Zoledronic Acid Exhibits Inhibitory Effects on Osteoblastic and Osteolytic Metastases of Prostate Cancer

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ABSTRACT

Purpose: In this study we have examined the effects of zoledronic acid (ZA), a new-generation bisphosphonate, on prostate cancer (CaP) cells in vitro, and on both osteoblastic and osteolytic CaP metastases in animal models.

Experimental Design: In vitro, CaP cells were treated with ZA, and the effects on proliferation, cell cycle, and apoptosis were determined. In vivo, PC-3, and LuCaP 23.1 s.c. and tibial tumors were treated with ZA. Effects on bone and tumor were determined by histomorphometry and immunohistochemistry.

Results: ZA decreased proliferation of CaP cells, and caused G1 arrest and apoptosis of CaP cells in vitro. In vivo, s.c. CaP tumor growth was not affected by ZA. However, growth of osteoblastic and osteolytic metastases of CaP was inhibited significantly in vivo. Matrix metalloproteinase-2, matrix metalloproteinase-9, and Cathepsin K levels were decreased in osteolytic bone metastases after ZA administration.

Conclusions: In conclusion, we have shown that ZA has significant antitumor effects on CaP cells in vitro and in vivo. Antiosteolytic activity and the antitumor effects of this compound could benefit CaP patients with bone metastases.

INTRODUCTION

Bone is a very common site of CaP3 metastases (1, 2), and bone metastases are responsible for most of the morbidity associated with this disease. In contrast to bone metastases of breast cancer and myeloma, which are mainly osteolytic, a high percentage of CaP metastases exhibit the radiographic appearance of osteoblastic lesions (3). Histomorphometric studies of CaP bone metastases have shown that some of the sclerotic lesions are actually mixed in nature, with increased activities of both osteoblasts and osteoclasts (4, 5). Roland (6) has introduced the hypothesis that every primary or metastatic cancer in bone begins with osteolysis. A number of studies have shown that patients with advanced CaP exhibit elevated levels of osteolytic bone resorption markers in urine and blood (7, 8). The preponderance of evidence indicates that osteolysis is present in CaP bone metastasis even when the overall character appears to be osteoblastic. It follows that the use of compounds inhibiting osteolysis could be beneficial to patients with advanced CaP.

BPs are nonhydrolyzable pyrophosphate analogs. BPs have been shown to have inhibitory effects on osteoclast generation, maturation, and activity, thereby reducing osteoclastic bone resorption (9–11). BPs are used to treat Paget’s disease, hypercalcemia associated with cancer, and lytic metastases of breast cancer and myeloma. Metabolic bone disease of CaP and the rationale for the use of BPs in CaP were reviewed recently (12).

ZA is a new-generation BP with a side chain containing an imidazole ring, and is one of the most potent BPs (13, 14). Berenson et al. (15) reported reduction of rates of skeletal events in metastatic patients by ZA. The value and potential of ZA in treatment of patients with cancer-related bone disease were also reviewed recently (16).

Over the past few years investigators have examined BPs for direct effects on cancer cells. Various studies support the hypothesis that there are such direct effects of BPs (17–21). Animal studies have demonstrated that pretreatment of mice with risedronate and ibandronate before breast cancer cell inoculation caused a significant reduction in tumor burden in bone (22, 23). Peyruchaud et al. (24) reported inhibitory effects of ZA on osteolytic lesions of breast cancer in vivo, and Mundy et al. (25) reviewed preclinical studies with BPs, finding that they reduced skeletal metastases, and that skeletal lesions and tumor burden were diminished by ZA. However, despite the extent of this work, the effects of ZA on CaP cells and CaP bone metastasis have not yet been studied in detail, although Lee et al. (26) have reported inhibition of CaP cell growth in vitro by ZA.

The mechanisms underlying BP effects on cancer cells are not well understood, and mechanistic studies have begun only recently. These mechanisms may involve apoptosis via interference with the mevalonate pathway (27–29) and activation of caspases (28), and/or effects on invasiveness of the target cells, mediated by changes in expression and activity of metallopro-
teinases. Tumor cell invasion and metastasis are at least partially mediated by MMPs, through their ability to digest basement membrane and extracellular matrix components. Two published studies have demonstrated convincingly that secretion of MMP-1 and MMP-2 is affected by BP (20, 30), whereas a study by Farina et al. (31) showed that BP affected the activation of MMP-2. The association of MMP-2 with PC-3 invasiveness and metastatic character also suggests a role for this proteinase in CaP metastasis.

We have examined the effects of ZA on CaP cells in vitro and in vivo. Our results show that ZA directly inhibits CaP proliferation and enhances apoptosis of CaP cells in vitro, and also reduces growth of CaP tumors in murine bone. These findings, combined with reports of increased osteolysis in CaP patients, indicate potential benefits of using ZA in treating CaP metastases, possibly involving a combination of effects on bone resorption and direct effects on tumor cells.

