Modulation of Bone Microenvironment with Zoledronate Enhances the Therapeutic Effects of STI571 and Paclitaxel against Experimental Bone Metastasis of Human Prostate Cancer

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Abstract
Prostate cancer cells metastasize to the bone where their interaction with osteoclasts and osteoblasts can lead to alterations in the structure of the bone. We determined whether the systemic administration of the bisphosphonate, zoledronate, could prevent bone lysis and halt the proliferation of human prostate cancer cells injected into the tibia of nude mice. Zoledronate did not affect the in vitro proliferation of human prostate cancer PC-3MM2 cells. The in vivo administration of zoledronate produced significant bone preservation but did not inhibit the progressive growth of PC-3MM2 cells. The systemic administration of STI571 (imatinib mesylate, Gleevec), an inhibitor of phosphorylation of the platelet-derived growth factor receptor, in combination with paclitaxel, produced apoptosis of tumor cells and bone-associated endothelial cells. The systemic administration of zoledronate with STI571 and paclitaxel produced a significant preservation of bone structure, a decrease in tumor incidence and weight, and a decrease in incidence of lymph node metastasis. This therapeutic activity was correlated with inhibition of osteoclast function, inhibition of tumor cell proliferation, and induction of apoptosis in tumor-associated endothelial cells and tumor cells. Cancer is a heterogeneous disease that requires multimodality therapy. The present data recommend the combination of a bisphosphonate agent with protein tyrosine kinase inhibitor and an anticycling drug for the treatment of prostate cancer bone metastasis. (Cancer Res 2005; 65(9): 3707-15)

Introduction
Prostate cancer frequently produces metastasis to the lymph nodes and bones, leading to morbidity and mortality (1, 2). The ideal treatment for prostate cancer bone metastasis must eradicate tumor cells while preserving the structure of the bone. The establishment and progressive growth of metastases depend on the interaction of tumor cells with the organ microenvironment (3-5). Once cancer cells reach the bone microenvironment, a reciprocal cellular interaction between cancer cells and bone stroma cells results in the proliferation of both (6, 7), and the activity of osteoclasts is a critical element to the formation of both osteolytic and osteoblastic lesions (8-10) due to differences in the milieu of cytokines (11). In the majority of prostate cancer patients, bone metastases are associated with osteoblastic changes, whereas in other patients the lesions are osteoblastic and osteolytic (1, 2, 5).

Recently, bisphosphonates have emerged as an important class of drugs to inhibit both normal and pathologic bone resorption and thus to have a major effect on the treatment of metastases to the bone (12-15). Among the bisphosphonates, analogues of PPI, nitrogen-containing bisphosphonates, inhibit protein prenylation, resulting in the inhibition of osteoclast function and the induction of apoptosis in osteoclasts (16), myeloma cells (17), breast cancer cells (18), osteosarcoma cells (19), and prostate cancer cells (20, 21). The analogues of PPI have also been reported to inhibit the invasion of breast and prostate carcinoma cells (22) and to inhibit testosterone-stimulated vascular regrowth in the ventral prostate of castrated rats (23) and recruitment of osteoclasts (24). Zoledronate (zoledronic acid, Zometa [2-(imidazole-1-yl)-hydroxy-ethylidene-1,1-bisphosphonic acid, disodium salt, 4.5 hydrate], Novartis Pharmaceuticals Corp., Basel, Switzerland) is a new-generation, nitrogen-containing bisphosphonate shown to be highly potent in preclinical models (25) and clinical studies (26). The interaction of platelet-derived growth factor (PDGF) with its receptor (PDGFR) can stimulate cell division (27, 28), cell migration (29), angiogenesis (30, 31), and survival of neuronal cells (32). PDGF is expressed by prostate cancer cells (31, 33). We have reported that inhibition of phosphorylation of the PDGFR by systemic administration of STI571 (imatinib mesylate, Gleevec) combined with paclitaxel induces significant apoptosis of tumor-associated endothelial cells in human prostate cancer cells growing in the bone of nude mice and, consequently, therapy of experimental bone metastasis (31). These data recommended investigating the therapeutic potential of experimental prostate cancer bone and lymph node metastases by systemic administration of zoledronate combined with STI571 and paclitaxel in experimental prostate cancer bone and lymph node metastasis. We report that this therapy produces a significant decrease in tumor incidence and size, a significant preservation of bone structure, and a highly significant decrease in incidence of lymph node metastasis.

