

Anticancer mechanisms of doxorubicin and zoledronic acid in breast cancer tumor growth in bone

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Abstract

Patients with advanced breast cancer frequently develop bone metastases, and at this stage, the disease is considered incurable. Here, we show that a 6-week course of weekly administration of doxorubicin (2 mg/kg), followed 24 hours later by the bisphosphonate zoledronic acid (100 µg/kg), causes substantial inhibition of MDA-MB-436 breast tumor burden in bone of immunocompromised mice, compared with administration of the single agents. Molecular analysis of tumors from animals treated sequentially with doxorubicin followed by zoledronic acid showed reduced numbers of proliferating tumor cells and decreased expression of cyclins E1, B, D1, and D3 as well as cdk2 and cdk4. Tumors from the sequential treatment group also displayed increased levels of apoptosis, increased expression of bcl2-associated X protein, decreased expression of B-cell chronic lymphocytic leukemia/lymphoma 2, and activation of caspase 3, 8, and 9. Zoledronic acid caused a small reduction in tumor volume, reduced tumor cell proliferation, and decreased expression of cyclins D1 and D3, compared with tumors from animals treated with saline or doxorubicin. Doxorubicin had no effect on tumor growth, cell cycle, or apoptosis *in vivo*, but did cause increased accumulation of a bisphosphonate in MDA-MB-436 cells *in vitro*, suggesting that doxorubicin may affect subsequent uptake of zoledronic acid. In support of this, accumulation of unprenylated Rap1A, a surrogate marker of zoledronic acid, was only detected in tumors following sequential treatment, and not following treatment with zoledronic acid alone. Our data are the first to show the specific molecular pathways by which sequential treatment with doxorubicin and zoledronic acid

induce tumor cell apoptosis and inhibit proliferation in an *in vivo* model of breast tumor growth in bone. [Mol Cancer Ther 2009;8(10):2821–32]

Introduction

Cancer-induced bone disease is a common and incurable complication of advanced breast cancer, highlighting the need for more effective treatment strategies, and increased understanding of the mechanisms by which these exert their anticancer effects (1).

The third generation nitrogen containing bisphosphonate, zoledronic acid, is the only bisphosphonate licensed for the treatment of 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 (3) including farnesyl pyrophosphate synthase (4, 5) and geranylgeranyl pyrophosphate synthase (6), leading to incomplete posttranslational prenylation of signaling GTPases, including Ras, Rho, and Rac (7), which ultimately causes osteoclasts to undergo apoptosis (8). The mevalonate pathway is not unique to osteoclasts; it is an important part in the metabolic process leading to cholesterol synthesis, which is ubiquitous to all nucleated cells. Thus, inhibition of this pathway may also induce apoptosis in a variety of other cell types. *In vitro* studies have clearly shown the potential for zoledronic acid to induce direct antitumor effects including inhibition of tumor cell growth, induction of apoptosis, (9–11), inhibition of adhesion and invasion (12–14), and antiangiogenic activity (15, 16). However, the concentrations of bisphosphonates used in these studies range from 5 to 20 µmol/L for up to 72 hours, compared with the 2 µmol/L circulating plasma concentration detectable in breast cancer patients for 1 to 2 hours following the standard 4-mg infusion of zoledronic acid (17). Antitumor effects of bisphosphonates have also been reported in *in vivo* model systems that mimic tumor-induced bone disease from a variety of cancer types including breast (18, 19), prostate (20), leukemia (21), and multiple myeloma (22). These studies also used high doses and frequent administration of zoledronic acid (from 120 µg/kg 2× per week to 120 µg/kg daily), whereas a clinically relevant dose of zoledronic acid (100 µg/kg 1× per month) was reported not to exert anticancer effects in a model of breast cancer bone metastases (23).

There is increasing evidence that combining bisphosphonates with other anticancer agents has the potential to significantly enhance the effects seen following treatment with either drug alone. Zoledronic acid has been shown to synergistically increase cancer cell death when combined with doxorubicin, paclitaxel, or cyclophosphamide in breast cancer cells (24–26); paclitaxel, etoposide, cisplatin, and irinotecan in lung cancer cells (27); and dexamethasone in

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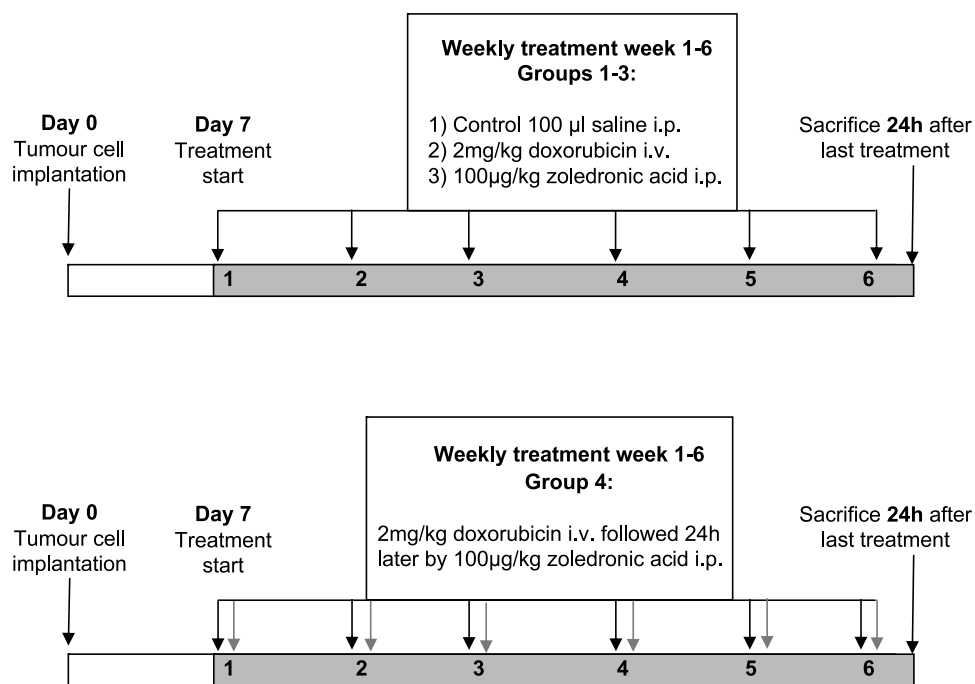


Figure 1. Treatment schedules. Diagram showing an outline of the treatment schedules used in this study. Following intratibial implantation of 1×10^5 MDA-MB-436/GFP tumor cells on day 0, animals were divided into four different groups receiving weekly treatment as indicated. *Top*, treatment groups 1 to 3; *bottom*, group 4. *Gray arrows*, administration of zoledronic acid 24 h after doxorubicin in animals receiving sequential drug treatment (group 4). Animals were sacrificed 24 h following the final treatment.

myeloma cells (28) *in vitro*. Combining zoledronic acid (250 $\mu\text{g}/\text{kg}$) with the oral fluoropyrimidine UFT (20 mg) daily decreased the number of bone metastases compared with single agents in the 4T1 mouse mammary tumor model (29). Increased inhibition of tumor growth was detected when zoledronic acid was combined with imatinib mesylate and paclitaxel in the PC-3MM2 model of prostate cancer in bone (30), and combining zoledronic acid with ifosfamide reduced tumor growth in a rat model of osteosarcoma (31). However, these studies used high doses of zoledronic acid ranging from a single injection of 250 to 100 $\mu\text{g}/\text{kg}$ 2 \times per week. We have recently shown that administration of a clinically relevant dose of doxorubicin (2 mg/kg) followed by a single injection of zoledronic acid (100 $\mu\text{g}/\text{kg}$) reduces intraosseous tumor growth in the B02 model of breast cancer bone metastases (32). Optimal antitumor effects were observed when doxorubicin was administered 24 hours before zoledronic acid, whereas giving the two drugs at the same time had no effect on tumor growth, indicating that the sequence in which these agents are administered may be important to achieve maximal therapeutic potential. In agreement with this, a 6-week course of weekly treatment with 2 mg/kg doxorubicin followed 24 hours later by 100 $\mu\text{g}/\text{kg}$ zoledronic acid eliminated growth of s.c. implanted MDA-MB-436 tumors (33). Although sequential administration was more effective than simultaneous in both models, the molecular mechanisms underlying this effect were not determined.

