Bone-Induced Expression of Integrin β3 Enables Targeted Nanotherapy of Breast Cancer Metastases

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Abstract

Bone metastases occur in approximately 70% of metastatic breast cancer patients, often leading to skeletal injuries. Current treatments are mainly palliative and underscore the unmet clinical need for improved therapies. In this study, we provide preclinical evidence for an antimetastatic therapy based on targeting integrin β3 (β3), which is selectively induced on breast cancer cells in bone by the local bone microenvironment. In a preclinical model of breast cancer, β3 was strongly expressed on bone metastatic cancer cells, but not primary mammary tumors or visceral metastases. In tumor tissue from breast cancer patients, β3 was significantly elevated on bone metastases relative to primary tumors from the same patient (n = 42). Mechanistic investigations revealed that TGFβ signaling through SMAD2/SMAD3 was necessary for breast cancer induction of β3 within the bone. Using a micelle-based nanoparticle therapy that recognizes integrin αvβ3 (αvβ3-MPs of ~12.5 nm), we demonstrated specific localization to breast cancer bone metastases in mice. Using this system for targeted delivery of the chemotherapeutic docetaxel, we showed that bone tumor burden could be reduced significantly with less bone destruction and less hepatotoxicity compared with equimolar doses of free docetaxel. Furthermore, mice treated with αvβ3-MP-docetaxel exhibited a significant decrease in bone-residing tumor cell proliferation compared with free docetaxel. Taken together, our results offer preclinical proof of concept for a method to enhance delivery of chemotherapeutics to breast cancer cells within the bone by exploiting their selective expression of integrin αvβ3 at that metastatic site.

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Introduction

Bone metastases occur in approximately 70% of metastatic breast cancer patients (1), often leading to the development of significant skeletal complications, such as pathologic fracture, spinal cord compression, or bone pain (2). Current treatments, radiation, surgery, chemotherapy, and antiresorptive drugs, can improve the quality of life but are rarely curative, with limited effect on overall survival (3–5). Treating breast cancer bone metastases has proven difficult due to frequent dissemination of metastases throughout the skeleton, a lack of tumor-specific targets expressed across breast cancer subtypes, and the chemoprotective nature of the bone microenvironment (2, 6, 7).

One approach to overcome these problems has been to enhance drug delivery to bone, most commonly by using bone matrix–targeted hydroxyapatite-avid bisphosphonates (8–10). Bisphosphonates are standard of care in patients with bone metastases or osteoporosis for their ability to inhibit osteoclast function and formation (1, 2, 10). While osteoclast inhibition can attenuate tumor-associated bone destruction with secondary effects on bone tumor growth, it does not improve overall survival in metastatic breast cancer patients (11, 12). In line with this, bisphosphonate-targeted chemotherapy delivery can reduce mouse models of breast cancer–associated bone loss more effectively than free-drug chemotherapy (13, 14).
However, bisphosphonate-targeted drug delivery indiscriminately accumulates on all skeletal bone matrix and primarily targets osteoclasts and neighboring marrow cells near the bone matrix (8, 15). To improve drug uptake by bone metastases, we sought to target tumor cells in the bone microenvironment more directly.

One targeting candidate of interest was the integrin αvβ3. Integrins are heterodimeric cell surface receptors, composed of an α and β subunit from a large family of subunits, that bind to components of the extracellular matrix or to other cells (16). Integrins are expressed in a cell-specific and context-dependent manner, and are critical to all aspects of cancer progression and metastasis through effects on migration, invasion, and cell survival (16–18). Specifically, integrin αvβ3 is composed of the tightly regulated integrin subunit β3 and the more widely expressed αv subunit. Expression of αvβ3 on most cells in the body is typically low, but is elevated on several cancer types (17), as well as a variety of cell types that are important within the tumor microenvironment, such as osteoclasts (11, 12), neoangiogenic endothelium (19), and tumor-promoting macrophages (20). The expression of αvβ3 on these cells led to the investigation of αvβ3 as a therapeutic target for cancer treatment (21). In several clinical trials, pharmacologic inhibition with αvβ3 antagonists as a single-agent cancer therapy did not demonstrate a significant clinical effect (22, 23). We, and others, have demonstrated that the inefficiency of αvβ3 inhibitors in advanced cancer may be due to the inherent complexities of integrin signaling and off-target effects of αvβ3 antagonism (20, 24).

Rather than aiming to antagonize αvβ3 function, we sought to utilize αvβ3 as a molecular target for enhancing drug delivery to cells of interest within the bone. Integrin β3 and αvβ3 expression has been observed on human breast cancer bone metastases (25–27), and ectopic overexpression of tumor β3 on breast cancer has been demonstrated to enhance tumor establishment in bone (26, 28). However, for the purpose of targeting αvβ3, physiologic expression on breast cancer during tumor growth and metastasis has not been defined. Here, we report that the bone microenvironment preferentially induces integrin β3 on breast cancer metastases, as compared with the primary tumor or visceral metastases, and we identify TGFβ to be responsible for this induction. Utilizing this information, we evaluated nanoparticledrugged delivery targeted against integrin αvβ3. We recently developed phospholipid/polysorbate-80 micelle nanoparticles (MPs, ~12.5 nm) for their small size and unique mechanism of “contact-facilitated drug delivery” (29); in this study, we demonstrate that integrin αvβ3-targeted micelle nanoparticles (αvβ3-MPs) carrying the chemotherapeutic docetaxel reduce bone metastases and tumor-associated bone destruction more effectively than free docetaxel. Collectively, we demonstrate that αvβ3 is a tumor target on breast cancer bone metastases and provides support for safer, more effective therapies against this often incurable disease by targeting integrin αvβ3.

Materials and Methods

Animals

Animal studies were performed according to the guidelines established by the Washington University Institutional Animal Care and Use Committee. PyMT-Bo1 tumor cells were implanted into female C57BL/6 mice, or 4T1 tumor cells were implanted into female BALB/c mice. All mice were obtained from The Jackson Laboratory, and injected at 6–7 weeks of age. All rodents were housed according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine (St. Louis, MO). In collaboration with Dr. T.A. Guise, histologic bone sections from female athymic nude mice injected with MDA-MB-231 human breast cancer cells were obtained from an experiment described previously (30).

Cell lines

The murine C57BL/6 PyMT-Bo1 luminal B breast cancer cell line (stably expressing GFP and firefly luciferase genes) was originally isolated from a transgenic MMTV-PyMT breast tumor, as validated and described previously (20). The murine BALB/c 4T1 triple-negative breast cancer cell line was purchased from ATCC, as described previously (31). Human MDA-MB-231 breast cancer cells (HTB-26) were purchased from ATCC (30). All cells were maintained at subconfluence in DMEM with 10% FBS and 0.5% penicillin–streptomycin, in a humidified chamber at standard culture conditions. Low-passage stocks were used and regularly tested for Mycoplasma and maintenance of growth characteristics.

