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Award Number: W81XWH-06-1-0435

TITLE: Enhancing Quality of Life for Breast Cancer Patients with Bone Metastases

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REPORT DATE: April 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-03-2007	Annual	15ar 2006 – 14 Mar 2007

4. TITLE AND SUBTITLE

Enhancing Quality of Life for Breast Cancer Patients with Bone Metastases

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0435

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Research Foundation of the State University
of New York
Syracuse, NY 13210

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Current treatment for osteolytic breast cancer bone metastasis typically involves radiation therapy (RTX) to palliate bone pain and a bisphosphonate to inhibit osteoclastic bone resorption. The goal of this research was to determine the effects of zoledronic acid as an adjunct to RTX on restoring bone strength and density. We also investigated whether the combination of ZA and an anabolic agent (parathyroid hormone, PTH) as adjuncts to RTX further enhanced bone strength, and thus decrease the risk of subsequent pathological fractures.

Using our mouse model of breast cancer bone metastasis, we were able to demonstrate that adjunct treatment of a high dose of ZA (600 µg/kg, accumulative dose) alone or in combination with PTH (800 µg/kg, accumulative dose) was capable of restoring bone mineral density (BMD) and mechanical strength to that of a normal bone. This was a significant improvement compared to treatment with RTX alone in which BMD was 36.6% lower compared to normal ($p<0.0001$) and mechanical strength was 2.4-fold lower compared to normal ($p<0.0001$). There was no significant difference in BMD or mechanical strength between mice treated with RTX and ZA or ZA/PTH. In a second series of experiments we were able to demonstrate that a clinically relevant of ZA (100 µg/kg, accumulative dose) as an adjunct to RTX, not only significantly increased BMD compared to treatment with RTX alone ($p=0.026$), but also significantly increased BMD by 12.7% compared to normal ($p=0.049$). Based on the results we have seen so far, we have decided that it is not necessary to combine ZA and PTH in order to restore the strength and bone properties of tumor-burdened bone to that of a normal bone. These results have also led us to investigate the effects of ZA on breast cancer cells and osteoblasts *in-vitro*.

15. SUBJECT TERMS
Breast cancer, bone metastasis, animal model, radiation therapy, anabolic agents, bisphosphonates, biomechanical testing, histology

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	28	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

The skeleton is the most common site of metastasis for women with advanced stage breast cancer (5, 8, 22). Severe osteolysis that is often associated with this stage of the disease can lead to skeletal related events such as pathological fracture, severe bone pain and hypocalcaemia. The current standard of care for treating osteolytic bone metastases includes palliating bone pain through radiation therapy and blocking ongoing osteoclastic bone resorption with a bisphosphonate. Results from clinical trials indicate that anywhere from 6 to 26% of metastatic bone lesions treated with radiation therapy will go on to develop pathological fractures (11, 23). Recent clinical data has shown that zoledronic acid, a third generation bisphosphonate, decreased the number of skeletal related events by 39% compared to placebo (12). Clinicians recognize that prevention of skeletal complications considerably improves the quality of life for these patients (4); and although bisphosphonates can decrease the chances of a patient developing a skeletal complication many are still at risk. In an effort to address this issue we **hypothesized** that the combination of an anabolic agent, parathyroid hormone (PTH 1-34) in conjunction with a bisphosphonate (zoledronic acid, ZA) and radiation therapy (RTX) for the treatment of metastatic bone lesion from breast cancer would stimulate new bone formation, improve mechanical properties, and thus decrease the risk of subsequent pathological fractures.

2. Body

2.1: *Task 1: Evaluation of PTH and ZA as adjuncts to RTX for the treatment of focal breast cancer bone metastasis in the mouse.*

In order to study the effects of PTH and ZA as adjuncts to RTX for the treatment of an established osteolytic bone metastasis from breast cancer, we have utilized a murine model developed in our lab (1), Appendix A. To date, two full studies have been completed and a third study is currently underway (Table 1). Below is a brief description of the materials and methods used for these three studies, followed by results and justifications for the next study. The number of mice used for histology (H) and for mechanics (M) is included in the table.

Table 1: Experimental Design for *In Vivo* Studies

Mouse Studies			
	Study 1	**Study 2**	**Study 3**
# of tumor cells injected (F10)	1 x 10^5	2 x 10^4	2 x 10^4
Duration	9 Weeks	12 Weeks	12 Weeks
Therapies			
Week initiated post-RTX	3 weeks	4 Weeks	4 Weeks
Drug Dose: All injections were given subcutaneous	ZA: 100µg/kg, weekly for 6 wks PTH: 80µg/kg, daily for 4 wks	ZA: 12.5µg/kg, weekly for 8 wks Or ZA: 25µg/kg weekly for 4 wks, followed by PTH (80µg/kg) daily for 4 wks	ZA: 11µg/kg, weekly for 9 wks
0 Gy	H: n = 0 M: n = 6	H: n = 6 M: n = 8	H: n =6
20 Gy	H: n = 6 M: n = 6	H: n = 6 M: n = 8	H: n =6
20 Gy + ZA	H: n = 6 M: n = 6	H: n =6 M: n = 8	H: n =6
20 Gy + PTH	H: n = 6 M: n =6		
20 Gy + ZA + PTH	H: n = 6 M: n = 6	H: n = 6 M: n = 8	
ZA	H: n = 6 M: n =6		H: n = 6
Status	Currently in submission	Completing histology and analyzing data; should be ready for publication early summer ‘07	Mice currently on study; should be ready for publication fall ‘07

H = number of mice for histology, M = number of mice for mechanics.

Materials and Methods:

Tumor Cells

F10 cells, a bone-adapted clone derived from the human MDA-MB-231 breast carcinoma cell line (27)(Dr. Toshiyuki Yoneda, UT San Antonio, TX) were used for this study. Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% penicillin-streptomycin-glutamine (Gibco, Grand Island, NY) and maintained at 37ºC in an atmosphere of 5% CO_2 in air.

Tumor Cell Inoculation

Female NCr homozygous nude mice (8 to 9 weeks old**,** 20-25 grams body weight) (Charles River, Wilmington, MA) were anesthetized with an injectable drug cocktail consisting of telazol (45 mg/kg, IM) and xylazine (7.5 mg/kg, IM). The mice were weighed and the hind limbs scrubbed with betadine followed by 70% ethanol. With the knee flexed, a small incision was made at the joint and the patella carefully moved to expose the distal end of the femur. Using a sterile 26G needle, twenty microliters of cell suspension (containing 1×10^5 or 2×10^4 F10 breast cancer cells) were injected through the intercondylar fossa of the right femur to a depth of approximately 5 mm. Mice were housed in micro-isolators with a 12-hour day/night cycle and were fed a diet of autoclaved food and water *ad libitum*.

Bone Densitometry

Dual-energy X-ray absorptiometry (DEXA) measurements of the left and right femora were obtained immediately following tumor inoculation and at three, six, nine, and twelve weeks using a dedicated mouse bone densitometer (PIXImus 2; GE Lunar; Madison, WI). Bone mineral density (BMD) values (g/cm^2) were determined from a region of interest that encompassed the entire femur.

Mechanical Testing

Whole-limb torsional testing was performed in order to asses the mechanical strength of tumor burdened bones in which the primary site of osteolysis was in the distal femur. This method has been previously described (1). Briefly, the proximal femur and the distal tibia were embedded in poly-methylmethacrylate (PMMA) using an alignment fixture. The bone was then placed in a torsion testing jig and loaded in external rotation at a rate of 180°/min. The resulting torque (N-mm) was measured. Energy to failure (N-mm-degree) was determined by calculating the area under the torque-rotation curve. Initial stiffness (N-mm/degree) was defined as the first 50% of the slope to torque at failure.

Histological Confirmation of Tumor

Limbs that were selected for histology were embedded in methylmethacrylate (MMA) according to the method described by Erben (6). Embedded bones were then trimmed on a low-speed diamond saw (Buehler, Lake Bluff, IL) and 5-µm sections were cut using a microtome. Sections were stained with modified Masson's trichrome stain and examined by light microscopy to confirm the presence of tumor.

Total Bone Analysis

Prior to sectioning, embedded femora were scanned using a micro-CT scanner (SCANCO µCT 40, SCANCOMedical, Zürich, Switzerland). X-ray acquisition settings were 55 kVp and 145 µA with an integration time of 200-ms. Scans were performed with an isotropic voxel size of 12 µm and images were reconstructed in 1024 x 1024 pixel matrices. A global threshold of 35% maximal grayscale was determined by visual inspection to best distinguish between bone and non-bone material. Measurements of total bone volume in the distal 5 mm of the femur (BV_{tot}) were obtained from transverse slices in which the region of interest (ROI) included both cortical and trabecular bone. For measurements of fractional trabecular bone (BV/TV), the distal femur was reconstructed in the sagital plane. The trabecular bone ROI was drawn to include all cancellous bone in the metaphysis region extending 1 mm from the base of the growth plate.

Statistics

Analysis of variance along with Fisher's PLSD post-hoc was performed to analyze differences between groups. Statistical significance was taken at $P < 0.05$.

Study 1: Response of High Dose ZA with and without anabolic agent (PTH)

The specific aim of this first study was to determine if bone that had been affected by both tumor-induced osteolysis and radiation was capable of regaining normal bone density, mechanical strength and bone morphology. Based on preliminary data, a study period of nine weeks was thought to be sufficient time to see significant improvement in bone density and mechanical strength in tumor-burdened bones treated with ZA, PTH, or ZA/PTH as adjuncts to radiation. Previous work with this model has shown that mice that do not receive treatment rarely survive to nine weeks; therefore, for this study we chose not to have an untreated, 0 Gy group for histology. Since the focus of this study was to determine if diseased bone was capable of "repair", we selected a dose of ZA that was much higher than the dose given clinically. The rationale behind this was due to the fact that this model system produces significant osteolysis that progresses rapidly. Therefore, it was thought that a higher dose of ZA may be required to combat this fast paced bone destruction. In this first study the ZA and PTH were administered concurrently. There is conflicting data in the literature as to whether patients who have been on bisphosphonates and then switch to PTH for treatment of osteoporosis experience a blunted anabolic affect from the PTH (2, 3, 9, 14, 15, 28). Based on this information it was rationalized that giving the two drugs concurrently may provide the best environment for bone "repair". In this first study we also wanted to determine if PTH was capable of restoring bone density and mechanical strength when used in combination with RTX only. Although the use of PTH in patients with cancer is not clinically applicable, it was used in this study to specifically address whether an anabolic agent was capable of "repairing" bone that was affected by both tumor and radiation. The goals of study one were to establish if tumor-burdened bone treated with radiation was capable of being restored to normal in terms of both bone density and mechanical strength; and whether this "repair" could be accomplished with an anti-resorptive agent, such as ZA, an anabolic agent, such as PTH, or if it would require the combined features of an anti-resorptive and an anabolic agent.

Summary of Key Results

Analysis of terminal BMD values revealed no significant difference between mice that received no therapy and those that received 20 Gy (p=0.083). However, mice that received 20 Gy along with either PTH, ZA, or the combination of PTH and ZA all had significantly higher BMD compared to both the 0 Gy and 20 Gy groups at the end of the 9 week study (p<0.05) (Figure 1). Further analysis indicated that there was no significant difference in BMD between normal bones and those treated with either 20 Gy and ZA or 20 Gy and PTH/ZA (p>0.05). However, the BMD was still significantly lower than normal in mice that were treated with 20 Gy and PTH (p=0.002), as well as those that received 0 Gy (p<0.0001) or 20 Gy only (p<0.0001).

