

Bisphosphonate Treatment Inhibits the Growth of Prostate Cancer Cells¹

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ABSTRACT

The presence of skeletal metastases in patients suffering from cancer leads to a variety of clinical complications. Bisphosphonates are a class of drugs with a potent bone resorption inhibition activity that have found increasing utility in treating and managing patients with metastatic bone disease. Several clinical trials have demonstrated that bisphosphonates have clinical value in the treatment and management of skeletal metastases derived from advanced prostate cancer. Currently, the mechanism(s) through which bisphosphonates exert their activity is only beginning to be understood. We have studied the effects of bisphosphonate treatment on the growth of prostate cancer cell lines *in vitro*. Treatment of PC3, DU145, and LNCaP cells with pamidronate or zoledronate significantly reduced the growth of all three cell lines. Using flow cytometry, pamidronate treatment (100 μM) was shown to induce significant amounts of cell death in all three cell lines studied. In contrast, treatment with zoledronate (100 μM) did not induce cell death, instead exerting dramatic effects on cell proliferation, as evidenced by a major increase in cells present in the G₀-G₁ and S phase. Although both drugs reduced prostate cancer cell growth in the presence of serum, zoledronate was more potent under these conditions, disrupting growth at doses as low as 25 μM in the presence of 5% fetal bovine serum. These results raise the intriguing possibility that the observed clinical utility of bisphosphonates in managing skeletal metastases may in part derive from direct inhibition of prostate cancer cell growth in the bone microenvironment.

INTRODUCTION

Skeletal metastases are a common occurrence in patients with advanced prostate carcinoma, affecting ~70% of patients with metastatic disease (1). More than 95% of prostatic metastases are osteoblastic in nature, with the remaining being of mixed type usually appearing in the terminal phase of the disease (2). It has been demonstrated that the majority of these metastases are osteosclerotic, with abnormal osteoblastic bone formation being preceded by osteoclastic activation, which in turn appears to be associated with bone pain (1). Over the past decade, these observations have served as the rationale for treating a variety of skeletal metastases with bisphosphonates. Bisphosphonates have proven useful in the management of patients with multiple myeloma, metastatic prostate, and breast carcinoma to bone as demonstrated in a number of clinical trials (1, 3–7).

It is well-appreciated that aminobisphosphonates are potent inhibitors of bone resorption. Current experimental evidence suggests that aminobisphosphonates inhibit squalene synthase, disrupting prenylation of small GTP proteins. Other potential targets for bisphosphonate action include inhibition of tyrosine phosphatase activity and disruption of metalloproteinase secretion (8–12). Several studies have also demonstrated that various bisphosphonates induce osteoclast apoptosis *in vitro* and *in vivo* (13, 14). Recent clinical studies have raised the intriguing possibility that bisphosphonates may also be capable of

interfering with the growth and survival of metastatic cancer cells in bone (15–17). A recent study demonstrated that three different aminobisphosphonates were capable of inducing a dramatic increase in apoptosis in the J774 macrophage cell line *in vitro* (18). Another study reported the induction of apoptosis in myeloma-derived cell lines *in vitro*, following treatment with the aminobisphosphonates pamidronate (PAM)³ and zoledronate (ZOL) (19).

Many studies have demonstrated varying effects of bisphosphonate treatment on several cancer cell lines *in vitro*. Several studies have demonstrated that breast and prostate cancer cell lines demonstrated reduced cell adhesion to nonmineralized bone matrices following bisphosphonate treatment (20). In one study, bisphosphonate treatment also reduced adhesion to bovine cortical bone slices. In another *in vitro* study, bisphosphonate treatment also reduced adhesion of the MDA-MB-231 breast cancer cell line to bovine cortical bone slices (21). One possible mechanism for bisphosphonate activity is based on its ability to inhibit some of the enzymes involved in the pathway for cholesterol synthesis. In particular, inhibition of geranylgeraniol PP_i (GGPP) synthase has been implicated in several studies involving induction of apoptosis following bisphosphonate treatment (9, 18, 22–24). GGPP is required for the prenylation and subsequent membrane localization of proteins, particularly small GTP proteins of the Rho/Rac/Rab family (25). The Rho/Rac/Rab family of proteins is involved in a number of cellular processes including vesicular trafficking, signal transduction, and cell adhesion. The ability to inhibit GGPP synthase appears to be restricted to the aminobisphosphonates, suggesting that different bisphosphonates may have different targets (18, 23). Several studies have provided evidence that bisphosphonates may also exert their effects via inhibition of protein tyrosine phosphatases (PTP) (11, 26). The inhibition of PTP1B by alendronate was investigated *in vitro*, with oxidation of the catalytic cysteine implicated in inhibition of phosphatase activity (27). Given the two PP_i groups present in each bisphosphonate structure, inhibition of enzymes that bind phosphate groups as part of their substrates is not unexpected. Further demonstration that inhibition of GGPP synthase or PTP is a necessary requirement for bisphosphonate activity will require more in-depth functional studies.

We have conducted experiments examining the effect of two aminobisphosphonates, PAM and ZOL, on the growth and survival of prostate cancer cell lines *in vitro*. Prostate cancer cell lines were chosen because of the common clinical problem of metastatic prostate cancer to bone and because we were unaware of previous studies of this type on prostate cancer models. Our preliminary results indicate that both PAM and ZOL are capable of inducing cell death and/or cytostasis in three different prostate cancer cell lines, significantly inhibiting their growth *in vitro*.

MATERIALS AND METHODS

Cell Lines, Reagents, and Antibodies. Three prostatic carcinoma cell lines, DU145, PC3 (androgen insensitive), and LNCaP (androgen sensitive) and the osteosarcoma cell line MG63 were obtained from American Type Culture Collection (Manassas, VA). All cell culture grade reagents including media, fetal bovine serum (FBS), and growth factors were obtained from either

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³ The abbreviations used are: PAM, pamidronate; ZOL, zoledronate; GGPP, geranylgeraniol pyrophosphate; PTP, protein tyrosine phosphatase; FBS, fetal bovine serum.

Life Technologies, Inc. (Gaithersburg, MD), Sigma (St. Louis, MO), or Summit Biotechnology (Fort Collins, CO). The DU145 and PC3 cell lines were cultured and routinely passaged in DMEM/F12 media containing 10% FBS. The LNCaP cell line was cultured and routinely passaged in RPMI 1640 containing 10% FBS, supplemented with 100 nM testosterone. The MG63 cell line was cultured and routinely passaged in DMEM/F12 containing 10% heat-inactivated FBS, supplemented with 0.1 mM sodium pyruvate. All cell lines were incubated in a humidified environment containing 5% CO₂ at 37°C. The neutralized sodium salts of PAM and ZOL were dissolved in sterile ddH₂O at final concentrations of 75 and 100 mM respectively (Novartis Pharma, Basel, Switzerland). Stock solutions of the bisphosphonates were aliquoted and kept at -20°C for long-term storage.