Here, we investigated the molecular mechanisms by which doxorubicin and zoledronic acid exert their com-

bined antitumor effects, using an *in vivo* model of breast tumor growth in bone. Sequential administration of doxorubicin followed by zoledronic acid resulted in significantly decreased bone tumor burden, accompanied by activation of intrinsic and extrinsic apoptotic pathways in the tumors and increased accumulation of unphosphorylated Rap1a. Decreased expression of cyclins and cyclin-dependent kinases, responsible for cell progression through G_1 , G_1 -S, G_2 , and G_2 -M phases of the cell cycle, were detected. *In vitro*, pretreatment of the MDA-MB-436 breast cancer cells with doxorubicin for 24 hours caused increased accumulation of bisphosphonate. This is the first report showing that *in vivo* administration of doxorubicin followed by zoledronic acid causes specific changes in gene/protein expression and inhibition of the mevalonate pathway in breast tumors in bone.

Materials and Methods

Maintenance of Cell Lines

Human MDA-MB-436 breast cancer cells stably expressing green fluorescent protein (eGFP) were maintained in DMEM supplemented with 4,500 mL/L glucose, L-glucosamine, and pyruvate and 10% fetal calf serum (Invitrogen-Life Technologies). This estrogen receptor (ER)-negative cell line is originally isolated from pleural effusion of a 43-year-old woman diagnosed with a breast adenocarcinoma, and was chosen as grows well in our mice without the need for implantation of estrogen pellets.

Inoculation of Bone Tumors and Drug Treatment

MDA-MB-436/GFP cells (1×10^5) were injected directly into the rear right tibiae of female CD1 nu/nu mice under general anesthesia induced by 100 mg/kg ketamine (Fort Dodge Animal Health Ltd) and 15 mg/kg xylazine (Bayer plc). All experiments were carried out in accordance with local guidelines and with Home Office approval under project license 40/2343 held by Professor N.J. Brown, University of Sheffield, United Kingdom.

Experiments were carried out in triplicate with each experimental group consisting of five animals. Tumor growth was detected in the tibiae of 80% of animals using a Lighttools GFP imaging system (Lighttools) 7 d following inoculation. At this point, animals were divided into four groups with equal tumor burden giving a total of 12 mice per treatment group. Following detection of intratibial tumor growth (day 7), mice were given the following: saline (control) via i.p. injection; 2 mg/kg doxorubicin (Pharmachemie B.V.) by i.v. injection; 100 μ g/kg zoledronic acid ([1-hydroxy-2-(1H-imidazoledronic acid-1-yl) ethylidene] biphosphonic acid; Novartis Pharma AG) i.p. or doxorubicin followed 24 h later by zoledronic acid, 1 \times per week for 6 wk (outlined in Fig. 1). The limited number of groups was based on our previous work showing that giving the two agents in combination only has an intermediary effect, and that the reverse sequence does not induce increased antitumor effects compared with the single agents (32, 33). The doses used in this study are comparable with clinical doses of both compounds. Doxorubicin (2 mg/kg) is equivalent to a dose of 6.5 mg/m² in patients¹ and 100 μ g/kg zoledronic acid were similar to the 4 mg clinical dose. Maximal doses of either drug were deliberately not included, to be able to see any additional effects of sequential administration. All animals were sacrificed by cervical dislocation 24 h following administration of their final treatment. Intratibial tumors extracted from 4/12 legs per treatment group and half of each tumor placed in cell lysis buffer from an MCL1-1KT mammalian cell lysis kit (Sigma) and protein was extracted according to manufacturer's instructions; the other half was placed in RNA later (Ambion) and stored at -20°C before RNA extraction. The remaining 8/12 tibiae per treatment group were simultaneously fixed and decalcified in a solution of 1% paraformaldehyde and 0.5% EDTA in PBS for 4 wk and then embedded in paraffin wax.

Bone Histology and Measurement of Tumor Volume

Histologic sections of decalcified tibiae (5 μ mol/L) were stained with H&E. Tumor volume was measured by drawing round tumors on four nonserial histologic sections per sample using Osteomeasure software (Osteometrics, Inc.) and a computerized image analysis system.

Immunohistochemistry

Immunohistochemistry for caspase-3 was done with the use of a rabbit polyclonal antibody that recognizes active human caspase-3 (AF835, 1:750 dilution; R&D Systems),

followed by a biotin-conjugated anti-rabbit secondary antibody (1:200 dilution; Vector laboratories) as described by Marshman et al. (34). Immunohistochemistry for the cell proliferation antigen Ki67 was carried out as previously described (34) using a mouse monoclonal antibody specific for human Ki67 (MIB-1, 1:125 dilution; DakoCytomation) followed by a biotin-conjugated anti-mouse secondary antibody (1:200; Vector laboratories).

Four nonserial sections per tumor sample were stained with each antibody and the numbers of caspase-3- or Ki67-positive cells in five randomly chosen 750 μm^2 fields of view per section were scored using a Leica BMRB upright microscope and OsteoMeasure software (Osteometrics).

Osteoclast Histochemistry

Osteoclasts were detected in sections of tumor-bearing tibiae by toluidine blue and tartate-resistant acid phosphatase staining as previously described (32). Two 5- μm sections per sample were scored; the numbers of osteoclasts per millimeter of cortical bone surface and the area of bone surface in contact with osteoclasts were quantified using a Leica RMRB upright microscope and OsteoMeasure software (Osteometrics) as previously described (32).

DNA Microarray Analysis

Biopsies from four tumors from mice in each treatment group were pooled before mRNA extraction using a SuperArray, ArrayGrade mRNA isolation kit (Tebu-Bio), and the manufacturer's instructions. Two micrograms of mRNA were used to produce biotin-labeled cRNA riboprobes with a SuperArray TrueLabeling-AMP 2.0 kit (Tebu-Bio) and biotinylated-UTP (Perkin-Elmer). Riboprobe from each treatment group was hybridized separately to an Oligo GEArray Human Cell Cycle DNA microarray (OHS-020) and to an apoptosis pathway-specific DNA microarray (OHS-012; Tebu-Bio). Gene expression was analyzed using GEArray Expression Analysis Suite.² Pathway Architect software (Stragagene) was subsequently used to generate gene maps linking genes to specific pathways.

Real-time PCR

Total RNA was extracted with Trizol (Invitrogen AB), before reverse transcription using Superscript II (Invitrogen), the resulting cDNA was used as a template for real-time quantitative PCR. Three tumors per treatment group were analyzed separately for relative mRNA expression compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1, Applied Biosystems) using an ABI 7900 PCR System (Perkin-Elmer, Applied Biosystems) and Taqman universal master mix (Applied Biosystems). Relative levels of apoptosis and cell cycle-related genes were assessed using the following Taqman gene expression assays (Applied Biosystems): *Caspase 2* (Hs00154240_m1); *Caspase 2* and *RIPK1* domain-containing adaptor with death domain (*CRADD*; Hs00388731_m1); Fas-Associated protein with Death Domain (*FADD*; Hs00538709_m1) *tumor protein 53*

¹ Calculated using the conversion table available at www.fda.gov/downloads/Drugs/GuidanceComplianceRegulator.

