Published in final edited form as:

Pain. 2018 April ; 159(4): 684–698. doi:10.1097/j.pain.0000000000001139.

Disease modifying actions of IL-6 blockade in a rat model of bone cancer pain

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Abstract

Metastasis of cancer to the skeleton represents a debilitating turning point in the lives of patients. Skeletal metastasis leads to moderate to severe ongoing pain along with bone remodeling that can result in fracture, events that dramatically diminish quality of life. IL-6 levels are elevated in metastatic breast cancer patients and are associated with a lower survival rate. We therefore determined the consequences of inhibition of IL-6 signaling using a novel small molecule antagonist, TB-2-081, on bone integrity, tumor progression, and pain in a rodent model of breast cancer. Rat MAT B III mammary adenocarcinoma cells were injected and sealed within the tibia of female Fischer rats. Growth of these cells within the rat tibia elicited increased IL-6 levels both within the bone exudate and in the plasma, produced ongoing pain and evoked hypersensitivity, and bone fracture that was observed by approximately day 12. Systemic TB-2-081 delivered by subcutaneous osmotic mini-pumps starting at tumor implantation prevented tumor-induced ongoing bone pain and evoked hypersensitivity without altering tumor growth. Remarkably, TB-2-081 infusion significantly reduced osteolytic and osteoblastic bone remodeling and time to fracture likely by decreasing osteoclastogenesis and associated increase in bone resorption. These

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Conflict of Interest:

The authors have no conflicts of interest to disclose.

Author contributions:

BR, DS, and AN performed intratibial surgeries.

BR and DS collected behavioral data, and the serum and bone exudate data.

BR, AN, and NL collected cellular protein, conducted western blots and XTT cell viability assays, and harvested bones for further analyses.

BR collected and conducted osteoblast assay, and performed analyses on Western blot, XTT, and osteoblast results.

FL, KC and KCR synthesized and purified TB-2-081.

TK prepared the bones for the μ CT and H&E cores, received, and analyzed the data.

TK and BR performed statistical analyses and prepared figures for the manuscript.

BR, TK, and FP designed experiments, and wrote and edited the manuscript.

findings indicate that blockade of IL-6 signaling may represent a viable, disease-modifying strategy to prevent tumor-induced bone remodeling allowing for stabilization of bone and decreased fractures as well as diminished ongoing pain that may improve quality of life of patients with skeletal metastases. Notably, anti-IL-6 antibodies are clinically available allowing rapid testing of these possibilities in humans.

Introduction

Commonly diagnosed cancers, including breast cancer, have a propensity to metastasize to the bone [46]. Within the bone, tumor growth is associated with inflammation, and osteolytic or osteoblastic bone remodeling that can lead to fractures [43]. Cancer-induced bone pain is also prominently characterized by persistent ongoing pain that is generally characterized as moderate to severe [51]. Metastasis of cancer to the bone is a drastic life-changing event for which only palliative options are available [10; 51]. Opioids remain the gold standard for care in these patients, but they are associated with many severe adverse side effects that contribute to diminished quality of life such as constipation, nausea, somnolence and mental confusion that produce dose-limiting effects [7; 51]. Preclinical evidence suggests that opiates can enhance bone loss possibly leading to increased fractures though this has not been demonstrated in humans [35]. Adjuvants such as bisphosphonates are used to counter tumor-induced bone loss and have been demonstrated to diminish bone loss and fracture, along with the onset of pain [64]. Bisphosphonates, however, are not sufficient to block bone cancer pain usually resulting in the need other pain relievers including opioids. The discovery of therapies that could impact disease progression related to tumor and bone remodeling, as well as providing adequate pain control, would be of high therapeutic significance [64].

Interleukin-6 (IL-6) is a pleiotropic cytokine that is upregulated in states of injury, inflammation, and infection [3; 32]. Several studies have demonstrated that serum levels of IL-6 are elevated in cancer patients, with higher levels correlated with advanced stage cancer, multidrug resistance, and shortened survival [5; 28; 38]. Within the tumor bearing bone, IL-6 has been demonstrated to signal through membrane bound glycoprotein 80 (gp80) found on osteoblasts, B-cells, and macrophages [3; 13; 23]. Dimerization of gp80 with gp130 induces transactivation and autophosphorylation of Janus kinases (Jak) that phosphorylates signal transducer and activator of transcription 3 (STAT3). IL-6 has been implicated in multiple components of disease progression associated with tumor growth within the bone [3]. IL-6 signaling leads to expression and release of receptor activator of NF- κ B ligand (RANKL) from osteoblast/stromal cells that promote osteoclast differentiation and maturation, resulting in increased bone resorption promoting bone loss and eventual fracture [6; 58]. IL-6 has also been directly implicated in the sensitization of nociceptive fibers and evoked pain and has been shown to mediate both peripheral and spinal sensitization indicating that it may play a role in tumor-induced bone pain [11; 22; 25; 26; 45; 49; 50; 56; 60; 61; 63; 65]. In addition, IL-6 has been linked to tumor growth, cell migration, invasion, and evasion of apoptosis [3]. Given these observations, we examined the hypothesis that blockade of IL-6 signaling will diminish tumor-induced pain and disease progression including tumor-induced bone loss and tumor growth within the bone using a