MATERIALS AND METHODS

CaP Cell Lines and Xenografts. LNCaP and PC-CaP cell lines, and CEM, an acute lymphoblastic leukemia cell line (American Type Culture Collection, Rockville, MD), were maintained under standard culture conditions. LuCaP 23.1, a PSA-producing human CaP xenograft (32), is passaged s.c. in male athymic mice (BALB/c nu/nu; Simonsen Laboratories, Gilroy, CA).

Cell Proliferation. LNCaP and PC-3 cells were allowed to adhere overnight in 96-well plates. RPMI 1640 without phenol red with 10% or 2% FBS was used for the experiments. ZA was dissolved in 100 mM PBS (pH 7.2) and added to the medium. Cells were treated with 4.1 mM to 340 μM ZA for 1, 2, 3, or 4 days. The Quick Cell Proliferation Assay kit (BioVision Laboratories, Mountain View, CA) was used to measure the effects of ZA on cell proliferation. Experiments were repeated three times and done in triplicate; Student’s t test was used to determine statistical significance.

Effects of ZA on Apoptosis and Cell Cycle Distribution. LNCaP and PC-3 cells were grown in RPMI 1640 without phenol red with 10% FBS and 68 μM ZA for 1, 2, or 3 days. Control cultures did not contain ZA. Semiconfluent attached and floating cells were harvested, counted, and resuspended to yield 10⁶ cells in 1 ml of PBS. CEM cells were incubated with 0.3 μM camptothecin (Sigma, St. Louis, MO) for 3 h and used as a positive control for apoptosis.

TUNEL Assay. The TUNEL assay was used to determine effects of ZA on DNA fragmentation (33). One × 10⁶ cells were fixed in 2% paraformaldehyde and refrigerated for 15 min. Cells were spun down and washed with PBS. Ice-cold ethanol was added drop-wise (3 volumes of cells), and the samples were stored at −20°C until the analysis. Untreated cells stained with DAPI (5 μg/ml; Accurate Chemical & Scientific Corporation, Westbury, NY) were used for each cell type as a control for autofluorescence. Treated and untreated cells (0.5 × 10⁶) were mixed with 50 μl of TdT reaction mixture with or without 5 units of TdT enzyme (Boehringer Mannheim, Indianapolis, IN) and incubated for 1.5 h at 37°C. After washing with 1 ml of 15 mM EDTA and 0.1% NP40, cells were resuspended in 200 μl of DAPI. For each sample, 40,000 particles were analyzed (Coulter Epics Elite cytometer; Beckman Coulter, Fullerton, CA) using 10 mW UV excitation at 360 nm and 15 mW excitation at 488 nm. UV-excited DAPI fluorescence was collected with a 450/35 nm filter, whereas 488 nm-excited FITC fluorescence was collected with a 525/40 filter. All of the data were analyzed using custom software available commercially (Phoenix Flow3 Systems, San Diego, CA). Exact Fisher tests were used to determine statistical significance of the differences.

CMXRosamine/MitoTracker Green FM Assay. Determinations of numbers of cells with compromised mitochondrial potential and cell cycle analysis were performed using Mitotracker Green FM, CMXRosamine, and Hoechst 33342 dyes, as described by Poot and Pierce (34). One μl of 200 μM CMXRosamine in DMSO, 1 μl of 200 μM MitoTracker Green in DMSO, and 20 μl of 1 mM Hoechst 33342 in water were added to 1 × 10⁶ cells in 1 ml of medium, and the mixture was incubated at 37°C for 30 min in the dark. After incubation, cells were kept on ice until the flow cytometric analysis. Cells with lowered mitochondrial membrane potential (reduced red:green fluorescence ratio) were quantified by flow cytometry as described above. Hoechst staining was used to examine cell-cycle distribution. Only cells with unchanged mitochondrial potential were used to assess changes in cell-cycle distribution.

Animal Studies. For in vivo experiments we used PC-3 cells, yielding osteolytic bone lesions, and the xenograft LuCaP 23.1, which gives rise to osteoblastic lesions. All of the proce-
dures were performed in compliance with the University of Washington Institutional Animal Care and Use Committee and NIH guidelines. Six-to-8-week-old male mice (Fox Chase SCID mice; Charles River, Wilmington, MA) were used for the s.c. injections, and 4–6-week-old male mice were used for tibial injections.