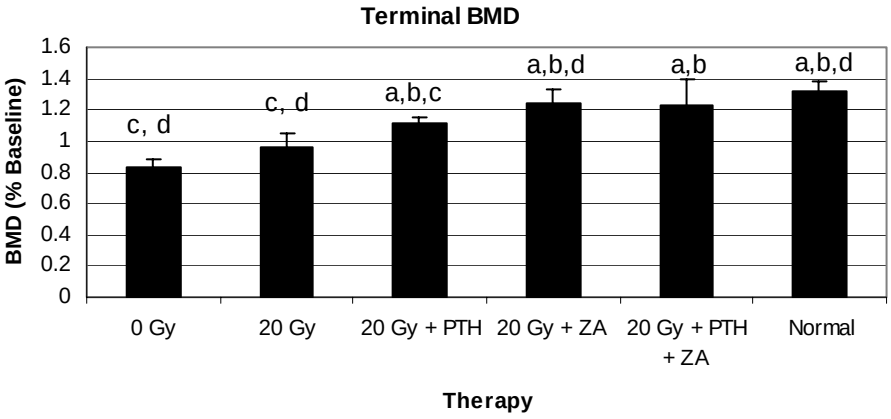

Figure 1: Effects of human breast cancer cell on bone mineral density. Terminal DEXA scans demonstrated a significant increase in BMD in mice treated with PTH, ZA or the combination of PTH/ZA as adjuncts to RTX, compared to treatment with either 0 Gy or 20 Gy alone (p<0.0001 and p<0.01, respectively). Terminal DEXA analysis also revealed that there was no significant difference between mice treated with either ZA or the combination of PTH/ZA as adjuncts to RTX, compared to normal (p=.182 and p=.164, respectively). There was no significant difference in BMD between mice treated with ZA or PTH/ZA (p=.951). (Alpha characters represent a significant difference between groups: a = different from 0 Gy, b = different from 20 Gy, c = different from normal, and d = different from PTH. Significance taken at p<0.05)

Analysis of the biomechanical data revealed that treatment with 20 Gy combined with either ZA or PTH/ZA was able to restore mechanical strength to that of a normal bone. (Figure 2). The results also indicated that mice treated with 20 Gy

and ZA or 20 Gy and PTH/ZA had significantly improved mechanical strength with regards to peak torque compared to mice treated with 0 Gy ($p<0.0001$ for both), 20 Gy only ($p<0.001$ for both), and 20 Gy plus PTH ($p=0.003$ for both). There was no significant difference between mice treated with 20 Gy only and those treated with 20 Gy and PTH ($p=0.497$). There was also no significant difference between mice that were treated with 20 Gy and ZA or 20Gy and PTH/ZA ($p=0.952$).

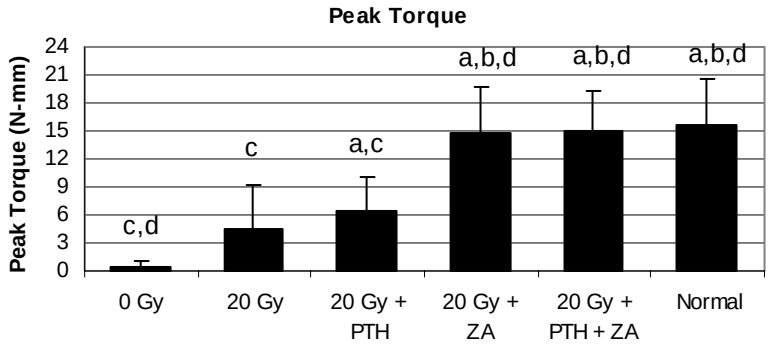

Figure 2: Effects of ZA and PTH as adjuncts to RTX on mechanical strength of tumor-burdened bones. Adjuvant treatment with ZA or PTH/ZA significantly improved mechanical strength compared to treatment with 20 Gy alone ($p=0.0009$ and $p=0.0008$, respectively). Adjuvant treatment with PTH did not significantly improve mechanical strength compared to 20 Gy alone ($p=0.499$). Further analysis indicated that there was no significant difference in mechanical strength in mice treated with adjuvant therapies of ZA or PTH/ZA compared to normal bones ($p=.763$ and $p=.811$, respectively). (Alpha characters represent a significant difference between groups: a = different from 0 Gy, b = different from 20 Gy, c = different from normal, and d = different from PTH. Significance taken at $p<0.05$)

Taken together these results indicate that radiation therapy when given alone for the treatment of a bone metastasis is not sufficient for restoring BMD or mechanical strength to that of a normal bone. Adjunct treatment with PTH was able to significantly increase both BMD and mechanical strength compared to 20 Gy alone, but these properties still remained significantly below normal. This study revealed that adjunct treatment with ZA or the combination of ZA/PTH was effective at restoring BMD and mechanical strength to that of a normal bone. Based on these results the second mouse study was designed to address the following questions: Is the clinical dose of ZA as effective as the high dose used in the first study in restoring BMD and mechanical strength? Does the order in which the combination of PTH and ZA are given affect BMD and mechanical strength? How is the bone microarchitecture affected by the use of these drugs? Due to the fact that there was not a significant improvement in mechanical strength compared to treatment with 20 Gy only in mice treated with PTH, we will no longer study the effects of PTH on its own as an adjunct to RTX.

Study 2: Response of Clinical Doses of ZA with and without Anabolic Agent (PTH)

The specific aim of this second study was evaluate the effectiveness of a clinically relevant dose of ZA as an adjunct to RTX, with regards to restoring BMD and mechanical properties to that of a normal, non-tumor bearing bone. Based on the results of the first study we wanted to see if the anabolic effects of PTH were affected by administering it after the mouse had been treated with ZA for four weeks. Taking into consideration that the mineralization process requires time to develop, we decided to extend the study period from nine weeks to twelve weeks. This lengthened study time also allows us to evaluate the safety aspects of these drugs in terms of secondary tumor growth in distant organs. In addition to the outcome measures analyzed in the first study, the second study included a more detailed analysis of bone microarchitecture using a microCT scanner that was acquired after the conclusion of the first study. These data allowed us to evaluate the effects of these adjunct therapies on bone geometry and possibly correlate features of the microarchitecture with mechanical strength. This study will also include a more detailed look at the bones histologically to determine whether ZA and PTH have an effect on the cellular level, such as causing apoptosis in tumor cells or bone cells.

Summary of Key Results to Date: The amount of bone present in each bone was measured several different ways, which included total BMD of the femur (using DEXA), total bone volume of the distal 5 mm of the femur (using μCT), and trabecular bone in the area 1 mm below the growth plate (using μCT). Analysis of the BMD data revealed that there was no significant difference between normal bones and tumor-burdened bones treated with 20 Gy ($p=0.314$). Mice treated with 20 Gy and ZA or 20 Gy and

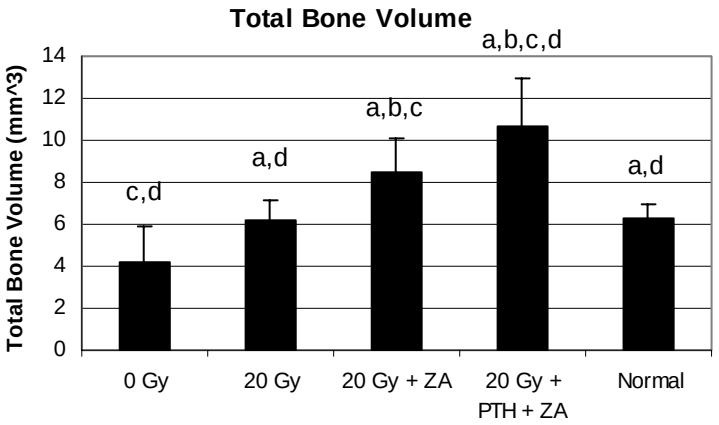

Figure 3: Effects of ZA and ZA/PTH on total bone volume in the distal 5 mm of tumor-burdened femora. Total bone volume was significantly increased in mice treated with ZA or ZA/PTH in combination with 20 Gy compared to treatment with 20 Gy alone ($p=0.013$ and $p<0.0001$, respectively). In this study there was no significant difference in total bone volume in mice treated with 20 Gy compared to normal bones ($p=0.866$). Mice treated with ZA or ZA/PTH exhibited significantly higher bone volume than normal bones ($p=0.015$ and $p<0.0001$, respectively). (Alpha characters represent a significant difference between groups: a = different from 0 Gy, b = different from 20 Gy, c = different from normal, and d = different from PTH. Significance taken at $p<0.05$)

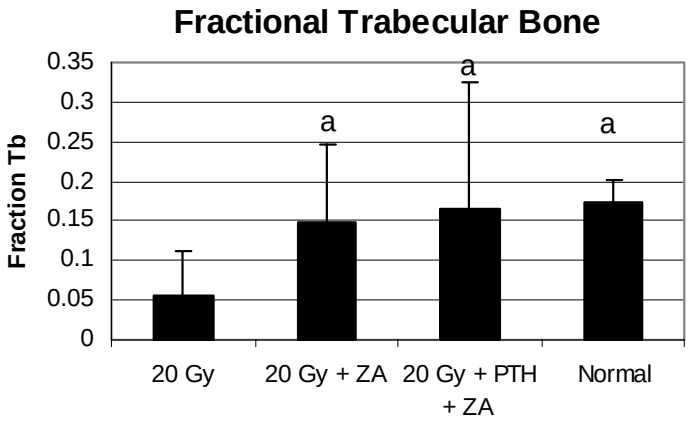

Figure 4: Fraction of trabecular bone located 1 mm below the growth plate in tumor-burdened bones. A significant increase in trabecular bone was observed in mice treated with ZA or ZA/PTH compared to those treated with only 20 Gy ($p=0.028$ and $p=0.012$, respectively). There was no significant difference in trabecular bone between mice treated with ZA or ZA/PTH and normal bones ($p=0.654$ and $p=.883$, respectively. (a=significantly higher than 20 Gy. Significance was taken at $p<0.05$).

ZA/PTH both had significantly higher BMD compared to treatment with 20 Gy alone ($p= 0.0005$ and $p<0.0001$, respectively). Mice treated with 20 Gy and ZA/PTH also had significantly higher BMD compared to normal bones ($p=0.010$). Similar results were seen for total bone volume measurements obtain through µCT. The only difference between the results for the two outcome measures was that mice treated with 20 Gy and ZA demonstrated significantly higher bone volume compared to normal bones ($p=0.004$) (Figure 3), where there was not a significant difference in BMD. Based on previous studies and what is seen clinically we also wanted to look at the changes in the amount of trabecular bone. Trabecular bone is typically more affected than cortical bone, and changes in the amount of trabecular bone as well as its structure may have a substantial impact on the mechanical strength of the bone. Our initial analysis revealed that trabecular bone was significantly affected by tumor osteolysis. The destruction in the 0 Gy group was so dramatic that there was virtually no trabecular bone to analyze, therefore no further evaluation of trabecular bone parameters could be obtained from this group. Mice that were treated with 20 Gy only also displayed significantly lower trabecular bone fraction compared to normal bones ($p=0.038$) (Figure 4). Adjunct treatment with ZA or ZA/PTH significantly improved the trabecular bone fraction compared to treatment with only 20 Gy ($p=0.028$ and $p=0.012$, respectively). There was no significant difference in trabecular bone fraction between normal bones and those treated with either 20 Gy plus ZA or 20 Gy plus ZA/PTH ($p=0.654$ and $p=0.883$, respectively). Mice treated with 20 Gy plus ZA/PTH were not significantly different from mice treated with 20 Gy and ZA for any of these parameters.

In this study were able to demonstrate that treatment with 20 Gy plus ZA significantly increased mechanical strength compared to treatment with 20 Gy alone ($p=0.034$). However, mice that were treated with 20 Gy plus ZA/PTH did not have significantly better mechanical strength compared to mice treated with only 20 Gy ($p=0.234$). Mechanical testing on normal bones has not been completed yet for this study.