Murine cancer models

To establish onthoptic mammary fat pad (MFP) tumors, 0.1 × 106 tumor cells in 50-μL PBS were injected into MFP tissue of 7-week-old female mice. To establish experimental secondary metastases, 0.05 × 106 tumor cells in 50-μL PBS were intracardially injected into the left ventricular chamber of 6-week-old female, with one exception; in collaboration with Dr. T.A. Guise, human MDA-MB-231 tumor cells were intracardially injected (0.1 × 106 tumor cells in 100-μL PBS) into 4-week-old female athymic nude mice, as described previously (30).

Synthesis of αvβ3-targeted micelle nanoparticles

Phospholipid/polysorbate-80 micelle nanoparticles (MP) were prepared as a microfluidized suspension of 20% (v/v), combining polysorbate Tween 80 (Sigma Aldrich, Inc.) with a 2.0% (w/v) of a surfactant comixture, and 1.7% (w/v) glycerin in pH 6.5 carbonate buffer, as described previously (29). Optionally, the surfactant comixture included 2.28 mol% of docetaxel-prodrug (DTX-PD), and/or 0.15 mol% of αvβ3-targeted quinoline nonpeptide coupled to phosphatidylethanolamine-PEG2000, with the remaining mol% lecithin. Docetaxel was modified into an Sn2 lipase-labile phosphatidylcholine DTX-PD as described previously (32). For fluorescent labeling, rhodamine-conjugated to phosphatidylethanolamine (0.1 mol%) was incorporated into the lipid surfactant. The surfactant components for each formulation were combined with the polysorbate, buffer, and glycerin mixtures and were homogenized at 20,000 psi for 4 minutes at 4°C with a microfluidics (M110s; Microfluidics, Inc.). The nanoparticles were preserved under inert gas in sterile sealed vials until use. Dynamic light scattering showed a nominal particle size of 12.5 ± 0.8 nm, with polydispersities 0.290 ± 0.03, and an average electrophoretic zeta potential of −3.82 ± 1.23 mV.

The nanoparticle-targeting quinoline nonpeptide specific for integrin αvβ3 was originally developed by Bristol–Myers Squibb Medical Imaging (US patent 6,511,648 and related patents) and coupled to phosphatidylethanolamine-PEG2000, as previously described (29). The quinoline nonpeptide was initially characterized as the 111In-DOTA conjugate RP478 and cyan 5.5 homolog TA145 (33). This αvβ3-targeting ligand is selective for cells...
expressing αvβ3 (IC_{50} = 12 nmol/L), as compared with IC_{50} > 10 μmol/L for αvβ5, αvβ1, or αvββ3 (34). Furthermore, the affinity of the αvβ3-targeting ligand increases 15-fold for activated integrin αvβ3 receptor in the presence of Mn2+ (33). In vitro, pretreating human endothelial cells with the human-specific integrin αvβ3 antibody (LM609) competitively inhibited binding of this αvβ3-targeting ligand (33). This αvβ3-targeted ligand has been applied to the surface of large, vascular-constrained perfluorocarbon nanoparticles (~250 nm), which displayed a specific affinity for αvβ3-expressing angiogenic blood vessels and αvβ3-expressing melanoma cells, in contrast to nanoparticle controls (32, 35, 36). The in vivo specificity of this αvβ3-targeting ligand has been further demonstrated through competitive pretreatment with unlabeled αvβ3-targeting ligand, which inhibited αvβ3-targeted nanoparticle binding (35).

**Bioluminescence imaging**

In vivo and ex vivo bioluminescence imaging (BLI) was performed on IVIS50 (PerkinElmer) as described previously (20). Total photon flux (photons/sec) was measured from fixed regions of interest (ROI) over entire mouse or manually around ex vivo organs using Living Image 2.6, as indicated. Mice with outstanding chest BLI intensity indicative of a failed intracardiac injection or with ineffective β-luciferin administration were excluded from all analyses. Investigators were blinded to treatment groups during all BLI analyses.

**Rhodamine-labeled MP colocalization with breast cancer bone metastases**

Tumor-bearing mice day 8 postintracardially injected with PyMT-Bo1 cells, or age-matched tumor-free mice, were treated with a single dose of rhodamine-labeled MPs (either nontargeted or αvβ3-targeted) at a nanoparticle dose of 2 mmol/g mouse weight. Investigators were blinded to treatment groups during intravenous MP and αvβ3-MP injections. MPs were allowed to circulate for 3 hours in vivo, before sacrifice and tissue collection. Unbound MPs were cleared from circulation via cardiac perfusion with 30 mL of PBS.

For fluorescent analysis of nanoparticle colocalization within bone, fresh-frozen long bones in optimum cutting temperature embedding medium (Tissue-Tek) were sectioned 5-μm thick onto cryofilm tape (Section Lab Co., Ltd) at the histology core of the Washington University Musculoskeletal Research Center. Cryofilm tape sections were fixed in −20°C acetone for 5 minutes and air-dried for 10 minutes. Slides were briefly rehydrated with PBS, and then mounted with ProLong Gold with DAPI (Invitrogen).

**Fluorescent imaging and analysis**

Fluorescent images were captured on a Photometrics CoolSNAP MYOcamera connected to a Nikon Eclipse Ti-E microscope, acquired with the 4 × Plan Fluor PhL DL objective through a TxRED HQx filter cube and DAPI filter cube. The TxRED fluorescence channel was equally set using fluorescent look-up-table (LUT) to the same minimum and maximum values, gamma = 1. Fluorescent colocalization analysis was performed using NIS-Elements AR (Nikon Canada, Inc.). Bone metastatic region of interest (ROI) was drawn at the tumor/marrow boundary, as defined by DAPI visualization of nuclei density. Using the object count function in NIS-Elements AR, fluorescent nanoparticle colocalization was calculated via the ratio of rhodamine-positive pixel area by the total tumor ROI pixel area [(number of pixels of positively labeled objects within the fixed tumor area)/(total number of pixels within the fixed tumor area)] × 100.