Materials and Methods

Human prostate cancer PC-3MM2 cell line. The metastatic PC-3MM2 cell line was isolated as described previously (34) and maintained as monolayer cultures in Eagle’s MEM supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Grand Island, NY), L-glutamine, pyruvate, nonessential amino acid mixture, 2-fold vitamins, and penicillin-streptomycin (Invitrogen, Carlsbad, CA) and incubated in 5% CO2 with balance of air at 37°C. All reagents used for tissue culture were free of endotoxins, Mycoplasma, and viral pathogens. Cells used in this study were from frozen stocks, and all experiments were carried out within 10 in vitro passages after thawing.
Animals. Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care that met all current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. The mice were used in accordance with institutional guidelines when they were 6 to 8 weeks old.

Therapeutic agents. Zoledronate was dissolved in 5% glucose (in water). STI571, a novel PDGF tyrosine kinase inhibitor (Novartis Pharmaceuticals), was dissolved in distilled water. Paclitaxel (Taxol) purchased from Bristol-Myers Squibb (Princeton, NJ) was diluted in distilled water for i.p. injection.

In vitro effects of zoledronate on PC-3MM2 cells. PC-3MM2 cells (1.2 × 10^5) were seeded into each well of 96-well plates and allowed to adhere overnight. The medium was replaced and removed with either medium with 0.1% FBS or medium with 0.1% FBS containing 1, 2.5, 5, 10, 100, 500, and 1,000 nM/LL zoledronate. Medium with 0.1% FBS containing 1, 2.5, 5, 10, 100, 500, and 1,000 nM/LL paclitaxel was used to compare cytotoxicity. After 96 hours of incubation, the number of metabolically active cells per well (triplicate wells per group) was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Stock solution of MTT (M2128; Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 5 mg MTT in 1 ml PBS and filtering the solution to remove particulates. The solution was protected from light and stored at 4°C. Following a 2-hour incubation period in medium containing 0.42 mg/ml MTT, the PC-3MM2 cells were lysed in DMSO. The conversion of MTT to formazan by metabolically active cells was monitored by Ceres UV9000C, a 96-well microplate reader at 570 nm (Bio-Tek Instruments, Inc., Winooski, VT). Growth inhibition was calculated by the formula: cytostasis (%) = [1 - (A / B)] × 100, where A is the total number of cells and B is the number of cells in the test group. IC_{50} was obtained from the plotted curves.

Intratrabecular injection of tumor cells. To produce tumors in the tibia, PC-3MM2 cells were harvested from confluent cultures by a 2-minute exposure to 0.25% trypsin and 0.02% EDTA. Then, medium containing 10% FBS was added and the cells were washed once in serum-free medium and resuspended in Ca^{2+}- and Mg^{2+}-free HBSS. Cell viability was determined by trypsin blue exclusion, and only single-cell suspensions of >95% viability were used for injection. Male nude mice were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL), and tumor cells were injected intraosseously into the tibia as described previously (31, 35). The animals tolerated this procedure well, and no anesthesia-related deaths occurred.

Therapy of human prostate cancer cells growing in the tibias of athymic nude mice. Zoledronate dissolved in water containing 5% glucose was injected s.c. twice daily. STI571 dissolved in distilled water at 6.25 mg/ml was given orally once daily. Paclitaxel diluted in water was injected i.p. once weekly. In the first set of experiments, we determined the optimal dose and schedule of zoledronate necessary to prevent osteolysis. Three days after the implantation of PC-3MM2 cells into the tibia, five mice were killed and the presence of actively growing cancer cells in the bone cortex was ascertained by histologic examination as described previously (39, 43). Injected mice were randomized into six treatment groups (n = 10): (a) control mice receiving twice daily s.c. injections of 5% glucose in water and once weekly i.p. injections of distilled water; (b) mice receiving twice weekly i.p. injections of paclitaxel (8 mg/kg) and twice daily s.c. injections of 5% glucose in water; (c) mice receiving twice daily s.c. injections of zoledronate (125 μg/kg) and once weekly i.p. injections of distilled water; (d) mice receiving twice daily s.c. injections of zoledronate (25 μg/kg) and once weekly i.p. injections of distilled water; (e) mice receiving twice daily s.c. injections of zoledronate (5 μg/kg) and once weekly i.p. injections of distilled water; and (f) mice receiving twice daily s.c. injections of zoledronate (1 μg/kg) and once weekly i.p. injections of distilled water. All of the mice were treated for 5 weeks when tumor size, lymph node metastasis, and osteolysis of the injected bone were evaluated. This experiment was done twice.

