The Bisphosphonate YM529 Inhibits Osteolytic and Osteoblastic Changes and CXCR-4–Induced Invasion in Prostate Cancer

Sotaro Miwa,1 Atsushi Mizokami,1 Evan T. Keller,2 Russell Taichman,3 Jian Zhang,2 and Mikio Namiki1

1Department of Urology, Kanazawa University, Kanazawa, Japan and 2Department of Urology, School of Medicine, and 3Department of Periodontics, Prevention and Geriatrics, School of Dentistry, University of Michigan, Ann Arbor, Michigan

Abstract

Bisphosphonates are useful for the treatment of prostate cancer bone metastasis. However, the role of bisphosphonate on the development of the osteoblastic component of prostate cancer bone metastases is not defined. In the present study, the third-generation bisphosphonate, YM529 (minodoronate), was tested for its effects on the osteolytic PC-3 and novel osteoblastic LNCaP-SF cell lines. YM529 inhibited both osteolytic and osteoblastic changes in an intratibial tumor injection murine model. In vitro, YM529 inhibited both the proliferation and the invasion of both prostate cancer cell lines. The stromal cell–derived factor-1 (or CXCL12)/CXCR-4 pathway is believed to play an important role in the development of prostate cancer bone metastases. Thus, we determined if YM529 affected this pathway. YM529 suppressed CXCR-4 expression in PC-3 and LNCaP-SF in vitro and in vivo and this was associated with decreased in vitro invasion. These results suggest that YM529 may inhibit cancer cell invasion into the bone matrix by repressing the expression of CXCR-4 in bone metastasis lesions. (Cancer Res 2005; 65(19): 8818-25)

Introduction

Prostate cancer has a great predilection for metastasizing to bone resulting in great morbidity to prostate cancer patients (1, 2). In contrast to myeloma and breast cancer, which show osteolytic changes in bone, a high percentage of patients with prostate cancer show osteoblastic changes in their bones. Roland proposed the hypothesis that osteolysis is an essential component of the progression of every primary or metastatic tumor in bone (3). In support of this proposal, several studies have shown that patients with advanced prostate cancer express elevated levels of osteolytic bone resorption markers in their urine and blood (4, 5). These data indicate that although the radiographic appearance of prostate cancer may be osteoblastic a significant feature of prostate cancer metastatic bone disease is osteolysis (6). These observations suggest that the use of compounds that inhibit osteolysis could have a therapeutic role for the treatment to metastatic bone disease in prostate cancer patients.

Bisphosphonates are potent inhibitors of bone resorption and have been used successfully in osteoporosis and other accelerated bone turnover diseases. Bisphosphonates have a high affinity for bone minerals and can quickly accumulate in bone tissue. Bisphosphonates that contain nitrogen atoms in their structural formula (N-bisphosphonates) have been reported to block the mevalonate pathway by inhibiting the biosynthesis of isoprenoids, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, resulting in apoptosis of osteoclasts (7). In addition to effects on bone tissue, bisphosphonates have been reported to have direct antitumor effects (8–10). The antitumor activities of bisphosphonates include induction of tumor apoptosis, inhibition of tumor cell proliferation, decreased tumor cell adhesion, and decreased invasion into bone. These observations suggest that bisphosphonates not only act on osteoclast-mediated bone resorption but also may affect the invasive behavior of metastatic prostate cancer cells in bone. Our group has shown that YM529, a newly developed third-generation N-bisphosphonate that has a strong anti–bone resorption effect, has direct antiproliferative and apoptotic effects on prostate cancer cells in vitro (4).

The chemokine receptor CXCR-4 and its ligand, stromal cell–derived factor-1 (SDF-1 or CXCL12), are known to be expressed in prostate cancer cells (11–16). The SDF-1/CXCR-4 pathway has been shown to participate in the proliferation, differentiation, and metastasis of many cancers, including prostate cancer (11, 12, 17–19). Accordingly, we hypothesized that bisphosphonates may inhibit prostate cancer cell invasion through the SDF-1/CXCR-4 pathway. We tested this hypothesis by using YM529 to determine if it had an effect on the development of intraosseous prostate cancer lesions and if this was associated with regulation of the SDF-1/CXCR-4 invasion pathway.

Materials and Methods

Chemicals. YM529 [1-hydroxy-2-(imidazo-[1,2-α]pyridin-3-yl) ethylidene bisphosphonate acid monohydrate] was supplied from Astellas Pharma, Inc. (Tokyo, Japan). The bisphosphonate was dissolved in saline (pH 7) and used for various assays described below.