Ongoing analysis for this study includes histological examination of the tumor bearing bones, more detailed analysis of the microarchitecture, and possible correlations to mechanical strength.

2.2: *Task 2: Determining the safety of anabolic therapy for the treatment of metastatic breast cancer by quantification of tumor burden and potential recurrence or metastasis using bioluminescent imaging.*

This task has changed focus over the past year. After many attempts, we were unable to find a luciferase-transfected breast cancer cell line that would work for this model. The first attempt involved transfecting our F10 cell line with luciferase. We were able to observe osteolysis using X-ray, but the cells did not reliably remain transfected for the duration of the study. We purchased a luciferase transfected cell line from XENOGEN. This cell line did produce a bioluminescent signal, but did not cause tumor-induced osteolysis. Our final attempt involved using a mouse breast cancer cell that was transfected with luciferase. This cell line was able to produce a bioluminescent signal and tumor-induced osteolysis, but it was extremely aggressive. Most of the mice died within four weeks of tumor injection due to lung metastases or severe osteolysis. These pilot studies were being performed in parallel with Study 1 described above; therefore for Study 2 we performed very detailed necropsies to try and identify secondary tumor growth in distant organs. Tissue samples were collected from the mice and were reviewed by a veterinary pathologist. Although this analysis is postmortem, it does allow us to explore the question as to whether the agents we are using are causing an increase in secondary tumor growth. By the end of the second study we were also to determine that there was no apparent benefit for using PTH, and therefore we are no longer studying this agent *in vivo*. However, to remain thorough in our evaluation of ZA, we are continuing to collect tissue samples from the mice and having them reviewed by a board certified pathologist.

Based on these findings we have decided to explore the effects of ZA in more detail. Study 3 describes the rationale and study design of the next mouse experiment. In combination with the *in vivo* study, we have also added an *in vitro* study to examine the effects of ZA on individual cell types. Details of this study are described in Study 4.

Study 3: Response of Tumor-Burdened Bone to ZA
This study was not in the original 'Statement of Work.'

The focus of this third study was to gain a better understanding of the "mechanisms" of ZA in bone repair. Based on the results from the first two studies we have not seen a significant benefit to using PTH as an adjunct to radiation therapy either alone or in combination with ZA. Therefore we did not use PTH in this third study. Although ZA is predominately viewed as an anti-resorptive agent, our studies have shown that even in the presence of tumor, mice treated with ZA have been able to obtain, and sometimes surpass, qualities of normal bone. There have also been reports in the literature of bisphosphonates having anti-tumor effects (13, 18-20) as well as promoting mineralization (10, 16, 17). The specific aim of this study was determine whether ZA was capable of inducing tumor cell apoptosis *in vivo*, as well as enhancing bone mineralization compared to treatment with 20 Gy alone. In order to examine these effects the following treatment groups were used: 0 Gy, 20 Gy, ZA alone, and 20 Gy combined with ZA. In addition to radiographs, DEXA scans, and μCT; mice will receive two series of bone labels which will be used to examine bone formation. As in study two, this will be a twelve-week study. These mice are currently on-study.

Study 4: Effect of ZA on Osteoblasts, Mesenchymal Stem Cells, and Breast Cancer Cells In-Vitro
This study was not in the original 'Statement of Work.'

The specific aim of this study was to examine the effects of ZA on individual cell types, specifically mesenchymal stem cells, osteoblasts, and human breast cancer cells. There are conflicting reports in the literature as to the effect ZA has on these various cell lines. It has been shown that bisphosphonates are able to induce apoptosis in breast cancer cell lines *in vitro* and *in vivo* (7, 18, 20, 25, 26). However, the sensitivity of the tumor cell to the bisphosphonate seems to depend on both the characteristics of the breast cancer cell line as well as the bisphosphonate being studied. Therefore we thought it was important to determine the effect of ZA on the F10 human breast cancer cell line that we use in our

mouse studies. If we can determine that ZA is effective at causing tumor cell apoptosis, we may be able to obtain a synergistic effect when ZA is combined with radiation therapy. The second arm of this study is to examine the effects of ZA on osteoblasts and mesenchymal stem cells. Based on our first two *in-vivo* studies where we were able to measure increased bone volume and bone density, it seemed reasonable to hypothesize that ZA is influencing mineralization. The purpose of our study is to determine whether the increased mineralization is due to an increased number of osteoblasts, an up-regulation of factors that increase mineralization, or if it is the result dystrophic mineralization caused by osteoblasts undergoing apoptosis. Although the dose and duration of exposure to ZA *in vivo* is not known, results of this study will give us an insight into the mechanistic of ZA on osteoblast cells.

Materials and Methods:
Cell Lines: Two metastatic human breast cancer cell lines will be used in the cell culture experiments: MCF-7 (estrogen-receptor-positive) and F10 (a bone-adapted clone derived from the MDA-MB-231 cell line, estrogen receptor-negative). Murine MC3T3 cells will be used as the standard osteoblastic cell line. Murine ST-2 cells will be used as the standard marrow stromal cell. Murine marrow stromal cells (MSCs) will also be isolated from primary cultures of murine bone marrow. Results in the murine models will subsequently be confirmed with primary cultures of human osteoblasts and MSCs (approved under IRB #4086).

Cells will be grown in the appropriate medium supplemented with 10% (v/v) fetal calf serum and 1% antibiotic solution. For mineralization experiments, medium will be supplemented with 50 µg/ml ascorbic acid and 5 mM sodium phosphate in order to promote mineral formation.

Cell Proliferation and Apoptosis: Samples will be assayed for cell proliferation (MTT assay; Sigma Chemical, St Louis, MO) and apoptosis (EnZChek Caspase-3 Assay kit; Molecular Probes Inc., Eugene, OR). Caspase activity will be normalized to total protein concentrations (BCA Assay; Pierce, Rockford, IL). All data from treated wells will be normalized to that of the control wells.

Flow Cytometry: Apoptosis will be confirmed by annexin-V/propidium iodide staining (Molecular Probes; Eugene, OR). In a subset of experiments, cell cycle analysis will be performed to determine the effects of radiation and ZA on tumor cells, osteoblasts, and MSCs.

Mineralization Assay: Osteoblast and MSC cultures will be set up at a density of 1×10^5 cells/well in 6-well plates. Once the cultures become confluent (3-4 days), the medium will be replaced with media containing ascorbic acid (50 µg/ml) and sodium phosphate (4 mM). After 21 days, mineral formation will be measured with a quantitative assay for Alizarin Red S (21).

Radiation Therapy: Radiation will be administered with a Phillips MGC-30 therapeutic X-ray machine operating at 300 kilovolts and 10 milliamps (effective dose rate 2.04 Gy/minute at a source-object distance of 15 cm). Cells will be irradiated with 0-20 Gy 24 hours after cell seeding.
Clonogenicity Assays: Cells will be seeded at a density of 100 to 10,000 cells/well in 6-well plates. After 24 hours, plates will be irradiated with 0, 1, 2, 5, 10 or 20 Gy. Medium changes will be performed twice weekly. After 14 days, colonies will be fixed with ice-cold ethanol, stained with 1% crystal violet and counted. Log-lin plots of survival fraction versus radiation dose will be constructed and the D_0 value (a measure of radiation sensitivity) will be determined.

Drug Therapies: In-vitro experiments will be carried out to determine the direct effect of ZA on breast cancer and osteogenic cells at concentrations from 0 to 100 µM (Table 2) (24).

Table 2: Experimental Design for *In Vitro* Experiments

Specific Aim	Test Conditions	Outcome Measures
ZA induces apoptosis in human breast cancer cells	F10 (ER-) or MCF-7 (ER+) HBC cells treated with ZA (0, 1, 10, 25, 50, 100 µM)	MTT Western blot – BAX/ BCL2 Cell cycle, Annexin V/PI
ZA induces apoptosis in osteoblasts	MC3T3, ST2, and human osteoblast cells treated with ZA (0, 0.05, 0.5, 5, 10 and 20 µM)	MTT Western blot – BAX/ BCL2 Cell cycle, Annexin V/PI
ZA increases mineralization	MC3T3 and ST2 cells treated with ZA (5 µM)	Alizarin Red S Western blot - ALP
ZA sensitizes breast cancer cells to radiation	F10 (ER-) and MCF-7 (ER+) HBC cells treated with 50 µM ZA prior to radiation (20 Gy)	Clonogenicity MTT Western Blot Annexin V/PI, cell cycle

Summary of Key Results to Date:
Clonogenicity experiments have been performed on the F10 and MC3T3 cell lines. The average Do value for the F10 cells was 2.39 Gy, where the MC3T3 cells had an average Do value of 5.84 Gy. These results confirmed that breast cancer cells are more sensitive to radiation then osteoblasts. Several experiments have been performed to examine the effects of ZA on the F10 and MC3T3 cell lines. F10 cells were treated for 48 or 72 hours with various concentrations of ZA ranging from 1 to 100 µM. Preliminary analysis of the data indicates that after exposure to 50 µM ZA for 48 hours there is a significant 21% decrease in cell viability ($p<0.0001$) (Figure 5). After exposure to 100 µM ZA for 48 hours there is a 61.5% decrease in cell viability ($p<0.0001$). Similar results were seen at 72 hours, with a 47.4% decrease in cell viability with 50 µM ZA and a 72.5% decrease in cell viability with 100 µM ZA. None of the other concentrations of ZA tested at these time points significantly decreased cell viability compared to the untreated controls.

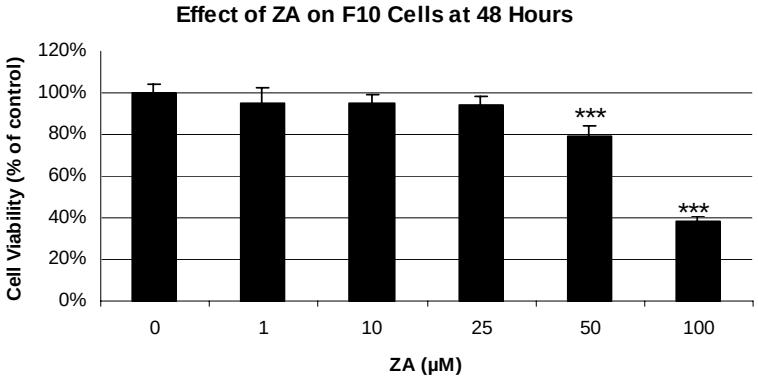

Figure 5: The effect of ZA on F10 human breast cancer cells *in vitro*. After exposure to various concentrations of ZA for 48 hours, cell viability was only significantly decreased from the control at doses above 50 µM (*** = $p<0.0001$).

MC3T3 cells were treated with ZA concentrations ranging from 0.05 to 20 µM. Cell viability was measured using the MTT assay as described above. Analysis of this data revealed that the lowest concentration of ZA used (0.05 µM) significantly decreased cell viability by 16.5% compared to the control ($p=0.0018$) at 7 days (Figure 6). The highest concentration of ZA tested (20 µM) significantly decreased cell viability by 71% compared to the control ($p<0.0001$). ZA at a concentration of 5 µM was able to decrease cell viability by 49.3% ($p<0.0001$). Since this concentration was able to decrease the cell viability by approximately 50% this dose was selected for use in future experiments.

Comparing the results ZA exposure to F10 human breast cancer cells and to MC3T3 mouse osteoblast-like cells it is important to note that the MC3T3 cells appear to be much more sensitive to ZA then the breast cancer cells. Future experiments will focus on determining why there is a decrease in MC3T3 cell viability when treated with ZA; are the cells dying through apoptosis? Is ZA causing cell cycle arrest? Are similar results seen when the cells are treated for shorter periods of time? An addition, a second breast cancer cell line will be evaluated to see if estrogen receptor status has a role in breast cancer cell sensitivity to ZA. Studies are also planned to examine the effect of radiation in combination with the individual cell lines to determine whether ZA causes cells to be more sensitive to radiation therapy. Outcome measures will include cell viability, clonogenicity, cell cycle analysis, and Annexin V/PI tests for apoptosis.