In the next set of experiments, we determined the therapeutic effects of combining zoledronate, STI571, and paclitaxel against PC-3MM2 tumors into the tibia of nude mice and preservation of the bone. To do so, mice were injected with PC-3MM2 in the tibia and 3 days later were randomized into eight groups (n = 10) as follows: (a) daily oral administrations of distilled water, twice daily s.c. injections of 5% glucose in water, and once weekly i.p. injections of distilled water; (b) once weekly i.p. injections of paclitaxel (8 μg/kg), daily oral administrations of distilled water, and twice daily s.c. injections of 5% glucose in water; (c) daily oral administrations of STI571 (50 μg/kg, biological optimal dose as determined previously; ref. 31), twice daily s.c. injections of 5% glucose in water, and once weekly i.p. injections of distilled water; (d) twice daily s.c. injections of zoledronate (25 μg/kg, optimal biological dose determined from the first set of experiments), twice daily oral administrations of distilled water, and once weekly i.p. injections of distilled water; (e) daily oral administrations of STI571 (50 μg/kg), once weekly i.p. injections of paclitaxel (8 μg/kg), and twice daily s.c. injections of 5% glucose in water; (f) twice daily s.c. injections of zoledronate (25 μg/kg), once weekly i.p. injections of paclitaxel (8 μg/kg), and daily oral administrations of distilled water; (g) daily oral administrations of STI571 (50 μg/kg), twice daily s.c. injections of zoledronate (25 μg/kg), and once weekly i.p. injections of distilled water; and (h) daily oral administrations of STI571 (50 μg/kg), twice daily s.c. injections of zoledronate (25 μg/kg), and once weekly i.p. injections of paclitaxel (8 μg/kg). All of the mice were treated for 5 weeks when tumor size and osteolysis were evaluated by gross observation, necropsy, and digital radiography. Incidence of bone tumors and lymph node metastasis were confirmed by histologic examination and H&E staining. Immunohistochemical analyses for various markers and tartrate-resistant acid phosphatase (TRAP) staining for functioning osteoclasts were done as described below. This experiment was repeated twice.

Digital radiography and harvest of tumors. After 3 or 4 weeks of treatment, mice were anesthetized with Nembutal (0.5 mg/g body weight) and placed in a prone position. Digital radiography of the hind legs was carried out using the Faxitron MX-20 X-ray machine (Faxitron X-ray Corp., Wheeling, IL). On week 6 of the study, the mice were euthanized by injection with Nembutal and weighed. Digital radiography of the hind limbs of each mouse was carried out and incidence of bone tumors was recorded. The legs with tumor and the unjected contralateral legs were resected at the head of the femur and weighed. Net tumor weight was calculated by subtracting the weight of the control leg from that of the leg with tumor. The legs with tumors were prepared for histologic studies as described below. Mice were autopsied and all enlarged lymph nodes were resected and fixed in 10% formalin. To evaluate the presence of tumor cells in the bones and lymph nodes was confirmed by histology following H&E staining.

Reagents for immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Antibodies for immunohistochemistry were purchased as follows: rabbit anti-fibroblast growth factor-2 [basic fibroblast growth factor (bFGF)]; rabbit anti-interleukin-8 (IL-8) (Biosource International, Camarillo, CA); rabbit anti-vascular endothelial growth factor (VEGF)/vascular permeability factor; rabbit anti–epidermal growth factor (EGF); rabbit anti-EGF receptor (EGFR); rabbit anti-PDGF-A/B and rabbit anti-PDGFRβ/β (goat polyclonal anti-phosphorylated PDGFRβ) (activated PDGFRβ); Santa Cruz Biotechnology, Santa Cruz, CA); rat anti-mouse CD31-platelet/endothelial cell adhesion molecule 1 (PECAM-1; Pharmingen, San Diego, CA); mouse anti–proliferating cell nuclear antigen (PCNA) clone PC-10 (DAKO A/S, Copenhagen, Denmark); peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated goat anti-rat IgG, Texas red-conjugated goat anti-rat IgG, and FITC-conjugated goat anti-rabbit IgG (Jackson Research Laboratories, West Grove, CA); peroxidase-conjugated rat anti-mouse IgG2a (Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN); and Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Stable 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) and Gills hematoxylin (Sigma Chemical) were used for visualization of immunohistochemical reaction and counterstaining, respectively. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was done by using a commercial apoptosis detection kit (Promega Corp., Madison, WI) with modification (31, 35).
Preparation of tissues. The tibia and surrounding muscles were fixed in 10% buffered formalin for 24 hours, washed with PBS for 30 minutes thrice, decalcified for 7 to 10 days at 4°C with 10% EDTA (pH 7.4), and sectioned and embedded in paraffin. Tissues were processed exactly as described previously (31, 35).