Effects of ZA on s.c. Tumors. Animals (10 in treatment groups and 5 in control groups) were injected s.c. with 2 × 10^6 PC-3 cells or implanted with LuCaP 23.1 tumor bits. After tumors reached ~200 mg, 5 μg of ZA was injected twice weekly s.c. Tumor volume (TuV) was measured twice weekly and calculated as $L \times H \times W \times 0.5236$. Animals were sacrificed when tumors reached ~1000 mg or when animals were becoming compromised.

Effects of ZA on Intratibial Tumors. Single-cell suspensions (2 × 10^5 cells in ~10 μl, PC-3 and LuCaP 23.1) were injected directly into the tibiae of intact male mice (SCID; Ref. 35). Two regimens of ZA administration were used: (a) for prevention, administration of ZA was started simultaneously with injection of tumor cell; this regimen was chosen to simulate potential effects of ZA on cells arriving at the metastatic site, thereby revealing potential interference with establishment of metastases; and (b) for treatment, administration of ZA was started when the tumor was established, simulating treatment of established metastatic disease (LuCaP 23.1: PSA level ~5–10 ng/ml, 33 days after injection of tumor cells; PC-3: 7 days after injection of tumor cells). Under each regimen, 5 μg of ZA was injected twice weekly s.c. Blood was drawn every 2 weeks for determination of PSA serum levels (LuCaP 23.1 only). Before sacrifice, the animals were anesthetized, and a flat plate radiograph was taken with a diagnostic mammography unit (35 kV, 2.3 s, 20.7 mA/s, small focal setting). Of the animals with PC-3 cells in tibiae, the control group and 5 animals from each ZA-administration group were sacrificed 4 weeks after injection of cells when the control animals were becoming compromised. The remaining animals bearing PC-3 cells in tibiae, which were all ZA-treated animals, were sacrificed at a later time, when the animals were becoming compromised (7–9 weeks after injection). Animals bearing LuCaP 23.1 were sacrificed based on radiographic appearance of the tibiae indicating extensive osteoblastic reaction. Tumor growth of LuCaP 23.1 in tibia depends significantly on the single-cell preparation; therefore we implanted 5 animals of the control group with the same preparation as animals of the prevention group, and, separately, 5 animals of the control group with the same preparation as animals of the treatment group. Animals of the prevention group and the corresponding control animals were sacrificed 130 days after injection of LuCaP 23.1 cells. ZA-treated animals under the treatment regimen and corresponding control animals were sacrificed 90 days after injection of the LuCaP 23.1 cells. After sacrifice the tumor-bearing tibiae were harvested. Samples were divided for histomorphometric analysis (embedding in methacrylate; Ref. 36) or for histology and immunohistochemistry (fixed in 10% neutral NBF for 24 h, decalcified in 10% formic acid as described (37), and embedded in paraffin). The contralateral tibiae of animals were harvested, embedded in methacrylate, and used as controls for histomorphometric analysis.

Fig. 2 Effects of ZA on apoptosis and cell-cycle distribution of CaP cells. LNCaP and PC-3 cells were treated with 68 μM ZA for 1, 2, and 3 days. Cells were processed for determination of apoptosis as described in “Materials and Methods.” A, percentage of apoptotic cells as determined by the TUNEL assay. A representative sample of results is shown. ZA treatment caused a significant increase in apoptosis of LNCaP, which rose with the duration of exposure. ZA also caused a significant increase in apoptosis of PC-3 cells, but the largest percentage of apoptotic cells was detected after 1 day of treatment. B, percentage increase in compromised cells (cells with diminished mitochondrial potential) after treatment with 68 μM ZA. C, Hoechst staining was used to perform cell-cycle analysis of the portion of LNCaP and PC-3 cells with unchanged mitochondrial potential after treatment with 68 μM ZA for 1, 2, and 3 days. A representative example of the results is shown. ZA treatment caused G1 arrest in LNCaP cells after 1, 2, and 3 days of exposure, and also in PC-3 cells after 2 and 3 days of exposure. One day of treatment of PC-3 cells caused a significant increase in the cell population in S phase.
Fig. 3  Radiography and histology of PC-3 and LuCaP 23.1 in tibiae after ZA treatment. A, representative radiographs of PC-3 tumors in tibiae. Tibiae of SCID mice were injected with PC-3 cells, and ZA was administered under prevention and treatment regimens as described in “Materials and Methods.” Animals were sacrificed 4 weeks after injection and radiographs were taken using a mammography unit. PC-3 cells cause a strong osteolytic reaction in the bone, with destruction of a large portion of mineralized tissue (control). ZA administration (prevention and treatment) decreased the bone lysis caused by PC-3 cells; radiographs show conservation of tibiae with increased density of bone. B, representative histology.
Materials and Methods.