² <http://geasuite.superarray.com/>

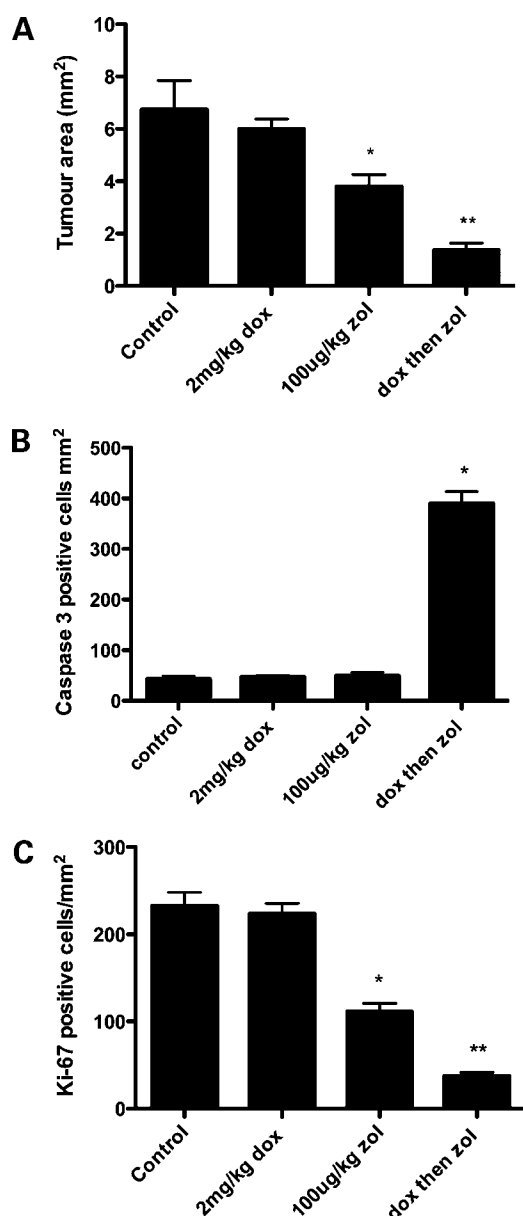


Figure 2. Effects of doxorubicin and zoledronic acid on tumor growth, apoptosis, and proliferation of breast tumors in bone. Columns, mean; bars, SEM. **A**, intraosseous tumor area. **B**, numbers of active caspase 3-positive tumor cells. **C**, numbers of Ki67-positive tumor cells. Mice ($n = 8$ /group) were treated with saline (control), 2 mg/kg doxorubicin, 100 μ g/kg zoledronic acid, or doxorubicin followed 24 h later by zoledronic acid, weekly for 6 wk. *, $P < 0.005$; **, $P < 0.0005$ by Kruskal-Wallis followed by Dunn's multiple comparisons test.

(Hs99999147_m1); *B-cell chronic lymphocytic leukemia/lymphoma 2* (Hs00236808_s1); *BCL2-associated X protein* (Hs99999001_m1) *cyclin-dependent kinase inhibitor 1A* (P21; Hs00355782_m1); *cyclin-dependent kinase inhibitor 1B* (P27; Hs00153277_m1); *CYCLIN D1* (Hs99999004_m1); *CYCLIN D3* (Hs 01017690_g1); *CYCLIN E* (Hs010518-94_m1); *cell division cycle 2, G₁-S, and G₂-M*; (CDC2; Hs00364293_m1); *cyclin-dependent kinase 7* (CDK7;

Hs00387062_m1); *cyclin-dependent kinase 2* (CDK2; Hs01548894_m1); and *cyclin-dependent kinase 4* (CDK4; Hs00364847_m1). Relative mRNA were determined using the formula $2^{-\Delta CT}$ (CT; cycle threshold) where $\Delta CT = CT$ (target gene) - CT (GAPDH). To check that the Taqman assays did not cross-react with mouse cells present in the tissue samples, cDNA reverse transcribed from RNA extracted from the bone marrow of naïve female CD1 nude mice was assessed for cross-reactivity. MDA-MB-436 human breast cancer cells were used as a positive control. With the exception of *CDK2* and *CDK4*, the human Taqman assays did not cross-react with the corresponding mouse genes and thus any expression detected in the tumor samples was assumed to be by the human cells.

Western Blotting

Tumor lysates from four mice per treatment group were pooled and protein quantified with bicinchoninic acid and copper sulfate as previously described (35). Ten micrograms of protein from each treatment group were run on a 10% polyacrylamide gel and transferred onto imibilon-P nitrocellulose membrane (Millipore). Nonspecific antibody binding was blocked with 1% casein (Vector Laboratories) before incubation with the relevant primary antibody. Proteins were detected using the following antibodies: Mouse monoclonal p53 (ab1101; 1:1,000), rabbit polyclonal caspase 9 (ab25758; 1:1,000), rabbit monoclonal (E6) caspase 8 (ab32125; 1:750), mouse monoclonal bcl2 (ab692; 1:100), rabbit polyclonal bax (ab10813; 1:1,000), rabbit monoclonal (E83-77) caspase 3 (ab32042; 1:500), mouse monoclonal p21 (ab54562; 1:10,000), mouse monoclonal cyclin E (ab3927; 1:250), mouse monoclonal cyclin D1 (ab6152; 1: 500), rabbit monoclonal cyclin D3 (ab52598; 1:5,000), mouse monoclonal Cyclin B (1:1000; BD Biosciences), mouse monoclonal cdk2 (ab6433; 1:2,500), mouse monoclonal cdk 4 (ab6315; 1:1,000), and goat polyclonal Rap1a (1:200; Santa Cruz). All antibodies were from AbCam unless otherwise specified and were made up in 1 \times PBS/1% casein and incubated with the membrane for 16 h at 4°C. Secondary antibodies used were sheep anti-mouse-horseradish peroxidase (HRP; 1:15,000), anti-goat-HRP (1:30,000), and donkey anti-rabbit-HRP (1:30,000; GE Healthcare Life Sciences). HRP was detected with Supersignal chemiluminescence detection kit (Pierce).

Flow Cytometry

For evaluation of bisphosphonate uptake 24 h following treatment with doxorubicin, MDA-MB-436 cells were treated with 25 μ mol/L alendronate-FITC (kind gift from Dr. Keith Thompson, University of Aberdeen, Aberdeen, UK), 1 or 25 nmol/L doxorubicin alone, or 25 μ mol/L alendronate-FITC 24 h following administration of either 1 or 25 nmol/L doxorubicin. Twenty-four hours after treatment, cells were suspended in PBS containing 10% fetal calf serum. Cells were analyzed using a 488-nm laser on a FACS-Calibur (BD) and CellQuest software.

Statistical Analysis

Statistical analysis was by one-way ANOVA and Dunnett's two-sided multiple comparison test (real-time reverse transcription-PCR analysis) or Kruskal-Wallis followed by

Dunn's multiple comparisons test. Statistical significance was defined as *P* value of <0.05. All *P* values are two sided.

