. The majority of these studies, however, have investigated the effects of bisphosphonates on tumors growing within the marrow cavity. There are currently only two studies in which detailed analysis of the effects of bisphosphonates have been carried out on both intraosseous and extraosseous tumor areas: Van der Pluilm et al. (10) reported that pamidronate and olpandronate caused an increase in tumor growth in soft tissues and suggested that this is due to the lack of available space in the bone marrow cavity following inhibition of tumor-associated bone resorption. In addition, a recent study by Peng et al. (41) reported a significant decrease in tumor cell proliferation and an increase in tumor cell apoptosis inside the femur of mice treated with a combination of zoledronic acid and cyclophosphamide/topotecan compared with control or cyclophosphamide/topotecan. In this study, no alterations in tumor cell proliferation or apoptosis were observed in tumors outside of the bone (41). In our study, we also observed single tumors growing both in the marrow cavity as well as spilling out and expanding along the outside surface of the bone. Similarly to the study carried out by Peng et al., the parts of the tumors growing extraosseously were of roughly equal volume in each treatment group, and all extraosseous tumor areas expressed similar numbers of BrdUrd-positive proliferating cells. These findings imply that either sequential administration of doxorubicin followed by zoledronic acid exerts more potent antitumor effects within the bone microenvironment or that BO2 cells are more sensitive to treatment when they are growing intraosseously. Alternatively, the concentration of the drugs varies between the two sites, perhaps with zoledronic acid being present in higher concentrations within bone compared with outside. Areas of tumors growing in the intraosseous environment preferentially grow around the trabecular bone and are consequently in contact with more resorption pits on the bone surface (where bisphosphonate concentrations are highest; ref. 2) compared with tumor that has grown out of the bone. Another possible explanation for the increased cytotoxicity of sequential treatment with doxorubicin then zoledronic acid in the marrow cavity compared with the extraosseous bone surface could the ability of bisphosphonates to inhibit the release of growth factors from bone marrow stromal cells. Zoledronic acid has been shown to inhibit the secretion of interleukin-6 and inhibit the interleukin-1-stimulated production of matrix metalloproteinase-1 by human bone marrow cells in culture *in vitro* (42). However, we were unable to further investigate this hypothesis as levels of mouse interleukin-6 and interleukin-1 were below the level of detection in the plasma of mice from all experimental groups when assayed by ELISA. Considering that treatment with zoledronic acid alone was insufficient to cause anticancer effects, it is apparent that this is not the only mechanism by which these drugs are able to reduce tumor growth. Prior exposure of breast cancer cells to doxorubicin may facilitate the subsequent uptake of zoledronic acid; however, further studies are needed to elucidate the molecular pathways by which sequential administration of doxorubicin then zoledronic acid exert their synergistic antitumor effects.

In the clinic, breast cancer patients with bone metastasis treated with zoledronic acid show a trend toward improved survival and delayed progression of bone lesions (43, 44). It has been suggested that their effects on bone metabolism and prevention of skeletal events could provide additional benefits beyond the palliation of bone pain and these could contribute to increased survival (43). In our study, we showed an increase in survival of animals treated with zoledronic acid and in mice treated simultaneously with doxorubicin and zoledronic acid. Although tumor growth was not reduced in either of these treatment groups compared with control, there was a significant reduction in osteolytic lesion area. The endpoint for our survival studies was defined as hind limb paralysis/moribund state; thus, it is possible that this increased survival is an artifact of decreased bone destruction observed in these animals. Administration of doxorubicin followed by zoledronic acid delayed onset of hind limb paralysis significantly compared with all other treatment groups, indicating that both reduced intraosseous tumor burden and osteolytic bone disease contribute to increased survival observed in these animals.

In conclusion, this is the first study to show that administration of clinically relevant doses of doxorubicin and zoledronic acid exert synergistic antitumor effects in breast tumors growing within bone. Sequential administration of doxorubicin followed by zoledronic acid was superior to simultaneous treatment, and the single agents had no effect at the doses used. Our data also show a differential effect of the anticancer agents on intraosseous versus extraosseous tumor growth, suggesting that response to therapy may depend on the tumor location as well as on the surrounding nonmalignant tissues. Furthermore, we show that administration of doxorubicin followed by a single dose of zoledronic acid significantly increases the survival compared with the individual agents or simultaneous administration. Taken together, these results imply that the way commonly used anticancer agents are used to treat breast cancer–induced bone disease may not be optimized to achieve maximum benefit for the patients and that clinical trials using sequential treatment protocols may be required.

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