**Treatment of murine breast cancer metastases**

Using the experimental metastasis model, PyMT-Bo1 metastases were established within female C57BL/6 mice. BLI analysis confirmed the establishment of bone metastases on day 3 postintracardiac injection, and metastatic-bearing mice were randomly sorted and treatment started. Docetaxel (LC Laboratories) was prepared at 10 mg/mL in a Tween 80/ethanol/saline (20:13.67: v/v/v) solution for drug solubility, and diluted to 0.5 mg/mL docetaxel or 1.0 mg/mL in saline for intravenous administration. For nanoparticle treatment, mice received either a cumulative dose of 5.55 mg/kg docetaxel, an equimolar dose of nanoparticle-encapsulated docetaxel-prodrug (2.2 μmol/kg), or an equal amount of saline or cargo-free αvβ3-MP, fractionally administered intravenously every three days. A priori comparisons of interest were between saline-treated mice as compared with docetaxel or αvβ3-MP/DTX-PD, or between saline-treated mice compared with the nanoparticle control treatments.

In collaboration with Dr. T.A. Guise, histologic bone sections were obtained from female athymic nude mice bearing MDA-MB-231 bone metastases, treated daily with the TGFβ receptor I kinase inhibitor, 60 mg/kg/day SD-208 (Epichem Pty Ltd, Murdoch University, Australia) or vehicle (1% methylcellulose), from an experiment published previously (30).

**Serum chemistry and hematologic analysis**

Blood was collected by submandibular vein puncture into Microtainer EDTA tubes (BD Biosciences) for hematologic analysis (Hemavet 950 FS, Drew Scientific, Inc.) or Microtainer serum separator tubes (BD Biosciences) for serum chemistry analysis on a Liasys 330 AMS Diagnostic liquid chemistry analyzer. Investigators were blinded to treatment groups during analysis.

**MTT assay**

The MTT assay was performed as described previously (31). Signal intensity normalized to 0% for the viability of cells at time of drug addition, and 100% for the viability of vehicle control treated cells at 72 hours.

**Radiography**

Osteolytic lesions were imaged by X-Ray imaging system (Faxitron), and lesion area within the tibiofibular joint was quantified using ImageJ (NIH, Bethesda, Maryland).

**Immunohistologic staining**

Freshly removed tissue was fixed in 10% neutral buffered formalin for 24 hours. Bone was decalcified in 14% EDTA for 10 days. Tissue was paraffin embedded and sectioned 5-μm thick at the histology core of the Washington University Musculoskeletal Research Center. Standard tartrate-resistant acidic phosphatase (TRAP) staining or hematoxylin and eosin (H&E) staining was performed by the musculoskeletal histology core of the Washington University Musculoskeletal Research Center.

For IHC, all slides were stained in parallel, using identical staining conditions. Paraffin tissue slides were prepared by immersing slides in xylene and rehydrating tissue in 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, and deionized water washing steps. Slides were immersed in EDTA antigen retrieval buffer (1 mmol/L EDTA, 0.05% Tween 20, PH 8.0) at 50°C overnight. Slides were treated with dual endogenous
enzyme block (Dako), TBS/0.1% Tween-20 (TBST) wash buffer, and 10 minutes of serum-free protein block (Dako). Slides were stained with the following primary antibodies: anti-integrin β3 antibody (D7 × 3P, 1:200, Cell Signaling Technology), anti-integrin αv (ab179475, 1:500, Abcam), isotype control rabbit IgG (ab227472, Abcam) antibody, or biotinylated anti-PCNA antibody (PC10, 1:100, eBioscience). Following primary antibody incubation, slides were extensively washed in TBST. Either Anti-Rabbit EnVision+/System-HPRT (Dako) or Vectastain Elite ABC HRP kit (Vector Laboratories) was used as the secondary antibody, followed by Liquid DAB+ (3,3’-Diaminobenzidine) Substrate system (Dako), according to the manufacturer’s protocol. Nuclear hematoxylin counterstain was applied, followed by dehydration through 70% ethanol, 95% ethanol, 100% ethanol, and xylene. Slides were mounted with Cytoseal XYL (Thermo Scientific).

**Histologic imaging and analysis**

Histologic slides were imaged on either an Olympus NanoZoomer 2.0-HT System or on a Zeiss Axioscan.Z1. In each experiment, postimage analysis was limited to changes in brightness or contrast, gamma = 1, which were applied equally to all images. Sections stained with integrin β3 or proliferating cell nuclear antigen (PCNA) were quantified using Visiopharm software, which allows for recognition and quantification of DAB-stained tissue areas. A supervised Bayesian pixel classifier was used to classify an image based on three distinct categories: DAB staining, hematoxylin staining (nuclei), and unstained tissue and other background structures. Integrin β3 expression from each sample was calculated within 5–10 random high-powered fields of 100–200 cells within the tumors. Values expressed as the percentage of integrin β3 expression (area of total DAB-positive staining) divided by the tumor area in the high-powered field. PCNA proliferation index was quantified by calculating the number of PCNA-positive cells divided by the total number of cells within the entire bone metastatic region. ImageJ software (NIH, Bethesda, Maryland) was used to measure tumor area, and to quantitate osteoclasts (OC; defined as a TRAP positive, multinucleated cell on a bone surface) per millimeter of bone surface (mmB.S.) at the tumor/bone interface (N.OC/mmB.S.). Histologic analysis of bone metastatic tumor burden (tumor area/total bone marrow area) was calculated by measuring the tumor area within the metaphysis of the tibiofemoral joint, divided by the total marrow area of the metaphysis.

**Flow cytometry and FACS**

*In vitro* tumor cells were lifted with 1× Versene (Invitrogen) and prepared into single-cell suspensions for flow cytometry analysis as described previously (20). *Ex vivo* cells were stained with PE-conjugated anti-mouse integrin β3 (1:200, clone: 2C9.02, BD Pharmingen), fixed, and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s protocol, and then with AlexaFluor488-conjugated anti-human/mouse cytokeratin 18 (1:100, clone LDK18, eBioscience). Data acquisition was performed on the LSRFortessa (BD Biosciences) and FlowJo software version 10.1 (Tree Star) was used for analysis, and fluorescence compensation using UltraComp eLeads (eBioscience) according to the manufacturer’s protocol. All flow cytometry data are presented as median fluorescent intensity. *Ex vivo* flow cytometry analysis of bone samples with insufficient number of tumor cells (<500 events) were excluded.

For FACS, *in vitro* tumor cells were lifted with 1× Versene (Invitrogen) and stained for surface expression of integrin β3 as described above. Tumor cells were sorted into two populations using a BD FACSAria-II cell sorter (BD Biosciences): integrin β3 negative (β3−) cells (based on the fluorescent intensity of unstained cells) and integrin β3-expressing (β3+) cells. In addition, control cells sorted without β3 discrimination were also collected (β3-all). After sorting, each population was counted for live/dead cells, and 0.05 × 10⁶ live tumor cells in 50-μL PBS were intracardially injected into the 6-week-old female mice.