Results

Sequential Administration of Doxorubicin and Zoledronic Acid Inhibits Breast Tumor Growth in Bone

The main aim of this study was to investigate the molecular mechanisms by which sequential administration of doxorubicin and zoledronic acid exert their antitumor effects in bone. We therefore determined the effects of weekly treatment with 2 mg/kg doxorubicin followed 24 hours later by 100 µg/kg zoledronic acid on growth of MDA-MB-436 tumor cells implanted directly into tibiae of nude

mice. In this model, the tumors grow relatively slowly, allowing us to determine the effects of a 6-week course of treatment. As shown in Fig. 2A, MDA-MB-436 tumor growth was significantly reduced in the tibiae of mice treated sequentially with doxorubicin followed by zoledronic acid, compared with those treated with saline (*P* < 0.0005), doxorubicin (*P* < 0.0005), or zoledronic acid alone (*P* < 0.005). There was no significant effect on bone tumor burden following administration of doxorubicin weekly for 6 weeks, whereas administration of zoledronic acid resulted in a small but significant reduction in intratibial tumor volume compared with control mice (*P* < 0.005) or mice treated with doxorubicin (*P* < 0.05). Having established that sequential administration of doxorubicin followed by zoledronic acid reduced tumor

Table 1. Effects of doxorubicin and zoledronic acid on apoptosis and cell cycle gene expression in bone metastases *in vivo*

A. Relative expression of apoptosis-related genes measured by real-time reverse transcription-PCR and normalized to GAPDH					
Gene		Control	Doxorubicin	Zoledronic acid	Doxorubicin then zoledronic acid
<i>Bcl-2</i>	Fold change		-1.10 ± 0.20	-1.37 ± 0.30	-3.89 ± 0.76*
	ΔCT	11.58 ± 0.27	12.03 ± 0.22	12.45 ± 1.59	13.78 ± 0.67
<i>Caspase 2</i>	Fold change		-1.31 ± 0.20	-1.08 ± 0.21	4.44 ± 0.34*
	ΔCT	11.41 ± 0.88	11.33 ± 0.25	11.07 ± 0.29	9.86 ± 1.24
<i>CRADD</i>	Fold change		4.38 ± 1.97[†]	1.60 ± 0.81	5.57 ± 1.41*
	ΔCT	11.21 ± 0.78	8.91 ± 0.61	10.61 ± 0.96	8.62 ± 0.74
<i>FADD</i>	Fold change		1.33 ± 0.82	1.52 ± 0.68	2.58 ± 0.24*
	ΔCT	12.05 ± 1.10	9.91 ± 0.85	9.53 ± 0.76	9.02 ± 0.96
<i>Bax</i>	Fold change		-1.14 ± 0.26	1.32 ± 0.36	4.28 ± 0.57*
	ΔCT	10.81 ± 0.39	11.03 ± 0.43	10.45 ± 0.49	8.64 ± 0.20
B. Relative expression of cell cycle-related genes measured by real-time reverse transcription-PCR and normalized to GAPDH					
Gene		Control	Doxorubicin	Zoledronic acid	Doxorubicin then zoledronic acid
<i>P53</i>	Fold change		-1.39 ± 0.07	1.36 ± 0.24	2.18 ± 0.03*
	ΔCT	6.24 ± 0.10	6.75 ± 0.24	5.84 ± 0.30	5.21 ± 0.16
<i>P21</i>	Fold change		-1.15 ± 0.22	-1.04 ± 0.05	4.54 ± 0.98*
	ΔCT	5.45 ± 0.08	5.72 ± 0.31	5.51 ± 0.08	3.35 ± 0.36
<i>P27</i>	Fold change		-1.31 ± 0.87	-1.05 ± 0.41	1.98 ± 0.74
	ΔCT	7.03 ± 1.21	6.97 ± 0.87	6.26 ± 0.18	5.53 ± 0.80
<i>GADD 45A</i>	Fold change		1.87 ± 1.54	1.16 ± 0.90	-1.16 ± 1.47
	ΔCT	12.19 ± 1.84	10.45 ± 1.32	11.34 ± 1.61	12.50 ± 1.85
<i>Cdk7</i>	Fold change		-1.56 ± 0.32	1.34 ± 0.43	-2.00 ± 0.64
	ΔCT	6.89 ± 0.36	7.53 ± 0.35	6.57 ± 0.54	7.98 ± 0.48
<i>Cyclin D1</i>	Fold change		-1.21 ± 0.12	1.75 ± 0.33	-4.26 ± 1.24*
	ΔCT	8.22 ± 0.15	8.51 ± 0.15	7.46 ± 0.28	10.57 ± 0.60
<i>Cyclin D3</i>	Fold change		1.14 ± 0.20	-1.72 ± 0.37	-10.98 ± 4.12*
	ΔCT	7.39 ± 0.74	7.28 ± 0.27	7.89 ± 0.33	10.81 ± 0.76
<i>Cdk4</i>	Fold change		-1.44 ± 0.27	1.32 ± 0.66	-4.53 ± 1.39*
	ΔCT	8.61 ± 0.53	8.71 ± 0.37	8.44 ± 0.80	11.61 ± 1.50
<i>Cyclin E</i>	Fold change		-2.89 ± 1.38	-1.27 ± 0.20	-7.02 ± 1.76*
	ΔCT	8.46 ± 0.46	10.51 ± 1.15	8.69 ± 0.24	12.77 ± 2.16
<i>Cdk2</i>	Fold change		-1.03 ± 0.24	-0.68 ± 0.26	-4.28 ± 0.15*
	ΔCT	7.16 ± 0.29	7.25 ± 0.40	6.77 ± 0.55	9.43 ± 0.59
<i>Rb</i>	Fold change		-1.80 ± 0.62	1.79 ± 0.51	1.24 ± 0.18
	ΔCT	8.09 ± 1.14	8.59 ± 0.72	6.74 ± 0.47	7.17 ± 0.21

NOTE: CT values and fold changes compared with control, and are mean ± SEM. Data highlighted in bold show a 2-fold or more increase or decrease in gene expression.

**P* < 0.005 compared with any of the other treatment groups by one way ANOVA and Dunnett's two-sided multiple comparisons test.

[†]*P* < 0.005 compared with control.

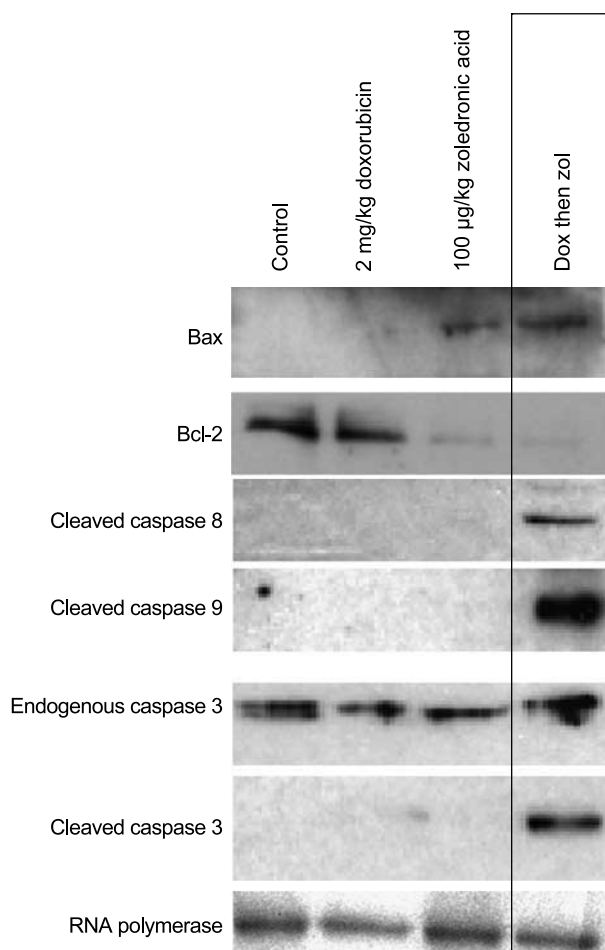


Figure 3. Effects of doxorubicin and zoledronic acid, alone and in sequence, on apoptosis-related protein expression. Tumor lysates were prepared from animals treated with saline (*control*), 2 mg/kg doxorubicin, 100 µg/kg zoledronic acid, or doxorubicin followed 24 h later by zoledronic acid, weekly for 6 wk. Representative Western blots from one of three experiments show expression of apoptosis-related proteins Bax and Bcl-2, and cleavage of caspases 8, 9, and 3. RNA polymerase is included to show equal sample loading.

volume in bone, at doses where the individual agents had little or no effect, we next investigated the ability of these treatments to induce apoptosis and cell cycle arrest in the tumors.