Cell lines and culture. Normal primary human osteoblasts (NH0st Clonetic Cambrex BioScience, Walkersville, MD) were maintained in Osteodist Growth Medium Bullet kit (Cambrex BioScience), PC-3 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO). LNCaP parental cells (American Type Culture Collection) and an androgen-independent LNCaP cell line (LNCaP-SF) that was established after long-term subculturing of the parental LNCaP cells in DMEM and 5% charcoal-stripped FBS were also used. LNCaP-SF cells express androgen receptor at the same level of parental LNCaP cells and secrete prostate-specific antigen (PSA), which is induced by androgen, but growth of LNCaP-SF cells is inhibited by androgen (data not shown). Each cell line was maintained at 37°C in a humidified atmosphere with 5% CO2.

Animal study. Severe combined immunodeficient (SCID) mice were maintained in a barrier facility and allowed free access to food and water.

Requests for reprints: Atsushi Mizokami, Department of Urology, Kanazawa University, 13-14 Takaramachi, Kanazawa-city, Japan 920-8640. Phone: 81-76-265-2393; Fax: 81-76-222-6726; E-mail: mizokami@med.kanazawa-u.ac.jp. ©2000 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-0540

4 Submitted for publication.
Subconfluent PC-3 and LNCaP-SF cell lines were harvested from culture and resuspended to 2 × 10^5 cells/mL in DMEM. The cells were combined with an equal volume of Matrigel (Collaborative Biomedical Products, Bedford, MA) such that 5 × 10^5 cells were prepared for injection into the left tibia of anesthetized (pentobarbital at 50 mg/kg body weight i.p.) 5-week-old intact or castrated male SCID mice for PC-3 and LNCaP-SF, respectively. Intrathelial injections were done using a 29-gauge, 0.5-inch needle inserted through the tibial plateaus of the flexed knee as described previously (20). After 1 week, YM529 in saline (pH 7.0) or saline as a control was injected i.p. (0.1 or 0.3 mg/kg) and each body weight was monitored at weekly intervals. Mice injected with the PC-3 cells were sacrificed and radiographed at 42 days following intrathelial injection. Mice injected with LNCaP-SF cells were sacrificed at 105 days following intrathelial injection. Mice that did not recover from anesthesia or died before these sacrifice times were excluded from the analysis. This protocol was approved by the Institutional Animal Care and Use Committee of the Graduate School of Medical Science, Kanazawa University.

**Immunohistochemistry.** After sacrifice, the tibias were removed and fixed in 10% buffered formalin for 24 hours and decalcified in 10% EDTA solution gently stirred for 2 weeks at room temperature. Section from the formalin-fixed, paraffin-embedded tissues were cut to 4 μm and were deparaffinized and rehydrated. Following rehydration, the sections were stained with either H&E for the purpose of detecting prostate cancer cells in bone or tartrate-resistant acid phosphatase (TRAP) for detecting osteoclasts. TRAP staining was done by using TRACP & ALP Double-Stain Kit (Takara, Tokyo, Japan) as directed by the manufacturer. Adherent TRAP staining, counter staining with hematoxylin solution was done. Osteoclasts were determined as TRAP-positive staining multinuclear cells (>3 nuclei) using light microscope. Osteoclast perimeter (osteoclast number per millimeter of bone) was compared between each control group and YM529-treated groups. Four tibias of each group were analyzed in this manner. Indirect immunohistochemistry was done for CXCR-4 protein detection. Antigen retrieval was done by incubating the section in Target retrieval solution (DAKO, Carpinteria, CA) for 20 minutes at 95°C. After blocking endogenous peroxidase, the sections were incubated with rabbit CXCR-4 polyclonal antibody (Biovision, Mountain View, CA) overnight at 4°C. Finally, the antigens were stained using DAKO Envision kit (DAKO).