Immunohistochemical determination of basic fibroblast growth factor, vascular endothelial growth factor, interleukin-8, proliferating cell nuclear antigen, CD31/platelet/endothelial cell adhesion molecule 1, epidermal growth factor, epidermal growth factor receptor, platelet-derived growth factor A/B, platelet-derived growth factor receptor α/β, and activated platelet-derived growth factor receptor α/β. The expression of bFGF, VEGF, IL-8, PCNA, EGF, EGFR, PDGF-A/B, PDGFRα/β, and activated PDGFRα/β was determined in sections (4-6 μm thick) of paraffin-embedded tissues exactly as described previously (31, 35). Negative control samples exposed to secondary antibody alone exhibited no specific staining.

Immunofluorescence double staining for CD31/endothelial cell adhesion molecule 1 (endothelial cells) and phosphorylated platelet-derived growth factor receptor or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling. Pyridoxal phosphate–fixed tissues were sectioned (8-10 μm), mounted on positively charged slides, and air-dried for 30 minutes. The samples were washed thrice with PBS, incubated for 20 minutes at room temperature with protein blocking solution containing 4% fish gel in PBS, and incubated for 18 hours at 4°C with a 1:400 dilution of rat monoclonal anti-mouse CD31 antibody (human cross-reactive). After the samples were rinsed four times with PBS (3 minutes each), the slides were incubated with a 1:200 dilution of secondary goat anti-rat conjugated to Texas red for 1 hour at room temperature in the dark. Tissues were then washed twice for 5 minutes with PBS containing 0.1% Brij and once for 5 minutes with PBS. Phosphorylated PDGFRα and PDGFRβ immunostaining was done following staining for CD31 as described previously (31, 35). TUNEL was done using an apoptosis detection kit (31, 35), and quantification of endothelial cells was done as described previously (31, 35).

Determination of tartrate-resistant acid phosphatase–positive cells. Tissue sections mounted on slides were fixed for 30 seconds at room temperature in fixative solution (25 mL citrate solution, 65 mL acetic acid, and 8 mL of 37% formaldehyde) and incubated for 1 hour at 37°C with protection from light in reaction solution (45 mL deionized water, 1 mL diazotized fast Garnet GBC solution-0.5 mL fast Garnet GBC base solution, and 0.5 mL sodium nitrite solution, 0.5 mL naphthol AS-BI phosphate solution, 2 mL acetate solution) with or without 1 mL tartrate solution (36–38). Slides were thoroughly rinsed in deionized water and then counterstained for 2 minutes in hematoxylin solution (Gill No. 3). To color the nuclei, the slides were soaked in alkaline tap water. Cells with purple or red fluorescence were counted as positive cells.

Quantification of tartrate-resistant acid phosphatase–positive cells, proliferating cell nuclear antigen–positive cells, and mean vessel density. Ten random 0.159 mm² fields (×100 magnification) of tumors in the bone were captured for each mouse. Mean vessel density was quantified according to the method described previously (31, 35). Cells stained with anti-PCNA antibody were counted in the same fields.

Statistical analysis. The significance of tumor incidence and incidence of lymph node metastasis was determined with the χ² test. The significance of differences in tumor weight was determined by the Mann-Whitney U test. The differences in TRAP-positive cells, PCNA-positive cells, and mean vessel density (CD31/PECAM-1) were determined by the unpaired Student’s t test.

Results

In vitro effects of zoledronate on PC-3MM2 cells. PC-3MM2 cells were incubated for 96 hours in medium containing 10% FBS and 0, 1, 2.5, 5, 10, 100, 500, and 1,000 nmol/L zoledronate. Paclitaxel at the same concentrations was used as a positive control. The number of metabolically active cells was determined by MTT assay. At the concentrations tested, zoledronate did not affect the proliferation of PC-3MM2 cells, whereas paclitaxel...
inhibited cell proliferation at IC$_{50}$ of 0.43 nmol/L. After 7 days of continuous incubation, the IC$_{50}$ of zoledronate was 23 μmol/L, which is similar to data reported previously (26), a concentration 3 to 4 times higher than that which can be achieved in vivo.