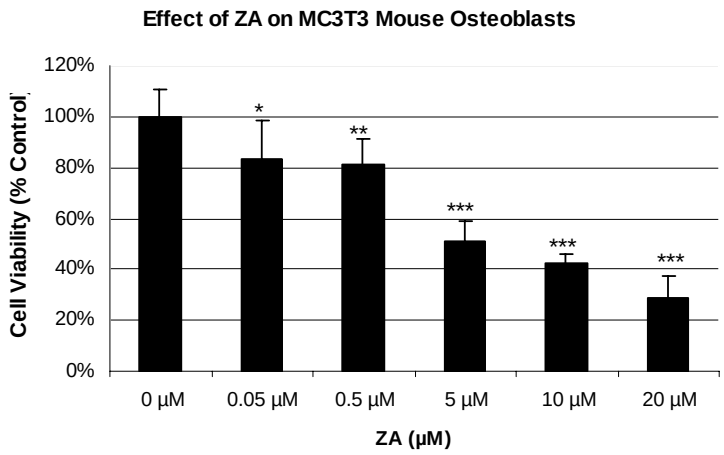

Figure 6: Effect of ZA on MC3T3 mouse osteoblasts at various concentrations for 7 days. Increasing concentrations of ZA caused a dose dependent decrease in cell viability. Low doses of ZA (0.05 µM) caused a significant 16.4% decrease in cell viability compared to the control ($p=0.0018$). (*** = $p<0.0001$, ** = $p<0.001$, and * = $p<0.01$)

2.3: *Task 3: Training and Professional Development*

The goal of Task 3 was to gain a strong foundation in technical laboratory skills, first-hand clinical exposure to cancer patients and current treatments, and to develop professional skills and collaborations. As stated in the original 'Statement of Work' most of the course work and training in various laboratory techniques has already been completed. However, with the aid of this grant I was able to receive advanced training on the use of our microCT scanner. For the clinical training aspect, I was able to shadow one of University Hospital's breast cancer surgeons both in the clinic and the operating room. I have also attended multidisciplinary breast conferences where I gained an appreciation for the complexity of treating patients with breast cancer. Due to structural reorganization within our institution I was not able to participate in the medical case study course as it was not offered. As listed in my reportable outcomes below, I have submitted two first-author abstracts to international meetings where I was also able to present my research as a poster. Through attendance at these meetings I was able to establish a collaboration with two senior researchers at Novartis, Switzerland. These collaborations have been extremely helpful in developing my thesis as unexpected results were observed during my *in vivo* studies.

3. Key Research and Training Accomplishments

A. Research Accomplishments

- Purchase of a SCANCO µCT 40 scanner (SCANCO Medical, Switzerland)
    - Worked with programmers at SCANCO to develop custom programs to evaluate the bone microarchitecture of severely diseased bone
- Learned how to do thin section histology on bone and developed a sampling scheme to acquire an accurate representation of the interaction between tumor and bone
- Developed a detailed testing metric for evaluating the effects of antiresorptive and anabolic agents on tumor-burdened bone in a mouse model of breast cancer bone metastases

B. Training Accomplishments

- Shadowed Dr Kara Kort M.D., breast cancer surgeon at University Hospital. Sat in on patient consults, reviewed mammograms and MRI's, discussed treatment options for various types of breast cancer (ER+ versus ER-, age of the patient, family and health history, etc.).
- Attended monthly multidisciplinary breast cancer conferences with medical oncologists, radiation oncologists, and surgeons discussed complex breast cancer cases and treatment options.
- Attended the Carol M. Baldwin Breast Cancer Seminar
- Attended the Eighth and Valedictory Workshop on Bisphosphonates – From the Laboratory to the Patient; Davos, Switzerland
    - Submitted an abstract (Appendix 2) and presented a poster at the workshop
- Received advanced training on the SCANCO µCT 40 scanner at SCANCO Medical; Basserdorf, Switzerland
- Attended the VI International Meeting on Cancer Induced Bone Disease; San Antonio, TX
    - Submitted an abstract (Appendix 3) and presented a poster at the meeting
    - Received a travel grant based on my abstract
- Gave a seminar at SUNY Upstate Medical University sponsored by the department of Neuroscience and Physiology entitled *Adjuvant Therapy with Zoledronic Acid and PTH: Safe and Effective for the Treatment of Osteolytic Bone Metastases in the Mouse*; October 19, 2006

4. Reportable Outcomes

1. Arrington, S.A.; Schoonmaker, J.E.; Damron, T.A.; Mann, K.A.; Allen, M.J. (2006). Temporal changes in bone mass and mechanical properties in a murine model of tumor osteolysis. BONE 38, 359-367.

2. Arrington, S.A.; Schoonmaker, J.E.; Mann, K.A.; Damron, T.A.; Sledz, T.; Willick, G.; and Allen, M.J. (2006) Zoledronic acid and PTH increase bone mass and mechanical strength following radiation therapy for osteolytic bone metastases • ABSTRACT BONE 38, Supplement 1, S43 Poster presented at the Eighth and Valedictory Workshop on Bisphosphonates: From the laboratory to the patient. Davos, Switzerland.

3. Arrington, S.A.; Fisher, E.R.; Gasser, J.A.; Willick, G.; Allen, M.J. (December 2006) Zoledronic Acid and PTH Increase Survival and Quality of Life Following Radiation Therapy for Osteolytic Bone Metastases. Poster Presented at VI International Meeting on Cancer Induced Bone Disease. San Antonio, Texas.

5. Conclusion

Through the experiments completed so far we have been able to demonstrate that adjuvant treatment with ZA following radiation therapy is successful in restoring the strength of irradiated bones to that of normal. Our results also demonstrated that there was no significant advantage in combining ZA and PTH for the treatment of an established bone metastasis, therefore this treatment combination will no longer be evaluated. Our *in vivo* work has also indicated that treatment with ZA can cause increased bone mineral density. This finding has led us to explore the cause for this increased mineralization using both *in vitro* and *in vivo* techniques.

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7. Appendices

Appendix 1: Arrington, S.A.; Schoonmaker, J.E.; Damron, T.A.; Mann, K.A.; Allen, M.J. (2006). Temporal changes in bone mass and mechanical properties in a murine model of tumor osteolysis. BONE 38, 359-367.

Appendix 2: Arrington, S.A.; Schoonmaker, J.E.; Mann, K.A.; Damron, T.A.; Sledz, T.; Willick, G.; and Allen, M.J. (2006) Zoledronic acid and PTH increase bone mass and mechanical strength following radiation therapy for osteolytic bone metastases • ABSTRACT BONE 38, Supplement 1, S43 Poster presented at the Eighth and Valedictory Workshop on Bisphosphonates: From the laboratory to the patient. Davos, Switzerland.

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Bone 38 (2006) 359–367

BONE

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Temporal changes in bone mass and mechanical properties in a murine model of tumor osteolysis

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Received 8 April 2005; revised 29 August 2005; accepted 13 September 2005
Available online 8 November 2005

Abstract

Pathological fracture is a devastating complication of osteolytic bone metastases. The progression of osteolysis and its effect on bone fracture risk are poorly understood. The goal of this study was to determine the temporal changes in bone strength following tumor inoculation in a preclinical model of tumor osteolysis. In addition, a predictive model was developed between non-invasive radiographic measures and bone strength. The right femora of female nude mice were injected with breast cancer cells; the left limb served as a sham-operated control. Radiographs and DEXA scans were obtained at the time of surgery and at 3, 6, and 9 weeks. Groups of mice were euthanized at each time point for mechanical assessment. Micro-CT analysis was performed on a sub-set of mice with advanced state disease to quantify bone loss. Radiographs documented an increase in tumor osteolysis over time, with 58% of the mice showing signs of osteolysis at 3 weeks, 75% at 6 weeks, and 81% at 9 weeks. BMD measurements revealed a 21.6% increase from baseline in the controls whereas tumor-injected femora failed to increase in BMD over the same time course. Tumor-bearing limbs exhibited statistically significant decreases in torque at failure (86%), energy to failure (88%), and initial stiffness (94%) compared to the controls. Both lysis scores and BMD measurements proved to be modest predictors of mechanical strength, accounting for approximately 73% and 41% of variation in torque at failure, respectively. Micro-CT analysis revealed decreases in both total bone volume in the distal femur (31%) and metaphyseal fractional trabecular bone (89%). We have shown that non-invasive radiographic techniques provide a useful tool for monitoring the progression of tumor osteolysis and for predicting the mechanical strength of tumor-bearing bones in this model. By integrating non-invasive measures of tumor osteolysis and fracture risk, we have validated a clinically relevant platform for evaluating new therapeutic approaches for preserving and/or restoring bone affected by metastatic disease.

Keywords: Bone metastasis; Animal model; Osteolysis; Biomechanical testing; Bone densitometry

Introduction

The skeleton is the most common site of metastasis in advanced breast cancer [1–3], with three quarters of all patients expected to develop bone involvement during the course of their disease [4,5]. Currently, the median survival for women with bone metastases from breast cancer is 3 years and the 5-year survival rate is 20% [6]. Major complications associated with bone metastases include severe bone pain, hypercalcemia, and increased risk of pathological fracture. Current treatment for metastatic disease typically involves systemic cytotoxic or endocrine chemotherapy combined with local radiation therapy to palliate bone pain. Bisphosphonates are now commonly used to suppress tumor-driven osteoclastic bone resorption and reduce the risk of skeletal complications in breast cancer patients with bone metastases [7–9]. However, even when treatments are effective in controlling tumor growth and palliating bone pain, over 50% of patients will still develop skeletal complications that require additional medical or surgical intervention [10–12]. Clinical data indicate that nearly half of all tumor-related pathological fractures are the result of breast cancer bone metastasis.

Animal models play an important role in the development and evaluation of new cancer therapies [13,14]. Several models are commonly used to study the process of breast cancer metastasis; including intracardiac [15], tail vein [16], and mammary fat pad [17] injections of breast cancer cells. In these models, the progression of tumor spread can be studied, and therapies

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doi:10.1016/j.bone.2005.09.013

targeted at preventing metastasis can be investigated. The intracardiac model is the most widely used method for evaluating the progression of bone metastases. Although this model has a high incidence for developing bone metastases, the exact location in bone and tumor burden cannot be controlled. Occasionally, these animals frequently develop spinal metastases which lead to paralysis and early euthanasia. In order to study the effect of therapies on established bone metastases, an alternative approach is required. The direct injection of tumor cells into the bone does not replicate the pathway by which tumor cells metastasize to bone, but it does allow for a relatively defined tumor burden in the same location, thus making experiments reproducible. This approach may also decrease the occurrence of metastatic spread to other organs, thereby making it possible to study animals with isolated bone metastases for relatively longer periods of time.

Wang et al. [18] demonstrated the temporal pathogenesis of human breast cancer cells in a murine model of tumor osteolysis. In this model, tumor cells were injected directly into the medullary canal and tumor progression was assessed qualitatively using histology. Clohisy et al. [19] used a similar approach to study the cellular response to tumor inoculation and identified osteoclasts as playing a key role in tumor osteolysis. However, neither of these studies explored the effects of tumor on bone strength and its clinical correlate, fracture risk. In more recent work, Kurth et al. [20] reported on a model in which a gel carrier was used to implant rat mammary carcinoma cells into the femur of Sprague–Dawley rats. Using this model, the authors demonstrated a weak to moderate correlation between BMD and mechanical strength in bones harvested at a single time point of 4 weeks following tumor inoculation.

