A Proteasome Inhibitor, Bortezomib, Inhibits Breast Cancer Growth and Reduces Osteolysis by Downregulating Metastatic Genes

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Running Title: Proteasome Inhibitors Prevent Tumor Growth in Bone

Key words: MDA-MB-231, bone metastasis, prevention of osteolysis, bone anabolic drug, VEGF, MMP9 and Runx2 genes

Grant Information: Studies reported were in part supported by National Institutes of Health grants P01CA082834 and S10RR023540. Core resources supported by the Diabetes Endocrinology Research Center grant DK32520 were also used. Jane Lian is a member of the

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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**STATEMENT OF TRANSLATIONAL RELEVANCE**

Breast cancer metastasis to the bone, and osteolytic lesions typically caused by these metastases, are particularly resistant to pharmacological therapy. This study shows that the FDA-approved proteasome inhibitor, bortezomib, strikingly decreases the size of breast cancer metastatic tumors and associated osteolytic lesions through multiple mechanisms: by inducing cellular necrosis and apoptosis; inhibiting tumor cell Wnt signaling, matrix degradation and reducing vascularization. In addition, the proteosome inhibitor provided a significant skeleton-wide bone anabolic effect, despite the presence of a metastatic lesion. Current treatment of metastases with bisphosphonates limits bone resorption, but does not rebuild bone volume lost to osteolysis. Our findings also provide evidence for increased capability to treat breast cancer osteolytic disease preemptively using bortezomib prior to tumor cell growth in bone by inhibiting tumor responses to the bone microenvironment and by providing protective anabolic effects on the skeleton.
Abstract

Purpose: The incidence of bone metastasis in advanced breast cancer exceeds 70%. Bortezomib (Bzb), a proteasome inhibitor used for the treatment of multiple myeloma, also promotes bone formation. We tested the hypothesis that proteasome inhibitors can ameliorate breast cancer osteolytic disease.

Experimental Design: To address the potentially beneficial effect of Bzb in reducing tumor growth in the skeleton and counteracting bone osteolysis, human MDA-MB-231 breast cancer (BrCa) cells were injected into the tibia of mice to model bone tumor growth for in vivo assessment of treatment regimens pre- and post-tumor growth.

Results: Controls exhibited tumor growth destroying trabecular and cortical bone and invading muscle. Bzb treatment initiated following inoculation of tumor cells strikingly reduced tumor growth, restricted tumor cells mainly to the marrow cavity, and almost completely inhibited osteolysis in the bone microenvironment over a 3-4 week period demonstrated by $^{18}$F-FDG PET, micro-CT scanning, radiography, and histology. Thus, proteasome inhibition is effective in killing tumor cells within bone. Pre-treatment with Bzb for 3 weeks prior to inoculation of tumor cells was also effective in reducing osteolysis. Our in vitro and in vivo studies indicate mechanisms by which Bzb inhibits tumor growth and reduces osteolysis result from inhibited cell proliferation, necrosis and decreased expression of factors that promote BrCa tumor progression in bone.

Conclusion: These findings provide a basis for a novel strategy to treat patients with breast cancer osteolytic lesions, and represent an approach for protecting the entire skeleton from metastatic bone disease.
Introduction

Metastatic osteolytic disease is prevalent in cancer patients. In advanced breast cancer (BrCa), 70% of women develop osteolytic lesions, resulting in pain, pathologic fracture, and increased morbidity. Dysfunction of the ubiquitin-proteasome system is associated with tumor growth and metastatic disease, providing the rationale for development of proteasome inhibitors as antineoplastic therapies [1, 2]. The proteasome is a ubiquitous enzyme complex that plays a critical role in the degradation of proteins involved in cell cycle regulation, apoptosis, and angiogenesis [2, 3]. Bortezomib (Bzb), a selective proteasome inhibitor used to treat multiple myeloma, has a potent anabolic effect on bone [4-9]. Bzb alters the bone marrow microenvironment by increasing the number and differentiation of resident mesenchymal stem cells (MSC) into osteoblasts, thereby increasing bone-formation rates within 4 weeks in normal mice and resulting in trabecular bone formation in bone loss model [7]. A similar enhancement of osteoblast-differentiation is found in myeloma patients treated with Bzb who show sustained increases in circulating osteocalcin, a marker of bone formation [6, 10]. Thus, Bzb treatment represents a novel and clinically feasible approach for increasing bone formation in the setting of the osteolytic bone disease accompanying metastatic breast, prostate, and lung cancers [4, 5, 11].

Nonsurgical treatment of bone metastatic lesions includes radiation therapy and bisphosphonates. Bisphosphonates were initially reported to reduce the risk of pathologic fracture and bone pain, although a recent study totaling over 7000 BrCa patients indicated no reduction in fracture risk as compared to placebo or no treatment groups [12-14]. In addition, bisphosphonates at doses required for cancer patients can have a significant side effect profile [15, 16] and alternate approaches to protect the skeleton are needed.