**Cell proliferation.** PC-3 (2.5 × 10^5 cells/mL) and LNCaP-SF (5 × 10^4 cells/mL) were allowed to adhere overnight in six-well plates. At this time, the cells were treated for an additional 24, 48, and 72 hours with various doses of YM529. To human osteoblast, YM529 was added at 0 to 30 μM for 48 hours. After addition of YM529, each cell line was harvested and washed with PBS. Total RNA extraction from cell cultures was done using Isogen (Nippon Gene, Tokyo, Japan). To make cDNA, total RNA (1-2 μg) was subjected to reverse transcription using ThermoScript RT (Invitrogen). Reverse transcription-PCR (RT-PCR) was done using Takara Ex Taq Hot Start Version (Takara Bio, Inc., Shiga, Japan). The sense and antisense primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GACCACGCTCATGCACTCA and 3′-TCCACCCCTGTGTGCTGTA; CXCR-4, 5′-GGGACGAGTGGACAAAGTGTA and 3′-TGATGACAGAGGAGGTCCG; and SDF-1, 5′-GTGTGAAGGAGGAGCTCG and 3′-GGTTCAGCAGGAACTCTG. RT-PCR conditions were as follows: GAPDH, 94°C for 1 minute followed by 18 cycles of the following varying conditions: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds; CXCR-4, 94°C for 1 minute followed by 28 cycles of the following varying conditions: 94°C for 15 seconds, 55°C for 45 seconds, and 72°C for 45 seconds; and SDF-1, 94°C for 1 minute followed by 18 cycles of the following varying conditions: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light. To quantify CXCR-4 expression, real-time quantitative PCR was done with 2 μL cDNA, 2 μL primer, and 4 μL Master Mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche Diagnostics, Alameda, CA) in a final volume of 20 μL. The primers used were same as above. LightCycler System (Roche Diagnostics) was used as follows: 94°C for 10 minutes followed by 45 cycles of the following varying conditions: 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. GAPDH expression was used as a normalization control. Experiments were done in triplicate.

**Western blot analysis.** Each prostate cancer cell line was cultured to subconfluence and washed in PBS and seeded at 2 × 10^5 cells per dish. YM529 was added (0-50 μM/L) for 48 hours to PC-3 and for 72 hours to LNCaP-SF. Each cell line was harvested and washed with PBS. Total RNA extraction from cell cultures was done using Isogen (Nippon Gene, Tokyo, Japan). To make cDNA, total RNA (1-2 μg) was subjected to reverse transcription using ThermoScript RT (Invitrogen). Reverse transcription-PCR (RT-PCR) was done using Takara Ex Taq Hot Start Version (Takara Bio, Inc., Shiga, Japan). The sense and antisense primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GACCACGCTCATGCACTCA and 3′-TCCACCCCTGTGTGCTGTA; CXCR-4, 5′-GGGACGAGTGGACAAAGTGTA and 3′-TGATGACAGAGGAGGTCCG; and SDF-1, 5′-GTGTGAAGGAGGAGCTCG and 3′-GGTTCAGCAGGAACTCTG. RT-PCR conditions were as follows: GAPDH, 94°C for 1 minute followed by 18 cycles of the following varying conditions: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds; CXCR-4, 94°C for 1 minute followed by 28 cycles of the following varying conditions: 94°C for 15 seconds, 55°C for 45 seconds, and 72°C for 45 seconds; and SDF-1, 94°C for 1 minute followed by 18 cycles of the following varying conditions: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light. To quantify CXCR-4 expression, real-time quantitative PCR was done with 2 μL cDNA, 2 μL primer, and 4 μL Master Mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche Diagnostics, Alameda, CA) in a final volume of 20 μL. The primers used were same as above. LightCycler System (Roche Diagnostics) was used as follows: 94°C for 10 minutes followed by 45 cycles of the following varying conditions: 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. GAPDH expression was used as a normalization control. Experiments were done in triplicate.

**Statistical analysis.** Student’s t test was used to determine the statistical significance of differences of proliferation assay and invasion assay between the control and others and differences in osteoclast perimeter among the control groups and the treatment groups. χ^2 test was used to determine the significance of differences of radiographic change between treated groups and control groups in vivo. A probability value P < 0.05 was considered to be statistically significant.