The specific aim of the present study was to perform a more complete investigation into the relationship between tumor growth, bone loss and changes in bone strength over time. For this purpose, we elected to use a validated murine model, based on the work of Wang et al. and Clohisy et al. [19], in which human breast cancer cells are injected into the distal femur of nude mice. The experimental work was conducted in two stages: an initial validation of the temporal progression of osteolysis, followed by a definitive longitudinal study in which serial radiographs and DEXA scans were used to monitor the effects of tumor on bone integrity and bone mass. At the conclusion of the study period, the effects of tumor cells on bone microarchitecture and bone strength were characterized by micro-CT analysis and destructive mechanical testing respectively. We hypothesized that expansion of tumor cells within the metaphyseal region would cause measurable disturbances in the normal pattern of age-related bone accrual. Furthermore, we hypothesized that BMD measurements could be used as a non-invasive predictor of the mechanical properties of diseased bones.

Materials and methods

Study design

An initial group of 34 mice was used to assess the temporal pattern of osteolysis and BMD changes in response to the tumor. Subsets of animals were used to document the relationship between histological evidence of tumor growth and radiographic signs of osteolysis (n = 6) and to provide micro-CT data on the effects of tumor on bone architecture (n = 6 mice).

Based on these data, a definitive study was then initiated in which 24 mice were radiographed and underwent DEXA scans at the time of tumor injection and at 3, 6, and 9 weeks. At each time point, subsets of animals were euthanized and bones evaluated by torsional mechanical testing. Mice were euthanized before their end of study time point if (1) their X-rays indicated impending fracture, (2) an animal displayed severe lameness, or (3) the animal had a 20% drop in weight. All experimental procedures used in this study were reviewed and approved by the local IACUC.

Tumor cells

F10 cells, a bone-adapted clone derived from the human MDA-MB-231 breast carcinoma cell line [21] (Dr. Toshiyuki Yoneda, UT San Antonio, TX) were used for this study. Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% penicillin–streptomycin–glutamine (Gibco, Grand Island, NY) and maintained at 37°C in an atmosphere of 5% CO_2 in air.

Tumor cell inoculation

Female NCr homozygous nude mice (8 to 9 weeks old, 20–25 g body weight, n = 58) (Charles River, Wilmington, MA) were anesthetized with an injectable drug cocktail consisting of telazol (45mg/kg, IM) and xylazine (7.5 mg/kg, IM). The mice were weighed and the hind limbs scrubbed with betadine followed by 70% ethanol. With the knee flexed, a small incision was made at the joint and the patella carefully moved to expose the distal end of the femur. Using a sterile 26G needle, 20 μl of cell suspension (containing 1×10^5 F10 breast cancer cells) were injected through the intercondylar fossa of the right femur to a depth of approximately 5 mm. The contra-lateral limb, which served as an internal control, was injected with 20 μl of vehicle (culture medium) following the same procedure. Mice were housed in micro-isolators with a 12-h day/night cycle and were fed a diet of autoclaved food and water ad libitum.

Radiographic evaluation of osteolysis

Anterior–posterior radiographs were obtained at time of surgery and then at 3, 6 and 9 weeks using a Faxitron MX-20 (Faxitron X-ray Corporation; Buffalo Grove, IL) and MIN-R 2000 mammography film (Kodak; Rochester, NY). Radiographs are used in clinical practice to evaluate the extent of osteolysis and to predict the risk of impending pathological fracture in patients with bone metastases. A standardized scoring system was used to evaluate radiographic lesions in the femur. This scoring system was selected because it not only distinguished between the size of the lesion, but also its location and whether or not the tumor perforated through the cortex. A grade of zero represented no lysis, 1+ indicated minimal but detectable lysis within the medullary canal, 2+ represented moderate lysis limited to the medullary canal, 3+ reflected severe medullary lysis with cortical involvement, and 4+ indicated massive lysis with cortical destruction and soft tissue extension [22]. Radiographs were evaluated by two independent reviewers, which were then averaged and used for analysis. A linear regression was performed between lysis scores and mechanical strength measures. Radiographs were also obtained following mechanical testing to document the site of limb failure.

Bone densitometry

Dual-energy X-ray absorptiometry (DEXA) measurements of the left and right femora were obtained immediately following tumor inoculation and at 3, 6, and 9 weeks using a dedicated mouse bone densitometer (PIXImus 2; GE Lunar; Madison, WI). Bone mineral density values (g/cm^2) were determined from a region of interest that encompassed the entire femur. Linear regression models were used to assess the change in BMD and mechanical properties over time. Regression analysis was performed to determine if there was a relationship between BMD and the mechanical properties in both normal and tumor-burdened bones.

Necropsy and tissue collection

At the time of euthanasia, all mice were weighed, radiographed, and scanned using DEXA. Complete post-mortem necropsies were performed to assess possible metastasis and other pathologies. The hind limbs from all mice were then explanted and the soft tissues removed. For mechanics, the entire limb was wrapped in saline-soaked gauze and frozen at −20°C until testing. For histology, the explanted limbs were disarticulated and the femora placed in 10% buffered neutral formalin overnight.

Histological confirmation of tumor

A subset of femora were embedded in methylmethacrylate (MMA) according to the method described by Erben [23]. Embedded bones were then trimmed on a low-speed diamond saw (Buehler, Lake Bluff, IL) and 5-μm sections were cut using a microtome. Sections were stained with modified Masson's trichrome stain and examined by light microscopy.

Mechanical testing

Torsional testing typically involves embedding ("potting") the ends of the bone in a solid support material (e.g., dental cement or liquid metal) that can be rigidly attached to the mechanical testing device [24]. However, in our model, the distal femur was the primary site of osteolysis and potting this region would affect structural properties. A new test protocol was, therefore, developed for this animal model. Prior to testing, bones were thawed at room temperature and the proximal femur and distal tibia of each mouse were potted in a mold using PMMA such that the knee was held at 90° flexion. Specimens were removed from the mold and placed in a custom testing apparatus in which the proximal femur was secured to a fixed spindle and the distal tibia was attached to a rotating spindle (Fig. 1a). The rotating spindle was attached by high-test line to a materials testing unit (QTest, MTS Corporation, Minneapolis, MN). The tibia was internally rotated at a constant rate (180°/min) and the applied torque and rotation angle were recorded. The primary outcome measures from mechanical testing were torque at failure (N-mm), energy to failure (N-mm-degree), and initial stiffness (N-mm/degree) (Fig. 1b). Torque at failure was defined as a 20% decrease in applied torque from a previous peak or a maximum rotation angle of 70°. Energy to failure (N-mm-degree) was determined by calculating the area under the torque–rotation curve. Initial stiffness (N-mm/degree) was defined as the first 50% of the slope to torque at failure.

Mechanical testing was conducted on 24 sham-operated control (left) limbs and 19 tumor-bearing (right) limbs. Due to increased bone fragility in the tumor-bearing limbs, 4 right femurs had fractured prior to mechanical testing and could not be tested. One right femur selected for mechanics showed no radiographic evidence of tumor, and was therefore excluded. All mechanical tests were conducted at room temperature; bones were kept moist during preparation and testing using saline-soaked gauze. Analysis of variance (ANOVA) with Fisher's PLSD post hoc was used to assess the mechanical strength of normal and tumor-bearing bones with early (0 to 3 weeks post tumor injection) and advanced (6 to 9 weeks post tumor injection) stage disease.

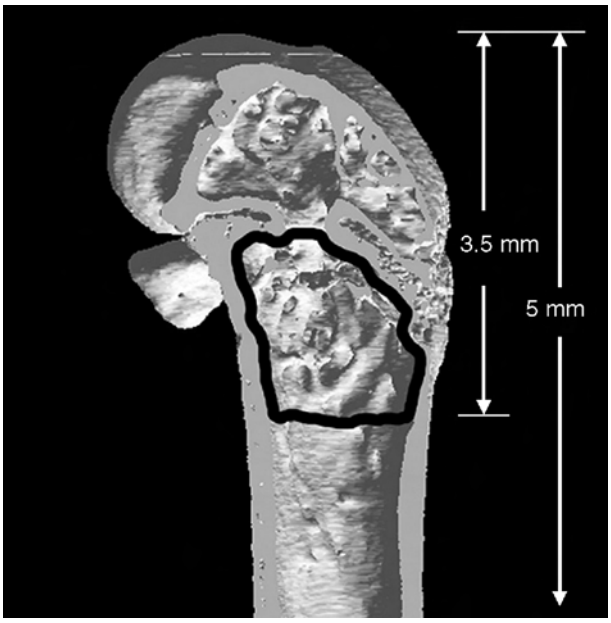

Fig. 2. Representative 2D image illustrating the ROI used to measure fractional trabecular bone volume in the metaphysis. Total bone volume was measured in the distal 5 mm of the bone and included both cortical and trabecular bone.

Micro-CT analysis

Micro-CT analysis was performed to obtain qualitative assessments and quantitative measurements of tumor-induced osteolysis in bones with advanced stage disease. Representative specimens from the control left limbs ($n = 6$) and tumor-inoculated right limbs ($n = 6$) were scanned on a high-resolution micro-CT scanner using a 10.46 μm pixel size (Skyscan 1072® Micro Photonics, Allentown, PA). Data sets were reconstructed using standardized cone-beam reconstruction software, (CONE REC V. 2.15, Skyscan). Measurements of total bone volume in the distal 5 mm of the femur (BV_{tot}) were obtained from transverse slices in which the ROI included both cortical and trabecular bone. For measurements of fractional trabecular bone volume (BV/TV), the distal 3.5 mm of the femora were reconstructed and 50 slices (0.52 mm) were sampled in the sagittal plane from the center of the bone using Ant: 3D-creator V. 2.2 (Skyscan). The trabecular bone ROI was drawn to include all cancellous bone in the metaphysis (Fig. 2). For tumor-burdened bones, where much of the morphology was distorted due to tumor osteolysis, the ROI was drawn to

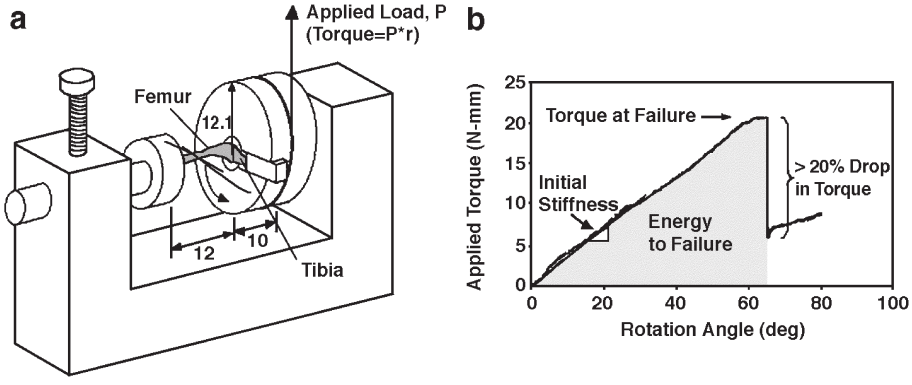

Fig. 1. (a–b) The torque–rotation response was determined for the hind limb using a test system that internally rotated the tibia relative to the femur. This produced axial torques on the femur with loads transmitted through the joint. All dimensions are in millimeters (a). Typical torque–rotation response of the hind limb indicating parameters used to describe structural strength of the mouse femur (b).

approximate the same area as in the control limbs. 2D and 3D analyses of total bone volume, fractional trabecular bone volume, and trabecular architecture measurements were performed using CTanalyzer (Skyscan, V.1.03.2). Paired *t* tests were performed to analyze total bone volume, fractional trabecular bone volume, and trabecular bone morphology between control and tumor-bearing bones.