Tumor cells in the bone microenvironment overcome the marrow compartment and inhibit the ability of sufficient stromal cells to differentiate into osteoblasts to replace lost bone. Cancer cells secrete factors that induce a vicious cycle of osteoclast activation and growth factor release that promotes tumor survival. Bzb contributes to the apoptosis of tumor cells and tumor-activated osteoclasts [17-19]; as well, low dose Bzb promotes bone anabolic effects in mice [7]. Given the aggressive osteolytic disease produced by BrCa cells that metastasize to bone, we
investigated the effectiveness of Bzb treatment for inhibiting rapid tumor growth within the bone, and the potential for retaining bone volume. We postulated that Bzb treatment could be an effective therapy by suppressing growth of the metastatic tumor [4, 7-9, 20-22], inhibiting osteoclastogenesis and survival [9, 17, 21, 23-26], and stabilizing osteoprogenitor cells within the marrow [26], or by a combination of all these effects.

Materials and Methods

Cell culture. The metastatic human BrCa MDA-MB-231 (ATCC # HTB-22) and mouse preosteoblast MC3T3-E1 (ATCC # CRL-2593) cell lines were used. Cells were cultured in α-MEM containing 10% fetal bovine serum (Invitrogen, Inc.). Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Animal care. Approval from the Institutional Animal Care and Use Committee was obtained. Six week old female severe combined immunodeficient (Ncr/SCID) mice were housed in pathogen free conditions and utilized for all experiments. Per experiment, groups contained n=3 mice, and all studies except for the Bzb pretreatment and continuous Bzb experiment comparison (Fig. 4) were repeated for a total of n=6-8 mice per group. The repeat studies were terminated at either 6 or 7 weeks. At sacrifice, all mice were analyzed radiographically at weekly intervals followed by either histology, micro-CT or with PET/SPECT imaging at sacrifice.

Tibial implantation of MDA-MB-231 cells and bortezomib treatment. Mice were anesthetized with 0.15 mg ketamine/0.015 mg xylazine IP per g body weight. A medial parapatellar incision was created and a needle was placed in the intramedullary canal of the tibia, by aid of fluoroscopy (XiScan 1000-1, XiTec, East Windsor CT). MDA-MD-231 cells (1x10⁵ in 100 µl of PBS) were slowly injected into the tibia and closed with 5-0 chromic suture (Ethicon Inc, Somerville, NJ). Mice were given 0.1 mg/kg buprenorphine SQ post-operatively. Bortezomib (Millennium Pharmaceuticals, Cambridge MA) IP injections at a dose of 0.3 mg/kg
body weight were begun 24 h after intra-tibial injection and continued 3 times a week, or as indicated in each study.

**Histologic analysis.** Following sacrifice, the lower extremities were dissected and were then fixed in 4% paraformaldehyde and decalcified in 18% EDTA pH 8.0. Paraffin sections were cut at 6 micron thickness and stained with routine H&E, TRAP or Ki67 immunohistochemistry [27]. Photomicrographs were acquired on a ZEISS Axioskop 40 microscope with attached AxioCam HRC and analyzed by AxioVision Rel 4.7 software (Carl Zeiss Micro Imaging, Thornwood NY).

**Radiographic analysis.** Osteolysis was monitored by serial radiographs using a Faxitron MX-20 (Faxitron, Lincolnshire, IL) X-ray machine unit. We devised a scale to measure the severity of osteolytic lesions based on their appearance by conventional radiography. Blinded radiographs were evaluated by seven different scorers, significant differences were determined by Student’s *t*-test. Osteolytic lesions were scored on a scale of 0-5 based on their severity: 0—no visible osteolysis; 5—most severe degree of osteolysis. A Grade 1 lesion was small, isolated and <10% of the width of the bone, Grade 2 included single or multifocal small lesions, less than 33% of the cortical width, Grade 3 lesions have a size of 33-75% the cortical width, extensive lesions with >75% of the cortical width are graded as 4, and pathologic fracture with extensive cortical destruction represents a Grade 5 lesion.

**Micro computed tomography analysis.** Specimens were scanned using a high-resolution desktop microtomographic imaging system (µCT40, Scanco Medical AG, Basserdorf, Switzerland) using an isotropic voxel size of 12 µm, as previously described [28-30]. In tumored bone, total bone volume is assessed by µCT by selecting the region of each tibia from the knee joint to the most proximal aspect of the proximal tibial-fibular joint, which ranges from 575 to 650 sections. In the non-tumored distal femur, bone was evaluated in the region starting 360 µm proximal to the growth plate and extending 1800 µm proximally. For bone regions without tumors, we assessed the trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, mm⁻¹), trabecular separation (Tb.Sp, µm), connectivity...
density (ConnD, 1/mm³) and structure model index (SMI). Results were analyzed for significant differences by Student’s t-test.

**PET/CT animal imaging.** Bioscan NanoSPECT/CT and Philips Mosaic HP PET cameras were used to collect CT and ¹⁸F-fluorodeoxyglucose (FDG) images of mice. Tumored mice were anesthetized and injected intravenously with 100 μCi of ¹⁸F-FDG and PET imaging was performed at 30 min post-administration. All data imaging and analysis was performed at the UMass Bioimaging Core Facility. After each PET acquisition, the mouse, immobilized on the Minerva bed (Bioscan) was transferred to NanoSPECT/CT camera for the CT acquisition. The CT acquisition was performed at standard frame resolution, 45 kVp tube voltage and 500 ms of exposure time. The CT reconstruction was performed using InVivoScope 1.37 software (Bioscan), and a PET/CT fusion image was created.