Histomorphometric Analysis. Analysis was performed on 5-μm sections of methacrylate-embedded tibiae stained with Goldner’s stain as described (38). Histomorphometric determinations of TuV of PC-3 tumors and BV were performed on a TV of 0.604 mm² adjacent to the growth plate using an Osteomeasure Image Analysis system (Osteometrics, Atlanta, GA). Percentages of BV and TuV in TV were calculated. Analysis of LuCaP 23.1 was performed by Skeletek, Inc. (Bothell, WA). Analysis was performed on mouse proximal tibiae. BV, TuV, Tb.N. (number/mm), Tb.Sp. (μm), Tb.Th. (μm), Ob.S/BS (%), and Oc.S/BS (%) were determined. Statistical analysis was performed using Student’s t test.

Immunohistochemical Analysis. Staining was performed to determine expression of Cathepsin K (anti-Cathepsin K chicken polyclonal antibody, 1:25 dilution; Immunodiagnostics System Ltd., Boldon, United Kingdom), MMP-2 (anti-MMP-2 rabbit polyclonal antibody, 5 μg/ml; Chemicon International Inc., Temecula, CA), and MMP-9 (anti-MMP-9 rabbit polyclonal antibody, 5 μg/ml; Chemicon International). Immunoperoxidase staining was performed as we described previously (39), with modifications to the serum blocking process. The serum block contained rabbit, horse, and goat serum (5% each) for the anti-Cathepsin K antibody, and horse, goat, and chicken serum (5% each) for the anti-MMP-2 and anti-MMP-9 antibodies, to prevent nonspecific binding in the xenograft tissues. Antigen retrieval was performed in 10 mM citrate buffer (pH 6) for 10 min before staining with the anti-MMP-2 and MMP-9 antibodies. The primary antibodies were diluted in the serum blocking solution, as were the appropriate negative controls (chicken IgY and rabbit IgG, respectively). Detection of immunoreactivity was performed using avidin-biotin complex method kits (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine as substrate.

RESULTS

Effects of ZA on Proliferation of CaP Cells. ZA (340 μM) inhibited proliferation of LNCaP and PC-3 cells in the presence of 10% FBS by up to 70% after 4 days of treatment (Fig. 1). Treatment with 13.6 μM ZA caused significant inhibition (15–45%) of proliferation after 2 or more days of exposure in both cell lines tested (P = 0.05–0.00001); however, 1 day of treatment was not effective. Similar effects were observed with 2% FBS (data not shown).

Effects of ZA on Apoptosis and Cell Cycle of CaP Cells. To evaluate effects of ZA on apoptosis we used 68 μM ZA, which decreased proliferation of all of the cell lines by ≥25% after 2 and 3 days of exposure. Using the TUNEL assay to detect DNA fragmentation, we observed 1.9, 2.2, and 2.6% apoptotic cells in the ZA-treated LNCaP cells after 1, 2, and 3 days of exposure, respectively, with no significant apoptosis detected in untreated cells (0.1, 0.0, and 0.5%; Fig. 2A). PC-3 cells treated with 68 μM of ZA showed an increase in apoptosis to 4.1% after 1 day of treatment, and 3% and 1.8%, respectively, after days 2 and 3 of treatment with no significant apoptosis detected in untreated cells (Fig. 2A). Differences were significant as determined by Fisher’s exact test (P < 0.001).

To confirm the increase in apoptosis we used an independent method. Detection of cells with compromised mitochondrial membrane potential (cells considered to be committed to the apoptotic pathway) also demonstrated an increase in the percentage of apoptotic cells after treatment with 68 μM of ZA (Fig. 2B). LNCaP cells exhibited significant increases after 1, 2, and 3 days of treatment (31.1 versus 25.3%, 35.2 versus 16.3%, and 35.7 versus 20.8%, respectively). Similar results were obtained with PC-3 cells, with the greatest difference appearing on day 1 (45.7 versus 10.8%, 31.4 versus 12.9%, and 30.5 versus 16.7%). All of the differences were significant as determined by Fisher’s exact test (P < 0.0001).

We also examined the cell-cycle distribution of CaP cells, using only the population of cells with unchanged mitochondrial potential. Treatment of LNCaP cells with 68 μM ZA for 1, 2, and 3 days decreased the proportion of cells in G1 phase by 9.8–12.6% over the control, indicating G1 arrest (Fig. 2C), with concomitant decreases in cell populations in S and G2 phases. Treatment of PC-3 cells with 68 μM of ZA caused a significant increase in percentages of cells in S phase after 1 day of treatment (17.5%), with a decrease in populations of cells in G2 phase. This was followed by increases in G1-phase cells after 2 and 3 days (24.3% and 17.7%, respectively), with concomitant decreases in S and G2 phases (Fig. 2C).