Bisphosphonate Dosage Studies. Cells from 80% confluent cultures were washed once with PBS and removed from the plates with trypsin/EDTA. Trypsin/EDTA reaction was stopped by addition of fresh media containing 10% FBS. Cells were pelleted by centrifugation, resuspended in fresh media containing 10% FBS, and counted using a hemocytometer. Five thousand cells (PC-3, DU145) and 10,000 cells (LNCaP) were seeded into each well of a 96-well plate and incubated for 24 h at 37°C to allow the cells to attach to the new culture plates. Following attachment, medium was aspirated and cells were gently washed once with PBS. Cells were treated with either PAM or ZOL in the range of 100, 50, 25, 10, 5, and 1 μM concentrations in DMEM/F12 (PC3, DU145) or RPMI 1640 (LNCaP) serum-free media containing 0.2% BSA. The effect of serum on bisphosphonate treatment was examined as described above by incubating cells in media supplemented with bisphosphonate and various percentages of FBS (0, 2, 5, and 10%). The dosage effect of bisphosphonate treatment on net cell loss per growth was monitored over a time course of 24, 48, and 72 h. After each 24-h period, the medium was removed and cells were gently washed once with PBS. Cells were fixed with 1% glutaraldehyde/PBS for 15 min at room temperature and stained with 0.1% crystal violet (w/v in ddH₂O) for 30 min at room temperature. Cells were destained under running tap water for 15 min and allowed to air dry completely. Cells were subsequently lysed with 100 μl/well of 0.2% Triton X-100 (v/v in ddH₂O) for 1.5 h. The absorbance (562 nm) for each well was measured using a Bio-Tek (Winooski, VT) FL600 plate reader. Each dose was tested in quadruplicate for each experiment including control wells containing no added bisphosphonate. Each experiment was repeated a minimum of four separate times, and the average values obtained were plotted ±SE. Statistical significance was determined using Tukey's Studentized range test with a 95% confidence interval.

Bisphosphonate Pulse Studies. The procedures used for the pulse study were identical to those described above with a few exceptions. Cells were treated with varying doses of bisphosphonate (100, 50, 25, and 10 μM) with DMEM/F12 media containing 2% FBS and 0.2% BSA for 6, 12, or 24 h. After each treatment period, bisphosphonate containing media was removed and replaced with DMEM/F12 media supplemented with 2% FBS and 0.2% BSA. The effect of duration of bisphosphonate treatment was then examined over a time course of 24, 48, and 72 h. Following each 24-h period, cells were fixed, stained, and processed as described above. Each experiment was repeated a minimum of four separate times, and the average values obtained were plotted ±SE. Statistical significance was determined using Tukey's Studentized range (HSD) test with a 95% confidence interval.

Flow Cytometry and Cell Cycle Analysis. Two million cells (PC3, DU145) were seeded into 60-mm dishes and incubated for 24 h at 37°C to allow the cells to attach to the new culture plates. Following attachment, cells were treated with bisphosphonate as described above for the dosage study. Cells were treated with either PAM or ZOL (100 μM) in DMEM/F12 serum-free media containing 0.2% BSA. The effect of serum on bisphosphonate treatment was examined as described above by incubating DU145 cells in media supplemented with bisphosphonate and 2% FBS/0.2% BSA. Following 48 h of treatment, cells were washed once with PBS and removed from the plates with trypsin/EDTA. Trypsin/EDTA reaction was stopped by addition of fresh media containing 10% FBS. All media, washes, and trypsinized cell solutions were pooled, pelleted by centrifugation, and resuspended in 200 μl of PBS. Cells were fixed and permeabilized by rapid addition of 2 ml of ice-cold 70% ethanol followed by 1-h incubation at -20°C. Cells were pelleted by centrifugation, resuspended in 800 μl of PBS, and incubated (protected from light) for 1 h at 37°C following addition of 100 μl of 1 μg/ml RNase A and 100 μl of 400 μg/ml of propidium iodide. Samples were analyzed for forward

and orthogonal light scatter and red fluorescence on a Becton Dickinson flow cytometer (Becton Dickinson, Mountain View, CA), measuring both area and peak width. DNA histograms were created using the MODFIT LT software for Macintosh version 2.0 (Becton Dickinson, Mountain View, CA).

Detection of Apoptosis using the Vybrant Assay. PC3 cells were plated and treated as described above for cell cycle analysis. Following 48 h of treatment, cells were washed once with cold PBS and removed from the plates with trypsin/EDTA. Trypsin/EDTA reaction was stopped by addition of fresh media containing 10% FBS. Apoptotic and necrotic cells were assayed in each sample using the Vybrant apoptosis assay kit 2 and the protocol supplied by the manufacturer (Molecular Probes, Eugene, OR). Briefly, cells were pelleted by centrifugation and resuspended in 1× annexin V binding buffer. Cells were stained first with propidium iodide to detect necrosis, washed, and then stained with Alexa fluor 488-labeled annexin V to detect apoptosis. Samples were analyzed for forward and orthogonal light scatter and green and red fluorescence on a Becton Dickinson flow cytometer. Histograms were created using the MODFIT LT software for Macintosh version 2.0.

RESULTS AND DISCUSSION

PAM and ZOL Inhibit the Growth of Prostate Cancer Cell Lines. The effect of two aminobisphosphonates, PAM and ZOL, on the DU145, PC3, and LNCaP prostate cancer cell lines was investigated *in vitro* using a simple cell number assay. Cells were seeded in 96-well plates in serum containing media and allowed to attach for 24 h. The medium was then removed and replaced with serum-free medium containing 0.2% BSA with or without bisphosphonates at various concentrations. The cells were incubated under these conditions for a time course spanning 72 h. Following incubation, the medium was removed, the cells were washed, and the loss or increase in cell number was assessed indirectly by staining with the dye crystal violet. In this assay the total amount of dye incorporated is proportional to the number of cells remaining in the well. A decrease in the absorbance would reflect the total amount of cells lost, whereas an increase in absorbance reflects the net growth of the original cells plated. The lack of a significant change in absorbance would indicate cytostasis or no net loss or growth of the cells. The results from these experiments are shown in Figs. 1-6.