**Real-Time Reverse Transcription-PCR Analysis.**

mRNA levels of metastatic and osteolytic-related genes were analyzed from MDA-MB-231 cells, MC3T3 cells or BrCa tumor following Bortezomib treatment. RNA was isolated using TRIzol (Invitrogen, Carlsbad CA) according to the manufacturer’s protocol. Oligo-dT primers were used in conjunction with the SuperScript First-Strand Synthesis System (Invitrogen) to synthesize cDNAs. Primer sequences are as follows: *AP*: forward, TTGTGCGAGAGAAAGAGAGAGA, reverse, GTTTCAGGGCATTTTTCAAGGT; *Col 1a*: forward, CCCAAGGAAAAGAAGACGTC, reverse, AGGTCAAGCTGATAGCAGATC; *DKK1*: forward, TCATTTCGAGGAGAAATTGAG, reverse, AACCTTTCTGTCTTTGTTG; *DKK3*: forward, TGCACCAGGAAGTTCACAAG, reverse, GGCCCACAGGTCTCTTG; *GAPDH*: forward, ATGTTCGTCATGGGTGTGAA, reverse, TGTGGTCATGAGTCCTTCCA; *LEF1*: forward, AGTGCAGCTATCAACCAGAT, reverse, TTCATAGTATTTGGCCTGCT; *MMP-9*: forward, CCTGGAGACCTGAGAACCAATC, reverse, CCACCCGAGTGTAACCATG; *RANKL*: forward, CACATCAGAGCAGAGAAAGC, reverse, CTTTATGGGAACCAGATGGG; *Runx2*: forward, CAGAATCCACAGATACAGCA, reverse, CAGCCTTTGAAATCCTCACC; and *VEGF*: forward, CAGCCTTTGAAACCTCTCCTC; reverse, CAGCCTTTGAAATCTTCTC.
forward, TCTTCAAGCCATCCTGTGTG, reverse, GCGAGTCTGTGTTTTTGAG. Real-time PCR analysis was performed to confirm expression levels by using an ABI machine and PRISM software (Applied Biosystems, Carlsbad CA); significant differences were determined by Student’s t-test.

**Annexin V binding and FACS analysis.** Bzb treated MDA-MB-231 cells were stained with Annexin V-FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). Stained live cells were submitted to the Flow Cytometry Core Lab at the University of Massachusetts for FACS analysis (BD Biosciences FACSCalibur™ [San Jose, CA]). All fluorescent parameters were acquired in logarithmic amplification, forward and side scatter parameters were acquired in linear. Data were analyzed using Cell Quest™ software.

**Results**

**Bortezomib slows the growth of osteolytic lesions caused by BrCa tumors in the bone microenvironment**

Given the anabolic effects of Bzb on bone in multiple myeloma, we investigated if Bzb treatment could ameliorate the osteolysis caused by breast cancer cells in bone. Mice that received intra-tibial inoculations of MDA-MB-231 BrCa cells were treated with 0.3 mg/kg body weight Bzb administered 3 times a week for 7 weeks. This dose and schedule represents the highest Bzb dose used in a study in which the serum osteocalcin level, a marker of bone formation, was found to increase in response to Bzb treatment [7]. We observed this dose to be non-toxic throughout the duration of the study (n>24 mice).

Radiographs in Figure 1A (3 representative tibias) show osteolytic lesions in controls at day 21 that are inhibited by Bzb treatment. By 35 days, small, focal areas of missing trabeculae were observed with Bzb treatment. With continued Bzb (to day 49 in Fig. 1A) there was evidence of a slow rate of progression of bone lysis in the majority (~70%) of the Bzb treated mice) in multiple independent experiments. At termination of studies, control mice had very large tumors causing significant morbidity, while the Bzb groups consistently had greatly
reduced bone loss in the region of the tumor cell inoculation when compared to control treated mice. These conclusions are in part based on a semi-quantitative evaluation of the radiographs based on a scoring system (see methods) (Fig. 1B). An aggressive increase in osteolysis was observed in controls after day 28 when tumor destroyed the cortical bone, invaded the surrounding tissue and contributed to osteolysis from the periosteal side of bone as well as from the medullary cavity. In the Bzb group, a clear inhibition of osteolytic disease is delayed until 4-5 weeks. After this point, low levels of osteolytic activity occurred indicated by the appearance of lesions at the end of the study (Fig. 1A). A similar late appearance of osteolytic lesions was observed in different experiments (data not shown).

The tumor containing tibias were further analyzed by ex vivo micro computed tomography (micro-CT) in n=3 mice. Figure 1C shows tibias of control mice with extensive osteolytic disease eroding through the cortex, while the tibias of the Bzb treated mice had minimal evidence of cortical erosion and mild osteolysis. Quantitative analysis by micro-CT shows that the tumor bearing tibias of the Bzb treated mice have greater than 2-fold higher bone volume than the tibias of control mice (Fig. 1D). Taken together, these findings show that the Bzb treated mice had a striking inhibition of osteolytic disease.