Effects of Doxorubicin and Zoledronic Acid on Tumor Cell Apoptosis in Bone

The levels of treatment-induced tumor cell apoptosis were assessed by analyzing the number of cells positive for active caspase 3 on histologic section of intratibial tumors following immunohistochemical staining. As shown in Fig. 2B, no significant differences were detected in numbers of caspase 3-positive cells in tumors from animals treated with saline, doxorubicin, or zoledronic acid. In contrast, sequential administration of doxorubicin, followed 24 hours later by zoledronic acid, resulted in a substantial increase in the proportion of tumor cells positive for active caspase 3, compared with control ($P < 0.0005$), doxorubicin ($P < 0.0005$), or zoledronic acid ($P < 0.0005$).

We next carried out a detailed analysis of the molecular mechanisms by which the sequential treatment schedule induced tumor cell apoptosis in the bone tumors. Pathway-specific microarrays were used to examine alterations in the expression of 112 apoptosis-related genes in tumors isolated from bones of mice treated with doxorubicin followed by zoledronic acid, compared with those treated with saline or single agents. Genes that showed a 2-fold or greater change in expression were clustered with the use of GEAsuite software, and Pathway Architect software was subsequently used to link these genes to specific pathways. We identified increased expression of proapoptotic *p53*, *bax*, *caspase 2*, *CRADD*, and *FADD*, as well as a decrease in the antiapoptotic gene *bcl-2*, in tumors from animals receiving doxorubicin followed by zoledronic acid, compared with those receiving saline or single agents. All apoptosis-related genes shown by microarray analysis to be specifically altered in intratibial tumors following sequential treatment were subsequently confirmed by real-time quantitative PCR, using assays that do not cross-react with mouse cDNA (Table 1A): Expression of *bcl-2* was reduced ($P < 0.005$), whereas expression of the proapoptotic genes *caspase 2*, *CRADD*, *FADD*, and *bax* were increased ($P < 0.005$, $P < 0.005$, $P < 0.05$, and $P < 0.005$, respectively). Expression of both *FADD*, which activates the extrinsic (nonmitochondrial) pathway via caspase 8 cleavage, and *CRADD*, which activates the intrinsic (mitochondrial dependent) pathway by binding to caspase 2 and causing cleavage of caspase 9, was increased in tumors following sequential treatment. Western blot analysis also showed that sequential administration of doxorubicin followed by zoledronic acid resulted in cleavage of caspases 8 and 9, supporting that both mitochondrial-dependent and mitochondrial-independent apoptotic pathways are activated in bone metastases following this treatment schedule (Fig. 3).

To confirm that apoptosis occurred via the mitochondrial pathway, the levels of proapoptotic *bax* and antiapoptotic *bcl-2* protein were determined by Western blot. We found that *bax* levels were increased in tumors treated sequentially with doxorubicin followed by zoledronic acid, accompanied by decreased levels of *bcl-2* (Fig. 3). Similar changes in the levels of *bax/bcl-2* were detected in tumors from animals treated with zoledronic acid, but without detectable levels of cleaved caspase 8 or 9. In agreement with the histologic scoring (Fig. 2B), cleavage of caspase 3 was only detected in tumors treated sequentially with doxorubicin followed by zoledronic acid (Fig. 3).

Effects of Doxorubicin and Zoledronic Acid on Tumor Cell Proliferation in Bone

The effect of sequential treatment with doxorubicin 24 hours before zoledronic acid on tumor cell proliferation was analyzed on histologic sections of tumors grown in bone by scoring of cells following immunohistochemistry for Ki67. As shown in Fig. 2C, tumors from animals having received sequential treatment had significantly lower levels of cell proliferation compared with tumors from animals treated with saline (control; $P < 0.0005$), doxorubicin

($P < 0.0005$), or zoledronic acid ($P < 0.005$). Tumors from animals having received zoledronic acid alone also displayed significantly decreased levels of tumor cell proliferation, compared with saline (control; $P < 0.0005$) or doxorubicin ($P < 0.0005$) treatment.

To elucidate the mechanisms by which cell proliferation is suppressed following sequential treatment, we used pathway-specific microarrays that included 112 cell cycle-related genes. Analysis of gene expression showed increased levels of *p21* and *p53*, and decreased levels of *cyclin D1*, *cyclin D3*, *cyclin E*, *cdk4*, and *cdk2*, in tumors from animals treated with doxorubicin followed by zoledronic acid, compared with tumors from all other treatment groups. Alterations in gene expression identified by microarray analysis were further investigated by real-time quantitative reverse transcription-PCR. Except for analysis of *cdk2* and

cdk4, the assays used did not cross-react with mouse cDNA (data not shown). Using this method, the cell cycle regulatory genes *p53* and *p21* were shown to be expressed at significantly higher levels in tumors from mice treated sequentially with doxorubicin followed by zoledronic acid, compared with tumors from control or single treatment groups ($P < 0.005$ for *p53* and $P < 0.0005$ for *p21*, respectively; Table 1B). Sequential drug administration also resulted in decreased expression of a number of other genes encoding for cyclins and cyclin-dependent kinases: *cyclin D1* ($P < 0.005$), *cyclin D3* ($P < 0.0005$), *cyclin E* ($P < 0.0005$), *cdk2* ($P < 0.001$), *cdk7* ($P < 0.005$), and *cdk4* ($P < 0.005$), compared with administration of saline, doxorubicin, or zoledronic acid alone (Table 1B), confirming the results obtained using the microarrays. No significant differences in gene expression were detected between tumors from control animals and those treated with either doxorubicin or zoledronic acid alone.

To investigate whether alterations in gene expression were translated into corresponding changes in protein levels, Western blot analysis was carried out on protein extracted from bone tumors from all treatment groups (Fig. 4). Intratibial tumors from mice treated sequentially with doxorubicin followed by zoledronic acid exhibited increased levels of the cell cycle regulatory proteins; *p53* and *p21*^{WAF1/CIP1} compared with tumors from any other treatment group. In addition, sequential treatment resulted in reduced tumor levels of cyclins D1, D3, B, and E, as well as their respective cyclin-dependent kinases *cdk4* and *cdk2*, compared with all other treatment groups. Zoledronic acid caused a reduction in the tumor level of cyclin D1 and cyclin D3, compared with control or doxorubicin alone, probably accounting for the decrease in Ki67-positive cells detected in these tumors (Fig. 2C).

Effects of Doxorubicin and Zoledronic Acid on Cancer-Induced Bone Disease

Zoledronic acid reduces the extent of lytic bone lesions by inhibition of osteoclast activity, thereby affecting progression of bone metastases. We therefore investigated the effects of weekly administration of 100 $\mu\text{g}/\text{kg}$ zoledronic acid, alone or in combination with doxorubicin, on osteoclasts lining the surface of both endocortical and trabecular areas of tumor-bearing bones. As shown in Fig. 5, zoledronic acid, alone or following doxorubicin, caused a significant reduction in both numbers of osteoclasts lining the surface of trabecular bone and in the % of trabecular bone surface in contact with osteoclasts, compared with bones from animals treated with saline or doxorubicin ($P < 0.005$ for both). There was no effect on osteoclasts lining the endocortical bone in any treatment group, but the numbers of osteoclasts detected in these areas were very low.