**Results**

Radiographic results of intrathelial injection of prostate cancer cell lines. Our first goal was to determine the effects of prostate cancer cells on bone in our animal models. Accordingly, we did intrathelial injection of the prostate cancer cell lines. For PC-3 cells, animals were sacrificed on day 42 after intrathelial injection at which time serum, radiographs, and tissues were obtained. Initially, we injected 15 mice with PC-3 cells; however, 2 mice died twenty-four hours later, YM529 was added (0-50 μM/L) for 48 hours to PC-3 and for 72 hours to LNCaP-SF. To human osteoblast, YM529 was added at 0 to 30 μM for 48 hours. After addition of YM529, each cell line was harvested and washed with PBS. Total RNA extraction from cell cultures was done using Isogen (Nippon Gene, Tokyo, Japan). To make cDNA, total RNA (1-2 μg) was subjected to reverse transcription using ThermoScript RT (Invitrogen). Reverse transcription-PCR (RT-PCR) was done using Takara Ex Taq Hot Start Version (Takara Bio, Inc., Shiga, Japan). The sense and antisense primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GACCACGCTCATGCACTCA and 3′-TCCACCCCTGTGTGCTGTA; CXCR-4, 5′-GGGACGAGTGGACAAAGTGTA and 3′-TGATGACAGAGGAGGTCCG; and SDF-1, 5′-GTGTGAAGGAGGAGCTCG and 3′-GGTTCAGCAGGAACTCTG. RT-PCR conditions were as follows: GAPDH, 94°C for 1 minute followed by 18 cycles of the following varying conditions: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds; CXCR-4, 94°C for 1 minute followed by 28 cycles of the following varying conditions: 94°C for 15 seconds, 55°C for 45 seconds, and 72°C for 45 seconds; and SDF-1, 94°C for 1 minute followed by 18 cycles of the following varying conditions: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light. To quantify CXCR-4 expression, real-time quantitative PCR was done with 2 μL cDNA, 2 μL primer, and 4 μL Master Mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche Diagnostics, Alameda, CA) in a final volume of 20 μL. The primers used were same as above. LightCycler System (Roche Diagnostics) was used as follows: 94°C for 10 minutes followed by 45 cycles of the following varying conditions: 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. GAPDH expression was used as a normalization control. Experiments were done in triplicate.
not recover from anesthesia and 1 mouse died of unknown cause before the termination of the study. There were no grossly observable pathologic lesions or tumor identified in these animals and they were excluded from further analysis. The PC-3 cells induced osteolytic lesions that developed in 10 of the 12 tibias of the vehicle-treated mice (Fig. 1A; Table 1). Histologic analysis showed that the two tibiae that failed to show any osteolytic changes did not have tumor cells present in them.

LNCaP-SF cells were injected directly into the tibia of castrated SCID male mice (n = 16 per group). The LNCaP-SF cells are slower to develop into tumors than the PC-3; therefore, the animals were sacrificed at 105 days after tumor injection. Radiographic evidence of osteoblastic lesions (Fig. 1B; Table 1) was observed in 13 of 16 animals. Histologic analysis revealed that the three tibiae that did not form osteoblastic changes did not have tumor cells in the tibiae. These findings suggest that the LNCaP-SF is a useful model to show osteoblastic bone changes that occur in prostate cancer and therefore represents a valuable tool to model prostate cancer bone metastasis.

Effects of YM529 on intratibial tumors. The effects of YM529 were examined using the administration of two doses [0.1 and 0.3 mg/kg/wk (or vehicle control)] on the growth of both PC-3 and LNCaP-SF cell lines.

Fifteen animals for each YM529 dose group were injected with tumor cells, but one mouse from each treatment group did not recover from anesthesia at the time of intratibial injection and these mice were excluded from further analysis. Mice injected with PC-3 cells were sacrificed on day 42. There was no difference in body weights among the vehicle-treated groups and the YM529-treated groups (data not shown). Radiographs revealed that in the group treated with 0.1 mg/kg wk dose of YM529 only 2 of the 14 tibiae developed osteolytic lesions and none of the 0.3 mg/kg wk dose treatment group (0 of 14 tibiae) developed radiographically detectable osteolytic lesions (Table 1). The radiographic data show that YM529 significantly inhibited the osteolytic changes that were observed in the vehicle-treated group (Fig. 1A; Table 1). In the vehicle-treated group, the majority of the intramedullary space was replaced by PC-3 cells and there was extensive osteolysis of trabecular and cortical bone. In the YM529-treated groups, the intramedullary space was also replaced by PC-3 cells, but trabecular and cortical bone was not destroyed (Fig. 1A). We observed a large number of TRAP-positive osteoclasts at the tumor-bone interface in the vehicle-treated group and only a few TRAP-positive osteoclasts were observed in the tumor-bone interface of the YM529-treated groups (Fig. 2A). Quantification of osteoclast perimeter (number of osteoclasts per millimeter bone) revealed that there were significantly less osteoclasts in YM529-treated groups than in the vehicle-treated group (Table 2). These data suggest that YM529 reduces PC-3-induced osteolysis through inhibiting the PC-3-induced increase of osteoclasts.