**Bortezomib reduces the size of BrCa tumors**

The volume of intra-tibial BrCa tumors was measured in vivo in Bzb and control treated mice by injecting the mice with $^{18}$F-fluorodeoxyglucose and visualizing the tumor using positron emission tomography (PET) imaging. Figure 2A shows representative PET images that identify a larger tumor volume and metabolic activity in controls as compared to Bzb treated mice. A 2 fold increase in tumor volume in the control was confirmed by histological examination of tibias with tumors from of Bzb and control mice (Fig. 2B and C, left panel). In controls there is aggressive lytic disease destroying trabecular and cortical bone with tumor growth invading muscle. In striking contrast, tumor growth in the Bzb-treated mice was initially restricted to the medullary cavity until a cortical break occurred allowing slow tumor growth in muscle (Fig. 2B). The lower panels of figure 2B illustrate this point. Viable tumor cells are found in muscle (panel 1) of Bzb treated mice, while areas of necrotic cells were evident within the medullary cavity.
(panel 2). Solid tumor tissue outside the bone exhibited similar cell morphology between Bzb and controls (panels 3 and 4). The fraction of actively growing cells within the tumor was examined by Ki-67 detection and found to be far less in Bzb treated tumor than control tumor (Fig. 2C right panel, p<0.05). The modest decrease in tumor growth fraction (% Ki-67 + cells) compared to tumor size can be accounted for by stimulated tumor growth in the Bzb treated mice after invasion into muscle.

The effect of tumor growth on bone osteolysis in controls is visualized by minimal detection of osteoclasts by TRAP staining, as there is little bone remaining to be resorbed in controls (Fig. 2D, panel 1). Only small remnants of bone remained with TRAP positive cells [panels 2 (10x) and 3 at 40x magnification of osteoclasts]. The Bzb group exhibited osteoclast activity on cortical surfaces where tumor growth was expanding along the periosteal side as well as on the endosteal surface. These findings suggest that Bzb treatment reduces osteolytic disease by killing off tumor cells and that remaining tumor cells can still locally secrete osteoclast activating factors and can survive as a solid tumor.

**Bortezomib promotes increased bone formation in the distal femur of the non-tumor bearing limb**

BrCa cells secrete many factors that cause local bone resorption and also circulate systemically to affect the entire skeleton. To assess the influence of Bzb on bone away from the area of the BrCa tumor, micro-CT analysis was performed and various parameters of bone growth were measured in the distal femur of Bzb treated and control mice. Figure 3 shows that there is increased trabecular bone formation in femurs of mice treated with Bzb as compared to control as evidenced by significant improvement in the parameters of bone growth including bone volume fraction, trabecular thickness, connectivity density and SMI (reflecting a more plate-like architecture in the Bzb treated bone). The trabecular number and spacing showed greater bone formation in Bzb treated mice, but did not reach significance. These results are entirely consistent with the previously described bone anabolic property of Bzb [7] and occur in the presence of metastatic tumor growth.
**Treatment with Bortezomib prior to tumor cell inoculation protects bone from osteolysis**

To assess clinical relevance, a study was designed to establish if Bzb given prior to tumor metastasis decreased tumor growth and osteolytic disease and would protect the bone from subsequent osteolysis. Therefore, we examined Bzb treatment of mice for a period of time prior to the intra-tibial inoculation of MDA-MB-231 cells. The rationale was twofold. First, the anabolic effect that Bzb treatment has on bone might render the bone less susceptible to subsequent osteolysis. Second, this has translational relevance as Bzb treatment might be used prophylactically to decrease the likelihood of BrCa patients developing osteolytic lesions. The study consisted of three groups: mice that were not treated prior to or following tumor cell inoculation, mice that were treated with Bzb (0.3 mg/kg body weight IP 3 times a week) both before and after tumor inoculation, and mice that were treated with Bzb only before, but not after injection of tumor cells (Bzb pretreatment) (Fig. 4A). All three mice in each group exhibited consistent results. Radiographic analysis of the tibias of mice pretreated with Bzb 3 weeks prior to time of inoculation with BrCa cells and mice treated continuously with Bzb both exhibited smaller osteolytic lesions than untreated mice (Fig. 4B). The radiographic grading of the tibias showed a statistically significant lower osteolytic lesion grade in the Bzb continuous and Bzb pretreatment groups compared to control mice with tumors (p < 0.0001) and a more effective inhibition of osteolysis in the continuously treated Bzb group (p< 0.05, Fig. 4C).