Effects of ZA on CaP in Vivo. For in vivo experiments we used two CaP models: PC-3 cells, which are responsive to ZA in vitro and yield lytic bone metastases, and LuCaP 23.1, which does not grow in vitro but is one of the few CaP xenografts that gives rise to osteoblastic bone metastases (40). We first examined effects on s.c. tumors. In contrast to the in vitro
results, we did not detect significant decreases in TuVs of s.c. PC-3 and LuCaP 23.1 xenographs treated with ZA versus untreated animals. However, when we examined the effects of ZA on both tumors and bones with PC-3 and LuCaP 23.1 grown in tibiae, we detected significant increases in BV and decreases in TuV under both regimens of administration (prevention and treatment). Representative examples of radiographs and histology are shown in Fig. 3, A and B (PC-3) and Fig. 3, C and D (LuCaP 23.1). The take rate of PC-3 in tibiae was 100% (10 of 10) in all three of the groups, and was unaffected by ZA administration. Percentage TuV/TV of PC-3 cells was significantly smaller in tibiae of ZA-treated animals versus control animals (Fig. 4A, control: 59.8 ± 11.6%, prevention: 20.7 ± 2.9%, P = 0.029, treatment: 18.3 ± 2.8%, P = 0.022). PC-3 cells caused the osteolytic reaction in bone, leading to a significant reduction in %BV/TV in comparison to contralateral tibiae (Fig. 4B, 5.9 ± 1.7%, versus 13.9 ± 1.4%; P = 0.0024). Comparison of %BV/TV of tumored and contralateral tibiae after administration of ZA showed that %BV/TV of tumored tibiae was not significantly different from %BV/TV of contralateral tibiae of the same animals (Fig. 4B, prevention: 26.9 ± 5.5% versus 19.9 ± 6.1%, P = 0.19, and treatment: 22.5 ± 4.6% versus 31.6 ± 5.9%, P = 0.26). Thus the effects of ZA were not restricted to the tumor site. However, there were significant increases in %BV/TV in both tumored and contralateral tibiae accompanying the ZA administration compared with vehicle alone (Fig. 4B; P < 0.01). The animals in the control group became compromised at week 4 and were, therefore, sacrificed; however, the ZA-treated animals under both regimens with PC-3 cells in tibiae did not become compromised until weeks 7–9, at which time they were sacrificed.

Because many CaP bone metastases have the radiographic appearance of osteoblastic reactions, we performed a more detailed analysis of the LuCaP 23.1 bone metastases. Take rates of LuCaP 23.1 were similar in control and ZA-treated groups (100%; 10 of 10), suggesting that ZA administration did not affect establishment of the cells in the bone milieu. The percentage of TuV/TV of LuCaP 23.1 in tibiae was significantly smaller in ZA-treated animals versus untreated animals (Fig. 4A, prevention: P = 0.001, treatment: P = 0.009; Table 1). Our data also showed that PSA serum levels in animals bearing LuCaP 23.1 xenographs were significantly decreased by ZA under prevention and treatment regimens versus control animals (Fig. 5), suggesting decreased tumor growth, based on the assumption that PSA serum levels are correlated positively with TuV. These results are in agreement with our observation that ZA in vitro had inhibitory effects on tumor growth. LuCaP 23.1 xenographs cause an osteoblastic reaction in bone, leading to a significant increase in %BV/TV versus contralateral tibiae (P = 0.0008). Administration of ZA under both regimens to animals bearing LuCaP 23.1 tumors in tibiae additionally increased the %BV/TV of tumored tibiae versus contralateral tibiae (prevention: P = 0.017, treatment: P = 0.017; Table 1). Analysis of tibiae from control animals (two groups, sacrificed 130 and 69 days after tumor cell injection) showed no significant differences in the parameters evaluated (%BV/TV, %TuV/TV, Tb.N., Tb.Th., Tb.Sp., Ob.S./BS, and Oc.S./BS). Therefore, for histomorphometrical analysis of the effects of ZA administration, the control tibiae were treated as one group. There were no significant changes in Tb.N. with ZA administration, but we observed significant decreases in Tb.Sp. and increases in Tb.Th., which did not reach significance. Ob.S./BS was greater in tibiae bearing LuCaP 23.1 versus contralateral tibiae, but remained approximately the same in ZA-treated animals versus untreated animals. However, in keeping with the known antiosteoclastic activity of ZA, the Oc.S./BS was lower in ZA-treated animals. However, when we examined the effects of ZA on both tumors and bones with PC-3 and LuCaP 23.1 grown in tibiae, we detected significant increases in BV and decreases in TuV under both regimens of administration (prevention and treatment). Representative examples of radiographs and histology are shown in Fig. 3, A and B (PC-3) and Fig. 3, C and D (LuCaP 23.1). The take rate of PC-3 in tibiae was 100% (10 of 10) in all three of the groups, and was unaffected by ZA administration. Percentage TuV/TV of PC-3 cells was significantly smaller in tibiae of ZA-treated animals versus control animals (Fig. 4A, control: 59.8 ± 11.6%, prevention: 20.7 ± 2.9%, P = 0.029, treatment: 18.3 ± 2.8%, P = 0.022). PC-3 cells caused the osteolytic reaction in bone, leading to a significant reduction in %BV/TV in comparison to contralateral tibiae (Fig. 4B, 5.9 ± 1.7%, versus 13.9 ± 1.4%; P = 0.0024). Comparison of %BV/TV of tumored and contralateral tibiae after administration of ZA showed that %BV/TV of tumored tibiae was not significantly different from %BV/TV of contralateral tibiae of the same animals (Fig. 4B, prevention: 26.9 ± 5.5% versus 19.9 ± 6.1%, P = 0.19, and treatment: 22.5 ± 4.6% versus 31.6 ± 5.9%, P = 0.26). Thus the effects of ZA were not restricted to the tumor site. However, there were significant increases in %BV/TV in both tumored and contralateral tibiae accompanying the ZA administration compared with vehicle alone (Fig. 4B; P < 0.01). The animals in the control group became compromised at week 4 and were, therefore, sacrificed; however, the ZA-treated animals under both regimens with PC-3 cells in tibiae did not become compromised until weeks 7–9, at which time they were sacrificed.