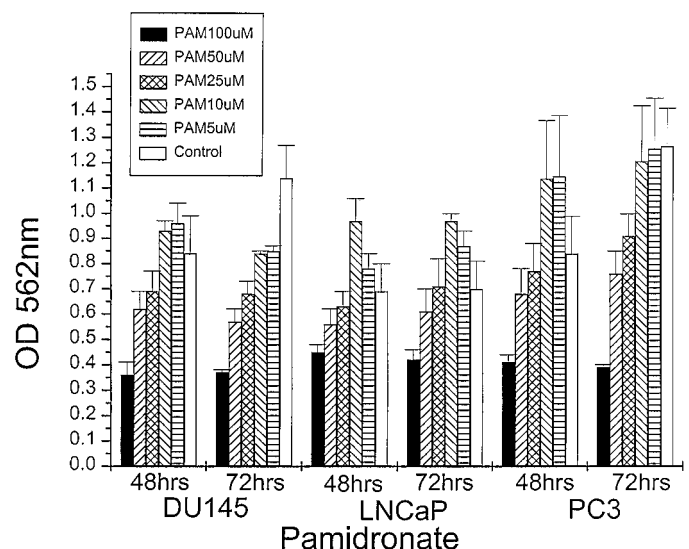


Fig. 1. PAM inhibits the growth of prostate cancer cells. DU145, LNCaP, or PC3 cells (5000/well) were plated in 96-well plates and incubated for 24 h. After 24 h, medium was removed and replaced with serum-free medium containing varying doses of PAM. After 24, 48, or 72 h of incubation, cells were fixed and stained with crystal violet as described in the text. The absorbance at 562 nm of each well was determined in a Bio-Tek FL600 plate reader. For each experiment, the average value obtained from four replicate wells was used to calculate mean absorbance at 562 nm for seven separate experiments. Results represent the mean value ($n = 7$) ± SE.

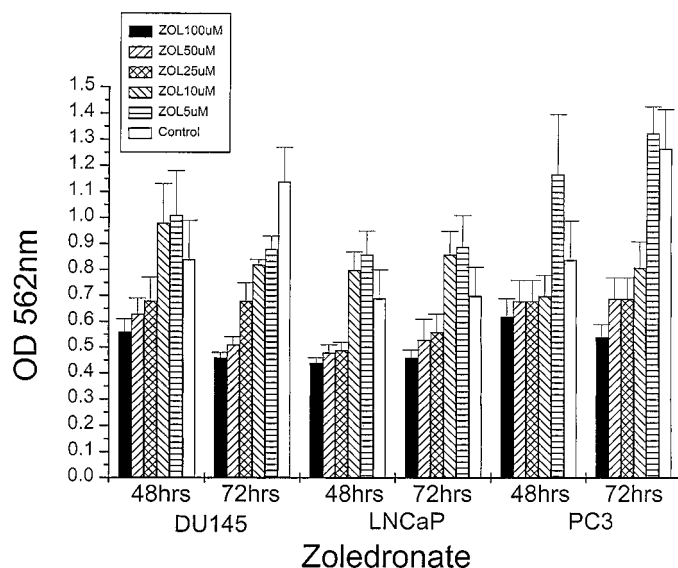


Fig. 2. ZOL inhibits the growth of prostate cancer cells. DU145, LNCaP, or PC3 cells (5000/well) were plated in 96-well plates and incubated for 24 h. After 24 h, medium was removed and replaced with serum-free medium containing varying doses of ZOL. After 24, 48, or 72 h of incubation, cells were fixed and stained with crystal violet as described in the text. The absorbance at 562 nm of each well was determined in a Bio-Tek FL600 plate reader. For each experiment, the average value obtained from four replicate wells was used to calculate mean absorbance at 562 nm for seven separate experiments. Results represent the mean value ($n = 7$) \pm SE.

Treatment of the PC3 cell line with 100 μ M PAM caused a significant decrease in total cell number after 48 h of treatment when compared to the untreated control cells ($P = 0.05$). The untreated cells in this assay were able to proliferate as evidenced by the dramatic increase in total cell number over time. Lower doses of PAM (50 μ M and 25 μ M) did not decrease the total cell number in contrast to the effect observed at 100 μ M PAM. The lower doses of PAM appear to cause cell cytostasis, since the overall cell number under these conditions was not significantly increased when compared to control cells (Fig. 1).

In contrast to the effects observed with treatment of the PC3 cells with PAM, treatment with 100 μ M ZOL did not cause as significant a decrease in cell number. Treatment of the cells with ZOL did however induce cell cytostasis at doses as low as 10 μ M, indicating it was more potent in this regard than PAM (Fig. 2). The results obtained using the DU145 and LNCaP cell lines were similar to those obtained with the PC3 cell line, with both PAM and ZOL inducing cytostasis in the DU145 and LNCaP cell lines in a fashion similar to that observed for the PC3 cell line (Figs. 1 and 2).

The ability of the bisphosphonates to act as chelating agents has been suggested as one possible explanation for their cellular effects. At the concentrations used in this study, it was unlikely that this was responsible for the observed effects of bisphosphonate treatment. This possibility was investigated by addition of 10 mM $MgCl_2$ and 10 mM $CaCl_2$ during PAM or ZOL treatment to overcome any chelation effects. Addition of 10 mM $MgCl_2$ and 10 mM $CaCl_2$ did not significantly alter the results, suggesting that chelation was unlikely to be a mechanism for the observed effects on PC3 and DU145 cells (results not shown).

Bisphosphonates Inhibit Prostate Cancer Cell Growth Even in the Presence of Serum. Our initial studies demonstrated that both PAM and ZOL induce cell death and/or cytostasis in three prostate cancer cell lines, namely, PC3, DU145, and LNCaP. These initial studies were performed in the absence of any trophic factors that would be provided by serum. We next investigated the ability of serum to rescue the effects of bisphosphonate treatment on prostate

cancer cell lines. Experiments were performed as described above with FBS included in varying concentrations during drug treatment. Even in the presence of 5% serum, both PAM and ZOL demonstrated cell death and/or cytostatic activity on the DU145 and PC3 cell lines ($P = 0.05$), suggesting that cellular response to these drugs is not solely due to the absence of trophic factors during treatment (Figs. 3 and 4). As would be expected, higher serum levels rescue the effects of bisphosphonate treatment, but not completely. Although both compounds are less effective in the presence of serum, PAM activity in particular was more abrogated under these conditions. Interestingly