Optimal dose and schedule of zoledronate necessary to prevent osteolysis. Male nude mice were injected in the tibia with PC-3MM2 cells. Treatment began 3 days later and continued for 5 weeks, when digital radiography revealed osteolysis of the tibia in control and paclitaxel-treated mice (Fig. 1). In additional preliminary experiments, groups of mice were injected s.c. with zoledronate at 50 μg/kg once or twice daily, 100 μg/kg once or twice daily, and 100 μg/kg every other day. Lytic bone lesions were observed in control mice, and mice were treated with zoledronate 50 μg/kg once daily or 100 μg/kg every other day. The mice tolerated all doses and schedules of the drugs. On necropsy, all organs were grossly normal. Lung, liver, heart, spleen, pancreas, intestine, and kidney were normal on histologic examination (H&E). These data recommend twice daily administrations of zoledronate. Treatment with zoledronate preserved the bones in a dose-dependent manner with complete preservation of bone structure in mice receiving 25 and 125 μg/kg. Minimal osteolysis was found in mice receiving 5 μg/kg zoledronate, whereas the administration of 1 μg/kg zoledronate did not prevent osteolysis. All of the mice were autopsied. Bone tumors were weighed and processed for histologic analyses as were lymph node metastases. The results of a representative experiment of two are shown in Table 1. All control mice had tumors in the tibia (median, 0.9 g; range, 0.1-7.1 g) with 100% lymph node metastasis, and 8 of 10 mice treated with paclitaxel had large tumors (median, 0.6 g; range, 0.5-3.3 g) and 7 of 10 had lymph node metastasis. Treatment with zoledronate did not significantly reduce the incidence of bone tumors nor the incidence of lymph node metastasis, but no destruction of the bone was found. The prevention of tumor cells from reaching the musculature where they readily proliferate significantly reduced the tumor size in mice treated with zoledronate. No significant differences in tumor incidence, tumor size, or lymph node metastasis were found among the groups treated with different doses of zoledronate. H&E staining of bone tumors of zoledronate-treated mice revealed preservation of the bony cortex even in the presence of growing tumors (Fig. 2).

Table 1. Treatment of human prostate carcinoma growing in the bone of nude mice

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>Incidence</th>
<th>Tumor weight (g), median (range)</th>
<th>Incidence of lymph node metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>0.9 (0.1-7.1)</td>
<td>10/10</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>8/10</td>
<td>0.6 (0.5-3.1)</td>
<td>7/10</td>
</tr>
<tr>
<td>Zoledronate 1 μg/kg</td>
<td>9/10</td>
<td>0.3 (0-4.2)</td>
<td>8/10</td>
</tr>
<tr>
<td>Zoledronate 5 μg/kg</td>
<td>7/9</td>
<td>0.2 (0.5-5.5)</td>
<td>7/9</td>
</tr>
<tr>
<td>Zoledronate 25 μg/kg</td>
<td>7/10</td>
<td>0.2 (0.5-5.5)</td>
<td>7/10</td>
</tr>
<tr>
<td>Zoledronate 125 μg/kg</td>
<td>9/10</td>
<td>0.3 (0-4.6)</td>
<td>8/10</td>
</tr>
</tbody>
</table>

*PC-3MM2 cells (2 × 10$^5$) were injected into the tibia of nude mice. Three days later, treatment with paclitaxel (8 mg/kg i.p. once weekly) or different doses of zoledronate (s.c. twice daily) began. The mice were killed after 5 weeks of treatment. Tumor incidence, tumor weight, and incidence of regional lymph node metastasis were determined.

Digital radiography of the hind legs revealed destruction of bone in control mice or mice treated with paclitaxel. Mice treated with
STI571 alone had diminished bone lysis, and the combination of STI571 with paclitaxel was associated with preservation of the bone structure. The combination of zoledronate with STI571 or paclitaxel led to clear preservation of the bone. The best therapeutic effects were found in mice treated with zoledronate, paclitaxel, and STI571 (Fig. 3).

Immunohistochemical analyses for expression of basic fibroblast growth factor, interleukin-8, vascular endothelial growth factor, epidermal growth factor, epidermal growth factor receptor, platelet-derived growth factor A/B, platelet-derived growth factor receptor \( \alpha/\beta \), proliferating cell nuclear antigen–positive, CD31-positive, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive cells. Treatment of mice with paclitaxel alone, STI571 alone, or zoledronate alone or in various combinations did not alter the expression of bFGF, IL-8, EGF, EGFR, VEGF, PDGF-A/B, and PDGFR\( \alpha/\beta \) by tumor cells growing in the bone microenvironment. Treatment with STI 571, but not zoledronate or paclitaxel, inhibited the phosphorylation of PDGFR\( \beta \) (Fig. 4A and B). The number of PCNA-positive cells (control group, 106 \( \pm \) 18) was significantly reduced in tumors from mice receiving a combination of STI571 and paclitaxel (43 \( \pm \) 9; \( P < 0.001 \), Student’s \( t \) test). Zoledronate alone did not decrease the number of PCNA-positive cells (98 \( \pm \) 17; \( P > 0.05 \), Student’s \( t \) test) and did not contribute to the antiproliferative effects of paclitaxel (paclitaxel + zoledronate, 82 \( \pm \) 15) or STI571 (STI571 + zoledronate, 87 \( \pm \) 11). Treatment with STI571 plus paclitaxel and STI571 plus zoledronate produced the most significant antiproliferative effects (39 \( \pm \) 12 PCNA-positive cells). The number of CD31-positive cells (control group, 46 \( \pm \) 14) was significantly reduced in tumors from mice treated with the combination of STI571 and paclitaxel (17 \( \pm \) 8; \( P < 0.01 \), Student’s \( t \) test). Treatment with zoledronate did not reduce the number of CD31-positive cells (41 \( \pm \) 10; \( P > 0.05 \), Student’s \( t \) test). The addition of zoledronate did not change the effects found for paclitaxel