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animals versus untreated animals and also in tibiae bearing LuCaP 23.1 versus contralateral tibiae, although these differences did not reach statistical significance.

Because ZA affects osteoclasts and their ability to degrade bone extracellular matrix, we examined levels of expression of Cathepsin K, a cysteine protease responsible for degradation of collagen in CaP cells. We detected immunoreactivity of Cathepsin K in PC-3 cells grown in tibiae, and this expression was decreased in PC-3 cells in tibiae from ZA-treated animals under both regimens (Fig. 6A; Table 2). Interestingly, Cathepsin K expression was not affected in LuCaP 23.1 xenografts in tibiae both regimens (Fig. 6B). Human bone with CaP metastasis used as a positive control exhibited strong staining in osteoclasts lining the BS. The same samples treated under the same conditions with chicken IgY were negative. A similar pattern was observed with MMP-2 and MMP-9. MMP-2 and MMP-9 expression was decreased in PC-3 cells in tibiae by ZA treatment (Fig. 7; Table 2), but no corresponding changes were observed in LuCaP 23.1 cells. The same samples stained with rabbit IgG exhibited no immunoreactivity.

**DISCUSSION**

Our data show that ZA has direct antiproliferative and apoptotic effects on CaP cells *in vitro*. The degree of inhibition of proliferation varied with the CaP cell line, but it is unclear whether this is connected with known phenotypic differences among these cell lines; mechanistic studies will be required to answer this question. Our data are in agreement with a recent report of Lee et al. (26), who also observed similar inhibitory effects of ZA on proliferation of CaP cells *in vitro*. The concentrations effective *in vitro* are higher than those used under clinical conditions, but because BPs accumulate in bone (41), the local concentrations might be considerably higher than those calculated on an organismal basis, conceivably reaching levels similar to those effective *in vitro*.

Several studies have suggested that BPs promote apoptosis of osteoclasts (11, 42). Apoptosis is characterized by various cellular changes, including DNA fragmentation, mitochondrial swelling, and chromatin condensation. We have used two independent assays to examine activation of apoptotic pathways. By detecting DNA fragmentation we observed an increase of up to 2.5-fold in apoptosis in LNCaP and PC-3 associated with ZA treatment, although the overall percentages of cells exhibiting DNA fragmentation were low. Assays of mitochondrial membrane potential as an indication of apoptosis also showed that ZA caused apoptosis of CaP cells. The time dependence of the results showed similar patterns: increased apoptosis was asso-

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*Table 1* Results of bone histomorphometrical analysis of LuCaP 23.1 in bone with and without ZA treatment

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* a P < 0.001.
  b P < 0.01.
  c P < 0.05.

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Fig. 5 Effects of ZA on PSA levels in animals with LuCaP 23.1 in tibia. Animals were injected with single-cell suspensions of LuCaP 23.1 in tibia. ZA was administered SC twice weekly under two regimens. 1. prevention: ZA injections started at the same time as cell injection. 2. treatment: ZA injection started when PSA levels reached approximately 5–10 ng/ml, indicating established tumor in the bone. Blood was drawn for determination of PSA every 2 weeks. Results are plotted as mean; bars, ±SE. Significant differences in PSA serum levels were detected under both regimens of ZA administration in comparison with untreated LuCaP 23.1 tumors.