Effects of Pretreatment with Doxorubicin on Bisphosphonate Uptake

The underlying mechanisms explaining why the observed antitumor effect requires administration of doxorubicin before zoledronic acid remains to be identified. We investigated whether pretreating breast cancer cells with

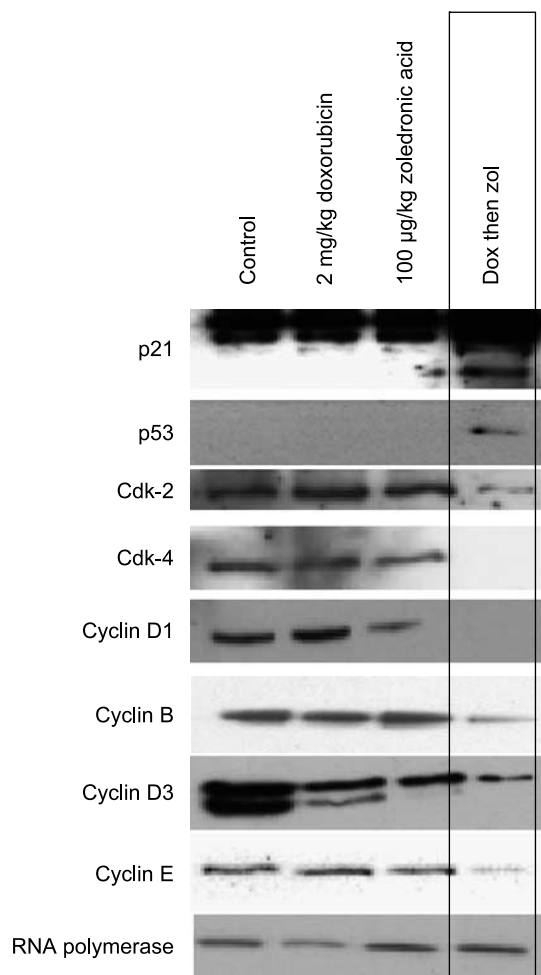


Figure 4. Effects of doxorubicin and zoledronic acid, alone and in sequence, on cell cycle-related protein expression. Tumor lysates were prepared from animals treated with saline (*control*), 2 mg/kg doxorubicin, 100 $\mu\text{g}/\text{kg}$ zoledronic acid, or doxorubicin followed 24 h later by zoledronic acid, weekly for 6 wk. Representative Western blots from one of three experiments show expression of cell cycle-related proteins P21, P53, Cdk-2, Cdk-4, Cyclin D1, Cyclin B, Cyclin D3, and Cyclin E. RNA polymerase is included to show equal sample loading.

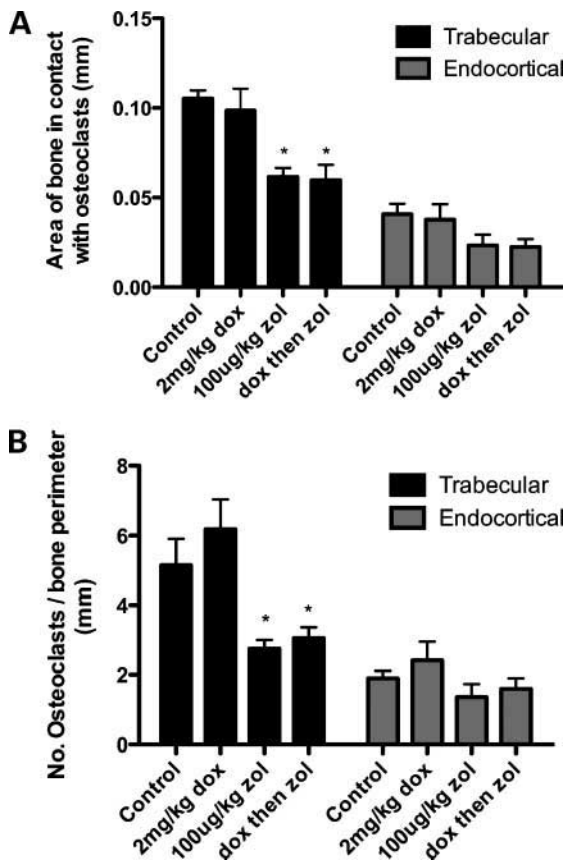


Figure 5. Effects of doxorubicin and zoledronic acid, alone and in sequence, on osteoclasts. *Columns*, mean; *bars*, SEM. **A**, area of endocortical and trabecular bone in contact with osteoclasts. **B**, number of osteoclasts perimeter (mm) of endocortical or trabecular bone following treatment with saline (*control*), 2 mg/kg doxorubicin, 100 μ g/kg zoledronic acid, or doxorubicin followed 24 h later by zoledronic acid, weekly for 6 wk. Four nonserial sections of bone from each animal were analyzed ($n = 12$); *, $P < 0.005$ by Kruskal-Wallis followed by Dunn's multiple comparisons test.

doxorubicin increased subsequent bisphosphonate uptake *in vitro* and *in vivo*. We used a fluorescently labeled alendronate (alendronate-FITC), as there is no commercially available equivalent of zoledronic acid. As shown in Fig. 6A, pretreatment of MDA-MB-436 cells with either 1 or 25 nmol/L doxorubicin for 24 hours before administration of alendronate-FITC resulted in a $54 \pm 0.14\%$ and $51 \pm 0.11\%$ increase in cellular uptake of alendronate-FITC, compared with administration of the bisphosphonate alone ($P < 0.005$).

In addition, indications of increased uptake of zoledronic acid following doxorubicin treatment were observed in intratibial tumors *in vivo*, using Western blot analysis for unprenylated Rap1a. This molecule is shown to accumulate as a result of bisphosphonate inhibition of posttranslational modification of small GTPases, and is commonly used as a surrogate marker of bisphosphonate uptake. A 22-kDa band indicative of unprenylated Rap1a was detected in tumor lysates from animals that had received sequential treatment with doxorubicin followed by zoledronic acid

(Fig. 6B). In contrast, we did not detect unprenylated Rap1a in tumors from animals receiving the same doses of zoledronic acid as single therapy.