For LNCaP-SF, there were 16 mice injected per treatment group. One mouse in the 0.3 mg/kg group did not recover from anesthesia. One mouse in the 0.1 mg/kg wk group and two mice in the 0.3 mg/kg wk group died of unknown causes before the end of the study. Necropsy did not reveal any gross lesions or tumor and these mice were excluded from the remaining analyses. On day 105 after intratibial injection, mice were sacrificed. There was no difference in body weights among the vehicle-treated groups and the YM529-treated groups (data not shown). In the group treated with the lower dose (0.1 mg/kg wk), 7 of 15 tibias developed osteolytic lesions (Fig. 2B). In the group treated with a 0.3 mg/kg wk dose, 6 of 13 tibias developed osteoblastic lesions. In the vehicle-treated animals, histology revealed that the LNCaP-SF cells grew throughout the marrow cavity and increased bone production was identified (Fig. 2B).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** YM529 diminishes osteolytic and osteoblastic bone changes induced by prostate cancer cells. A, representative radiographs of SCID mouse tibia 42 days after intratibial injection of PC-3 cells. Left, nonimplanted tibia side; middle, vehicle-treated tibia; right, PC-3-implanted tibia of mouse treated with YM529 (0.1 mg/kg wk). B, representative radiographs of castrated SCID mouse tibia implanted with LNCaP-SF at day 105. Left, nonimplanted tibia side; middle, vehicle-treated tibia; right, LNCaP-SF-implanted tibia of mouse treated with YM529 (0.1 mg/kg wk).

### Table 1. Radiographic analysis of tibia

<table>
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<tr>
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<th>PC-3 day 42 osteolytic change (+)</th>
<th>LNCaP-SF day 105 osteoblastic change (+)</th>
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<tr>
<td>Control</td>
<td>10/12 (83%)</td>
<td>13/16 (81%)</td>
</tr>
<tr>
<td>0.1 mg/kg</td>
<td>2/14 (14%)</td>
<td>7/15 (47%)</td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>0/14</td>
<td>6/13 (46%)</td>
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**NOTE:** \( \chi^2 \) test.
YM529 treatment of mice injected with LNCaP-SF cells blocked the tumor-induced bone changes, although tumor cells were present throughout the marrow cavity (Fig. 2B). TRAP staining showed that there were fewer osteoclasts present in the osteoblastic bone compared with osteolytic bone in the vehicle-treated animals. Furthermore, TRAP-positive osteoclasts were very rare in the YM529-treated groups as opposed to the vehicle-treated groups (Fig. 2G and H). These histologic observations were confirmed by measurement of osteoclast perimeter (number of osteoclasts per millimeter bone). Specifically, the osteoclast perimeter was lower in the osteoblastic bone of the LNCaP-SF-injected mice compared with the osteoclast perimeter in the osteoclastic bone of the PC-3-injected mice (Table 2). Furthermore, YM529 decreased the osteoclast perimeter in bone injected with either tumor type. Taken together, these data show that YM529 is an effective agent to block both prostate cancer–mediated osteolytic and osteoblastic changes.

YM529 decreases prostate cancer cell growth in vitro. To further explore the role of the mechanism by which YM529 inhibited tumor size in vivo, in vitro investigations were done. We initially evaluated for a direct effect of YM529 on prostate cancer cell growth in vitro. YM529 diminished cell numbers of both PC-3 and LNCaP-SF cells in a dose-dependent fashion (Fig. 3A and B). Especially, >10 μmol/L YM529 caused significant inhibition in PC-3 and >3 μmol/L YM529 caused significant inhibition in LNCaP-SF after ≥24 hours of exposure (P < 0.05-0.001).

YM529 inhibits prostate cancer cell invasion. In addition to changes in cell growth, modulation of other metastatic phenotypes, such as invasive ability, may affect the progression of prostate cancer growth in bone. Accordingly, we evaluated the ability of YM529 to modulate prostate cancer cell invasion. Both PC-3 and LNCaP-SF cells are able to spontaneously invade through Matrigel-coated chambers (Fig. 4). YM529 started to inhibit PC-3 invasion at 1 μmol/L and continued to inhibit it in a dose-responsive fashion (Fig. 4A). Similarly, YM529 decreased the LNCaP-SF invasion but required a slightly higher dose (3 μmol/L) than the PC-3 cells to see the initial inhibition (Fig. 4B).