novel small molecule antagonist TB-2-081 (3-O-formyl-20R,21-epoxyresibufogenin) previously demonstrated to block signaling at the sIL-6 receptor [37; 42; 60].

Materials and Methods

Experimental Design

Analysis of IL-6 induced intracellular signaling—The Jak/STAT signaling cascade was assessed to determine if TB-2-081 blocks IL-6 signaling. Cultured MAT B III cells were pre-treated with either vehicle (0.1% DMSO in Opti-Mem) or TB-2-081 (10ug) for one hour. Following pre-treatment, the testing media was removed and cells were challenged with 5ng of IL-6 recombinant protein in Opti-Mem for 1 hour. Cells were harvested, lysed, and the extracted protein underwent Western Blot analysis to investigate changes in pSTAT and STAT levels. Beta-actin served as the loading control. Untreated (naïve), vehicle pre-treated, and TB-2-081 pre-treated cellular protein was run in triplicate on every blot.

Analysis of intramedullary and systemic IL-6—Animals underwent surgical implantation of MAT B III cells or equivolume cell-free media (sham controls) into the tibia under anesthesia. For assessment of plasma IL-6 levels, rats were deeply anesthetized using ketamine/xylazine on day 12 post-intratribial surgery and terminal blood draws were performed by cardiac puncture. Samples were processed and plasma collected for ELISA analysis of IL-6. For assessment of intramedullary IL-6, rats were humanely euthanized and tibias collected. The intramedullary space of the bone was flushed with a 1mL mixture of protease inhibitors in PBS. Samples were processed for presence of IL-6 via ELISA analysis.

Analysis of acute TB-2-081 administration on tactile hypersensitivity—All rats had pre-surgery tactile sensory thresholds measured. Rats underwent surgical implantation of MAT B III cells or equivolume cell-free media (sham controls) into the tibia under anesthesia. Twelve days later, tactile sensory thresholds were retested. Rats were then treated with TB-2-081 (10 mg/kg, i.p.) or the vehicle (PEG400) and tactile sensory thresholds were measured 30, 60 and 90 min post administration. Experimental design was 2 (sham vs cancer) × 2 (TB-2-081 × vehicle) with time as a within-subject variable. Initial tactile hypersensitivity testing was conducted using 1mg/kg TB-2-081 based on previously published data from our lab showing reversal of tactile hypersensitivity in a rat model of acute pancreatitis [60]. This dose failed to reverse tactile hypersensitivity in cancer-bearing rats, suggesting that cancer pain is associated with a different dose-respons relationship. For this reason, a 10-fold higher dose was selected.

Analysis of acute TB-2-081 administration on ongoing pain—Rats underwent surgical implantation of MAT B III cells or equivolume cell-free media (sham controls) into the tibia under anesthesia. Blockade of ongoing pain by TB-2-081 was measured using the 3-day single trial conditioned place preference (CPP) procedure 11-13 days post-surgery as previously described [52]. Since the pain alleviating effects of TB-2-081 have a slow onset, as demonstrated by its peak reversal of tactile hypersensitivity in cancer-bearing rats at 30 min post-administration, we rationalized that pre-treatment with TB-2-081, if effective at

alleviating ongoing pain, would prevent CPP to saphenous nerve block (given at the time of peak effect; 20 min post-TB-2-081). This indirect method has been used to examine whether systemic administration of duloxetine blocks ongoing pain measured by peripheral nerve block in a rat model of osteoarthritis [29]. On conditioning day (D12 post-surgery) of the single trial CPP procedure, rats were pre-treated with vehicle (PEG400, i.p.), and 20 min later were given a saphenous saline injection under light isoflurane anesthesia during the morning pairing. For the afternoon pairing, rats received TB-2-081 (10mg/kg, i.p.) followed 20 min later by saphenous lidocaine administered under light isoflurane anesthesia. All rats awoke within 1 min of isoflurane removal. Failure of saphenous lidocaine to induce CPP in TB-2-081 treated rats would indicate that TB-2-081 successfully blocked cancer-induced ongoing pain. In contrast, equivalent CPP to the lidocaine-paired chamber observed in both vehicle and TB-2-081 treated rats would indicate failure of TB-2-081 to block tumor-induced ongoing pain. Experimental design is 2 (sham $\times$ cancer) $\times$ 2 (TB-2-081 $\times$ vehicle).