Patients with metastatic breast cancer have a potential for continuously developing new bony lesions. Therefore, we addressed if the anabolic effect of Bzb treatment on bones that did not contain tumor (shown in Fig. 3) would provide protection to the skeleton in a patient with advanced breast cancer, and if this effect would remain over time despite the presence of tumor at a remote site. To determine if treatment with Bzb may protect the skeleton from subsequent osteolysis, micro-CT analysis of the femurs of the 3 groups of mice described above (Fig. 4A) was performed examining both femurs of each group. Several significant changes are observed in trabecular bone structure between the control and the 2 Bzb groups and between the left femur and the right femur (Fig. 5). Bzb treatment supports increased trabecular bone formation in both tumor bearing (right) and contralateral (left) limbs reflected in all parameters of trabecular bone,
in both Bzb continuous and pre-treatment groups compared to control (Fig. 5 continuous vs control, p<0.05 to p<0.005). Both pretreatment and continuous Bzb groups exhibited a significant improvement in bone formation (approximate 60% increase in bone volume) as compared to control. However, it is noteworthy that the femurs from the tumor affected right leg of both Bzb and control mice consistently have values which indicate less bone when compared to the left femurs (Fig. 5, right vs. left). This suggests mice develop disuse osteopenia in the right femur as a result of decreased weight bearing on the tumored leg. The bone anabolic effects of Bzb were not attenuated during the subsequent tumor growth period. Mice receiving a 21 day course of BZB pre-treatment (prior to inoculation of tumor cells) did not have significantly different bone parameters compared to the mice receiving continuous treatment (Fig. 5, continuous vs. pretreatment, p>0.05). This effect is significant in that a pulsed dose of Bzb prior to inoculation of cancer cells was sufficient to confer an anabolic effect on bone, and provide protection from osteolysis. We conclude, from the increased bone volume following 3 weeks pretreatment in the non-tumored femur and the reduced tibial osteolysis post-inoculation during the following 25 days of tumor growth, that Bzb could contribute to protecting the skeleton in advanced breast cancer.

**Bortezomib decreases survival of MDA-MB-231 BrCa cells and affects gene expression**

To gain insight into the mechanisms of Bzb on the cellular activities of BrCa cells, cell growth and mRNA levels of metastatic and osteolytic genes were examined. MDA-MB-231 proliferating cells were treated *in vitro* with varying doses of Bzb ranging from 0 to 50 nM to determine the effects on cell proliferation and survival. Initial cell count studies performed after 24 h showed that adherent cell counts decreased beginning at 10 nM Bzb (21% of control) with a 62% and 74% cell loss at 20 and 50 nM Bzb, respectively (data not shown). The loss of cell viability by Bzb was the result mainly of necrosis of the cells as determined by an annexin V binding assay performed on proliferating cells treated with varying doses of Bzb (Fig. 6A). FACS analysis of the cells (represented in Fig. 6B) showed that the percentage of viable cells (neither stained with propidium iodide nor bound to annexin V, figure 6A solid line, diamond; 6B lower left quadrant) decreased steadily to 60% at a dose of 20 nM Bzb then remained
constant to a dose of 50 nM Bzb. The percentage of cells undergoing apoptosis (bound to annexin V, but not stained with propidium iodide, figure 6A dashed line, open square; figure 6B, lower right quadrant) did not exceed 10%. The amount of cells undergoing necrosis (bound to annexin V and stained with propidium iodide, figure 6A solid line, circle; figure 6B upper right quadrant) was greater than that of apoptotic cells, and remained relatively constant from Bzb doses of 10 nM to 50 nM. These results indicate that both necrosis and apoptosis contribute to death of MDA-MB-231 cells by Bzb treatment, but also suggests that the MDA-MB-231 cell line may have a small population of Bzb resistant cells.

We determined the contribution of Bzb in preventing osteolytic disease by analyzing dose dependent expression of marker genes associated with bone resorption and formation in MDA-MB-231 cells treated 24 h with Bzb (Fig. 6C). Expression of genes promoting tumor growth (Runx2, MMP9 and VEGF) decreased at increased Bzb concentrations, while GAPDH, an internal marker of cellular RNA levels, remained constant. In response to Bzb, we found a steady increase in expression of the Wnt antagonist DKK1 [31], with a concomitant decrease in the canonical Wnt transcription factor LEF1. This finding indicated that Bzb is inhibiting tumor cell growth mediated by the Wnt pathway, as well as reducing the levels of genes in MDA-MB-231 cells related to osteolytic activity in bone [32].

To evaluate the mechanism for the anabolic effect of Bzb on bone formation, we examined a pre-osteoblast cell line (MC3T3) analogous to osteoprogenitor cells within the marrow for its responsiveness to the same dose range that affects BrCa cells (Figure 6D). The high doses of 20 and 50 nM were toxic to both cell types, osteoblasts and BrCa cells, resulting in loss of expression of bone formation markers (collagen Type 1 and alkaline phosphatase in the osteoblasts). A striking difference between BrCa and MC3T3 cells was the opposite effects on the Wnt pathway which was inhibited in the tumor cells, but stimulated in osteoblasts at lower doses for bone formation. This finding is consistent with the anabolic effect of Bzb in multiple myeloma [33]. We next determined if the same effects occurred in response to the in vivo dose of Bzb. Tumor tissue excised from the tibia showed a striking inhibition of MMP9 with a modest increase in DKK, consistent with the in vitro effect of Bzb in BrCa cells. Additionally, in the
tumor tissue, RANKL, promoting bone resorption, was stimulated. This finding reflects the histological analyses of the Bzb treated group (see Fig. 2D) exhibiting osteoclastic activity.

We conclude from these studies that metastasis associated osteolytic disease is reduced in vivo by Bzb through multiple mechanisms in the bone microenvironment: by inducing cellular necrosis, decreasing the tumor response to Wnt signaling, reducing expression of a key factor in tumor vascularization, and decreasing the Runx2 and MMP-9 osteolytic cascade. Together, these modifications in tumor cell activity by Bzb contribute to inhibiting tumor growth to the bone microenvironment.