Table 2. Treatment of human prostate carcinoma growing in the bone of nude mice

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>Incidence</th>
<th>Tumor weight (g), median (range)</th>
<th>Incidence of lymph node metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20/20</td>
<td>2.6 (0.9-5.2)</td>
<td>20/20</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>19/19</td>
<td>2.2 (0.9-4.8)</td>
<td>19/19</td>
</tr>
<tr>
<td>STI571</td>
<td>13/19(^c)</td>
<td>1.2 (0-3.2)</td>
<td>13/19(^c)</td>
</tr>
<tr>
<td>Zoledronate</td>
<td>18/20</td>
<td>0.7 (0-3.2)</td>
<td>18/20</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>8/20(^x)</td>
<td>0.5 (0-1.9)</td>
<td>6/20(^x)</td>
</tr>
<tr>
<td>Zoledronate + paclitaxel</td>
<td>16/20</td>
<td>0.9 (0-3.2)</td>
<td>16/20</td>
</tr>
<tr>
<td>STI571 + zoledronate</td>
<td>13/19(^c)</td>
<td>0.9 (0-3.2)</td>
<td>13/19(^c)</td>
</tr>
<tr>
<td>STI571 + zoledronate + paclitaxel</td>
<td>5/20</td>
<td>0.2 (0-2.9)</td>
<td>2/20(^x)</td>
</tr>
</tbody>
</table>

*PC-3MM2 cells (2 \( \times \) 10\(^5\)) were injected into the tibia of nude mice. Three days later, treatment with paclitaxel (8 mg/kg i.p. once weekly), STI571 (50 mg/kg oral daily), or zoledronate (25 \( \mu \)g/kg s.c. twice daily) or different combinations began. The mice were killed after 5 weeks of treatment. Tumor incidence, tumor weight, and regional lymph node metastasis were determined.

\(^c\) \( P < 0.05 \),

\(^x\) \( P < 0.01 \),

\(^c\) \( P < 0.001 \).

Figure 2. H&E staining of tumor-bearing hind legs of nude mice. Control mice and mice treated with paclitaxel had significant lysis of the tibia injected with PC-3MM2 prostate cancer cells 5 weeks earlier. In contrast, the structure of the tibia in mice treated with zoledronate was well preserved even in the presence of growing cancer cells.
The STI571 plus paclitaxel plus zoledronate group produced the best reduction in CD31-positive cells (15 ± 6). The number of apoptotic (TUNEL-positive) cells (control group, 13 ± 4) increased by treatment with paclitaxel (36 ± 11; P < 0.01, Student’s t test) and more so with STI571 and paclitaxel (66 ± 16; P < 0.001, Student’s t test). Zoledronate did not induce significant apoptosis (15 ± 5; P > 0.05, Student’s t test) and did not show additive effects (paclitaxel + zoledronate, 40 ± 9; STI571 + zoledronate, 20 ± 3; paclitaxel + STI571 + zoledronate, 59 ± 15; P > 0.05, Student’s t test).

Phosphorylated platelet-derived growth factor receptor-β and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive (apoptosis) endothelial cells. Endothelial cells in the vasculature of PC-3MM2 bone lesions express PDGFR (31). Treatment of mice with zoledronate or paclitaxel did not inhibit phosphorylation of the PDGFRβ on tumor cells or tumor-associated endothelial cells nor induce apoptosis of endothelial cells in tumors of mice treated with either paclitaxel or zoledronate. Endothelial cells (CD31-positive; red) of tumor-associated vessels from control mice treated with vehicle solution, paclitaxel, or zoledronate expressed phosphorylated PDGFR (green) on their surface (yellow; Fig. 4A and B). Administration of STI571 inhibited phosphorylation of PDGFR and treatment with STI571 and paclitaxel induced significant apoptosis of tumor-associated endothelial cells (Fig. 4A and B). The median percentage of apoptotic endothelial cells in control group mice was 1% (range, 0-4%) and only the combination of STI571 with paclitaxel significantly increased the median percentage of apoptotic endothelial cells [STI571 + paclitaxel, 17% (range, 9-22%); STI571 + paclitaxel + zoledronate, 14% (range, 4-21%); Table 3].