Discussion

In the current study, we have investigated the efficacy of sequential treatment with doxorubicin and zoledronic acid in an intratibial model of breast cancer growth in bone. In contrast to previous studies using a single administration of zoledronic acid (32), a 6-week course of weekly sequential treatment was given, and a detailed analysis of the molecular mechanisms by which doxorubicin and zoledronic acid exert their anticancer effects was carried out. Histologic analysis showed that weekly dosing with 2 mg/kg doxorubicin had no effect on intraosseous tumor growth, reflecting that a suboptimal dose was chosen to allow us to explore the benefits of combined therapy. Doxorubicin is associated with cardiotoxicity, limiting its clinical use, and hence exploring the option of low-dose combination therapy may be of patient benefit. Weekly administration of 100 μ g/kg

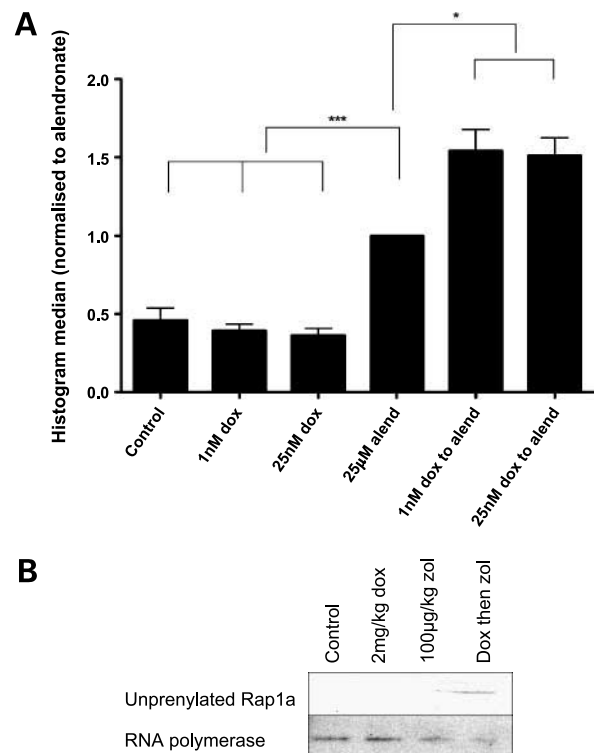


Figure 6. Effects of doxorubicin on uptake and activity of bisphosphonates, *in vitro* and *in vivo*. **A**, median fluorescently labeled MDA-MB-436 breast cancer cells treated with control (saline), doxorubicin, alendronate-FITC, or doxorubicin followed 24 h later by alendronate-FITC. Fluorescence was normalized to that in cells treated with alendronate-FITC alone. **B**, representative Western blots from one of three experiments showing accumulation of unprenylated Rap1a in bone tumor samples from animals treated with saline (*control*), 2 mg/kg doxorubicin, 100 μ g/kg zoledronic acid, or doxorubicin followed 24 h later by zoledronic acid. *, $P < 0.05$; and ***, $P < 0.001$ by Kruskal-Wallis followed by Dunn's multiple comparisons test.

zoledronic acid resulted in significantly reduced tumor growth and tumor cell proliferation, and no extrasosseous tumor growth was evident in any of the animals in this study. Zoledronic acid has previously been shown to exert anticancer effects on tumors growing in the bone environment. Corey et al. (20) reported that twice weekly treatment with 200 $\mu\text{g}/\text{kg}$ zoledronic acid from weeks 4 to 9 significantly reduced the volume of established prostate tumors in bone, and Peyruchard et al. (36) have shown reduced growth of established B02 breast tumors in bone following administration of 120 $\mu\text{g}/\text{kg}$ for 12 consecutive days. Both reports involved higher doses and more frequent administration of zoledronic acid compared with our study, and significantly higher than those used in the clinical treatment of cancer-induced bone disease (4 mg infusion; ref. 17). Although the dose of 100 $\mu\text{g}/\text{kg}$ research grade zoledronic acid (disodium salt, 4.75 hydrate) we used equates to the 4-mg clinical dose, this was administered weekly as opposed to every 3 to 4 weeks in clinical use. This difference can to some extent be justified by the higher level of overall bone turnover in young mice, compared with adult humans. Treatment of established B02 breast tumors in bone with a single injection of 100 $\mu\text{g}/\text{kg}$ zoledronic acid has been shown to have no effect on tumor volume, tumor cell apoptosis, or proliferation (23, 32). However, an accumulative dose of 100 $\mu\text{g}/\text{kg}$, where mice were given zoledronic acid daily or weekly, significantly reduced intraosseous tumor burden (23). Thus, it seems that multiple doses of zoledronic acid are required to induce anticancer effects *in vivo*.

Combining bisphosphonates with cytotoxic drugs *in vivo* has been shown to increase the anticancer effects compared with either drug alone (29, 37–39). We have shown that clinically relevant doses of doxorubicin (2 mg/kg) combined with a single administration of zoledronic acid (100 $\mu\text{g}/\text{kg}$) reduced intraosseous tumor growth in the B02 model of breast cancer bone metastases (32). As the B02 cells are derived from MDA-MB-231 cells, the increased antitumor effect of this combination treatment does not seem to be cell line specific, although the data should ideally also be confirmed in a model using hormone-dependent breast cancer cells. The treatment regimen chosen for the current study has been shown to completely inhibit growth of subcutaneous MDA-MB-436 tumors (33). Our current data are in agreement with these previous reports, showing significantly reduced MDA-MB-436 tumor burden in the tibiae of mice treated sequentially with doxorubicin followed 24 hours later by zoledronic acid, compared with animals treated with either drug alone.

Administration of doxorubicin combined with zoledronic acid induces apoptosis and inhibits proliferation of breast cancer cells *in vitro* and *in vivo*, with maximum effects detected when the chemotherapeutic agent is given 24 hours before the bisphosphonate (24, 32, 33). Here, we have shown the molecular pathways by which these two drugs interact to increase apoptosis, reduce proliferation, and ultimately inhibit MDA-MB-436 breast tumor growth in bone. Analysis of protein levels revealed increased proapoptotic BCL2-associated X protein and decreased antiapoptotic

BCL2 expression in tumors from animals treated with zoledronic acid, but these alterations were not sufficient to induce caspase cleavage or subsequent induction of apoptosis. Zoledronic acid caused significant reductions in tumor cell proliferation associated with decreased expression of the cell cycle regulatory proteins Cyclin D1 and D3, which may account for the decreased tumor growth observed in this treatment group. Numerous *in vitro* studies have reported direct proapoptotic and antiproliferative effects of bisphosphonates (reviewed in 40). Exposure of colon cancer and leukemia cell lines to zoledronic acid reduced expression of cyclin D3 (41), whereas in osteosarcoma cells, expression of cyclin D1 was decreased (42). The current study is the first to show effects of zoledronic acid on cell cycle-related proteins in breast tumors in bone *in vivo*. These anticancer effects seem to be site specific; in previous studies where animals with s.c. implanted breast tumors were treated weekly with 100 $\mu\text{g}/\text{kg}$ zoledronic acid, no inhibition of tumor growth or tumor cell proliferation were detected (33).

It has been postulated that bisphosphonates delay progression of bone metastases via the inhibition of osteoclast-mediated bone resorption, leading to a decrease in the release of tumor-promoting growth factors (22). Our current data show significant reductions in the area of bone exposed to resorbing osteoclasts in treatment groups receiving zoledronic acid. However, in a B02 model of breast cancer metastases, a single 100 $\mu\text{g}/\text{kg}$ dose of zoledronic acid inhibited bone resorption without reducing tumor burden, suggesting that these tumors grow independently of bone-derived growth factors (32). Whether combining zoledronic acid with other chemotherapy agents (e.g., paclitaxel) also results in inhibition of breast tumor growth in bone may be the topic of future *in vivo* studies. Our data suggest that the molecular mechanisms by which doxorubicin in sequence with zoledronic acid exert their effects are a complex combination of induction of proapoptotic proteins and suppression of cell cycle proteins (Fig. 7). Doxorubicin inhibits cellular proliferation via p53-dependent and p53-independent mechanisms (43–45). In p53 wild-type MCF7 breast cancer cells, doxorubicin treatment causes an accumulation of p53 (45–48), p53 recruits p21 (46) leading to decreased expression of cyclin B and cdc2 resulting in a cell cycle block at G₂-M (47, 48). However, MDA-MB-436 cells express mutated p53 and it is unlikely that increases in p53 expression seen in these tumors are capable of recruiting p21 (49). In the absence of functional p53, p73 and p63 have been shown to replace p53 inducing p21 in doxorubicin-treated breast cancer cells (44), and this is a possible mechanism by which p21 accumulates following sequential administration of doxorubicin and zoledronic acid. In addition to causing a cell cycle block at G₂-M, experiments in colon cancer cells has shown that doxorubicin can also block G₁ by reducing expression of cyclin D1 (43). Both doxorubicin (43) and zoledronic acid (42) have been shown to reduce expression of cyclin D1. Zoledronic acid has been shown to reduce expression of cyclin E (50), cyclin D3, and cyclin B (41) in a p53-independent manner. There is