**Pharmacologic inhibition of signaling pathways**

Tumor cells were pretreated for 1 hour with pharmacologic inhibitors: cells were pretreated for 1 hour with the following pharmacologic inhibitors: TGFβ-Receptor 1 kinase inhibitor, specific for the site necessary for SMAD2/SMAD3 phosphorylation (SMAD2/3i, SB431542, 20 μmol/L, Sigma-Aldrich); p38 MAP kinase (MAPK) inhibitor (p38i, SB203580, 20 μmol/L, Cell Signaling Technology); MEK1/2 (MAPK/ERK Kinase) inhibitor (MEK1/2i, U0126, 20 μmol/L, Cell Signaling Technology); c-Jun N-terminal kinase (JNK) inhibitor (JNKi, SP600125, 50 μmol/L, Sigma-Aldrich). After 1 hour of pretreatment, cells were treated with 2 ng/mL of murine TGF-β1 (R&D Systems) or vehicle treatment. Cells were all cultured in 0.1% DMSO.

**Western blot analysis**

Whole cell lysates from tumor cells were collected in RIPA buffer (Cell Signaling Technology) in the presence of Halt phosphatase inhibitor cocktail (Thermo Scientific) at 4°C. Protein samples were separated on 10% polyacrylamide gels (Bio-Rad) and transferred onto an Immobilon-P polyvinylidene difluoride membrane (EMD Millipore). Membranes were incubated with phosphorylated-SMAD 2/3 (pSMAD2/3, pS46/47, R&D Systems) or vehicle treatment. Cells were all cultured in 0.1% DMSO.

**qPCR analysis**

Total RNA from cells was isolated with the RNeasy Mini Plus Kit (Qiagen). Complementary DNA was made using the SuperScript II first-strand synthesis system for qPCR (Invitrogen). qPCR was performed using SYBR Advantage mix (Bio-Rad) as described previously (20), with mouse-specific primers for mRNA genes of interest: Itgb1 forward: 5’-CAAGGTTGCGTTTTGGAATGCT-3’, Itgb1 reverse: 5’-ACAAGTTGCCCCTTGAACACTTGG-3’, Itgb3 forward: 5’-TCTCAATGCACCTGCTCAACAT-3’, Itgb3 reverse: 5’-ACGCACCTGCTTCTGATCAAAA-3’, Itgd5 forward: 5’-TCAGCATTCTCAGATCGGCGC-3’, Itgd5 reverse: 5’-TACAC-CTTCTTCACTGTCGCCAT-3’, Itgb6 forward: 5’-TGGATGACTGCTTTCGTCCTCG-3’, Itgb6 reverse: 5’-CTGCTCAGGTGTCCTCAGACT-3’, Itgd8 forward: 5’-CAATCTGCTACGAGCAGGCTA-3’, Itgd8 reverse: 5’-AACCAAGCGGAGGTACCG-3’, Itgd2 forward: 5’-GACGCTTGATTCGCTCCTAACC-3’, Itgd2 reverse: 5’-GACGCCACAGATCCTGAA-3’.
5′-ACATTGAGGCCTTGGAGGCT-3′, Itgav reverse: 5′-TTGGCACACAGCCTAGTACC-3′, Itga3 forward: 5′-CCGGAAGGA-CITGGAGTTAT-3′, Itga3 reverse: 5′-GATCATTCCTGGCCATGTAG-3′, Itgav forward: 5′-AAGAAGCCTGCGCCTATTG-3′, Itgav reverse: 5′-TCTAAGGCCACTGGAAGTTAG-3′. Gapdh forward: 5′-AGGGCTGTGAAACCGAATTG-3′; Gapdh reverse: 5′-TGTA-GACCATGTAGTTCGCTGC-3′. Target gene expression was normalized against the housekeeping gene GAPDH (Gapdh), and data were analyzed using the ΔΔCt method.

Panel of cytokines and growth factors
Tumor cells were cultured for 24 hours with the following murine factors: 2 ng/mL TGFβ1 (R&D Systems), 2 ng/mL TGFβ2 (R&D Systems), 50 ng/mL Sonic Hedgehog (Shh; PeproTech, #315-22), 50 ng/mL FGF2 (PeproTech, #315-20), 100 ng/mL insulin-like growth factor 1 (IGF-1; PeproTech, #250-19), 50 ng/mL epidermal growth factor (EGF; PeproTech, #315-09), 50 ng/mL fibroblast growth factor 2 (FGF2 or bFGF; PeproTech, #450-33), 100 ng/mL osteopontin (OPN; Leinco, #0121), 10 ng/mL IL4 (R&D Systems), 200 ng/mL stromal cell−derived factor 1α (Stef-D1α or CXCL12; Biolegend), 10 ng/mL Il6 (R&D Systems).

Patient breast cancer and matching bone metastatic biopsies
Matching primary breast and bone metastatic biopsies were both taken at the time of metastatic diagnosis, from patients without detectable bone metastases at diagnosis but detectable bone metastases at least 6 months after diagnosis. Data were obtained in accordance with the guidelines established by the Washington University Institutional Review Board (IRB #201102394) and WAIVER of Elements of Consent per 45 CFR 46.116 (d). All patient information was deidentified prior to sharing with investigators. All of the human research activities and all activities of the IRBs designated in the Washington University (WU) Federal Wide Assurance (FWA), regardless of sponsorship, are guided by the ethical principles in "The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects Research of the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research."

Tissue samples all displayed detectable tumor cells, as previously determined by examination of cellular morphology by a board-certified pathologist. IHC for integrin β3 was performed as described in the previous section. Tumor-associated β3 expression was semiquantitatively scored in a blinded manner, in which the scorer did not have access to disease classification or clinical annotation, using the histoscore (H-score) system: H-score = \( \sum (i \times k) \), where "i" is the staining intensity (0–3 scale), and "k" is the percentage of tumor cells stained at each intensity, ranging 0% to 100%. We observed an expression pattern of tumoral β3 consistent with a previous literature description of β3 expression in human breast cancer: primarily localized along the cell plasma membrane at sites of tumor−tumor and tumor−stroma contact (37). In addition to the β3 expression on bone metastases, we also observed β3 staining on some host cells within the primary and bone tumor microenvironments, including tumor-infiltrating immune cells (β3 expression colocalized with CD68-stained cells, predominately expressed on monocytes and macrophages), tumor-associated blood vessels, and osteoclasts, consistent with previous observations (11, 12, 19, 20).