Analysis of sustained TB-2-081 administration on tactile hypersensitivity—All rats had pre-surgery tactile sensory thresholds measured. Rats underwent surgical implantation of MAT B III cells or equivolume cell-free media (sham controls) into the tibia under anesthesia. While still under anesthesia, rats had Alzet osmotic mini-pumps implanted (s.c.) to deliver TB-2-081 or vehicle (PEG400) at a dose of 1 mg/kg/day across 7 days. On day 7, pumps were replaced with freshly prepared pumps to ensure continuous drug delivery. Tactile sensory thresholds were retested on days 10 and 12 post-tibial surgery. Experimental design is 2 (sham $\times$ cancer) $\times$ 2 (TB-2-081 $\times$ vehicle) with time as a within-subject variable.

Analysis of sustained TB-2-081 administration on ongoing pain—Rats underwent surgical implantation of MAT B III cells or equivolume cell-free media (sham controls) into the tibia under anesthesia. Blockade of ongoing pain by TB-2-081 was measured using the 3-day single trial CPP procedure 11-13 days post-surgery as previously described [52]. On conditioning day (D12, conditioning), rats were lightly anesthetized with isoflurane and given a vehicle treatment of saphenous saline followed by immediate (<1 min) confinement into the appropriate pairing chamber for 30 min, following which they were returned to their home cage. Four hours later, rats were lightly anesthetized with isoflurane and treated with saphenous lidocaine followed by immediate (<1 min) confinement to the opposite pairing chamber for 30 min. All rats awoke within 1 min of isoflurane removal. If the sustained TB-2-081 blocks ongoing pain, then the tumor-bearing TB-2-081 treated rats should not demonstrate CPP to the saphenous lidocaine-paired chamber. In contrast, equivalent saphenous lidocaine-induced CPP in vehicle and TB-2-081 treated rats would indicate failure of sustained TB-2-081 delivery to block tumor-induced ongoing pain. Experimental design is 2 (sham $\times$ cancer) $\times$ 2 (TB-2-081 $\times$ vehicle).

Analysis of sustained TB-2-081 administration on tumor-induced bone remodeling—Radiographs were performed prior to tibial surgery and following behavioral testing on days 12-13 post-intratibial surgery for all rats examining the effects of sustained TB-2-081 on tumor-induced tactile hypersensitivity and ongoing pain. Experimental design is 2 (sham $\times$ cancer) $\times$ 2 (TB-2-081 $\times$ vehicle). A subset of tibias from each sustained administration treatment group were collected and sent for microCT analysis of tumor-

induced bone remodeling. Experimental design is 2 (sham × cancer) × 2 (TB-2-081 × vehicle).

Analysis of IL-6 and TB-2-081 administration on tumor growth—The impact of IL-6 and TB-2-081 on growth of MAT B III cells was performed in vitro. MAT B III cells were plated at a pre-determined density of 20,000 cells/well in a 96-well plate and allowed to adhere for a 24-hour period before any testing. Cell viability was assessed following administration of a range of concentrations of IL-6 (0.1–50 ng/ml) at 1, 2, 12, and 24 hrs, and TB-2-081 (0.01 – 1,000 µM) at time points 2 and 24 hours, by XTT assay. The impact of sustained TB-2-081 on tumor growth in vivo was measured using Hematoxylin and Eosin (H&E) histological analysis of tibias collected following behavioral and radiograph analysis D12 post-tibial surgery.

Effects of IL-6 on RANKL expressing osteoblasts—To determine if IL-6 promotes RANKL expression that is blocked by TB-2-081, cultured osteoblasts were exposed to 0.1% DMSO or TB-2-081 pre-treatment prior to challenge of IL-6 recombinant protein. Osteoblasts were harvested from 24–48 hour old neonatal Fischer F344/NhSD pups. Osteoblasts were allowed to grow until confluent (~3 days), at which time they were collected and plated at a density of 1×10^6 cells/well in a 6-well plate and allowed to grow again to confluence. Osteoblasts were pre-treated with either vehicle (0.1% DMSO in Opti-Mem) or drug (10 µg of TB-2-081) for 2 hours. Following pre-treatment, the media was removed and cells were challenged with 5 ng of IL-6 recombinant protein in Opti-Mem for 1 hour. After treatment, osteoblasts were immediately prepared for RANKL immunofluorescence to observe membrane-bound expression levels.