Stromal cell–derived factor-1 promotes invasion of prostate cancer cells. We next wanted to explore the possibility that YM529 modulated the SDF-1/CXCR-4 pathway, which has been implicated in tumor invasion. We first verified that SDF-1 enhanced invasion in the in vitro assays. When placed only in the lower well of the invasion chamber, SDF-1 stimulated invasion of both prostate cancer cell lines, but the effect was eliminated when SDF-1 was added to both upper and lower wells of the invasion chamber or when anti-CXCR-4-neutralizing antibody was added to the upper chambers (Fig. 4C and D). Based on these results, we hypothesized that YM529 may affect SDF-1/CXCR-4–mediated tumor metastasis in bone.

YM529 inhibited CXCR-4 expression on prostate cancer cells. Because SDF-1 enhanced prostate cancer cell invasion and YM529 blocked invasion, we next determined if YM529 modulated the SDF-1/CXCR-4 axis. We initially identified using RT-PCR and real-time RT-PCR that both PC-3 and LNCaP-SF expressed CXCR-4 mRNA and protein (Fig. 5A and B). YM529 decreased CXCR-4 expression in a dose-dependent fashion.

Table 2. Histomorphometric quantification of osteoclast at bone/tumor interface

<table>
<thead>
<tr>
<th>PC-3 Osteoclasts (number/mm)</th>
<th>LNCaP-SF Osteoclasts (number/mm)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>13.8 ± 2.06</td>
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<tr>
<td>0.1 mg/kg</td>
<td>4.5 ± 0.86</td>
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<td>0.3 mg/kg</td>
<td>1.8 ± 0.85</td>
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NOTE: Student’s t test.

Figure 2. YM529 decreases prostate cancer–induced osteoclast numbers. A, PC-3 at 42 days. B, LNCaP-SF cells at 105 days. Top, H&E staining was done as described in Materials and Methods to detect tumor in the marrow. Bottom, TRAP staining was done as described in Materials and Methods to detect osteoclasts (arrows), which were determined as TRAP-positive staining multinuclear cells (>3 nuclei). T, tumor cells. Magnification, ×200.
As a control, we assessed both CXCR-4 and SDF-1 expression in mature primary human osteoblasts. The osteoblasts did not express CXCR-4 mRNA (data not shown) but expressed SDF-1 mRNA (Fig. 5C). YM529 did not alter SDF-1 levels in the osteoblasts (Fig. 5C). Based on these findings, we hypothesized that YM529 decreased CXCR-4 expression in prostate cancer cells but not SDF-1 expression from osteoblasts.

**YM529 inhibited CXCR-4 expression in PC-3 and LNCaP-SF in bone marrow.** To provide further support for the possibility that YM529 alters the CXCR-4/SDF-1 axis in prostate cancer bone metastases, we evaluated CXCR-4 expression in the intratibial tumors from the *in vivo* studies. Using immunohistochemistry, we identified strong CXCR-4 expression in PC-3 and LNCaP-SF cells in the tibia (Fig. 6A and B). YM529 markedly decreased CXCR-4 expression in both prostate cancer cell lines within the tibia (Fig. 6C and D). These results confirm that YM529 can modulate the CXCR-4/SDF-1 axis in intraosseous prostate cancer tumors *in vivo*.

**Discussion**

In this study, we show four major findings. Firstly, we identified a novel osteoblastic prostate cancer model, LNCaP-SF. Secondly, we identified that a new third-generation bisphosphonate, YM529, which, in addition to being effective against prostate cancer-induced osteolytic changes, was also effective against prostate cancer-induced osteoblastic changes *in vivo*. Thirdly, YM529 inhibited proliferation and invasion ability of prostate cancer cells *in vitro* assay. Finally, YM529 suppressed CXCR-4 expression *in vitro* and *in vivo*, suggesting that YM529 achieved its ability to inhibit prostate cancer bone lesions, in part, through inhibiting the CXCR-4/SDF-1 axis.