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associated with longer exposure of LNCaP to ZA in each case, whereas the highest level of apoptosis of PC-3 was seen at 24 h. The difference between percentages of apoptotic cells as determined by TUNEL versus the mitochondrial assay is probably related to the kinetics of apoptosis. Mitochondria lose their function early in the apoptotic process, as reflected in a lowered mitochondrial membrane potential, whereas degradation of DNA and formation of apoptotic fragments are late events. Therefore, the time window during which a cell exhibits a lowered mitochondrial membrane potential is longer. Our data contrast with results published by Lee et al. (26), who reported that ZA did not cause apoptosis in CaP cells (DU 145 and PC-3). The differences between our results and those of Lee et al. (26) may be due to differences in the sensitivity of the methods used for determination of apoptosis, and/or to variations in the cultured cell lines.

There were significant differences in the responses of the LNCaP (hormone-sensitive) and PC-3 (hormone-insensitive) cell lines to ZA. The p53-deficient PC-3 (43) exhibited high levels of apoptosis and accumulation in S phase after 24 h, whereas LNCaP cells, with wild-type p53 (44), exhibited a slow rise in apoptotic cells, concomitant with a strong G1 phase.

Fig. 6 Presence of Cathepsin K immunoreactivity in PC-3 and LuCaP 23.1 tumors in tibiae with and without ZA administration. Immunoreactivity of Cathepsin K, an enzyme involved in degradation of bone extracellular matrix, in representative samples of untreated and treated tibiae with PC-3 (A) and LuCaP 23.1 (B). Staining was performed as described in “Materials and Methods.” Expression of Cathepsin K in PC-3 cells is decreased in tibiae after ZA administration under both prevention and treatment regimens versus control (untreated) tumored tibiae. In contrast Cathepsin K expression was not changed in osteoblastic, LuCaP 23.1 metastases.
arrest. We hypothesize that ZA induces apoptosis by distinct mechanisms, depending on the p53 status of the target cells. Cells that have lost their G1/S checkpoint undergo rapid apoptosis (PC-3), whereas cells with functional p53 (as observed by arrest at the G1/S border) still undergo ZA-induced apoptosis, albeit at a slower rate (LNCaP). A useful corollary is that the cytotoxic action of ZA on CaP cells does not appear to be dependent on p53 function.

To investigate in vivo the potential benefits of ZA to CaP patients we have examined the effects of ZA on s.c. CaP tumors and CaP bone metastases in animal models (40). The effects of ZA on s.c. tumors should be independent of the bone environment. In contrast with the in vitro inhibition data, we saw no significant effects of ZA on the growth of s.c. tumors. This may indicate that there are no direct effects of ZA in vivo under these conditions and that the observed effects on tumors in bone are all related to inhibition of osteolysis. An alternative hypothesis is that the lack of inhibitory effects on the s.c. tumors may be due to poor bioavailability of ZA, at least in part because the compound accumulates in bone. However, in osseous-CaP models, consisting of direct injection of CaP cells into tibiae, we observed inhibition of both tumor growth and bone degradation by ZA. It is not yet clear whether the two inhibitory phenomena are caused by a single or multiple activities of ZA. It has been suggested that factors released during degradation of bone extracellular matrix support growth of tumor cells (45). Zhang et al. (46) showed that administration of osteoprotegerin, a modulator of osteoclast recruitment and activity, can impede establishment of C4–2B metastases in bone when injected at the same time as the C4–2B cells, suggesting that osteoclastogenesis is an important mediator of establishment of CaP bone metastasis. The inhibition of tumor proliferation observed could be because of direct effects of ZA on tumor cells, indirect effects resulting from decreased bone lysis, a combination of these, or still other, unknown mechanisms. Our data indicate that ZA probably does not interfere with initial establishment of the tumors in bone, because the take rates were similar in control and prevention groups. Moreover, in the PC-3 intratibial tumors, ZA slowed but did not halt growth of the tumors, indicating that ZA may be palliative but not curative with osteolytic CaP metastases.

Results of BHM analysis also suggest that the increase in %BV/TV in ZA-treated animals bearing LuCaP 23.1 in tibiae versus nontreated animals appears to be due to increased thickness of trabeculae, which is consistent with decreased lysis and/or more bone formation in apposition to the existing trabeculae.