General procedures

Animals—Female Fisher F344/NhSD rats (Harlan Laboratories Inc., Indianapolis, IN, USA) weighing 150 to 200g were kept on a 12-hour dark/light cycle with food and water *ad libitum*. All experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee of the University of Arizona, the guidelines set forth by the National Institutes of Health, and the ARRIVE animal reporting recommendations.

Cell line—Rat 13762 MAT B III (CRL-1666, ATCC, Manassas, VA, USA) mammary adenocarcinoma cells were maintained in McCoy's 5A media (CellGro, Manassas, VA, USA) with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin at 37°C. Cells were harvested for use in this study between passages 15 and 35.

Intratibial Surgery and Cancer Implantation

This surgical procedure was performed as previously described [52]. Rats were anaesthetized using a mixture of ketamine and xylazine (intraperitoneal [i.p.] ketamine/xylazine, 80/12 mg/kg; Western Medical Supply, Arcadia, CA; Sigma, St. Louis, MO, USA). The right hindlimb of the rat was shaved and disinfected with 70% ethanol and betadine. The animal was placed on its back and a 1 cm incision was made horizontally across the femoral-tibial region to expose the patellar tendon, and surrounding skin retracted to expose the proximal end of the tibia. A small hole was drilled between the lateral and

medial condyles into the intramedullary canal followed by insertion of a 5 cm, 28-gauge guide cannula (Plastics One) attached to Tygon tubing (Cole-Palmer) to a 25 μ L syringe (Hamilton, Reno, NV), with location verified by x-ray imaging (Faxitron, Tucson, AZ). Injection of 5 μ L of MAT BIII cells, or cell free McCoy's serum free media (vehicle), was followed by sealing the drilled hole with bone cement (Stryker Orthopaedics, Simplex P Bone Cement, Mahwah, NJ). The area was flushed with sterile saline and the knee joint was reinforced with a vicryl 5-0 suture (Ethicon, Cornelia, GA) placed across the drilled area. Each rat received 1 mg/ml of gentamicin sulfate (Sparhawk Laboratories Inc, Lenexa, KS) via subcutaneous (s.c.) injection and was allowed to recover from anesthesia prior to return to the housing colony. Animals did not receive treatment with analgesics following tumor implantation as treatment with NSAIDs, specifically COX inhibitors, as well as morphine have been demonstrated to impact aspects of disease progression including tumor growth and tumor-induced bone remodeling [35; 53]. For ethical considerations, all experiments were terminated within 14 days of tumor inoculation into the tibia. A total of 83 tumor bearing rats and 50 sham rats were used across all studies.

Drug Administration

Saphenous lidocaine—Rats were anaesthetized with a 2% isoflurane O₂ mixture. To produce an effective peripheral nerve block, lidocaine (Roxane Laboratories, Columbus, OH) was administered over the saphenous nerve in a single s.c. injection (4% wt/vol, 350 μ L). Equivolume saline was given as a vehicle control. The saphenous nerve was chosen because it is the primary innervation of the tibia in rats and is completely sensory in function so that nerve block will not produce motor impairment. Proper injection of the anesthetic was identified as a small bubble appearing under the skin following administration before dispersing.

TB-2-081—Sustained administration of TB-2-081 was accomplished utilizing osmotic mini-pumps (model 2001, Alzet, Cupertino, CA, USA). The compound was made up in PEG400, and using sonication, was agitated and dissolved. Due to the viscosity of the solution, it was kept in a 37°C water bath prior to mini-pump filling. The pumps were primed with either TB-2-081 or PEG400 24-hours in advance TB-2-081 was prepared in a similar manner for acute administration, and was injected (i.p.).

In vivo Behavioral Testing

Experimenters blinded to the treatment conditions conducted all behavioral testing. Sample sizes were based on extensive literature using the von Frey test and conditioned place preference.

Tactile Hypersensitivity—Paw withdrawal thresholds were determined in response to probing with calibrated von Frey filaments (Stoelting, USA) with spaced increments ranging from 0.5 to 15 g. All animals were allowed to acclimate in suspended wire mesh cages for 30 minutes before the start of the study, and each filament was applied to the middle of the plantar surface of the paw using the “up and down” method and analyzed using a Dixon nonparametric test [16; 24].