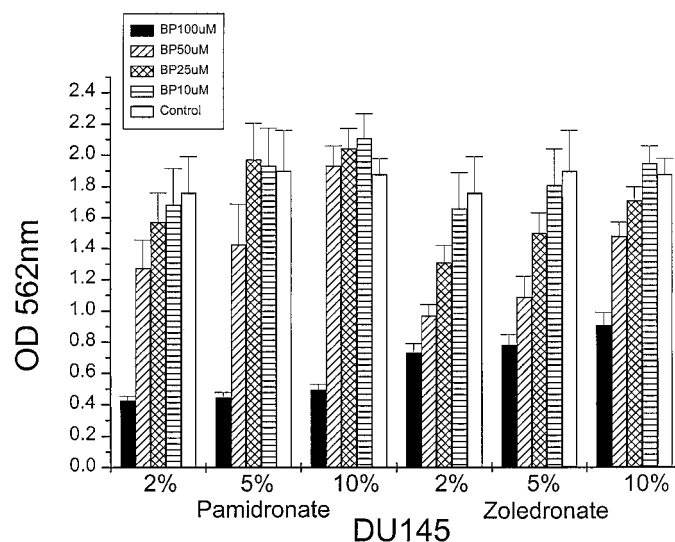


Fig. 3. PAM and ZOL inhibit the growth of DU145 cells in the presence of serum. DU145 cells (5000/well) were plated in 96-well plates and incubated for 24 h. After 24 h, medium was removed and replaced with media containing 2, 5, or 10% FBS and varying doses of PAM or ZOL. Following 72 h of incubation, cells were fixed and stained with crystal violet as described in the text. The absorbance at 562 nm of each well was determined in a Bio-Tek FL600 plate reader. For each experiment, the average value obtained from four replicate wells was used to calculate mean absorbance at 562 nm for four separate experiments. Results represent the mean value ($n = 4$) \pm SE.

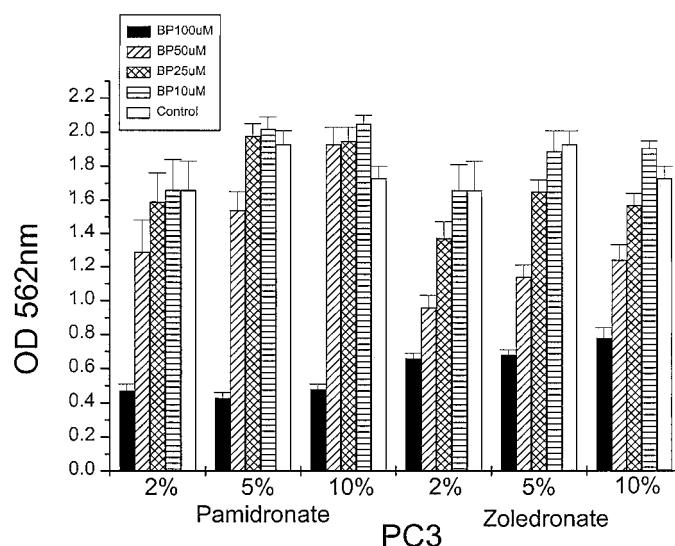


Fig. 4. PAM and ZOL inhibit the growth of PC3 cells in the presence of serum. PC3 cells (5000/well) were plated in 96-well plates and incubated for 24 h. After 24 h, medium was removed and replaced with medium containing 2, 5, or 10% FBS and varying doses of PAM or ZOL. After 72 h of incubation, cells were fixed and stained with crystal violet as described in the text. The absorbance at 562 nm of each well was determined in a Bio-Tek FL600 plate reader. For each experiment, the average value obtained from four replicate wells was used to calculate mean absorbance at 562 nm for four separate experiments. Results represent the mean value ($n = 4$) \pm SE.

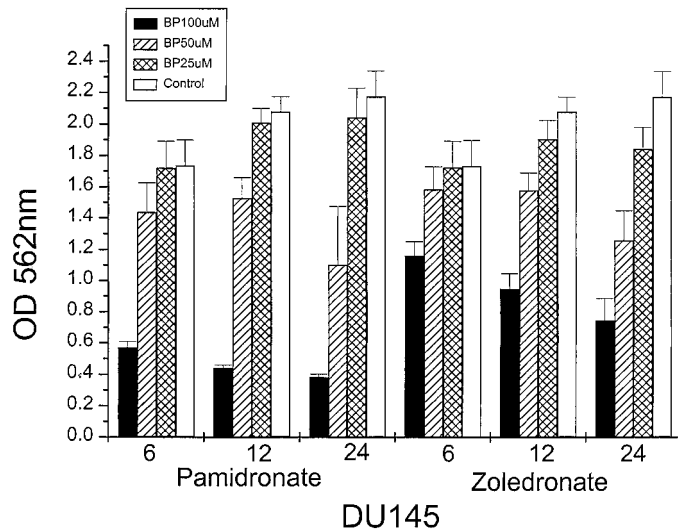


Fig. 5. PAM and ZOL pulse treatment inhibits the growth of DU145 cells. DU145 cells (5000/well) were plated in 96-well plates and incubated for 24 h. After 24 h, medium was removed and replaced with medium (DMEM/F12 + 2% FBS) containing varying doses of PAM or ZOL for 6, 12, or 24 h. After treatment, medium containing drug was removed and replaced with fresh DMEM/F12 + 2% FBS. After an additional 72 h of incubation, cells were fixed and stained with crystal violet as described in the text. The absorbance at 562 nm of each well was determined in a Bio-Tek FL600 plate reader. For each experiment, the average value obtained from four replicate wells was used to calculate mean absorbance at 562 nm for four separate experiments. Results represent the mean value ($n = 4$) \pm SE.

ZOL even at lower doses (25–50 μM) was still effective even in the presence of 5–10% FBS ($P = 0.05$). These observations may reflect the known difference in the skeletal potencies of these drugs *in vivo*, since ZOL is a more potent inhibitor of bone resorption than PAM.

Inhibition of Prostate Cancer Cell Growth Does Not Require Continuous Exposure to Bisphosphonate. Our initial studies had demonstrated that continuous exposure to both PAM and ZOL induce cell death and cytostasis in three prostate cancer cell lines, namely, PC-3, DU145, and LNCaP. These studies were performed by continuous exposure to the bisphosphonates over a 24- to 72-h time course. We next investigated whether shorter (6- to 24-h) pulse treatment with bisphosphonates could inhibit prostate cancer cell growth, since in typical clinical use these drugs are often administered over a period of hours (PAM) or minutes (ZOL) once every month. Pulse experiments were performed by incubating cells with bisphosphonate for 6, 12, or 24 h, after which the drug was removed and replaced with fresh media. Cells were then further grown for 24, 48, or 72 h, and overall growth compared to controls was assessed using the crystal violet assay. Experiments were performed as described above with 2% FBS included during drug treatment. Treatment of both DU145 and PC3 cells with high doses (100 μM) for 6 h inhibited the growth of both cell lines (Figs. 5 and 6). At lower doses, 24-h treatment with PAM or ZOL was the minimum time required to observe statistically relevant inhibition of prostate cancer cell growth ($P = 0.05$). As would be expected, shorter treatment times and lower doses were less effective at inhibiting prostate cancer cell growth with bisphosphonates. Our results suggest that exposure to concentrations of 25–50 μM ZOL, for a minimum duration of 12–24 h, can significantly inhibit the growth of prostate cancer cells even in the presence of 2% FBS. In general, the results from all our studies indicate that ZOL is more effective than PAM at inhibiting the growth of prostate cancer cells.