Breast cancer subtyping was based on ER, PR, HER2 status, where luminal A was ER, PR, HER2 (n = 18), luminal B was ER, PR, HER2 (n = 7). HER2-enriched was ER− PR− HER2+ (n = 2), and triple-negative was ER− PR− HER2− (n = 7). Eight breast cancer carcinomas had incomplete genetic subtyping and were excluded from molecular subtype analysis.

Statistical analysis
All data shown as mean with error bars representing SEM. All sample sizes reported in the study are the minimum number of samples. For animal studies, sample sizes were estimated according to our previous experience. Statistical differences were analyzed using either a two-tailed t test, ANOVA with Tukey test for a posteriori (post hoc) multiple comparisons, or a two-tailed unpaired t test with Bonferroni correction for a priori comparisons between a control group and experimental treatment groups of interest. Assumptions for ANOVA and t test (independent samples, approximately normal distributions) for samples n > 5 were sufficiently met, or used if a random sample of n ≤ 5 were selected from an approximately normally distributed population. Non-normally distributed data was analyzed using a two-tailed Mann–Whitney U test or a two-tailed Wilcoxon signed-rank test for matched pairs. All tests were considered significant at P < 0.05, or in case of k comparisons, P < 0.05/k. Data analyses were completed using Prism 6 (GraphPad Software).

Results
Breast cancer cells overexpress integrin β3 within the bone microenvironment
To determine the potential of targeting αvβ3 at different metastatic sites, we evaluated breast cancer expression of integrin αv or β3 at the primary site as compared with secondary metastases. Using the murine breast cancer cell line PyMT-B01 that models the luminal B subtype, primary MFP tumors were established by orthotopic injection, or metastases within the bone and visceral organs were established by intracardiac injection, into separate cohorts of mice. Tumors were removed 12 days post-injection and stained for integrin β3 or integrin αv expression using IHC. Bone metastases expressed elevated levels of β3, as compared with MFP tumors or visceral metastases within the lung or kidney (Fig. 1A; Supplementary Fig. S1A). Unlike the selective upregulation of integrin β3 on bone metastases, we observed expression of integrin αv across all primary and metastatic tumors (Supplementary Fig. S1B). To determine whether the observed β3 expression was tumor cell−specific, we used flow cytometry to quantify cell surface β3 expression. Using PyMT-B01 cells or murine 4T1 triple-negative breast cancer cells, MFP tumors or bone metastases were established by orthotopic injection or intracardiac injection, respectively. Isolated tumor cells were identified on the basis of cytokeratin 18 (CK18+) expression [Supplementary Fig. S1C]. In both cell lines, tumor cells from bone metastases expressed significantly elevated β3 levels, as compared with MFP tumor cells (Fig. 1B and C).

To assess the translational implications of this observation, we examined β3 expression on biopsies from 42 breast cancer patients, comparing patient-matched primary tumors to bone metastases. IHC for β3 was semiquantitatively scored on the basis of the extent and intensity of tumor-associated β3 expression. Across nearly all patients, human bone metastases expressed significantly higher levels of tumor-associated β3, as compared
with the matching primary tumor (Fig. 2A; Supplementary Fig. S1D). Furthermore, this elevated expression of bone metastatic β3 was observed across all breast cancer patient subtypes: luminal A, luminal B, HER2-enriched, and triple-negative (Fig. 2B).

Tumoral β3 is induced in the bone microenvironment and mediated by TGFβ signaling

We next asked whether bone metastases express elevated β3 due to preferential seeding and colonization of a β3hi tumor cell subpopulation in the bone. Using FACS, PyMT-Bo1 cells were sorted into three different subpopulations based on surface β3 expression: β3-negative cells (β3−), β3-expressing cells (β3+), and control cells sorted without β3 discrimination (β3-all). These PyMT-Bo1 subpopulations were collected and injected intracardially into separate groups of mice, which all developed bone metastases (Fig. 3A). Flow cytometry revealed that regardless of initial β3 status, each FACS subpopulation developed into bone metastases that strongly expressed tumoral β3 (Fig. 3B). This result demonstrates that elevated expression of bone metastatic β3 is not due to preferential seeding of β3hi tumor cells to the bone, and suggests that tumoral β3 is induced in the bone microenvironment.

To ascertain pathways that might be responsible for this induction, we evaluated a panel of cytokines and growth factors present in the bone microenvironment for their effect on β3 expression. Of the tested factors, only members of the TGFβ family upregulated integrin β3 expression, observed in both PyMT-Bo1 and 4T1 cells (Fig. 3C and D). To assess the specificity of TGFβ-mediated upregulation of integrin β3 as compared with other related integrin subunits, we evaluated how TGFβ alters expression of integrin αv, as well as a subfamily of β-integrin subunits that can also heterodimerize with integrin αv (integrin subunits β1, β5, β6, and β8). In addition, we evaluated integrin αβ1 (glycoprotein-Ilb), which is largely restricted to platelets and megakaryocyte-lineage cells but can also heterodimerize with integrin β3 (16), and integrin α2, which facilitates adhesion to type I collagen and is upregulated on prostate cancer lines in response to TGFβ (38).

Collectively, we evaluated gene expression of integrin subunits β1, β3, β5, β6, β8, αv, αβ1, and αvβ2 on PyMT-Bo1 and 4T1 cells, following 24 hours of in vitro TGFβ stimulation. Integrin β3 was the most upregulated subunit in both PyMT-Bo1 and 4T1 cells (Supplementary Fig. S2A and S2B). In PyMT-Bo1 cells, integrin α2 was the next most upregulated subunit, although 5-fold less than the level of β3 induction (Supplementary Fig. S2A). In 4T1 cells, β5 and α2 were the next most upregulated subunits, although 4-fold less than the level of β3 induction (Supplementary Fig. S2A). TGFβ-mediated changes in αv, αβ1, and the other β-integrin subunits were either unchanged, reduced, or displayed a less-than 2-fold upregulation (Supplementary Fig. S2A and S2B). These results provide support for TGFβ as a potent inducer of integrin β3 as compared with these other integrin subunits.