Results

The original study design consisted of three time points (3, 6, and 9 weeks post-tumor injection) at which radiographic evidence of osteolysis, BMD, and mechanical strength were to be assessed. Due to the extensive bone loss that occurred in the metaphysis by 6 weeks, 24% of the mice selected to go to 9 weeks had to be euthanized due to impending fracture. By week 8, 56% of the 9-week mice were removed from the study (data not shown). This significant loss of animals at the late time points required that the mice be placed in one of two groups, early stage disease (0 to 3 weeks) and advanced stage disease (6 to 9 weeks). It should be noted that none of the mice presented with lesions in distant organs at the time of necropsy.

Radiographic and histological evidence of tumor osteolysis

Tumor osteolysis was progressive and focused in the distal femur (Figs. 3a–d). At 3 weeks post tumor injection, over 58% of the mice presented with radiographic evidence of osteolysis (Table 1). At 6 weeks, 75% of the mice presented with lesions, with almost 44% displaying severe osteolysis with cortical disruption and some extension to soft tissue (lysis rating 3+ to 4+). Of the mice surviving beyond 6 weeks, approximately 81% showed radiographic lesions, with 40% having a lysis rating of 3+ or 4+. Histology was performed on a subset of mice to verify tumor progression. Analysis at 3 weeks revealed small clumps of tumor in the metaphysis with minimal bone derangement (Fig. 4a). At 6 and 9 weeks, progressive tumor growth was observed and associated with advanced bone destruction (Figs. 4b and c). Histology performed in the preliminary study confirmed that mice with no evidence of osteolysis on radiographs by 9 weeks did not have successful tumor take (Fig. 4d). Therefore, in the definitive study, mice that exhibited no signs of osteolysis on radiographs at 9 weeks (1 of 24 or 4%) were excluded.

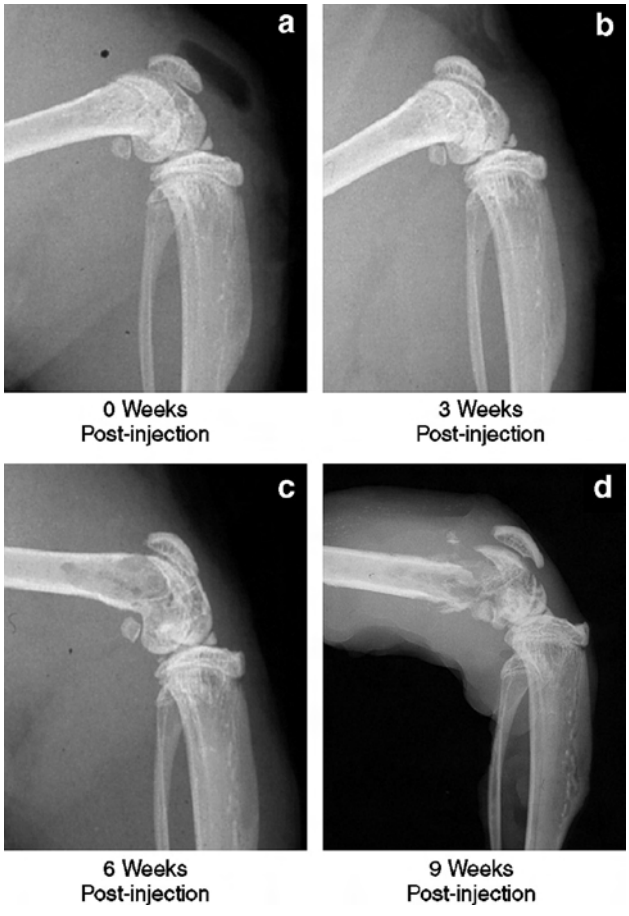

Fig. 3. (a–d) The progression of osteolysis as assessed by radiographs from the time of tumor injection (a) at 3 weeks (b), 6 weeks (c), and 9 weeks (d).

Table 1
Lysis scores over time

Lysis score	0 weeks $N = 58$ (%)	3 weeks $N = 58$ (%)	6 weeks $N = 36$ (%)	>6 weeks $N = 16$ (%)
0	98.3	39.7	25	18.8
1	1.7	55.2	16.7	25
2	–	5.2	13.9	0
3	–	–	30.6	18.8
4	–	–	13.9	37.5

Data represent the percentage of mice with a given lysis score at each time point.

Bone mineral density

Control femora exhibited a time-dependent increase of 21.6% in BMD compared to baseline over the course of the 9-week study ($P < 0.0001$) (Fig. 5). In tumor-injected femora, in which radiographic lesions were observed, no significant change in BMD from baseline was measured. At the 3-week time-point, both the control and tumor-injected femora exhibited a significant increase in BMD ($P < 0.0001$). However, by 6 weeks there were clear differences between controls and femora with radiographic evidence of osteolysis (Fig. 5). Furthermore, by 6 weeks, it was possible to distinguish between femora that would go on to develop radiographic changes and those that would not based on their BMD values. DEXA scans at 6 weeks may therefore provide a means of classifying femora into those that did have successful tumor take and those that did not. Animals that displayed substantial lesions at 6 weeks were often removed from the study prior to 9 weeks due to impending fracture. Therefore, a statistical correlation between the extent of osteolysis, as determined by BMD, and time following tumor inoculation was not possible due to the varying rate of tumor progression between mice.

Mechanical assessment of bone strength

An initial analysis (Fig. 6) of bone strength over time for tumor-burdened bones indicated that data could be separated into two groups: (1) early disease state (0 to 3 weeks) and (2)

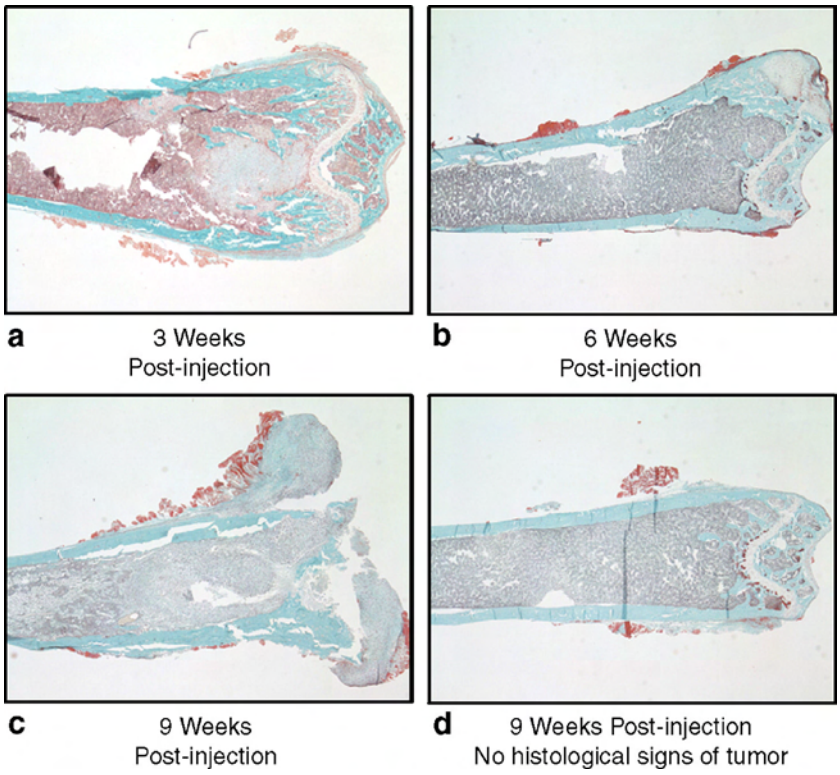

Fig. 4. (a–d) Representative histological sections confirming the presence of tumor in the metaphysis of the femur. At 3 weeks post-injection the tumor is confined to the medullary canal and most of the trabecular bone is unaffected (a). At 6 weeks, tumor has expanded within the medullary canal, but the cortex is still preserved (b). At 9 weeks massive osteolysis is present with cortical destruction and extension of tumor outside of the bone (c). 10% of the animals injected with tumor did not show radiographic signs of lesions, which was confirmed by histology (d).

advanced disease state (6 to 9 weeks). Given the general clustering of data, all statistical analysis was performed by grouping data into these two groups. There was no significant difference in mechanical strength between control limbs and those with early stage disease (Table 2). However, tumor-bearing limbs with advanced stage disease exhibited a significant decrease of 86% in torque at failure (N-mm) (Fig. 6), an 88% decrease in energy to failure (N-mm-degree), and a 94% decrease in initial stiffness in the tumor-bearing limbs compared to the control limbs ($P = 0.0004$).

Post-mechanical test failure analysis revealed that at early time points both the control and tumor-bearing limbs had a 50% chance of failing through either the bone or the ligaments of the knee. This suggests that at early time points the strength of the

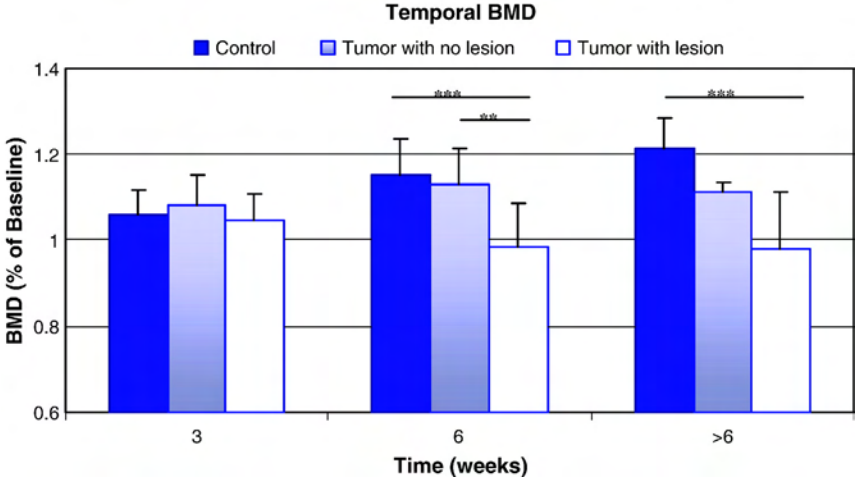

Fig. 5. BMD measurements taken at 3 weeks post tumor-injection demonstrated a significant increase in bone mass compared to baseline (0 weeks) for both control and tumor-injected limbs ($P < 0.0001$). At 6 weeks, femora displaying lesions on radiograph (tumor with lesion group) exhibited a significantly lower BMD compared to both control femora ($P < 0.0001$) and tumor-injected limbs that did not display lesions on radiograph (tumor with no lesion group) ($P < 0.0144$). By 9 weeks, BMD had increased by 21.6% from baseline ($P < 0.0001$) in the control femora, whereas tumor-bearing femora exhibited no change in BMD from baseline. (**$P < 0.01$, ***$P < 0.001$).

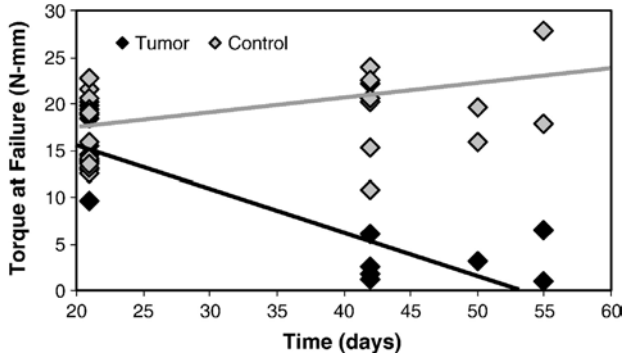

Fig. 6. A representative plot of mechanical strength with time in both sham-injected control limbs ($n = 24$) and limbs injected with 1×10^5 F10 breast cancer cells ($n = 19$).

bone may be higher than was reported here. Beyond 6 weeks, only 16% of the control limbs failed through the bone, suggesting that the control limbs were becoming stronger in comparison to the knee ligaments. The tumor-bearing limbs at late time points had 100% failure through the bone indicating that this model system is measuring bone strength as opposed to knee ligament strength.