PAM and ZOL Induce Cell Death and/or Cell Cycle Inhibition in Prostate Cancer Cell Lines. Using the model system above, we then investigated the effect of bisphosphonate treatment on the PC3 and DU145 cell lines using flow cytometry. Cell cycle analysis was

performed on control and PAM- or ZOL (100 μM)-treated cells following 48 h of incubation under serum-free conditions or in the presence of 2% FBS (DU145). Untreated control cells have little or no cells in the apoptotic peak and have a distribution of cells in G_0 - G_1 , S, and G_2 -M phase typical of a proliferating cancer cell line (Fig. 7, A–C). PAM treatment clearly induces a significant increase in the apoptotic sub- G_0 - G_1 peak in both prostate cancer cell lines (Fig. 7, D–F). PAM treatment induced predominantly cell death, whereas ZOL was more effective at inducing cell cytostasis as evidenced by the dramatic increase in cells trapped in G_0 - G_1 or S phase (Fig. 7, G–I). However, as indicated above, ZOL was more active at preventing the overall growth of the prostate cancer cell lines, exerting effects at lower doses, even in the presence of serum. Furthermore, both ZOL- and, to some extent, PAM-treated cells appear to have a decrease in the number of cells in the G_2 -M phase, with an associated increase in the number of cells in G_0 - G_1 or S phase. These results indicate that the effect of PAM treatment is dose dependent, inducing cell death at high doses, while reducing the number of cells capable of completing the cell cycle, thereby leading to reduced proliferation and cytostasis at lower doses.

The mechanism of PAM- and ZOL-induced inhibition of prostate cancer cell growth was investigated further using the Vybrant cell death assay kit on treated cell lines. PC3 cells treated for 48 h with 100 μM PAM exhibited an increase in red fluorescence, typical of necrotic cells, with a minimal increase in green fluorescence typical of apoptotic cells when compared to the untreated control cells (Fig. 8, A and C). In contrast, PC3 cells treated with Adriamycin (1 μM) as a cell death control show a large amount of both apoptotic and necrotic cells, with a dramatic increase in cells stained for green and red fluorescence, respectively. These results suggest that PAM at 100 μM induces necrosis, with a minimal amount of apoptosis. In contrast, ZOL, even at a high dose (100 μM ; Fig. 8D), does not appear to induce significant amounts of cell death, but does however induce cell cytostasis as evidenced by our previous cell cycle analysis results (see Fig. 7). Our experiments indicate that only high doses of PAM induce

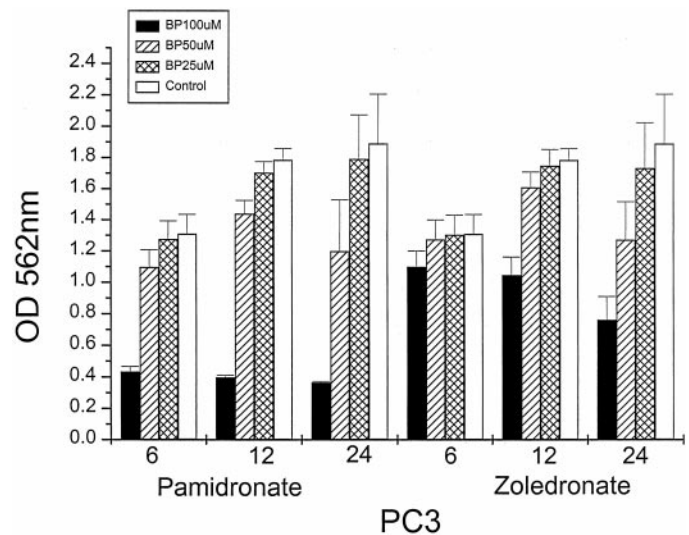


Fig. 6. PAM and ZOL pulse treatment inhibits the growth of PC3 cells. PC3 cells (5000/well) were plated in 96-well plates and incubated for 24 h. After 24 h, medium was removed and replaced with medium (DMEM/F12 + 2% FBS) containing varying doses of PAM or ZOL for 6, 12, or 24 h. After treatment, medium containing drug was removed and replaced with fresh DMEM/F12 + 2% FBS. After another 72 h of incubation, cells were fixed and stained with crystal violet as described in the text. The absorbance at 562 nm of each well was determined in a Bio-Tek FL600 plate reader. For each experiment, the average value obtained from four replicate wells was used to calculate mean absorbance at 562 nm for four separate experiments. Results represent the mean value ($n = 4$) \pm SE.