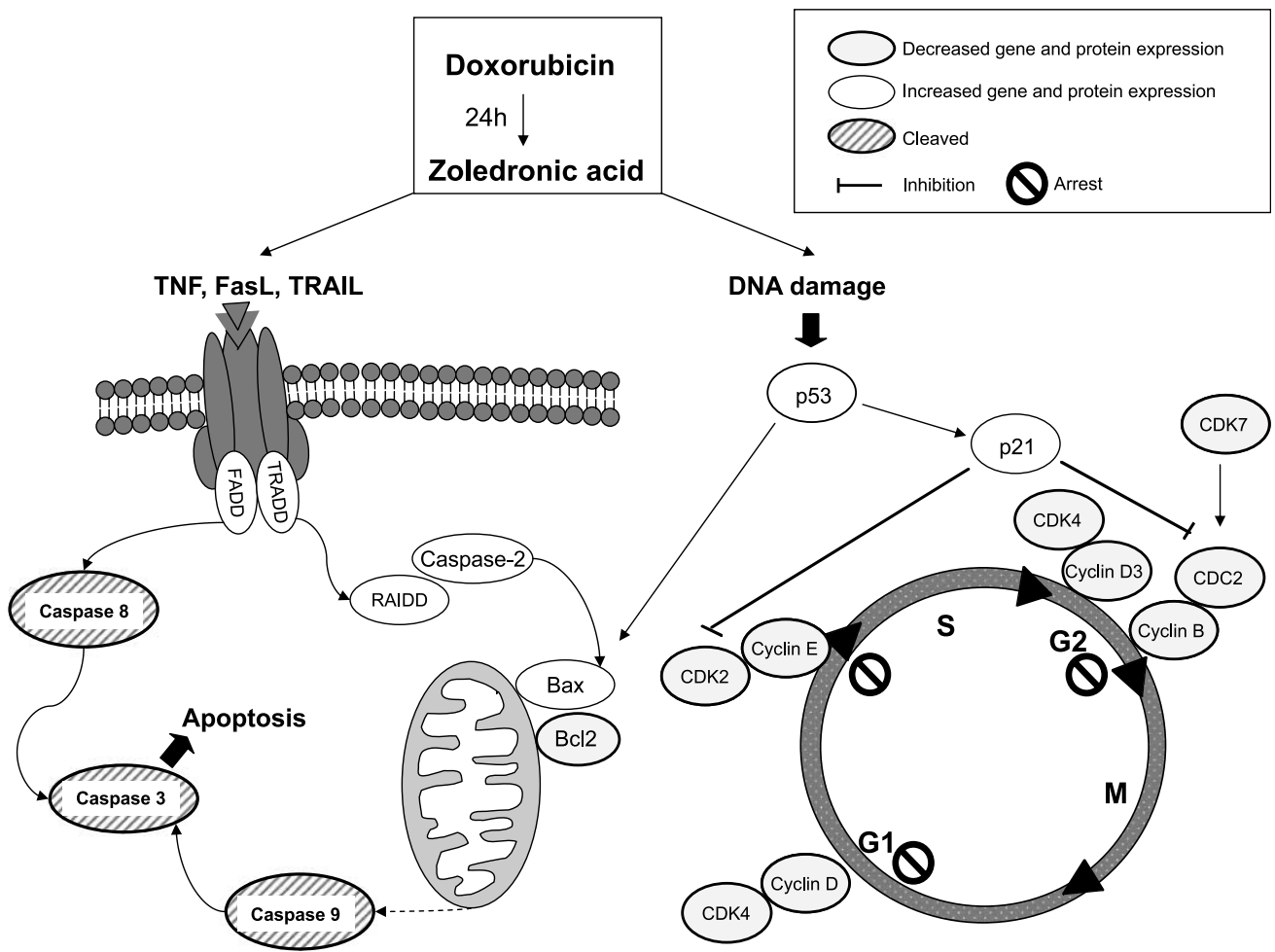


Figure 7. Proposed mechanism of antitumor effects of doxorubicin and zoledronic acid in breast tumors in bone. Diagram showing the links between the observed effects of doxorubicin and zoledronic acid on factors involved in regulating tumor cell apoptosis and proliferation.

evidence reported from leukemia, colon cancer (41), and osteosarcoma models (50) that zoledronic acid does not alter p53 or p21 expression, but acts independently of these proteins.

The roles of doxorubicin and zoledronic acid in induction of apoptosis of tumor cells growing in bone are less clear. Both drugs have been shown to induce apoptosis by uncoupling the bcl-2 family protein balance and disrupting the mitochondrial membrane potential (50, 51), leading to activation of caspase 9 (51, 52) and cleavage of caspase 3 (53, 54). In addition to activating the mitochondrial pathway, both drugs can induce cleavage of caspase 8 (53, 54) resulting in activation of the mitochondrion-independent pathway. Therefore, induction of apoptosis following sequential treatment with doxorubicin followed by zoledronic acid could be caused by one drug increasing the cytotoxicity of the other, or be the result of an additive effect of both drugs acting on the same pathway.

In vitro studies have shown that the antiproliferative and proapoptotic effects of zoledronic acid can be reversed by simultaneous addition of zoledronic acid and geranylgera-

niol (GGOH), an intermediate of the mevalonate pathway (42). Restoration of geranylgeranylation has been shown to rescue G₁ arrest caused by inhibitors of mevalonate synthesis resulting in increased expression of cyclin D1, cyclin E, and cdk2 (reviewed in 55). Similar reversal experiments cannot be done *in vivo*, preventing direct demonstration of a key role for inhibition of the mevalonate pathway in the observed antitumor effects. Our data show that pretreatment of MDA-MB-436 breast cancer cells with doxorubicin for 24 hours leads to increased cellular uptake of a subsequently added bisphosphonate *in vitro*, but the mechanism mediating this remains to be identified. Bisphosphonates are taken up by fluid phase endocytosis, and it is possible that doxorubicin affects this process. The detection of increased accumulation of unprenylated Rap1a in intrasosseous tumors only following sequential treatment indicates that there may be increased uptake of bisphosphonate following exposure of tumor cells to doxorubicin also *in vivo*. Alternatively, zoledronic acid may increase the retention time of doxorubicin, thereby increasing the effect of this potent chemotherapy agent.

This is the first report showing that weekly administration of doxorubicin 24 hours before zoledronic acid for 6 weeks reduces the growth of breast tumors in bone by induction of tumor cell apoptosis, suppression of tumor cell proliferation, and inhibition of the mevalonate pathway *in vivo*, whereas single agent therapy had no effect. The expression profiles of apoptosis- and cell cycle-related genes and proteins detected in tumors following sequential treatment support that this schedule causes specific antitumor effects. Our results suggest that breast cancer patients with confirmed bone metastases may benefit from a treatment schedule of doxorubicin followed 24 h later by zoledronic acid.

Disclosure of Potential Conflicts of Interest

R.E. Coleman: commercial research grant, Novartis; honoraria, Novartis; advisory board, Novartis; expert testimony, Novartis. I. Holen, honoraria, Novartis; advisory board, Novartis. No other potential conflicts of interest were disclosed.

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