Conditioned Place Preference (CPP) to Pain Relief by Saphenous Lidocaine

Rats in this study underwent single trial CPP to saphenous lidocaine on days 11 through 13 post-intratumoral surgery as previously described (Remeniuk et al, 2015). The 3-chamber CPP apparatus consists of 2 conditioning chambers with distinct tactile, visual, and olfactory cues, connected by a smaller neutral chamber that was brightly lit. Rats for this study were not handled before the start of this experiment to minimize the potential to produce pain by inadvertent movement of, or damage to, the cancer-bearing limb. White noise was played to provide background noise and block out any extraneous sounds. On the first day of the experiment (D11, preconditioning), rats were introduced to the neutral chamber and allowed to explore all 3 chambers for 15 min. Baseline time spent in the chambers was measured using ANYmaze tracking software (Stoelting, USA). Exclusion criteria for rats were spending less than 20% (180 sec) or more than 80% (720 sec) of total time (15 min) in a single chamber. Rats were assigned treatment–chamber pairings using a counterbalanced design for the following day. Care was taken so that group means for the morning (vehicle) and afternoon (drug) chamber pairings were not significantly different (unbiased CPP design). On the second day (D12, conditioning), rats underwent conditioning. Rats were lightly anesthetized with isoflurane and given a vehicle treatment of saphenous saline followed by immediate (<2 min) confinement into the appropriate pairing chamber for 30 min, following which they were returned to their home cage. Four hours later, rats were lightly anesthetized with isoflurane and treated with saphenous lidocaine followed by immediate (< 2 min) confinement to the opposite pairing chamber for 30 min. All rats awoke within 1 min of isoflurane removal. On the final day (D13, testing), rats were once again allowed to freely explore the apparatus for 15 min. Time spent in the chamber was recorded by ANY-maze. Preference for the lidocaine-paired chamber was calculated as difference scores, subtracting the baseline time from the testing time (test-baseline). A positive score indicates preference.

Collection of intramedullary and systemic IL-6

Bone Exudate Collection—Prior to the start of bone exudate collection, a stock solution of 1X PBS + 1X PIC was pre-loaded into 1mL syringes (309659, Becton Dickinson, USA) with 25-5/8 gauge needles (305122, Becton Dickinson, USA) and placed on ice. Rats were humanely euthanized following the University of Arizona IACUC and the American Veterinary Medicine Association's guidelines. The skin surrounding tibia and proximal end of the femur were removed. Bone shears were used to cut through the femur to detach the limb from the body. The muscle surrounding the tibia was removed, and using the bone shears, the distal and proximal ends of the tibia were clipped off to reveal the intramedullary space. Tweezers held the bone in place over a 1.5mL centrifuge tube, and the needle of the syringe was inserted into the proximal end of the tibia. Lightly pressing down on the plunger of the syringe, the bone marrow was forced through the distal end of the tibia until all of the contents of the syringe were emptied. Bone exudates were homogenized via sonication and spun down at 11,500 rpm for 5 min at 4°C. The supernatant was collected in 1.5mL tubes and stored at –20°C to analyze IL-6 levels using ELISA. Multiple samples were collected for analysis, with each sample run in duplicate.

Plasma Collection—Following all behavioral experiments and radiograph analysis D12 post tibial surgery, rats were deeply anesthetized using ketamine/xylazine and terminal blood draws were performed by cardiac puncture and placed in anticoagulant (EDTA)-treated tubes (Becton Dickinson, USA). Cells were removed from plasma by centrifugation at 3,000 rpm for 10 minutes at 4°C. The supernatant (plasma) was collected for ELISA detection of IL-6. All samples were run in duplicate.

In Vitro Assays

Western blot—MAT B III cells were grown in 25cm² flasks and allowed to reach 80% confluency. Cells were pre-treated with either vehicle, 0.1% DMSO in Opti-Mem, or 10ug of TB-2-081, for one hour. Following pre-treatment, the testing media was removed and cells were challenged with 5ng of IL-6 recombinant protein in Opti-Mem for 1 hour. The media was removed following testing, and the cells were washed with chilled phosphate buffered saline (PBS). Cells were harvested and lysed with a mixture of RIPA buffer (Thermo Scientific, USA), phosphatase inhibitors (Thermo Scientific, USA), and protease inhibitor cocktail (PIC) (Roche Pharmaceuticals, USA) for 10 min. The mixture underwent sonication for 5 seconds and centrifuged at 11,500 rpm for 5 min at 4°C (5804R, Eppendorf, USA). Supernatant was collected, and a bicinchoninic acid (BCA) assay was performed to determine total protein concentration. Protein was stored in aliquots at –80°C. Proteins were separated via electrophoresis on 10% SDS-PAGE gels (456-1034, Bio-Rad, USA) and transferred to a PVDF membrane (IPVH00010, EMD Millipore, USA). Membrane was blocked with 5% bovine serum albumin (BSA), then incubated overnight at 4°C in 1% BSA with dual primary antibodies: mouse monoclonal to pSTAT3 (4113S, Cell Signaling Technologies, USA) and rabbit polyclonal to beta-actin (ab8227, Abcam, USA); or rabbit monoclonal to Stat3 (4904S, Cell Signaling Technologies, USA) with mouse monoclonal to beta-actin (ab8226, Abcam, USA). Secondary IRdye antibodies were incubated at room temperature for 1 hour: goat anti-mouse 680RD (926-68070, Li-Cor, USA) and goat anti-rabbit 800CW (926-32211, Li-Cor, USA). Blot was dried and imaged via Li-Cor Odyssey with channels 600, 700, and 800 for 1 min each. Protein quantification was determined with Image Studio.