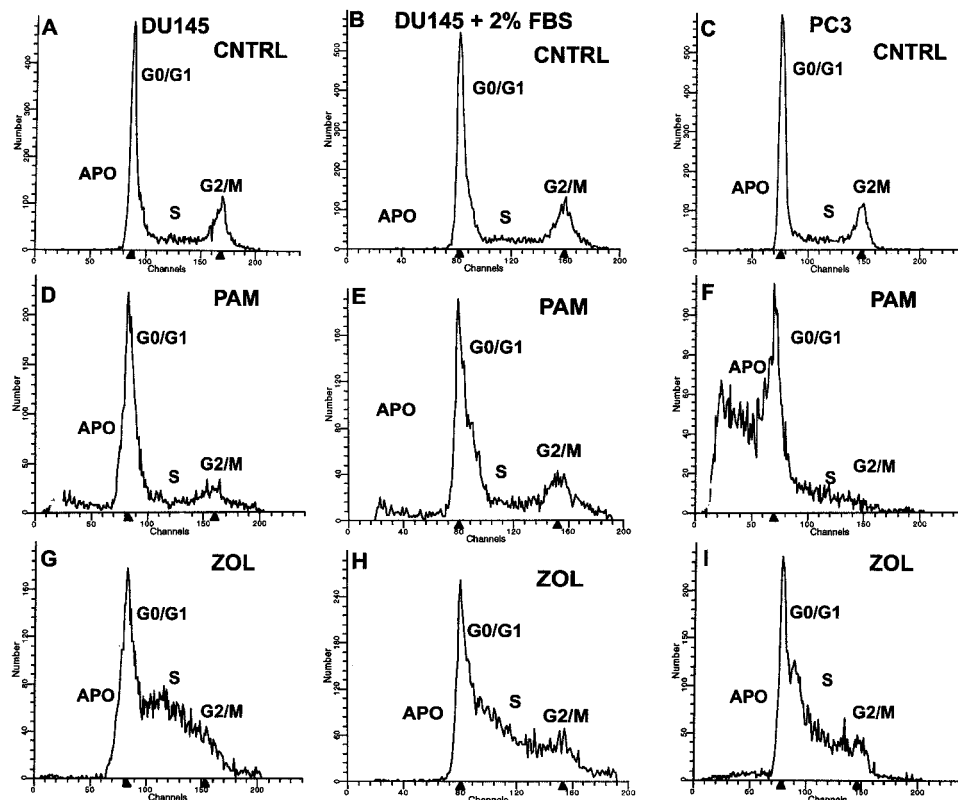


Fig. 7. Flow cytometry analysis of bisphosphonate-treated prostate cancer cells. DU145 (A, B, D, E, G, and H) or PC3 (C, F, and I) cells (1.0×10^5) were plated on 60-mm dishes and incubated for 24 h. After 24 h, medium was removed and replaced with serum-free medium (A–C), serum-free medium with $100 \mu\text{M}$ PAM (D–F), or $100 \mu\text{M}$ ZOL (G–I). DU145 cells (B, E, and H) were treated in the presence of 2% FBS. Each dish of cells was then incubated for 48 h, harvested, fixed, and stained with propidium iodide. Apoptotic (APO), G₀-G₁, S, and G₂-M regions are labeled on each DNA histogram. DNA histogram for each sample was created using MODFIT LT version 2.0.

necrosis, and that in our assay system the predominant effect of lower doses of both bisphosphonates is a reduction in cell growth mediated by inhibition of the cell cycle.

Bisphosphonates have previously been demonstrated to inhibit squalene synthase, an enzyme in the cholesterol synthesis pathway that uses farnesyl diphosphate as a substrate (8). Both farnesyl diphosphate and GGPP are important substrates in the prenylation of many G proteins involved in signal transduction pathways in cytoskeletal function and vesicular trafficking. The effects of alendronate treatment on osteoclasts can be partially rescued by geranylgeraniol but not farnesol, indicating selectivity in their mechanism of action based on inhibition of protein isoprenylation (10). We were unable to test this possibility as a mechanism for bisphosphonate activity on prostate cancer cell growth, since in our hands addition of $50 \mu\text{M}$ geranylgeraniol alone caused significant cell detachment.⁴

Several studies have presented evidence that the total net effect of bisphosphonate treatment *in vivo* may reflect their ability to disrupt or alter paracrine interactions in the bone microenvironment (13, 28, 29). Treatment of osteoblast/osteoclast cocultures with bisphosphonates produces results more closely aligned with their respective *in vivo* potencies (29). In one study, treatment of osteoblasts with bisphosphonates resulted in significantly reduced secretion of the growth factor interleukin 6 (30, 31). In other experiments, treatment of osteoblasts with bisphosphonates induced the secretion of a soluble inhibitor of osteoclast activity into conditioned media (32). Several studies have shown that prostate cancer cells release growth factors capable of modulating osteoblast activity and function (33). In turn, osteoblasts have been shown to produce growth factors which modulate the growth and activity of prostate cancer cells (34–36). It is clear that the sum activity of bisphosphonate action in the bone microenvironment will reflect alterations of these interactions in a

dynamic fashion. Our results from preliminary coculture studies (data not shown) suggest that ZOL (and possibly other bisphosphonates) is potentially capable of directly inhibiting the growth of prostate cancer cells even when grown in the presence of the osteoblast cell line MG63. Our results, and those of others, raise the intriguing possibility that the observed clinical utility of bisphosphonates in managing skeletal metastases may in part derive from direct inhibition of prostate cancer cell growth even in the bone microenvironment.

The increased use and success of bisphosphonate treatment in managing patients with metastatic bone disease has increased the importance of elucidating their mechanism of action. The mechanism of growth inhibition differs between the two bisphosphonate analogues PAM and ZOL. PAM induces cell death at higher doses and cytostasis at lower doses. In the presence of serum, only the $100 \mu\text{M}$ dose of PAM was capable of abrogating the growth of prostate cancer cell lines. In contrast, ZOL induces minimal amounts of cell death, but instead produced cytostasis, even at lower doses and in the presence of serum. This is intriguing since ZOL has been shown to be a more potent inhibitor of bone resorption than PAM *in vivo*. It is unclear however whether the mechanism for inhibition of bone resorption and inhibition of prostate cancer cell growth are functionally related. Results from our experiments are similar to recently published work conducted by Boissier *et al.* (37) that demonstrated that treatment of the prostate cancer cell line PmPC3 with ZOL does not induce cell death.

One caveat to our work, as well as that of others, is that the doses required to achieve inhibition of cell growth (typically 10 – $50 \mu\text{M}$) may not be achieved *in vivo* using current dosage and treatment regimens. Several studies have determined that serum levels of PAM range from 0.5 to $8 \mu\text{M}$ depending on the dosage and duration of infusion (38–42). Most *in vitro* studies, including our own, appear to demonstrate growth inhibitory effects at doses ~ 10 -fold higher (10 – $50 \mu\text{M}$) than levels currently measured in the serum of patients.

⁴ Lee *et al.*, unpublished results.