Lysis ratings and BMD measurements are moderate predictors of mechanical strength of tumor-bearing bones

Regression analysis revealed that 73% of torque at failure variation ($P = 0.0002$) (Fig. 7a), 59% of energy to failure variation ($P = 0.0003$), and 61% of initial stiffness variation ($P = 0.0002$) could be explained by lysis ratings (Table 3). There was no significant correlation between the mechanical properties of sham-injected control bones and BMD. Tumor-burdened bones however, exhibited a moderate correlation between BMD with both torque at failure ($r^2 = 0.41$, $P = 0.016$)

Table 2
Effects of tumor on the mechanical properties of the femur

	Disease stage	Control		Tumor		ANOVA P value
		Mean	SD	Mean	SD	
Torque at failure, N-mm	Early	17.4	3.98	15.77	3.42	<0.0001
	Advanced	19.8	4.44	2.74	2.09	
Energy to failure, N-mm-degree	Early	541	237	340	108.6	<0.0001
	Advanced	651	214	78.9	46.3	
Initial stiffness, N-mm/degree	Early	0.319	0.098	0.287	0.141	<0.0001
	Advanced	0.414	0.165	0.025	0.017	

No significant difference in mechanical strength was found between sham-injected control bones and tumor bones with early stage disease (0 and 3 weeks). Tumor-bearing bones with advanced stage disease (6 to 9 weeks) significantly decreased in torque at failure, energy to failure, and initial stiffness compared to the control limbs. Tumor-bearing limbs with advanced stage disease also had 100% failure through the bone, where as the control limbs had only 16% failure through the bone. A modified Bonferroni correction was performed to account for multiple sampling. Statistical significance was taken at $P < 0.05$.

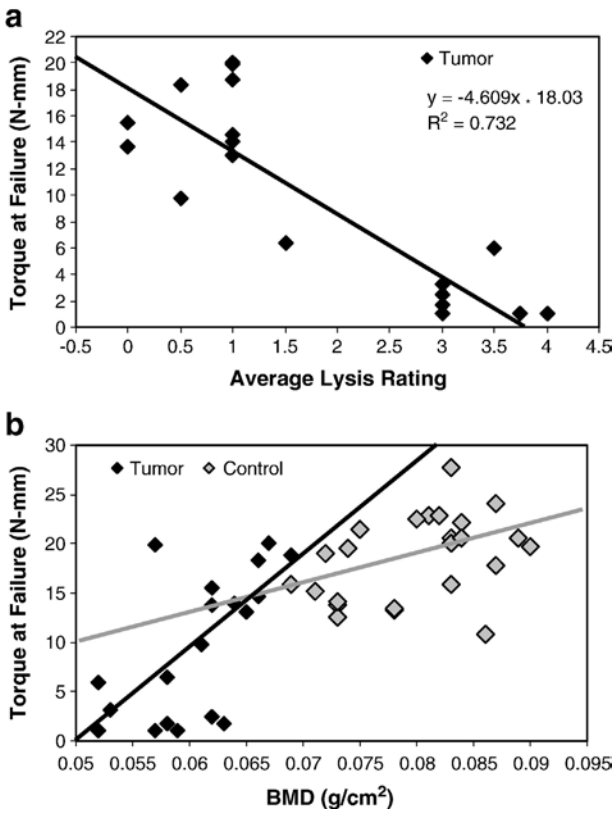

Fig. 7. Regression plots comparing torque at failure to lysis ratings (a) and measures of femoral BMD (b). Lysis ratings were found to predict approximately 73% of torque at failure where terminal BMD predicted about 41% of torque at failure in tumor-bearing bones. Tumor: Torque at Failure = −4.61 * (Lysis Rating) + 18.0, $r^2 = 0.73$. Tumor: Torque at Failure = 912 * (BMD) − 45.8, $r^2 = 0.41$. Control: Torque at Failure = 280 * (BMD) − 3.8, $r^2 = 0.16$.

(Fig. 7b) and energy to failure ($r^2 = 0.41$, $P = 0.016$). No significant correlation could be made with initial stiffness ($r^2 = 0.24$, $P = 0.1023$) (Table 4). Therefore in this model, lysis rating and BMD may be used as modest predictors of mechanical strength in tumor-burdened bones.

Micro-computed tomography analysis of tumor osteolysis

Three-dimensional micro-CT reconstructions of the bones clearly demonstrated severe cortical and trabecular destruction

Table 3
Summary of the correlation between mechanical strength and radiographic lysis ratings

	Group	A * Lysis + B		r^2	ANOVA P value
		A	B		
Torque at failure, N-mm	Tumor	−4.37	17.7	0.66	0.0002
Initial stiffness, N-mm/degree	Tumor	−0.10	0.62	0.62	0.0002
Energy to failure, N-mm-degree	Tumor	−86.6	376	0.55	0.0003

Analysis revealed that lysis ratings could predict between 59% and 73% of the mechanical strength of tumor-burdened limbs. A modified Bonferroni correction was performed to account for multiple sampling. Statistical significance was taken at $P < 0.05$.

Table 4
Summary of the correlation between BMD and the mechanical properties of normal and tumor-burdened femora

	Group	A * BMD (mg/cm^2) + B		r^2	ANOVA *P* value	ANCOVA *P* value
		A	B			
Torque at failure, N-mm	Normal	280	−3.8	0.16	0.136	<0.0001
	Tumor	912	−45.8	0.41	0.016	
Initial stiffness, N-mm/degree	Normal	7.77	−0.26	0.11	0.212	<0.0001
	Tumor	16.0	−0.81	0.24	0.156	
Energy to failure, N-mm-degree	Normal	9500	−163	0.07	0.227	<0.0001
	Tumor	19,732	−981	0.41	0.016	

No correlation was found between normal bones and BMD. In tumor-burdened bones, both torque at failure and energy to failure exhibited a moderate correlation to BMD. A modified Bonferroni correction was performed to account for multiple sampling. Statistical significance was taken at $P < 0.05$.

in tumor-bearing limbs (Fig. 8). Based on radiographic analysis of the tumor-burdened femora, most of the osteolysis occurred within the distal 5 mm; therefore, this region was selected for total bone volume assessment. Analysis using a paired *t* test showed that tumor-burdened bones had an average decrease of 31% in total bone volume (BV_{tot}, $P = 0.0416$). Trabecular bone located in the metaphysis may be more prone to tumor osteolysis than cortical bone due to its faster turnover rate. To evaluate the extent of damage caused by the tumor in the metaphysis of the femur, additional analysis of the trabecular bone was performed. Paired *t* tests revealed a significant decrease of 89% in fractional trabecular bone volume (BV/TV, $P = 0.0045$), trabecular number (Tb.N, $P = 0.0044$) and fractional trabecular surface area (BS/TV, $P = 0.003$) in tumor-burdened bones (Table 5). Further analysis revealed a 127% increase in trabecular pattern factor (TbPf, $P = 0.0486$), which represents a significant decrease in trabecular bone connectivity [25]. Two mice were excluded from this final analysis because no discernable trabecular bone was found in their femora; therefore a TbPf could not be calculated. Based on the significant decrease in trabecular bone, future experiments will focus on determining the correlation between trabecular bone architecture and mechanical strength.

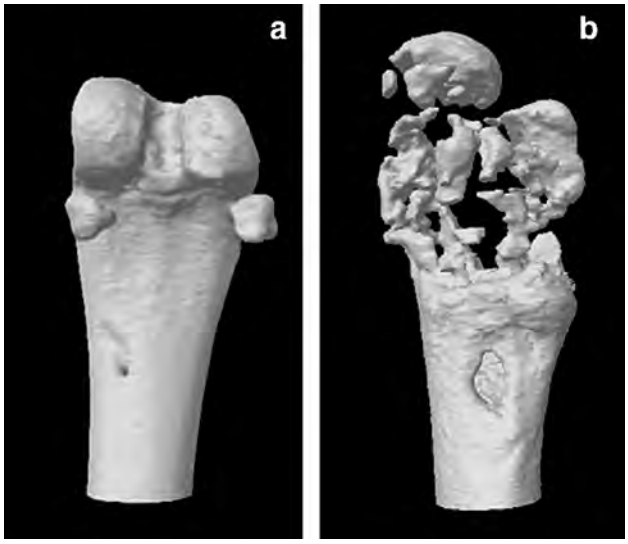

Fig. 8. Micro-CT images of the distal 5mm of a normal left femur (a) and a tumor-burdened right femur (b). Massive disruptions of the cortical wall and trabecular bone as well as extensive fragmentation were clearly evident in the tumor-bearing limbs.

Table 5
Micro-CT analysis of total bone (BV_{tot}) and trabecular bone parameters in normal and tumor-burdened femora

	Group	Mean	SD	*t* test *P* value
BV_{tot} (mm^2)	Normal	6.88	1.06	0.042
	Tumor	4.74	1.79	
BV/TV (%)	Normal	9.88	3.55	0.005
	Tumor	1.1	1.91	
BS/TV (%)	Normal	6.83	1.75	0.003
	Tumor	0.77	1.07	
Tb.N. (mm^{-1})	Normal	1.48	0.46	0.004
	Tumor	0.16	0.24	
Tb.Pf. (mm^{-1})	Normal	25.3	6.12	0.049
	Tumor	57.3	23.9	

Tumor-bearing bones exhibited a significant decrease in total bone volume, fractional trabecular volume, fractional trabecular bone surface, and trabecular number compared to the control at time points greater then 6 weeks. A significant increase in trabecular pattern factor was also observed in the tumor-bearing animals, indicating a loss of connectivity. A modified Bonferroni correction was performed to account for multiple sampling. Statistical significance was taken at $P < 0.05$.

Discussion

The specific aims of this study were to quantify the progression of tumor osteolysis using non-invasive measures (radiography and DEXA) and to quantify the mechanical strength of bones with both early and advanced osteolysis. We also sought to determine whether it was possible to correlate non-invasive estimates of tumor osteolysis with direct measures of the mechanical strength of diseased bones. For the mouse model to be clinically relevant, osteolytic changes should be centered on the metaphyseal region of the distal femur. Radiographic and histological evaluation confirmed that the metaphysis was the site of osteolysis in this model. This model had a high percentage of tumor take with 81% of mice exhibiting osteolysis at 9 weeks and was very aggressive, with 56% of the mice assigned to the 9-week group requiring early euthanasia due to impending fracture.

To assess the progression of tumor osteolysis longitudinally, X-rays and BMD measurements were taken every 3 weeks post tumor-injection for up to 9 weeks. Lysis scores demonstrated an increase in both the incidence and severity of lesions over time. Radiographic signs of osteolysis were seen in just over half of the animals at 3 weeks, in 75% by 6 weeks and in 81% of the animals that survived to 9 weeks. The scores also confirmed an increase in the severity of osteolysis over 9 weeks, with 56% of the animals presenting with massive destruction after 6 weeks. DEXA measurements indicated that non-tumor-bearing femora

increase their BMD from baseline by >20% over 9 weeks. In contrast, tumor-burdened bones exhibited no apparent change in BMD from baseline over the same time period. This observation can be explained by the way in which the data were analyzed. The small size of the mouse femur complicates bone density measurement in that it is extremely hard to generate very small regions of interest, for example, with the metaphyseal bone only. In tumor-burdened bones histological and radiographic analyses confirmed significant net bone loss, but this loss was generally confined to the metaphyseal region. As such BMD measurements that represent an average for the entire bone will under-represent the extent of focal bone loss in any one sub-region. Nevertheless, our finding of no net bone accrual in tumor-burdened bones provides clear evidence of a substantial and sustained disturbance in bone remodeling. These data highlight the potential value of non-invasive techniques, such as DEXA in assessing the effects of tumor on skeletal remodeling over time.