TGFβ-β signals through TGFβ receptor 1 (TGFβR1), which canonically phosphorylates the transcription factors SMAD2/SMAD3. In addition, TGFβR1 can activate ‘noncanonical’ signaling pathways, most commonly p38 MAP kinase (p38), MAPK/ERK kinase-1 and -2 (MEK1/2), and c-Jun N-terminal kinase (JNK; ref. 39). To evaluate how TGFβ induces β3, tumor cells were treated with TGFβ in combination with pharmacologic inhibitors for p38, MEK1/2, JNK, or TGFβR1 kinase activity at the site specific for SMAD2/SMAD3 phosphorylation (see Fig. 4 for details). In both PyMT-Bo1 and 4T1, only pharmacologic inhibition of SMAD2/SMAD3 phosphorylation completely ablated β3 upregulation. This was observed by flow cytometry for surface...
β3 expression (Fig. 4A and B) and qPCR analysis for β3 mRNA levels (Supplementary Fig. S3A). Western blot analysis confirmed selective inhibition of SMAD2/SMAD3 phosphorylation (Fig. 4C) and suppression of total β3 protein (Fig. 4D) with TGFβRII kinase inhibition. To determine whether TGFβ signaling was responsible for tumoral β3 induction in vivo, mice bearing human MDA-MB-231 triple-negative breast cancer bone metastases were treated daily with a pharmacologic TGFβRII kinase inhibitor. TGFβRII kinase inhibition significantly suppressed β3 expression on MDA-MB-231 breast cancer bone metastases (Fig. 4E), demonstrating the targeting potential and specificity of β3-MPs for bone metastases.

Next, we sought to evaluate the therapeutic efficacy of β3- MP–mediated drug delivery. Nanotherapeutics often suffer from a premature loss of drug payloads during circulation and diminished intracellular drug bioavailability due to endosomal entrapment (40). To overcome these problems, a lipase-labile phospholipid–prodrug concept was employed, in which drug cargo is coupled to the Sn2 acyl-chain of phosphatidylcholine. This phospholipid–prodrug can be stably incorporated into the MP membrane during self-assembly and provides drug retention during circulation (35). Upon β3-MP binding to activated β3 on target cells, a hemifusion complex forms between MPs and the plasma membrane, enabling endocytosis-independent “contact-facilitated drug delivery” (see Fig. 5B for details; refs. 36, 41).

The chemotherapeutic agent selected for nanoparticle delivery was docetaxel, a potent microtubule inhibitor employed as a first-line agent against breast cancer (42). We modified docetaxel as an

**Figure 2.** IHC for β3 on patient-matched primary breast cancer and bone metastatic biopsies. A, Semiquantitative analysis of the extent and intensity of tumor-associated β3 expression using the histoscore (H-score) system. n = 42 matched-pairs, two-tailed Wilcoxon signed-rank test. ***, P < 0.0001. B, Subdivision of A by molecular subtype (see Materials and Methods for details). Right, representative images of patient-matched primary tumors and bone metastases. Scale bar, 50 μm. Two-tailed paired t test; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data presented as mean ± SEM.
Bone microenvironment and TGFβ stimulation induce tumoral β3. **A**, in vitro PyMT-Bo1 cells were FACS sorted into three groups based on basal β3 expression: β3−, β3+, and control β3-all. Immediately after collection, cells were injected (intracardially) into separate groups of mice. **B**, Thirteen days post-injection, isolated bone metastatic PyMT-Bo1 cells were identified by CK18− and evaluated by flow cytometry for surface β3 expression. Left, representative samples. Right, n = 4 β3−; n = 4 β3-all; n = 5 β3+. One-way ANOVA with Tukey post hoc test; ns, not significant. **C** and **D**, qPCR analysis of β3 (Itgb3) mRNA expression by PyMT-Bo1 or 4T1 cells cultured in vitro, following 24 hours stimulation with the listed factors (see Materials and Methods for details). One of two biological replicates, each in technical duplicate. One-way ANOVA with Tukey post hoc test; *****, P < 0.0001. Data presented as mean ± SEM.

**Figure 3.**

Sn2 lipase-labile phospholipid-prodrug (DTX-PD) to enable αβ3-MP delivery (Fig. 5B; ref. 32). In vitro, PyMT-Bo1 cells were markedly sensitive to both docetaxel and DTX-PD, with a bioequivalent IC_{50} of 5.5 nmol/L (Fig. 5C). Unfortunately, chemotherapeutics such as docetaxel rarely eradicate bone metastases in human patients (3, 4) and can induce off-target toxicity such as hair loss, neuropathy, pancytopenia, and liver toxicity (42). To test the efficacy of docetaxel against PyMT-Bo1 cells in vitro, mice were intracardially injected with PyMT-Bo1 cells. BLI analysis confirmed equivalent establishment of bone metastases on day 3 post-intracardiac injection, and mice bearing PyMT-Bo1 metastases received intravenous docetaxel treatments beginning on day 0, with a cumulative dose of 5.55 mg/kg docetaxel or equimolar dose of MP-encapsulated DTX-PD (see Fig. 5D). On day 12, ex vivo BLI analysis of bone metastases revealed that αβ3-MP/DTX-PD significantly attenuated bone metastatic tumor burden, while no significant attenuation was observed with docetaxel treatment (Fig. 6A and B). Consistent with this result, histologic analysis of bone tumor burden (Fig. 6C) and X-ray analysis of tumor-associated bone loss (Fig. 6D) revealed a significant reduction with αβ3-MP/DTX-PD treatment as compared with saline, and no significant attenuation with docetaxel. BLI analysis of visceral metastases within the liver, lungs, kidneys, and brain revealed that neither αβ3-MP/DTX-PD nor docetaxel resulted in significant attenuation (Supplementary Fig. S5B), supporting our previous observations concerning bone-specific β3 expression and resistance of PyMT-Bo1 metastases to docetaxel.

Hepatotoxicity was tested by serum liver function tests for aspartate transaminase (AST) and alanine transaminase (ALT). Docetaxel treatment resulted in elevated levels of AST and ALT outside of the normal ranges, while αβ3-MP/DTX-PD treatment displayed no evidence of hepatotoxicity (Fig. 6E). Both renal function, assessed by blood urea nitrogen (BUN), and whole

**αβ3-MP–Mediated drug delivery of docetaxel attenuates bone metastases**

To evaluate the therapeutic potential of αβ3-targeted delivery of DTX-PD against PyMT-Bo1 bone metastases, mice were treated with docetaxel, or an equimolar amount of DTX-PD encapsulated by αβ3-MP (αβ3-MP/DTX-PD). In parallel, two control nano-particle treatments were evaluated to assess the in vivo specificity of αβ3-MP targeting, and the specificity of docetaxel-mediated tumor-suppressive effects: nontargeted MP/DTX-PD and cargo-free αβ3-MP without DTX-PD cargo, respectively.