Cathepsin K, a cysteine protease expressed by osteoclasts, appears to be essential for osteoclast-mediated collagen type-I degradation (47). Cathepsin K is expressed in breast cancer cells (48), and we have reported recently on detection of Cathepsin K and its proteolytic activity in CaP tissues and CaP cell lines (49). In the studies presented here, ZA treatment decreased expression of Cathepsin K in PC-3 cells in bone. Reduced expression of Cathepsin K could also play a role in reduced degradation of bone by PC-3 tumors treated with ZA. However, the significance of unchanged levels of Cathepsin K in LuCaP 23.1 under ZA treatment needs to be determined. The difference between these two CaP xenografts suggests that more than one mechanism may be involved in the action of ZA and that the effective mechanisms may depend on the type of tumor.

To characterize additionally the direct effects of ZA on CaP xenografts in vivo, we examined expression of metalloproteinases MMP-2 and MMP-9, which are implicated in tumor cell migration, invasion, and bone extracellular matrix degradation in CaP tumors. Boissier et al. (20) showed that BPs affect invasiveness of breast and CaP cells. They associated this with inhibitory effects of BPs on the proteolytic activities of MMP-2, MMP-9, and MMP-12, but the actual production of MMP was unaffected. Teronen et al. (50) also reported decreased activity without decreased expression of MMP after BP treatment. We were unable to detect any proteolytic activity of MMP-2 and MMP-9 in the culture medium of CaP cells (data not shown), but we did observe reduced expression of MMPs 2 and 9 in osteolytic PC-3 tumors (but not in LuCaP 23.1 tumors) by immunohistochemistry. This represents a possible mechanism whereby ZA could directly inhibit the osteolytic activity of CaP tumors. In agreement with our results, Nemeth et al. (51) reported recently inhibition of PC-3 tumor-induced bone lysis by MMP inhibitors, additionally suggesting the importance of MMP in CaP lytic lesions.

In conclusion, we have shown that ZA has antiproliferative, apoptotic, and cytostatic effects on CaP cells in vitro. Significantly, ZA also has inhibitory effects on the growth of CaP osteoblastic and osteolytic bone metastases in an animal model, but we observed no inhibitory effects on CaP tumors outside of the bone milieu. The observation that ZA causes a

<table>
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<th>Table 2</th>
<th>Immunohistochemical analysis of PC-3 and LuCaP 23.1 in tibiae after administration of ZA</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>PC-3</td>
<td></td>
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<tr>
<td>Cathepsin K</td>
<td>20–40%</td>
</tr>
<tr>
<td>MMP-2</td>
<td>70–90%</td>
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<tr>
<td>MMP-9</td>
<td>90%</td>
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<tr>
<td>LuCaP 23.1</td>
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<tr>
<td>Cathepsin K</td>
<td>80–90%</td>
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<td>MMP-2</td>
<td>80–90%</td>
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<tr>
<td>MMP-9</td>
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reduction in expression of enzymes involved in bone degradation by PC-3 cells suggests a range of mechanisms by which ZA may inhibit CaP-induced bone lysis.

ACKNOWLEDGMENTS

Zoledronic acid was kindly provided by Pharma Novartis AG, Basel, Switzerland, and PSA assays by Abbott Laboratories, Abbott Park, IL. We thank Dr. Jonathan Green for helpful discussions and Dr. Michael Corey for useful comments and editorial help.

REFERENCES


Erratum

In the article by Eva Corey et al., which appeared in the January 2003 issue of Clinical Cancer Research (pp. 295–306), there was an error in the printing of figures 6 and 7. Please see the corrected figures below.

Fig. 6  Presence of Cathepsin K immunoreactivity in PC-3 and LuCaP 23.1 tumors in tibiae with and without ZA administration. Immunoreactivity of Cathepsin K, an enzyme involved in degradation of bone extracellular matrix, in representative samples of untreated and treated tibiae with PC-3 (A) and LuCaP 23.1 (B). Staining was performed as described in “Materials and Methods.” Expression of Cathepsin K in PC-3 cells is decreased in tibiae after ZA administration under both prevention and treatment regimens versus control (untreated) tumored tibiae. In contrast Cathepsin K expression was not changed in osteoblastic, LuCaP 23.1 metastases.
Fig. 7  Presence of MMP-2 and MMP-9 immunoreactivity in PC-3 in tibiae after administration of ZA. Effects of ZA on expression of MMP-2 and MMP-9 enzymes involved in degradation of bone extracellular matrix were examined by immunohistochemistry. Representative examples of MMP-2 (A) and MMP-9 (B) immunoreactivity in tibiae after ZA administration are shown. Both regimens of ZA administration appear to reduce immunoreactivity of MMP-2 and MMP-9 in osteolytic PC-3 metastases, suggesting possible mechanisms of direct effects of ZA on PC-3 cells resulting in decreased bone lysis.
Zoledronic Acid Exhibits Inhibitory Effects on Osteoblastic and Osteolytic Metastases of Prostate Cancer

Eva Corey, Lisha G. Brown, Janna E. Quinn, et al.


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