In this study, we were able to distinguish between animals with early and advanced stage disease through both radiographic and mechanical analysis. Tumor-bearing bones analyzed between 0 and 3 weeks showed minimal signs of osteolysis on radiographs, had 50% fracture through the bone, and mechanical strength similar to that of the control bones. Analysis at 6 weeks and greater revealed advanced osteolysis on radiographs, 100% failure through the bone during mechanical testing and an average decrease of 86% in torque at failure compared to the control limbs. Mechanical testing of the limb through the intact knee joint therefore proved to be a sensitive measure for assessing the strength of tumor-burdened bones.

In a clinical setting, X-rays and DEXA scans are commonly used to determine bone mass and skeletal integrity. We were therefore interested in seeing whether these widely available non-invasive measures could predict mechanical strength in tumor-burdened bones in mice. Regression analysis revealed that lysis ratings could explain 73% of the variation in torque at failure, while BMD could explain 41% of the variation in torque at failure. One limitation of these clinical outcome measures is that they provide little information on the exact location and amount of destruction caused by tumor osteolysis. Detailed analysis regarding alterations to the bone micro-architecture as a result of tumor osteolysis would aid in the development of therapies aimed at improving bone strength.

A limitation of the current model system is that a single focal metastatic lesion to one site in the skeleton was used. In clinical practice, lesions are often multiple and are disseminated to numerous sites. Further, differences in bone morphology, loading direction, and relative distribution of cortical and trabecular bone may all confound the predictive capability of the non-invasive assessment of bone strength. Definitive proof of the relationship between these non-invasive measures and functional bone strength can only be confirmed by detailed post-mortem analyses in patients that die with metastatic disease. The relationships developed in this study between radiographic scoring, whole bone BMD, and bone strength would therefore only be relevant for this animal model system. However, this approach would be useful as a tool to monitor fracture risk with this laboratory animal model and could also point to mechanisms by which the pathological fractures occur. Ultimately, these data should lead to the development of algorithms that will accurately predict fracture risk in patients with focal or multifocal metastases.

In animal models, micro-CT provides a great deal of information about the structural properties of bone, including total bone volume, fractional trabecular bone volume, and the microarchitecture (connectivity, rod versus plate configuration) of trabecular bone [25]. Using our model we analyzed bones with advanced stage disease. We found that the bone-adapted human breast cancer cells caused a 31% decrease in total bone volume and an 89% decrease in fractional trabecular bone at the distal end of the femur as compared to control bones. This decrease in trabecular bone was further analyzed to gain information about the structure of the remaining bone. This analysis revealed a dramatic decrease in trabecular bone connectivity, which may have a significant affect on bone strength [26]. Future studies will be designed to more fully analyze the correlation between the architecture of trabecular bone, such as connectivity, and mechanical strength through whole-limb torsional testing.

This model also has the potential for investigating the effects of adjunct therapies to radiation aimed at preventing further bone resorption and the stimulation of new bone formation. Anti-resorptive agents, such as bisphosphonates, have become a standard of care for treating patients with bone metastases. Although bisphosphonates appear to be effective in preventing further bone destruction, little work has been done to assess how these agents impact the microstructure and mechanical properties of bone. Through the use of the mechanical test developed in this study and the use of micro-CT, future work will focus on addressing how various adjunct therapies to radiation affect the bone microenvironment and how these changes impact the mechanical properties of tumor-burdened bones.

Through this study we have confirmed that the intraosseous injection of breast cancer cells into the mouse femur provides a useful model in which to study therapies aimed at treating established bone metastases. We have shown that this model has high tumor take with 81% of the mice developing osteolysis. We have also demonstrated that non-invasive techniques, such as radiography and DEXA, provide a means of monitoring the progression of tumor osteolysis in this mouse model. Mechanical testing through the intact knee joint also allowed for a sensitive measure of bone strength in tumor-burdened bones. The use of micro-CT in bones with advanced osteolysis provided detailed information on the micro-architecture in 3D, which was not attainable by any other measure. Future work using micro-CT analysis combined with mechanical data will provide insight into how modifications in the bone micro-environment impact the strength of the bone. Correlations found between the extent of osteolysis, BMD and microstructure of bone and mechanical testing will allow for a more accurate prediction of fracture risk and improve the treatment of patients with bone metastases.

Acknowledgments

The authors thank Dr. Toshiyuki Yoneda, D.D.S., Ph.D. for providing the F10 breast cancer cells and for consultation regarding selection of the animal model. The authors would also like to thank Timothy Sledz at Micro Photonics for the use of his Skyscan 1072 micro-CT scanner. Grant support: New York State Department of Health, Orthopaedic Research and Education Foundation, Carol M. Baldwin Breast Cancer Research Fund.

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3

Optimal timing of bolus intravenous zoledronic acid in a rat fracture model

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This study investigated the use of single dose bisphosphonate therapy (Carbon-14 labeled zoledronic acid) administered at different time points to improve fracture repair by transiently interfering with bone catabolism.

A closed femoral fracture was created in male Wistar rats. Five treatment groups were investigated (n = 15 per group): saline control group, local ZA at the time of fracture (0.01mg/kg), single IV ZA at the time of fracture (0.1 mg/kg), single IV ZA one week after fracture (0.1 mg/kg), and single IV ZA two weeks after fracture (0.1 mg/kg). Rats were sacrificed at six weeks.

Regardless of administration time, single IV ZA significantly increased callus BMC, volume and mechanical strength. Intravenous ZA at the time of fracture resulted in a callus strength increase of 30% compared to controls ($P < 0.05$). In comparison, when the IV dose was given at one or two weeks after fracture, the mechanical strength was increased by 44% and 50% respectively compared to controls ($P < 0.05$). No significant differences were seen with local injection.

Autoradiographic analysis indicated that ZA binds to bone present at the time of administration; new bone formed after administration contains little ZA. Quantification ZA in fractured and non-fractured femoral diaphyses indicated that administration post-fracture significantly increased the uptake efficiency in the callus.

These results suggest that the optimal time for single dose ZA administration after a closed fracture is one to two weeks in this rat fracture model. Delayed administration appears to increase mechanical strength in addition to reducing systemic exposure due to increased ZA uptake in the callus.

doi:10.1016/j.bone.2005.12.024

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Zoledronic acid and PTH increase bone mass and mechanical strength following radiation therapy for osteolytic bone metastases

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The specific aim of this study was to quantify the effects of a bisphosphonate (zoledronic acid, ZA; Novartis Pharma AG, Basel) alone or in combination with an anabolic agent (PTH 1-34) on bone mass, microarchitecture, and mechanical properties following radiation therapy (RTX) for an osteolytic bone metastasis. The right distal femora of 22 female nude mice were injected with 1×10^5 F10 human breast cancer cells. Mice were divided into four groups: non-irradiated (0Gy, n = 4), 20Gy (20Gy, n = 6), 20Gy plus ZA (ZA, n = 6), and 20Gy plus PTH and ZA (PTH/ZA, n = 6). The non-operated limb of the 0Gy mice was the control (n = 4). RTX was administered 3 weeks post-injection. ZA (100 μg/kg sc) was given once weekly for 6 weeks starting three days before irradiation. PTH (80 μg/kg sc) was given 5 days a week for 4 weeks starting the day after irradiation. ANOVA and Fisher's PLSD were performed to analyze comparisons between groups. Mice in the ZA and PTH/ZA groups exhibited increased BMD (19% and 34%, respectively $P < 0.05$) and increased bone strength (115%, $P < 0.05$ and 92% $P = 0.065$, respectively) compared to 20Gy only, still mechanical strength was below normal. The PTH/ZA group exhibited a 3-fold increase in BV/TV compared to the 20Gy only group (P = 0.003). These results clearly demonstrate that ZA used as an adjuvant to radiation leads to clinically relevant increases in mechanical strength. Combination of an anti-resorptive drug and an anabolic agent as adjuncts to RTX significantly increase bone mass and trabecular bone volume. Future work will focus on improving bone quality and strength by altering the timing of adjuvant therapies.

doi:10.1016/j.bone.2005.12.025

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Effects of ibandronate and zoledronic acid in a model of inflammatory pain

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Bisphosphonates have well-documented effects on metastatic bone pain. However, agents differ regarding the extent and duration of their effects. A rat model of hindpaw inflammatory pain has been used to compare ibandronate and zoledronic acid. Animals were injected with complete Freund's adjuvant (CFA) on Day 0, with intraperitoneal injections of ibandronate 2.0 mg/kg or zoledronic acid 1.0 mg/kg administered on Days 1–3. Inflammatory edema (plethysmometry), hyperalgesia (Randall-Selitto paw-withdrawal test), and hindpaw levels of prostaglandin E2 (PGE2) and substance P (SP), were assessed on Days 3 and 7. Both ibandronate and zoledronic acid significantly reduced hindpaw edema, although effects were more prolonged with ibandronate ($P < 0.05$ vs CFA alone on Days 3 and 7, compared with Day 3 only for zoledronic acid). Hindpaw hyperalgesia was also significantly reduced by both agents, with a more rapid ibandronate effect ($P < 0.05$ vs CFA alone from Day 3 onwards compared with Day 7 for zoledronic acid). Neither ibandronate nor zoledronic acid affected PGE2 levels. However, both agents significantly attenuated CFA-induced increases in hindpaw SP, with ibandronate having a more rapid effect ($P <$ 0.05 vs CFA alone on Days 3 and 7 compared with Day 7 only for zoledronic acid). This study shows that both ibandronate and

Zoledronic Acid and PTH Increase Survival and Quality of Life Following Radiation Therapy for Osteolytic Bone Metastases

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The aim of this study was to evaluate the effectiveness of Zoledronic Acid (ZA) following radiation therapy (RTX) in decreasing bone loss and improving survival for patients with bone metastases. Additionally, we sought to determine if the beneficial effects of ZA could be enhanced by the sequential use of an anabolic agent, PTH 1-34. The right distal femora of 24 female nude mice were injected with $2x10^4$ F10 human breast cancer cells. Mice were divided into four groups: non-irradiated (0Gy, n=6), 20Gy only (20Gy, n=6), 20Gy plus ZA (ZA, n=6), and 20Gy plus ZA and PTH (ZA/PTH, n=6). The left non-operated limb of the 20Gy mice served as the control (n=6). RTX was administered to the right femur 4 weeks post-tumor inoculation. Mice were injected with either 12.5ug/kg of ZA weekly for 8 weeks (ZA) or 25ug/kg ZA weekly for 4 weeks (ZA/PTH). ZA/PTH mice were then injected with 80ug/kg of PTH 5 days/week for 4 weeks. X-rays and DEXA scans were obtained at 0, 3, 6, 9 and 12 weeks post-tumor inoculation. Mice were euthanized at 12 weeks or earlier if severe lameness or pathology occurred. ANOVA with Fisher's PLSD or Chi-Square were performed to compare groups. Mice treated with ZA or ZA/PTH survived an average of 39 days longer than the 0Gy mice ($p<0.05$) and 16 days longer than 20Gy only ($p<0.05$). Treatment with ZA/PTH significantly decreased the frequency of osteolysis, metastatic lung lesions, and the growth of extraosseous tumor compared to untreated mice ($p<0.05$). Bone mineral density (BMD) at 6 weeks was significantly lower in the untreated group compared to all of the treated groups. By 12 weeks mice treated with ZA or ZA/PTH had significantly higher BMD compared to those treated with 20Gy ($p<0.05$). Future work will investigate the effect of this treatment regime on bone microarchitecture and strength.