As before, BLI analysis on day 3 post-intracardiac injection confirmed PyMT-Bo1 bone metastatic establishment and treatment began on day 4, with a cumulative dose of 5.55 mg/kg docetaxel or equimolar dose of MP-encapsulated DTX-PD (see Fig. 5D). On day 12, ex vivo BLI analysis of bone metastases revealed that αβ3-MP/DTX-PD significantly attenuated bone metastatic tumor burden, while no significant attenuation was observed with docetaxel treatment (Fig. 6A and B). Consistent with this result, histologic analysis of bone tumor burden (Fig. 6C) and X-ray analysis of tumor-associated bone loss (Fig. 6D) revealed a significant reduction with αβ3-MP/DTX-PD treatment as compared with saline, and no significant attenuation with docetaxel. BLI analysis of visceral metastases within the liver, lungs, kidneys, and brain revealed that neither αβ3-MP/DTX-PD nor docetaxel resulted in significant attenuation (Supplementary Fig. S5B), supporting our previous observations concerning bone-specific β3 expression and resistance of PyMT-Bo1 metastases to docetaxel.
blood counts were within the normal range among mice treated with saline, docetaxel, or DMSO control in the presence of a pharmacologic inhibitor SMAD2/3i (20 μmol/L SB43552, MEK1/2i (20 μmol/L U0126), p38i (20 μmol/L SB203580), or JNKi (50 μmol/L SP600125)). After 48 hours, flow cytometry for surface β3 expression was evaluated on PyMT-Bo1 cells (A) or 4T1 cells (B). Left, representative experiment; right, n = 3 biological replicates. One-way ANOVA with Tukey post hoc test, with denoted significance in relation to DMSO control; *, P < 0.05; **, P < 0.001; ***, P < 0.0001. C and D, Western blot analysis of in vitro PyMT-Bo1 cells treated as described previously, for 3 hours (C) or 24 hours (D). E, Mice intracardially injected with MDA-MB-231 cells were treated daily with a TGFβRI kinase inhibitor of SMAD2/3 phosphorylation (SD-208, 60 mg/kg/d) or vehicle control (1% methylcellulose) for 28 days. IHC for β3 with representative images (left) and quantification of DAB-stained bone metastases, n = 4 (right). Scale bar, 100 μm. Two-tailed unpaired t test; ***, P < 0.001. Data presented as mean ± SEM.

**Discussion**

Breast cancer bone metastases often irreversibly damage the skeleton, severely impacting quality of life and overall survival (2). Treating bone metastases has proven difficult, in part due to the bone microenvironment’s status as a chemoprotective niche (7), and coupled with inefficient delivery of drug to tumor cells in bone (43). While drug or nanoparticle conjugation to hydroxyapatite-avid molecules like bisphosphonates have provided positive results against mouse models of bone metastases (13, 14), they lack direct specificity for tumor cells (8, 15). In this study, we provide new evidence for integrin β3 as a molecular target on breast cancer bone metastases in mice and humans, and demonstrate the potential for improving therapeutic efficacy against this metastatic site via αvβ3-targeted approaches.

We first investigated differences in the physiologic expression of integrin β3 on breast cancer tumors growing in different organs, in search of a tumor target on cells within the bone microenvironment. We demonstrated that orthotopic injection of murine breast cancer cell lines into the MFP establishes primary tumors...
with weak β3 expression. Intracardiac injection establishes metastases within the lung and kidney with similarly weak β3 expression, but establishes bone metastases with significantly stronger β3 expression, observed by both IHC and flow cytometry of tumoral surface expression. In contrast, integrin αv was strongly expressed on tumors at all evaluated sites.

We employed the widely published intracardiac injection model to establish experimental bone and visceral metastases in these experiments. While this model does not recapitulate all steps of the metastatic cascade, circulating tumor cells still need to extravasate from the vessels to establish within the bone and other organs. We also evaluated β3 expression on 42 human bone metastatic tissue samples, as compared with matching primary breast cancer biopsies from the same patient. Across nearly all patients, tumor-associated β3 expression was significantly higher on bone metastases. Furthermore, we show for the first time that β3 is highly expressed on breast cancer bone metastases of all subtypes, including those with the worst overall survival that have proven
Integrin αvβ3-targeted nanotherapy attenuates breast cancer bone metastases. Following the schematic described in Fig. 5D, mice were treated with either a cumulative dose of 5.55 mg/kg docetaxel, an equimolar dose of docetaxel-prodrug encapsulated by αvβ3-MP (αvβ3-MP/DTX-PD), or saline. Analyses completed on samples collected on day 12 postinjection.

**A**, Representative in vivo BLI of mice bearing PyMT-Bo1 bone metastases, matching day 3 and day 12 postinjected mice from each group. **B**, Ex vivo PyMT-Bo1 bone metastatic tumor burden by BLI analysis, n = 9. **C**, Histologic analysis of tumor burden within the tibiofemoral joint, n = 4. **D**, X-ray analysis of osteolytic bone destruction, n = 9. **E**, Scale bar, 1 mm, two-tailed unpaired t test with Bonferroni correction for a priori multiple comparisons between saline and each experimental treatment, *P < 0.05/2). **F**, Serum chemistry analysis of BUN, AST, and ALT, n = 5. Gray range illustrates the normal reference range (mean ± 2SD) for female C57BL/6 mice bearing PyMT-Bo1 metastases. **G**, TRAP staining for quantification of osteoclast number per millimeter of bone surface (N.OCC/mmB.S.) at the tumor (T), bone (B) interface, n = 4. **H**, Quantification of PCNA-positive cells within the bone metastatic region, n = 3. Scale bar, 50 μm, one-way ANOVA with Tukey post hoc test; *, *P < 0.05; **, *P < 0.01; ns, not significant. All images are representative and data presented as mean ± SEM.
difficult to target (44), supporting the translational potential of targeting β3 in bone metastatic breast cancer patients.

On the basis of previous studies demonstrating that ectopic overexpression of β3 enhances tumor cell establishment in bone (26, 28), we considered the possibility that breast cancer bone metastases display elevated β3 due to preferential bone colonization of a tumor cell subpopulation with high basal β3 expression. We established bone metastases from FACS-sorted tumor cell subpopulations, which were initially positive or negative for basal β3 expression. Here, we demonstrated that initial β3 expression is dispensable for establishing breast cancer bone metastases with elevated β3 expression. This finding does not contradict the role of tumoral αvβ3 in enhancing bone colonization (26, 28), as there are numerous ligands for αvβ3 within the bone microenvironment (17, 18); rather, it indicates that tumoral β3 can be induced during the establishment of bone metastases, suggesting that β3 expression might be regulated by the bone microenvironment itself.