We established the LNCaP-SF, primarily for the purpose of studying androgen-independent cancer, by culturing parental LNCaP in androgen-deplete environment for 6 months. When LNCaP was transplanted in SCID mouse tibia, it showed osteolytic change (21). In contrast, we observed that LNCaP-SF showed osteoblastic change 4 months after transplantation. It is
not clear at this time why LNCaP-SF caused osteoblastic change. It has been shown that prostate cancer cells secrete several osteoblastic factors, including bone morphogenetic protein (22), transforming growth factor-β (23), insulin-like growth factor (24), platelet-derived growth factor-BB (25), endothelin-1 (26), fibroblast growth factor (27), urokinase plasminogen activator (28), and PSA (29). We examined the expression of PSA in LNCaP-SF cells. They express PSA; however, the expression level in LNCaP-SF is lower than that of LNCaP cells in the absence of androgen (data not shown) and thus most likely does not contribute to the osteoblastic activity of LNCaP-SF. Unfortunately, the parental LNCaP cells do not readily form intratibial tumors, making it an extremely challenging tumor cell line to use for in vivo studies; thus, we could not do a direct in vivo comparison between LNCaP cells and LNCaP-SF cells.

Currently, there are no optimal models of prostate cancer bone metastases. Several prostate cancer osteoblastic cell lines or xenografts currently exist, including LUCaP-23.1 (21), LAPC-9 (30), MDA PCa2b (29), and C4-2B (31). However, these are associated with technical difficulties, such as a requirement to passage the tumors in vivo to maintain them (i.e., LUCaP-23.1 and LAPC-9) or a low frequency of development of osteoblastic tumors (MDA PCa2b) or mixed osteoblastic and osteolytic tumors (C4-2B). Yonou et al. succeeded in establishment of osteoblastic metastasis to human adult bone transplanted s.c. into nonobese diabetic/SCID mice using LNCaP (32). However, this method is complicated and expensive. In contrast to these challenges, the LNCaP-SF cells can be passed in vitro and the cells have a high probability (81%) of developing osteoblastic lesions. Moreover, because LNCaP-SF cells were established in vitro, their characteristics should be more similar to the parental LNCaP cells than C4-2B, which were derived from LNCaP cells by coincubating them with bone stromal cells in vivo. Therefore, LNCaP-SF cells transplanted into the tibia of SCID mouse is a very convenient model to investigate osteoblastic change.

Bisphosphonates have already been proven to be effective for the treatment of breast cancer bone metastasis and myeloma, which show osteolytic change with bone pain (33–35). Although a high percentage of prostate cancer bone metastasis shows osteoblastic change compared with breast cancer and myeloma, clinical efficacy of bisphosphonates for bone pain caused by prostate cancer bone metastasis has already been shown (36). It has been shown that a third-generation bisphosphonate, zoledronic acid, decreases skeletal-related events, such as fracture and bone pain, in men with prostate cancer and thus is an important treatment option for prostate cancer bone metastasis (37–39). Furthermore, we reported previously that a third-generation bisphosphonate, incadronate, decreased serum PSA levels in a hormone-refractory prostate cancer patient with bone metastases, suggesting that incadronate inhibits prostate cancer progression (40). One reason these bisphosphonates are effective in prostate cancer is that osteoclastic activity is an important component of osteoblastic invasion.

Figure 5. YM529 decreases CXCR-4 expression in prostate cancer cells in vitro. Twenty-four hours after PC-3 (A) or LNCaP-SF (B) cells were seeded, cells were treated with the indicated concentrations of YM529 and cultured for 24 or 72 hours, respectively. RT-PCR analysis of CXCR-4 and GAPDH mRNA was done as described in Materials and Methods. CXCR-4 mRNA expression levels were quantified using real-time quantitative RT-PCR of PC-3. CXCR-4 amounts were normalized to GAPDH mRNA expression. For Western blot analysis, cytosol proteins of PC-3 and LNCaP-SF cells were extracted as described in Materials and Methods and loaded on an 8% SDS-polyacrylamide gel for Western blot analysis. C. effect of YM529 on SDF-1 expression in human osteoblast. RT-PCR was done as described in Materials and Methods.

A

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Quantitative RT-PCR

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Western blotting

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<tbody>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
<tr>
<td>CXCR-4</td>
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C

Human Osteoblast

| YM529 | 0 | 3 | 10 | 30 | 50 | (μM) |

RT-PCR

<table>
<thead>
<tr>
<th>YM529</th>
<th>GAPDH</th>
<th>SDF-1</th>
<th>YM529</th>
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<td>0</td>
<td>3</td>
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prostate cancer bone metastases even in osteoblastic lesions (41). Many studies have shown that patients with advanced prostate cancer exhibit elevated levels of osteolytic bone resorption markers in urine and blood (4, 5). Additionally, histomorphometric studies of prostate cancer bone metastases have shown that osteoblastic lesions are actually mixed in nature, with increased activities of both osteoclasts and osteoblasts (42). These findings are consistent with the hypothesis stated by Roland that every primary or metastatic cancer in bone begins with osteolysis (3). These observations show that osteolysis is an important component of prostate cancer bone metastasis even if the radiographic appearance is primarily osteoblastic and thus suggest that bisphosphonate may play an important role in therapy of prostate cancer bone metastases.