IL-6 ELISA—In preparation for detecting levels of IL-6 via ELISA (BMS625, Affymetrix eBioscience, USA), bone exudates, plasma, and cancer cell medium were placed on ice and allowed to thaw. ELISA kits were followed according to the printed protocol provided. Absorbance was read at a wavelength of 450nm (Synergy 2, Biotek, USA). Standard curves were generated using Graphpad Prism 6, and concentrations of IL-6 were interpolated from the information provided.

XTT analysis of cell viability—MAT B III cells were plated at a pre-determined density of 20,000 cells/well in a 96-well plate and allowed to adhere for a 24-hour period before any testing. Prior to the start of the experiment, McCoy's complete media was collected and stored at –20°C to analyze for levels of IL-6. For all cell viability studies, Opti-Mem reduced serum media (31985-070, Life Technologies, USA) was used to allow for the observation of the compounds' effects on the cells without the interference of the FBS. XTT activated reagent was created by taking 100uL of activation reagent and adding it to a 5mL

aliquot of XTT reagent (30-1011K, ATCC, Manassas, VA, USA). Using a multi-channel pipettor, 50uL of the activated reagent was then added to each well. A sealing film was placed over the wells, and the plate was placed in a 37°C incubator for 2 hours. Absorbance was read at a wavelength of 475 nm for specific readings and 660 nm for non-specific readings (Synergy 2, Biotek, USA). The effects of IL-6 on viability of MAT B III cells were examined in a dose- and time- dependent manner using IL-6 rat recombinant protein (IL025, EMD Millipore, USA). A stock solution of 50ng of IL-6 was made in Opti-Mem with subsequent serial dilutions to make 10ng, 5ng, 1ng, 0.5ng, and 0.1ng solutions, respectively. Cells were treated with 100uL of each of the solutions for a time course of 1, 2, and 12 hours, and then analyzed for cell viability. Opti-Mem was used as a blank control. The effects of blocking IL-6 signaling on the viability of MAT B III cells was examined by application of the small molecule IL-6 antagonist, TB-2-081, for 12- and 24-hours. TB-2-081 was synthesized by Drs. Booth and Rice. The compound underwent sonication in a solution of 0.1% dimethyl sulfoxide (DMSO) and Opti-Mem and was pipetted onto cells at 100uL/well. Opti-Mem + 0.1% DMSO was used as a blank control for this study.

Osteoblast Assay—Osteoblasts were harvested from 24-48 hour neonatal Fischer F344/NhSD pups. The calvarias were extracted and each skull was placed in an individual 7mL scintillation vial. Calvarias were washed with PBS and 1mL of 0.25% trypsin was placed in the vials containing the calvarias for 10 min at 37°C to remove any remaining membrane debris. Trypsin was neutralized with supplemented DMEM (sDMEM; comprised of 10% FBS, 100ug/mL of penicillin and streptomycin, and 0.25ug/mL of amphotericin). Skulls were incubated in 1mL of 0.2% collagenase in Hank's balanced salt solution (HBSS) for 30 min at 37°C. This solution was discarded at the end of the time period, and fresh 1mL – 0.2% collagenase was added to each vial for 1 hour at 37°C. Skulls were washed with 5mL of sDMEM, and the collagenase/media mixture was put into a 15mL conical tube and spun down at 1500g for 5 min at room temperature. The supernatant was removed and the pellet re-suspended in 1mL of sDMEM. Suspension was then placed into a 75cm² flask (430641, Corning, USA) containing 20mL of sDMEM. Osteoblasts were allowed to grow until confluent (~3 days), at which time they were collected and plated at a density of 1×10^6 cells/well in a 6-well plate. Three milliliters of sDMEM was placed into each well and the osteoblasts were allowed to grow to confluency (~3-4 days, day 6-7 post-calvaria collection). Upon reaching confluency, sDMEM was removed and cells were washed with PBS. Osteoblasts were pre-treated with either vehicle (0.1% DMSO in Opti-Mem) or drug (10 µg of TB-2-081) for 2 hours. Following pre-treatment, the media was removed and cells were challenged with 5 ng of IL-6 recombinant protein in Opti-Mem for 1 hour. After treatment, osteoblasts were immediately prepared for RANKL immunofluorescence to observe membrane-bound expression levels. Cells were fixed with 4% paraformaldehyde for 10 minutes. Wells were washed twice with PBS and blocked with 5% BSA in 0.05% tris buffered saline with tween 20 (TBST) for 1 hour at room temperature. Osteoblasts were incubated overnight at 4°C with anti-sRANKL antibody (1:1000 dilution; ab62516, Abcam, Cambridge, MA, USA) in 1% BSA. The following day, cells were incubated with Alexafluor 488 secondary (1:1000 dilution; A-11070, Life Technologies, USA) in 1% BSA in the dark for 1 hour at room temperature. Wells were washed twice with TBS and imaged