The bone is a natural reservoir for TGF-β, which is stored in the matrix as an inactive latent complex that must be liberated for bioactivation (45). Bioactive TGF-β plays a critical role in both the homeostatic and pathologic bone microenvironment, regulating osteolytic destruction and pathologic muscle weakness (30, 46). TGF-β has been shown to induce β3 expression on normal and transformed mammary epithelial cells (47–49), but the physiologic regulation of tumoral β3 in vivo has not been explored. Here, we demonstrated that TGF-β strongly induces tumoral β3, and analysis of other integrin subunits of interest revealed that TGF-β induced the low expression of tumoral β3 more strongly than any other. Furthermore, we determined that TGF-β induces β3 through TGFβRI phosphorylation of SMAD2/SMAD3, evaluated at the transcriptional, total protein, and cell surface expression level. And for the first time, we demonstrate that TGFβ signaling is required for integrin β3 induction by breast cancer cells within the bone.

We were surprised by the lack of tumoral β3 upregulation in the MFP tumor or visceral metastases, where TGFβ can be present. This observation suggests that other microenvironments either lack a sufficient amount of bioactive TGFβ necessary for tumoral β3 induction, or contain inhibitors of TGFβ or SMAD2/SMAD3 signaling that prevent TGFβ-mediated induction of tumoral β3. Studies are in progress to define the molecular mechanism underlying the low expression of tumoral β3 outside of the bone.

Selective elevation of β3 on bone metastases prompted us to explore αvβ3-targeted nanotherapy as a means to overcome the barriers limiting effective treatment. We selected phospholipid/prodrug cargo directly to the target cell's plasma membrane, where bioactive free-drug is enzymatically liberated directly into the cytoplasm by phospholipase activity (36, 41). Furthermore, phospholipid–prodrug is stably incorporated into the MP–single lipid layer, thereby minimizing passive drug loss during circulation (35). Administering rhodamine-labeled MPs or αvβ3-MPs into mice bearing breast cancer bone metastases resulted in stronger αvβ3-MP colocalization with bone metastases, compared with a 6.5-fold reduction in colocalization with nontargeted MPs. To examine the importance of MP’s small size in bone metastatic colocalization, we also evaluated the colocalization potential of significantly larger αvβ3-targeted perfluorocarbon nanoparticles (~250 nm) (35). We observed that fluorescently labeled αvβ3-targeted perfluorocarbon nanoparticles were much less effective at localizing to the center of bone metastases (data not shown). These observations prompted us to proceed with αvβ3-MP–mediated drug delivery, and we selected the chemotherapeutic docetaxel as cargo for lipase-labile phospholipid–prodrug modification (DTX-PD).

While PyMT-Bo1 breast cancer cells are equally sensitive to both docetaxel and DTX-PD in vitro, PyMT-Bo1 metastases were strikingly resistant in vivo, with bone metastatic tumor burden essentially unaffected by docetaxel treatment even at the highest, most toxic dose tested (20 mg/kg/week). These observations highlight the difficulty of achieving an inhibitory dose of chemotherapy at metastatic sites in vivo. To test the efficacy of MP-encapsulated DTX-PD, mice bearing PyMT-Bo1 metastases were treated with a suboptimal docetaxel dose (5.55 mg/kg/week), 3.6-fold lower than the previously tested 20 mg/kg/week docetaxel dose, which did not significantly attenuate bone metastases. At this suboptimal dose, we observed a significant attenuation of bone metastatic tumor burden and tumor-associated bone loss with αvβ3-MP/DTX-PD treatment, as compared with no significant attenuation by equimolar docetaxel treatment. Furthermore, neither cargo-free αvβ3-MP treatment nor nontargeted MP/DTX-PD treatment significantly altered bone metastatic tumor burden or osteolytic bone destruction. The observation that micelle coating with the αvβ3-targeting ligand was necessary for bone metastatic colocalization, coupled with fact that αvβ3-MP/DTX-PD failed to attenuate tumor independent of β3-expressing bone metastases, provides additional support for the in vivo specificity of the αvβ3-targeting ligand for integrin αvβ3 in line with previous reports (32–36).

To examine the potential mechanism of αvβ3-MP–mediated activity, we quantified osteoclast number at the tumor/bone interface, which is reflective of osteoclast formation and serves as a useful indicator of bone resorption. Previous studies have found that osteoclast formation is markedly sensitive to inhibition by docetaxel, even at nanomolar concentrations (30). Osteoclast number at the tumor/bone interface was similar between αvβ3-MP/DTX-PD and saline treatment groups, demonstrating that αvβ3-MP/DTX-PD treatment attenuates bone metastases and tumor-associated bone loss independent of inhibiting osteoclast formation or direct osteoclast killing. It is possible that there remains an effect of αvβ3-MP/DTX-PD treatment on osteoclast function that was not revealed by histomorphometric analyses.

We evaluated tumor cell proliferation through expression quantification of PCNA, a DNA polymerase processivity factor required for DNA synthesis during replication. Docetaxel inhibits tumor proliferation through effects on microtubule stabilization and disruption of mitotic spindle assembly (42). We found that αvβ3-MP/DTX-PD treatment significantly decreased PCNA expression on bone-residing tumor cells, as compared with saline and docetaxel treatment. Taken together with the osteoclast histomorphometry result, these observations suggest that αvβ3-MP/DTX-PD attenuated bone metastases due to enhanced therapeutic efficacy against bone-residing tumor cells.

We observed that docetaxel administration resulted in increased hepatotoxicity, while hematologic values and liver function tests remained within normal limits following αvβ3-MP/DTX-PD treatment. This work thus provides support for safer, more effective drug delivery against bone metastases through
exploitation of αvβ3 expression on breast cancer cells within bone. Going forward, αvβ3-targeted drug cargo could be modified to fit specific treatment needs.

We recognize the limitation of αvβ3-targeted nanotherapy against breast cancer metastases outside of the bone. Nevertheless, bone metastases occur in approximately 70% of metastatic breast cancer patients and represent the only metastatic site in approximately 30% of patients, suggesting that a substantial number of patients could benefit from this approach (1, 44). Collectively, we provide support for integrin αvβ3-targeted drug delivery as a bone-specific therapeutic strategy to address the unmet clinical need for effective treatments against breast cancer bone metastases.

Disclosure of Potential Conflicts of Interest

D. Pan is a co-founder and CTO at Innsight Tech. Inc. and Kalocyte and has ownership interest (including patents) in Innisight Tech and Kalocyte. T.A. Guise is a consultant/advisory board member for Bayer and has received expert testimony from Novartis. No potential conflicts of interest were disclosed by the other authors.

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References


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Drug Targeting to Breast Cancer via Bone-Induced Integrin β3

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