In our study, YM529 suppressed not only osteolytic change but also osteoblastic changes. Moreover, YM529 reduced the number of osteoclasts in the tibia transplanted with PC-3 and LNCaP-SF cells, which cause osteolytic and osteoblastic changes, respectively. Our observation that the osteolytic PC-3 cell tumors had three times more osteoclasts than the osteoblastic LNCaP-SF tumors suggests that PC-3 secretes more substances, such as interleukin-6 or RANKL, which stimulate osteoclast proliferation than LNCaP-SF. Alternatively, LNCaP-SF may secrete more osteoclastic inhibitory factors, such as osteoprotegerin (20).

In contrast to our results, it was reported that zoledronic acid did not inhibit osteoblastic change induced by the LACP-9 prostate cancer xenograft in vivo, although it suppressed osteoclast numbers (43). The reasons for these different observations are not clear; however, differences of cell type and cell proliferation ability, cell’s response to bisphosphonate, or differences in direct antitumor effects or effects on osteoblasts between the different bisphosphonates may account for these contrasting observations.

The role that bisphosphonates play directly on tumors, including induction of tumor cell apoptosis, inhibition of proliferation, altering tumor cell adhesion, and invasive ability, has been receiving much attention (9, 10, 44, 45). Bisphosphonates have been shown to have direct antitumor effects on prostate cancer cells. For example, Corey et al. reported the apoptotic induction and suppression of proliferation by zoledronic acid in LNCaP and PC-3 cells (46). In addition, bisphosphonates inhibited prostate cancer adhesion to bone in vitro (46). These observations, therefore, suggest that bisphosphonates not only act on osteoclast-mediated bone resorption but also may affect the invasive behavior of metastatic prostate cancer cells in bone. Our results are consistent with these previous reports and identified that YM529 has a direct antitumor effect, including inhibition of proliferation and invasion.

Tumor invasion is one of the key mechanisms through which prostate cancer proliferates in the limited bone marrow space. In the present study, we showed that YM529 inhibited prostate cancer cell invasion. Our in vitro and in vivo data suggest that it mediates this effect, in part, through decreasing the ability of prostate cancer cells to respond to SDF-1 by decreasing CXCR-4. This is consistent with our previous report that SDF-1 and CXCR-4 confers a bone-specific phenotype on the metastasis of prostate cancer (11) and the report by Singh et al. that showed the SDF-1/CXCR-4 pathway modulated migration, invasion, and secretion of several matrix metalloproteinases (MMP) by prostate cancer (14). SDF-1 activation of CXCR-4 confers invasive ability through several mechanisms. Fernandis et al. reported that SDF-1 activates several proinvasive factors, including focal adhesion kinase, RAFTK/Pyk2, phosphatidylinositol 3-kinase, Cbl, SHP2, MMP-2, and MMP-9, in breast cancer (18). Consistent with our results, Denoyelle et al. showed that zoledronic acid inhibits the chemotactic effect induced by SDF-1 both by reducing cell motility through inhibiting RhoA and by decreasing CXCR-4 expression (47).

Clinically, bisphosphonates provide effective relief for bone pain (38, 48). Tumor burden and its effects on bone remodeling as well as tumor-mediated cytokine production may contribute to pain. Recently, Oh et al. published that CXC chemokines produced pain via direct action on chemokine receptor expressed by nociceptor neurons (49). Therefore, the observations that YM529 can decrease CXCR-4 expression and inhibit osteoclastogenic bone remodeling suggest that YM529 may be effective for decreasing bone pain.

In summary, our work suggests that following models: YM529 decreases CXCR-4 expression resulting in decreasing the prostate cancer’s invasive ability, which then would result in inhibiting tumor progression. Additionally, YM529 inhibits osteoclastogenesis, which in turn will diminish the release of growth factors from the bone microenvironment and inhibit the prostate cancer–induced bone remodeling. In conclusion, our results suggest that YM529 has dual effect on prostate cancer growth in bone (i.e., a direct antitumor effect and an indirect effect on the bone microenvironment). Our results suggest that YM529 may be effective for decreasing bone pain.

Acknowledgments

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