**Discussion**

Here we established that the proteasome inhibitor Bzb effectively suppresses BrCa tumor growth within bone, and stimulates new bone formation in the presence of metastatic disease. Anti-tumor growth effects by Bzb occur in the bone microenvironment where the vicious cycle of tumor growth and osteolytic disease is activated in response to BrCa cells. We show that the anti-osteolytic effects of Bzb are primarily due to significantly decreased tumor size as evidenced by histology, radiographic monitoring and micro-CT quantitation of bone volume. Lastly, we have defined mechanisms contributing to the inhibition of tumor growth in the bone microenvironment and osteolytic disease by Bzb that include (i) the sensitivity of highly metastatic breast cancer cells to necrosis, (ii) reduction in expression of metastatic and tumor growth related genes, and (iii) promotion of bone formation throughout the skeleton. These effects of Bzb provide a beneficial anti-tumor and bone anabolic effect.

In our continuously treated study, we find a striking inhibition of osteolytic disease that continued through 4 weeks post inoculation. However, at 5 weeks evidence of the onset of osteolysis was identified in the Bzb group, which slowly progressed until the study was terminated when tumor size became unbearable in the control group (7 or 8 weeks in repeat study). The Ki67 assays suggest that surviving tumor cells exposed to Bzb results in a delayed
onset of osteolysis. As our Bzb dose did not cause toxic effects in mice, perhaps a higher dose would be more effective in killing all BrCa cells, as indicated from our in vitro studies. Two mechanisms are contributing to reduced osteolytic disease: the killing of tumor cells by Bzb and Bzb inhibition of osteoclast activity through induction of apoptosis, which has been identified in several studies [17-19, 23-26]. BrCa cells are known to produce many different factors that induce osteolytic disease. Notably, at sacrifice after 7 weeks, we observe TRAP positive osteoclasts at the tumor-bone interface in Bzb treated mice and also found RANKL to be expressed in the tumor tissue. Our in vitro studies analyzing Bzb dose effects suggest a small population of MDA-MB-231 cells survive at high doses.

Early studies found mixed results in ongoing clinical trials evaluating the effect of Bzb on osteolytic lesions caused by solid tumors [34-37]. However, in one other study using the intratibial model of prostate cancer, it was suggested that osteoclast activity was diminished [38], similar to what has been shown in multiple myeloma patients [39]. In our studies, the net effect of systemic Bzb is that proteasome inhibition is effective in preventing BrCa tumor growth in bone and greatly diminishes the osteolytic disease. Thus, just as Bzb is used for multiple myeloma cells which have increased proteasome activity [40] inducing osteolytic bone disease (as does breast cancer [18]), we find Bzb has the similar anti-osteolytic effects in BrCa tumors.

We identified mechanisms that are directly attributed to Bzb modifying the properties of BrCa cells in vivo to facilitate an anabolic effect in the bone microenvironment in the presence of a tumor. Analysis of the tumor tissue revealed a reduction in the metastasis related genes Runx2, VEGF and MMP9 at high doses of Bzb. This profile reflects altered cellular properties, reduced tumor vascularization, migration, matrix destruction, and osteolytic disease [27, 30, 41-44]. Another mechanism of Bzb inhibiting tumor growth is by elevation of DKK1, an inhibitor of the Wnt signaling pathway, and a concomitant decrease in Lef-1, the transcriptional mediator of Wnt signaling, thereby decreasing tumor growth potential. In multiple myeloma patients, serum levels of DKK are very low, contributing to activated Wnt signaling [21, 22]. BrCa tumorigenesis and bone metastasis are linked to the Wnt signaling pathway, having downregulated DKK and dysregulation of β-catenin linked to progression and prognosis [31, 45-
These beneficial effects of Bzb are also observed in the tumor tissue by the changes in expressed genes (reduced LEF1 and MMP9). Notably, osteoblasts have the opposite response at low Bzb doses, exhibiting increase LEF1/TCF4, alkaline phosphatase, collagen Type 1 and reflecting bone formation activity. In multiple myeloma patients, Bzb stimulates osteoblast activity [22, 33], consistent with the significant anabolic effect of Bzb in the non-tumor bearing limbs of mice with bone tumors.

Our studies are significant as the anabolic effects of Bzb on BrCa-free bone in the setting of metastatic bone disease were demonstrated by a 1.5 fold increase in bone volume. Furthermore, we showed that the effects of a 3 week pre-treatment allowed accrual of bone throughout the skeleton from the analyses of the non-tumor bearing femurs. This suggests a pre-emptive, protective effect to the bone, thereby diminishing the effect of tumor mediated osteolysis. Thus we find an additional benefit to the anabolic bone effect, as pretreatment with Bzb has a repressive effect on subsequent tumor growth. The clinical implications of these results are considerable. Proteasome inhibitors could provide a protective effect on the skeleton if Bzb were to be combined with other treatments for BrCa prior to metastasis.