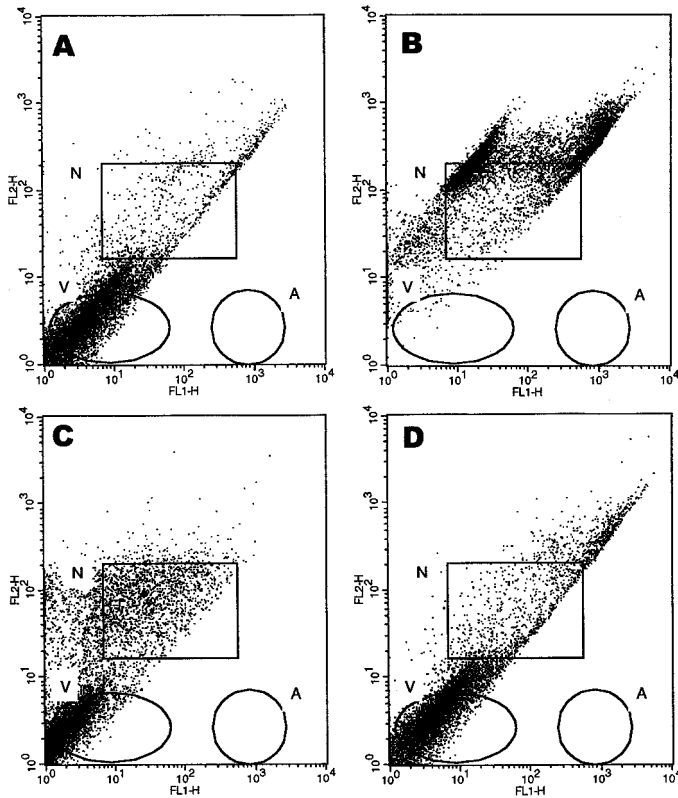


Fig. 8. Vybrant flow cytometry analysis of bisphosphonate-treated prostate cancer cells. PC3 cells (1.0×10^5) were plated on 60-mm dishes and incubated for 24 h. After 24 h, medium was removed and replaced with serum-free medium (negative control, A) or serum-free medium with $1 \mu\text{M}$ Adriamycin (positive control, B), $100 \mu\text{M}$ PAM (C), or $100 \mu\text{M}$ ZOL (D). Each dish of cells was then incubated for 48 h, harvested, stained with propidium iodide, and Alexa Fluor 488-labeled annexin V after the instructions provided by the manufacturer. Regions containing apoptotic (A), necrotic (N), and viable (V) cells are labeled on each dot plot. Dot plots for each sample, red fluorescence (propidium iodide, necrosis)/Y-axis, green fluorescence (annexin V, apoptosis)/X-axis were created using MODFIT LT.

It has been suggested however that the effective local concentrations of bisphosphonates at sites of active bone resorption may be much higher than serum levels due to concentration of bisphosphonates via their affinity for bone mineral (43). It is unclear however if the bioavailability of bisphosphonates in bone mineral, particularly for cancer cells, would allow effective concentrations in the $10\text{--}50 \mu\text{M}$ range. Despite these caveats, the low toxicity of bisphosphonates, coupled with promising results from their usage to treat bone metastases, warrants a more in-depth investigation into the potential effects these drugs may exert directly on cancer cells, especially in the bone microenvironment. More detailed studies on the mechanism of bisphosphonate-mediated inhibition of prostate cancer cell growth using coculture and *in vivo* model systems should further elucidate their potential use for improved treatment and therapy of advanced bone disease involving prostate cancer metastases.

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REFERENCES

- Adami, S. Bisphosphonates in prostate carcinoma. *Cancer (Phila.)*, *80*: 1674–1679, 1997.

- Galasko, C. S. Mechanisms of lytic and blastic metastatic disease of bone. *Clin. Orthop.*, *20*–27, 1982.
- Paterson, A. H., Kanis, J. A., Powles, T. J., McCloskey, E., Hanson, J., and Ashley, S. Role of bisphosphonates in prevention and treatment of bone metastases from breast cancer. *Cancer J. Oncol.*, *5* (Suppl. 1): 54–57, 1995.
- Berenson, J. R., Lichtenstein, A., Porter, L., Dimopoulos, M. A., Bordon, R., George, S., Lipton, A., Keller, A., Ballester, O., Kovacs, M. J., Blacklock, H. A., Bell, R., Simeone, J., Reitsma, D. J., Heffernan, M., Seaman, J., and Knight, R. D. Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma: Myeloma Aredia Study Group. *N. Engl. J. Med.*, *334*: 488–493, 1996.
- Conte, P. F., Latreille, J., Mauriac, L., Calabresi, F., Santos, R., Campos, D., Bonnetterre, J., Francini, G., and Ford, J. M. Delay in progression of bone metastases in breast cancer patients treated with intravenous pamidronate: results from a multinational randomized controlled trial. The Aredia Multinational Cooperative Group. *J. Clin. Oncol.*, *14*: 2552–2559, 1996.
- Kanis, J. A., Powles, T., Paterson, A. H., McCloskey, E. V., and Ashley, S. Clodronate decreases the frequency of skeletal metastases in women with breast cancer. *Bone*, *19*: 663–667, 1996.
- Lipton, A. Zoledronate in the treatment of osteolytic bone metastases. *Br. J. Clin. Pract. Suppl.*, *87*: 21–22, 1996.
- Amin, D., Cornell, S. A., Perrone, M. H., and Bilder, G. E. 1-Hydroxy-3-(methylpentylamino)-propylidene-1,1-bisphosphonic acid as a potent inhibitor of squalene synthase. *Arzneim.-Forsch.*, *46*: 759–762, 1996.
- Luckman, S. P., Hughes, D. E., Coxon, F. P., Graham, R., Russell, G., and Rogers, M. J. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. *J. Bone Miner. Res.*, *13*: 581–589, 1998.
- Fisher, J. E., Rogers, M. J., Halasy, J. M., Luckman, S. P., Hughes, D. E., Masarachia, P. J., Wesolowski, G., Russell, R. G., Rodan, G. A., and Reszka, A. A. Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation *in vitro*. *Proc. Natl. Acad. Sci. USA*, *96*: 133–138, 1999.
- Schmidt, A., Rutledge, S. J., Endo, N., Opas, E. E., Tanaka, H., Wesolowski, G., Leu, C. T., Huang, Z., Ramachandran, C., Rodan, S. B., and Rodan, G. A. Protein-tyrosine phosphatase activity regulates osteoclast formation and function: inhibition by alendronate. *Proc. Natl. Acad. Sci. USA*, *93*: 3068–3073, 1996.
- Stearns, M. E., and Wang, M. Effects of alendronate and Taxol on PC-3 ML cell bone metastases in SCID mice. *Invasion Metastasis*, *16*: 116–131, 1996.
- Nishikawa, M., Akatsu, T., Katayama, Y., Yasutomo, Y., Kado, S., Kugal, N., Yamamoto, M., and Nagata, N. Bisphosphonates act on osteoblastic cells and inhibit osteoclast formation in mouse marrow cultures. *Bone*, *18*: 9–14, 1996.
- Hughes, D. E., Wright, K. R., Uy, H. L., Sasaki, A., Yoneda, T., Roodman, G. D., Mundy, G. R., and Boyce, B. F. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. *J. Bone Miner. Res.*, *10*: 1478–1487, 1995.
- Diel, I. J., Solomayer, E. F., Costa, S. D., Gollan, C., Goerner, R., Wallwiener, D., Kaufmann, M., and Bastert, G. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. *N. Engl. J. Med.*, *339*: 357–363, 1998.
- Pelger, R. C., Hamdy, N. A., Zwiderman, A. H., Lycklama a Nijeholt, A. A., and Papapoulos, S. E. Effects of the bisphosphonate olpadronate in patients with carcinoma of the prostate metastatic to the skeleton. *Bone*, *22*: 403–408, 1998.
- Tamura, H., Ishii, S., Ikeda, T., Wada, N., Enomoto, K., and Kitajima, M. Therapeutic efficacy of pamidronate in combination with chemotherapy to bone metastasis of breast cancer in a rat model. *Surg. Oncol.*, *5*: 141–147, 1996.
- Benford, H. L., Frith, J. C., Auriola, S., Monkkonen, J., and Rogers, M. J. Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. *Mol. Pharmacol.*, *56*: 131–140, 1999.
- Aparicio, A., Gardner, A., Tu, Y., Savage, A., Berenson, J., and Lichtenstein, A. *In vitro* cytoreductive effects on multiple myeloma cells induced by bisphosphonates. *Leukemia*, *12*: 220–229, 1998.
- Boissier, S., Magnetto, S., Frappart, L., Cuzin, B., Ebetino, F. H., Delmas, P. D., and Clezardin, P. Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. *Cancer Res.*, *57*: 3890–3894, 1997.
- van der Pluijm, G., Vloedgraven, H., van Beek, E., van der Wee-Pals, L., Lowik, C., and Papapoulos, S. Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices *in vitro*. *J. Clin. Invest.*, *98*: 698–705, 1996.
- van Beek, E., Pieterman, E., Cohen, L., Lowik, C., and Papapoulos, S. Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their anti-resorptive potencies *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.*, *255*: 491–494, 1999.
- van Beek, E., Pieterman, E., Cohen, L., Lowik, C., and Papapoulos, S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem. Biophys. Res. Commun.*, *264*: 108–111, 1999.
- Luckman, S. P., Coxon, F. P., Ebetino, F. H., Russell, R. G., and Rogers, M. J. Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure-activity relationships in J774 macrophages. *J. Bone Miner. Res.*, *13*: 1668–1678, 1998.
- Seabra, M. C. Membrane association and targeting of prenylated Ras-like GTPases. *Cell Signal.*, *10*: 167–172, 1998.
- Endo, N., Rutledge, S. J., Opas, E. E., Vogel, R., Rodan, G. A., and Schmidt, A. Human protein tyrosine phosphatase-sigma: alternative splicing and inhibition by bisphosphonates. *J. Bone Miner. Res.*, *11*: 535–543, 1996.