on a Hamamatsu fluorescence microscope with a FITC filter. Membrane-bound RANKL expression was quantified via ImageJ.

Bone Image Analysis

Radiograph Analysis—Confirmation of tumor-induced bone remodeling was determined by radiograph imaging (Faxitron, Tucson, AZ, USA) as previously described (Remeniuk et al, 2015). Bones were rated according to a 3 point scale: 0=normal bone; 1=bone loss/pitting; 2=unicortical fracture. An observer blinded to the treatment evaluated radiographs.

Micro-Computed Tomography—Following behavioral and radiograph analyses, rats were euthanized and tibias were dissected. The tibias were post-fixed in 10% neutral buffered formalin across 3 days. They were then transferred to 70% EtOH. To characterize the cancer-induced changes in mineralized bone micro-architecture, the proximal tibias were analyzed with a SCANCO micro-computed tomography (μ CT). Bones were loaded into 12.3 mm-diameter scanning tubes and imaged using a vivaCT-40 scanner (SCANCO Medical AG, Basserdorf, Switzerland). Scans were integrated into three-dimensional voxel images with trabecular measurements scanned at 10.5 mm high resolution (2048×2048 pixel matrices) and cortical bone measurements scanned at 21 μ m medium resolution (1048×1048 pixel matrices). A threshold of 220 was applied to all scans at high resolution ($E = 55$ kVp, $I = 145 \mu$ A, integration time 300 ms). All trabecular measurements were made by drawing contours every twenty slices and using voxel counting for bone volume per tissue volume and sphere-filling distance transformation indices. Cortical thickness was measured at the proximal tibial metaphysis with an isotropic pixel size of 21 μ m and slice thickness of 21 μ m.

Hematoxylin and Eosin staining

To verify tumor growth, rat tibias were decalcified using 10% EDTA across ~3 weeks. Decalcification was verified using radiograph analysis. Bones were cut in half and each half embedded in paraffin wax in an automated tissue processor (Sakura, Torrance, CA, USA). Sections were collected using a microtome (Leica Biosystems, USA) at 3-5 μ m and directly mounted on slides for H&E staining. Slides were dried overnight and baked at 60° C for 1 hour. Slides were dewaxed, rehydrated, and stained with H&E on a Leica Autostainer XL, then dehydrated through graded alcohols, cleared with xylene and mounted with a resinous mounting medium (Mercedes Medical, Sarasota, FL, USA). Montage images of stained slides were acquired on a Leica DM2500 brightfield microscope. Region of the bone with tumor was assessed using FIJI software (FIJI is just ImageJ).

Statistical Analyses

Baseline tactile hypersensitivity responses were compared to post-administration time points by 2-way analysis of variance (ANOVA) followed by post hoc analysis using Bonferroni's multiple comparisons test using GraphPad Prism 6. A probability level of $p < 0.05$ was used to establish significance. Differences in tactile hypersensitivity between treatment groups at each time point were determined by 2-way ANOVA with Tukey's post-hoc analysis for multiple comparisons. For CPP, the effects of lidocaine treatment on cancer vs. sham rats were analyzed by 2-way ANOVA followed by post hoc analysis between pre-conditioning

(BL) vs. post-conditioning (testing) values within each treatment group using Sidak correction. Analysis of variance on the difference scores calculated as (post-conditioning) - (pre-conditioning) time spent in the drug-paired chamber was performed to determine differences between treatment groups. Bone pitting ratings were analyzed using Mann-Whitney U-test. Differences in treatment groups of micro-CT images and XTT assays were compared using a 2-way ANOVA with multiple comparisons, with Sidak and Bonferroni post-hoc, respectively. Western blot and RANKL group differences were analyzed with a one-way ANOVA with multiple comparisons and Dunnett correction. All graphs show mean $\pm$ SEM.