In summary, proteasome inhibitors may treat solid tumors and the osteolytic bone disease that accompanies metastasis by myriad effects that include: decreasing tumor cell proliferation and survival, inhibiting bone destructive pathways and enzymes and vascularity of the tumors, reducing osteoclast number and function [17, 24, 25, 39], and increasing osteoblast differentiation and bone formation [7, 9, 22, 33]. Our studies demonstrate inhibitory effects of Bzb on solid tumor (breast cancer) growth in bone and prevention of the early onset osteolytic disease. We conclude that proteasome inhibitors have multifactorial beneficial effects in prevention of BrCa growth in bone and induced osteolysis.
REFERENCES


FIGURE LEGENDS

Figure 1. Bortezomib slows growth of BrCa osteolytic lesions as seen by conventional radiographs and micro-CT. (A) Radiographs of NCr/SCID mice during progression of tumor growth. Mice (n=3) received intratibial injection of 100,000 MDA-MB-231 BrCa cells and were treated with Bzb or PBS (0.3 mg/kg i.p.; 3 times/week) until sacrifice at 49 days following BrCa injection. Interval radiographs are shown indicating a delay in the onset of osteolysis in Bzb treated mice. There is an absence of clearly defined lesions in Bzb treated mice until day 35, in contrast the control group has clear osteolysis by day 21 (box). Microlytic lesions are seen in the Bzb group on day 35 (box). (B) Quantitation of radiographs. Average score (see methods) from each time point and all reviewers for control and Bzb treated mice is presented. This shows presence of osteolysis from d21 in control treated mice, but delay of osteolysis until day 35 in the Bzb treated group. Additionally, the increase in osteolysis during the final week of the experiment (d42-49) is shown (days 21 and 28 p<0.05, days 35, 42 and 49 p<0.005). (C) Micro-CT images. Transverse section distal to the physis (left panels) and 3D reconstruction of the proximal tibia (right panels) of Bzb and control treated mice (n=3) 49 days following intratibial injection of MDA-MB-231 BrCa cells. The reduction in osteolysis is evident by an unresorbed periosteal surface in Bzb treated as compared to control group. (D) Quantitation of micro-CT images. Relative bone volume at time was assessed by micro-CT analysis of the proximal tibia in two separate experiments at 28 days (n=5) and 49 days (n=3). The relative bone volume was significantly lower in control treated as compared to Bzb treated mice (p<0.05, 28 days, p=0.001, 49 days).

Figure 2. Bortezomib treatment reduces the size of BrCa tumors implanted in the mouse tibia. (A) In vivo positron emission tomography (PET) imaging. 39 days following intratibial injection, mice were injected intravenously with 50 µCi 18F-fluorodeoxyglucose. Representative PET image reconstructions of the tumor containing tibial region of Bzb treated and control mice overlaid on CT image reconstructions of the same region. The color corresponds to the intensity of the 18F signal and is scaled from bottom to top (black to white) as shown in the color bars where black indicates 0% radioactivity uptake and white indicates 100%.
uptake. Higher intensity in the control (white area) reflects the 3-dimensional size of the tumor, while red areas are those tumor cells with less metabolic activity. Blue is normal tissue cellular activity. Controls exhibited a 50-100% (yellow) range of intensity, while the Bzb group showed isotope labeling within the 25-50% (orange) intensity range. Standard uptake value for the control mouse is 2.94 g/ml and for the Bzb treated mouse is 2.31 g/ml. (B) The effect of Bzb on tumor growth and progression (H & E stained sections). Upper Panels (5x): Representative histologic sections of proximal tibias from n=6 control (left panels) and Bzb treated mice (right panels) showing the tumor size and tibial bone loss. Lower Panels (10x): Representative sections of Bzb and control treated tibias. 1) Muscle of Bzb treated tibia showing tumor growth. 2) Marrow cavity of Bzb treated tibia with necrotic cells. 3-4) Area of similar tumor growth in Bzb (3) and control (4) tibias. (C) Quantification of tumor size and growth. Left panel: Areas of tumor were selected from multiple histologic sections (n=5-7) of Bzb treated and control tibias (n=3) and tumor size (mm²) was calculated. Bzb treated mice had significantly (60%) smaller tumors as compared to control mice (p<0.0001). Right panel: Ki-67 immunohistochemistry was performed on Bzb and control treated tumor sections. The Ki-67 growth fraction was calculated as the % of total cells that were positive for Ki-67. The fraction of growing tumor cells was also significantly less in Bzb tumors than controls (n=4, p<0.05). (D) The effect of Bzb on bone osteolysis as evaluated by TRAP staining. In controls, the cells of the bone surface were already resorbed (Panel 1), only small pieces of fractured bone remained with TRAP positive cells (Panels 2 and 3). In the Bzb treated group, bone architecture was preserved (Panel 1) and osteoclast activity was present on cortical surfaces (Panels 2 and 3). Sections are TRAP stained and presented at 5x (left), 10x (center) and 40x (right).

**Figure 3.** Bortezomib causes increased bone formation. Micro-CT analysis was performed on the distal femurs of NCr/SCID mice 49 days following injection of MDA-MB-231 BrCa cells into the right proximal tibia. Parameters of trabecular bone formation showed significant (p<0.05) increases in Bzb treated mice as noted by increases bone volume/total volume,
trabecular thickness and connectivity density. Structure model index showed a significant (p<0.05) decrease, indicative of a more plate-like architecture in the Bzb treated bone.