27. Skorey, K., Ly, H. D., Kelly, J., Hammond, M., Ramachandran, C., Huang, Z., Gresser, M. J., and Wang, Q. How does alendronate inhibit protein-tyrosine phosphatases? *J. Biol. Chem.*, 272: 22472–22480, 1997.
28. Giuliani, N., Pedrazzoni, M., Negri, G., Passeri, G., Impicciatore, M., and Girasole, G. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures *in vitro* and promote early osteoblastogenesis in young and aged mice *in vivo*. *Bone*, 22: 455–461, 1998.
29. Sahni, M., Guenther, H. L., Fleisch, H., Collin, P., and Martin, T. J. Bisphosphonates act on rat bone resorption through the mediation of osteoblasts. *J. Clin. Investig.*, 91: 2004–2011, 1993.
30. Derenne, S., Amiot, M., Barille, S., Collette, M., Robillard, N., Berthaud, P., Harousseau, J. L., and Bataille, R. Zoledronate is a potent inhibitor of myeloma cell growth and secretion of IL-6 and MMP-1 by the tumoral environment. *J. Bone Miner. Res.*, 14: 2048–2056, 1999.
31. Giuliani, N., Pedrazzoni, M., Passeri, G., and Girasole, G. Bisphosphonates inhibit IL-6 production by human osteoblast-like cells. *Scand. J. Rheumatol.*, 27: 38–41, 1998.
32. Vitte, C., Fleisch, H., and Guenther, H. L. Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated resorption. *Endocrinology*, 137: 2324–2333, 1996.
33. Koutsilieris, M., Reyes-Moreno, C., Sourla, A., Dimitriadou, V., and Choki, I. Growth factors mediate glucocorticoid receptor function and dexamethasone-induced regression of osteoblastic lesions in hormone refractory prostate cancer. *Anticancer Res.*, 17: 1461–1465, 1997.
34. Reyes-Moreno, C., Sourla, A., Choki, I., Doillon, C., and Koutsilieris, M. Osteoblast-derived survival factors protect PC-3 human prostate cancer cells from adriamycin apoptosis. *Urology*, 52: 341–347, 1998.
35. Ritchie, C. K., Andrews, L. R., Thomas, K. G., Tindall, D. J., and Fitzpatrick, L. A. The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. *Endocrinology*, 138: 1145–1150, 1997.
36. Giunciuglio, D., Cai, T., Filanti, C., Manduca, P., and Albini, A. Effect of osteoblast supernatants on cancer cell migration and invasion. *Cancer Lett.*, 97: 69–74, 1995.
37. Boissier, S., Ferreras, M., Peyruchaud, O., Magnetto, S., Ebetino, F. H., Colombel, M., Delmas, P., Delaisse, J., and Clezardin, P. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res.*, 60: 2949–2954, 2000.
38. Daley-Yates, P. T., Dodwell, D. J., Pongchaidecha, M., Coleman, R. E., and Howell, A. The clearance and bioavailability of pamidronate in patients with breast cancer and bone metastases. *Calcif. Tissue Int.*, 49: 433–435, 1991.
39. Dodwell, D. J., Howell, A., Morton, A. R., Daley-Yates, P. T., and Hoggarth, C. R. Infusion rate and pharmacokinetics of intravenous pamidronate in the treatment of tumor-induced hypercalcaemia. *Postgrad. Med. J.*, 68: 434–439, 1992.
40. Oiso, Y., Tomita, A., Hasegawa, H., Ariyoshi, Y., Niinomi, M., Yamamoto, M., Takano, T., and Sakiyama, N. Pamidronate treatment in patients with tumor-associated hypercalcemia: pharmacological effects and pharmacokinetics. *Endocrinol. J.*, 41: 655–661, 1994.
41. Berenson, J. R., Rosen, L., Vescio, R., Lau, H. S., Woo, M., Sioufi, A., Kowalski, M. O., Knight, R. D., and Seaman, J. J. Pharmacokinetics of pamidronate disodium in patients with cancer with normal or impaired renal function. *J. Clin. Pharmacol.*, 37: 285–290, 1997.
42. Leyvraz, S., Hess, U., Flesch, G., Bauer, J., Hauffe, S., Ford, J. M., and Burckhardt, P. Pharmacokinetics of pamidronate in patients with bone metastases. *J. Natl. Cancer Inst.*, 84: 788–792, 1992.
43. Sato, M., Grasser, W., Endo, N., Akins, R., Simmons, H., Thompson, D., Golub, E., and Rodan, G. A. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J. Clin. Investig.*, 88: 2095–2105, 1991.

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