Quantification of functioning osteoclasts (tartrate-resistant acid phosphatase–positive cells). The number of cells positively stained for TRAP was determined in 10 random 0.159 mm² fields in the bone at ×100 magnification. The contralateral, tumor-free bone serving as control had a median of 2 ± 1 TRAP-positive cells. The bones with tumors had 37 ± 15 TRAP-positive cells. Treatment with paclitaxel did not decrease the number of TRAP-positive cells (27 ± 10; P > 0.05, Student’s t test). Treatment with STI571 significantly reduced the number of TRAP-positive cells to 19 ± 2 (P < 0.01, Student’s t test), and the combination of STI571 and paclitaxel further reduced TRAP-positive cells (12 ± 6; P < 0.01, Student’s t test). Treatment with zoledronate significantly reduced the number of TRAP-positive cells to 4 ± 1, zoledronate plus paclitaxel to 3 ± 2, zoledronate plus STI571 to 3 ± 2, and zoledronate plus STI571 plus paclitaxel to 2 ± 1 (P < 0.001, Student’s t test).

Discussion
In the present study, we show that the systemic administration of zoledronate with STI571 and paclitaxel results in the significant preservation of bone structure, a decrease in tumor incidence and
weight, and a decrease in the incidence of lymph node metastasis. This therapeutic activity was correlated with inhibition of osteoclast function, inhibition of tumor cell proliferation, and induction of apoptosis in tumor-associated endothelial cells and tumor cells.

The establishment and progressive growth of metastases depends on multiple and continuous interactions between metastatic cells and host cells in specific organ microenvironments. To proliferate and remain viable, tumor cells like all other

![Figure 4](image-url)
cells require oxygen that can diffuse only 100 to 150 μm from the nearest blood vessel. Data from our laboratory showed that in metastases all proliferating tumor cells are located <150 μm from the nearest blood vessel, whereas apoptotic tumor cells are located at a distance exceeding 150 μm from the nearest blood vessel (39). These data clearly show that the progressive growth of primary tumors and metastases is dependent on the development and maintenance of adequate vasculature (i.e., angiogenesis; ref. 40). In the majority of patients, the growth of prostate cancer cells in bone leads to osteoblastic changes with some osteolysis. These changes are associated with symptoms of severe pain, nerve compression, and a decrease in quality of life (41). Zoledronate has been shown to preserve bone structure in patients, thus improving their quality of life (41). Zoledronate has been shown to modulate the effects of chemotherapeutic agents, such as doxorubicin, on prostate cancer cells (42). Activation of endothelial cells also leads to bone lysis (42). The systemic administration of a bisphosphonate drug, such as zoledronate, inhibits the function of osteoclasts and osteoblasts (41). In the present studies, zoledronate did not inhibit the proliferation of PC-3MM2 cells in culture unless it was added in concentrations exceeding achievable peak levels in plasma or tissues (20). Treatment of mice implanted with PC-3MM2 cells into the tibia with zoledronate did not decrease the incidence of tumors in the tibia nor incidence of lymph node metastasis. However, zoledronate did decrease the number of functioning osteoclasts (TRAP-positive cells), which resulted in impressive preservation of bone structure in the presence of proliferating prostate cancer cells.

Prostate cancer cells proliferating adjacent to bone tissue express high levels of PDGF (31). These tumor cells and tumor-associated endothelial cells, but not endothelial cells in the normal bone, express phosphorylated PDGF (31). The activation of PDGF on the surface of endothelial cells leads to up-regulation of mitogen-activated protein kinase, Akt, Bcl-2, and Bcl-x, and cell proliferation (43, 44). The phosphorylation of the PDGFR is therefore associated with cellular resistance to apoptosis mediated by many chemotherapeutic agents. In normal tissues, endothelial cells rarely divide, whereas in prostate tumors 2% to 3% of endothelial cells can divide daily (45); however, as stated above, the cycling endothelial cells in prostate cancer growing in the bone are more resistant to apoptosis. Indeed, treatment of mice with paclitaxel did not produce significant apoptosis in tumor cells nor in tumor-associated endothelial cells.