Figure 4. Treatment with Bortezomib prior to tumor cell inoculation protects bone from osteolysis. (A) Experimental design. The experiment consisted of 3 groups: a control group treated with saline from 21 days prior to intratibial inoculation with BrCa until termination of the experiment at 25 days following inoculation; a Bzb pretreatment group that received 0.3 mg/kg Bzb IP three times a week for the 21 days prior to BrCa cell inoculation with saline injection for the 25 days following inoculation; and a continuous Bzb treatment group that received 0.3 mg/kg Bzb IP three times a week in both the pre-inoculation and post-inoculation periods. (B) Radiographic analysis of NCr/SCID mice taken at 25 days following injection of MDA-MB-231 BrCa cells into the right proximal tibia and the treatment conditions described above. Presented are views of the entire tibia (n=3) and representative higher magnification views of the area in which the majority of the osteolysis occurred. Note the fibular head overlapping the tibia in the area of the osteolytic lesion. The tibias of Bzb pretreated and Bzb continuous treatment show smaller osteolytic lesions than those in the control group at both time points. (C) Radiographs were independently scored based on a grading scale developed for osteolytic lesions (n=7 reviewers). Bzb pretreatment and Bzb continuous groups had a lower grade than the control group. Significantly decreased grade was found in Bzb pretreatment and Bzb continuous groups as compared to control (***p<0.0001) and Bzb pretreatment as compared to Bzb continuous (*p<0.05).

Figure 5. Anabolic effects of Bzb in tumor bearing and contralateral femurs. Micro-CT analysis was performed on the bilateral distal femurs of the mice from the 3 treatment groups described in Figure 4 (n=3). In all measured parameters, there is a significant anabolic effect evident in both Bzb pre-treatment, and continuous treatment groups as compared to controls.
Note that the right femur, proximal to the tumor bearing tibia, has less bone than the contralateral femur. (* p<0.05, + p< 0.001, # p<0.0005)

**Figure 6. Bortezomib treatment reduces cell proliferation and decreases cell viability of BrCa cells in vitro but does not induce significant apoptosis.** (A) The relative number viable cells, necrotic cells, and cells undergoing apoptosis following treatment with Bzb in the range of 0 - 50 nM for 14 h were measured by binding of FITC conjugated annexin V and staining with propidium iodide (PI) followed by FACS analysis. The percentage of gated cells that were annexin V and PI negative (live cell, solid line, diamond); annexin V positive, PI negative (apoptotic cells, dashed line, open square); and annexin V and PI positive (necrotic cells, solid line, circle) are shown as a function of the increasing concentration of Bzb treatment. (B) Bivariate plots of the primary FACS data for ungated cells treated with 0 and 50 nM Bzb. The quadrants, which are defined using FACSCalibur software and represent cells that are annexin V and PI negative (bottom left), annexin V positive, PI negative (bottom right); annexin V negative, PI positive (top left); and annexin V and PI positive (top right), show a far greater proportion of cells in the quadrant representing necrosis in response to Bzb treatment. (C) The amount of transcript of various genes in MDA-MB-231 cells treated with Bzb in the range of 0 - 50 nM Bzb for 24 h was measured using qPCR and standardized to the amount of GAPDH transcript in the same cells. The amount of transcript relative to no Bzb treatment is shown for each concentration of Bzb treatment used. The CT values for monitored genes were between 18 and 25, except for MMP9 (CT 35). VEGF= vascular endothelial growth factor, DKK1=Dickkopf 1, MMP9 = matrix metallopeptidase 9, LEF1=lymphoid enhanced binding factor 1. (D) Gene transcript levels were measured in MC3T3 osteoblast-like cells following 24 h treatment with 0-50 nM Bzb using qPCR, as described above. The CT values were all less than 21. Col1a=collagen 1A, DKK3=Dickkopf 3, AP=alkaline phosphatase, TCF4=transcription factor 4 (E). Tumor tissue from mice treated with Bzb or control for 28 days was analyzed for relevant gene expression by qPCR. The amount of transcript of Bzb treated tumors relative to control is shown. The CT values were between 16 and 35 for all the monitored genes.
(note – all less than 30 except RANKL). Significant differences were seen in RANKL, MMP-9 and LEF1 (p<0.05, n=5) RANKL=Receptor activator for nuclear factor κβ ligand.
Figure 1
Figure 3

Bone Volume Fraction

Trabecular Number

Trabecular Thickness

Trabecular Separation

Connectivity Density

Structure Model Index

Control BZB

Control BZB

p = 0.03

p = 0.07

p = 0.02

p = 0.11

p = 0.03

p = 0.04
Figure 4

A Experimental Design

Control (PBS)  
BZB Pretreatment (-21d to + 6d)  
Continuous BZB (-21d to + 25d)

B Tibial Osteolysis (right limb)

25 days post-inoculation

Control  
BZB Pretreatment  
BZB Continuous

C Radiographic Assessment day 25 post-inoculation

Radiographic Grade

Ctrl  BZB Pretreat.  BZB Contin.
Figure 5

- Bone Volume Fraction
- Trabecular Number
- Trabecular Thickness
- Trabecular Separation
- Connectivity Density
- Structure Model Index

(left) vs (right) comparison with error bars.
Figure 6
A Proteasome Inhibitor, Bortezomib, Inhibits Breast Cancer Growth and Reduces Osteolysis by Downregulating Metastatic Genes

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Clin Cancer Res Published OnlineFirst September 15, 2010.

Updated version

Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-3293

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