Treatment of mice with STI571 blocks activation of the PDGFR and thus alters the phosphatidylinositol-3 kinase activity leading to a reduction in interstitial fluid tension and an increase in transcapillary transport of chemotherapeutic drugs (46, 47). Moreover, inhibition of PDGFR phosphorylation decreases the level of Bcl-2 and phosphatidylinositol-3-kinase/Akt and increases caspase-3 activity (44). Indeed, treatment of mice with STI571 and paclitaxel produced significant apoptosis of tumor-associated endothelial cells and tumor cells. Collectively, these data clearly support the conclusion that PDGF is a survival factor for endothelial cells.

Treatment of mice with zoledronate combined with STI571 and paclitaxel produced the best therapeutic results of a highly osteolytic human prostate cancer metastasis in the bones of nude mice. In mice treated with only saline and monotherapy with paclitaxel or STI571, the PC-3MM2 cells produced osteolysis and could then proliferate in extrasseous tissues. Treatment with only zoledronate inhibited osteolysis and thus restricted the growth of tumor cells to the bone, but zoledronate as a single agent had no effect on distant lymph node metastasis. Treatment of mice with all three agents prevented bone lysis and restricted the growth of PC-3MM2 cells to bone. PC-3MM2 cells growing in the bone microenvironment express PDGF and PDGFR, which is phosphorylated (4, 31). Treatment with STI571 and paclitaxel inhibited the phosphorylation of the PDGFR, hence inhibiting a survival pathway of tumor cells and tumor-associated endothelial cells, and produced apoptosis of tumor-associated endothelial cells and metastatic prostate cancer cells.

### Table 3. Treatment of human prostate carcinoma growing in the bone of nude mice

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>PCNA, mean ± SD</th>
<th>TUNEL, mean ± SD</th>
<th>CD31, mean ± SD</th>
<th>CD31/TUNEL, % median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>106 ± 18</td>
<td>13 ± 4</td>
<td>46 ± 14</td>
<td>1 (0-4)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>89 ± 15</td>
<td>36 ± 11</td>
<td>36 ± 8</td>
<td>2 (0-4)</td>
</tr>
<tr>
<td>STI571</td>
<td>89 ± 16</td>
<td>19 ± 4</td>
<td>34 ± 9</td>
<td>2 (0-4)</td>
</tr>
<tr>
<td>Zoledronate</td>
<td>98 ± 17</td>
<td>15 ± 5</td>
<td>41 ± 10</td>
<td>1 (0-4)</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>43 ± 9</td>
<td>66 ± 16</td>
<td>17 ± 8</td>
<td>17 (9-22)</td>
</tr>
<tr>
<td>Zoledronate + paclitaxel</td>
<td>82 ± 18</td>
<td>40 ± 9</td>
<td>37 ± 10</td>
<td>2 (0-4)</td>
</tr>
<tr>
<td>STI571 + zoledronate</td>
<td>87 ± 11</td>
<td>20 ± 3</td>
<td>32 ± 9</td>
<td>4 (0-6)</td>
</tr>
<tr>
<td>STI571 + zoledronate + paclitaxel</td>
<td>39 ± 12</td>
<td>59 ± 15</td>
<td>15 ± 6</td>
<td>14 (4-21)</td>
</tr>
</tbody>
</table>

*PC-3MM2 cells (2 × 10⁶) were injected into the tibia of nude mice. Three days later, treatment with paclitaxel (8 mg/kg i.p. once weekly), STI571 (50 mg/kg oral daily), or zoledronate (25 μg/kg s.c. twice daily) or different combinations began. The mice were killed after 5 weeks of treatment and autopsied. Tibia with tumors were resected, fixed in either 10% buffered formalin or pyridoxal phosphate solution, and frozen. Sections were stained with the different antibodies as described in Materials and Methods.

†P < 0.01.
‡P < 0.001.
Cancers are biologically heterogeneous and thus require multimodality therapy. The present results recommend the combination of nitrogen-containing bisphosphonates, such as zoledronate, to inhibit destruction of the bone, with STI571 to inhibit phosphorylation of the PDGFR on tumor cells and tumor-associated endothelial cells and anticoagulant agent, such as paclitaxel, for the treatment of prostate cancer bone metastasis.

References

32. Antivascular Therapy of Bone Metastasis
Modulation of Bone Microenvironment with Zoledronate Enhances the Therapeutic Effects of STI571 and Paclitaxel against Experimental Bone Metastasis of Human Prostate Cancer

Sun-Jin Kim, Hisanori Uehara, Sertac Yazici, et al.


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