Results

TB-2-081 blocks IL-6 induced activation of the Jak/Stat3 signaling cascade

Application of rat recombinant protein IL-6 to cultured MAT B III cells significantly increased phosphorylation of STAT3 compared to naïve (untreated) cells (Fig. 1A,B, $^{**}p<0.01$). Pretreatment of the cultured cells with TB-2-081 blocked the IL-6 induced phosphorylation of STAT3 (Fig. 1A,B). Paralleling this, application of recombinant protein IL-6 reduced total STAT in cultured MAT B III cells (Fig. 1C,D, $^{**}p<0.01$). Pretreatment with TB-2-081 blocked the IL-6 induced reduction in total STAT (Fig. 1C,D, $p>0.05$).

Tumor growth in the tibia elevates local and plasma IL-6

Bone exudates harvested 12 days post-intratibial surgery revealed that tumor bearing tibias had significantly elevated levels of IL-6 within the bone microenvironment, with an approximate 5-fold increase observed in tumor bearing rats compared to naïve rats (Fig. 2A, $^{*}p<0.05$ vs. naïve). Plasma levels of IL-6, measured 12 days post-intratibial surgery revealed that IL-6 was also significantly elevated with an approximate 15-fold increase compared to naïve controls (Fig. 2B, $^{**}p<0.01$ vs. sham).

Acute blockade of IL-6 signaling inhibits tumor-induced tactile hypersensitivity, but not ongoing bone pain

Tumor-induced tactile hypersensitivity observed day 12 post cancer surgery (Fig. 3A, $^{#}p<0.05$ vs. BL) was fully, but transiently, reversed by administration of a single dose TB-2-081 (10 mg/kg, i.p) within 30 min of administration ($^{***}p<0.001$ vs. D12 pre-drug thresholds). Hypersensitivity re-emerged at 60 min post-TB-2-081 administration ($^{#}p<0.05$ vs. BL, $^{*}p<0.05$ vs. BL), with sensory thresholds returning to pre-drug levels at 90 min post-administration.

Using conditioned place preference (CPP), tumor-induced ongoing pain was revealed by determination of increased time spent in a saphenous lidocaine paired chamber in animals that received vehicle or TB-2-081 (i.p.) 20 min prior to saphenous nerve block ($^{**}p<0.01$ vs. sham vehicle). Ongoing pain was also observed in animals that received TB-2-081 (10 mg/kg, i.p.) 20 min prior to saphenous nerve block, as a significant increase in time spent in the saphenous nerve block chamber was observed ($^{*}p<0.05$ vs. sham-TB-2-081). No significant difference was observed between saphenous nerve block induced CPP between vehicle and TB-2-081 treated tumor bearing rats, with equivalent increases in time spent in

the saphenous nerve block chamber observed between these treatment groups ($p>0.05$)(Fig. 3B).

Sustained blockade of IL-6 signaling inhibits tumor-induced bone pain

Comparison of paw withdrawal thresholds demonstrate that tumor-bearing rats developed tactile hypersensitivity between days 7 and 10 post-tumor inoculation that progressively worsened by day 12 post surgery (Fig. 3C, **** $p<0.001$ vs. D7 with Bonferroni's multiple comparison's test). Treatment with the TB-2-081 significantly blocked development of tumor-induced tactile hypersensitivity (** $p<0.05$ vs. cancer vehicle D12). Group comparisons revealed that tumor-bearing rats treated with TB-2-081 had elevated thresholds compared to cancer vehicle treated rats (Fig. 3C, **** $p<0.0001$ vs. cancer vehicle with Tukey's multiple comparison's test); however, these thresholds were still significantly lower compared to sham treated rats ($\#p<0.05$).

Tumor bearing rats also demonstrated ongoing pain as indicated by CPP to saphenous nerve block administered on day 12 post tumor inoculation into the tibia. Difference scores for the saphenous lidocaine paired chamber demonstrate a significant increase in time spent in the saphenous nerve block paired chamber (### $p<0.01$ t-test vs. null hypothesis). Group comparisons confirmed that vehicle treated tumor-bearing rats demonstrated significantly more time in the saphenous nerve block chamber compared to sham controls (Fig. 3D, ** $p<0.01$ vs. sham vehicle). Sustained treatment with TB-2-081 prevented peripheral nerve block induced CPP indicating a block of ongoing bone cancer pain ($p>0.01$ vs. null hypothesis). Difference scores for the saphenous lidocaine paired chamber demonstrated no change in the nerve block paired chamber day 12 post-tumor inoculation, with difference scores equivalent to sham treated rats (Fig. 3D, $p>0.05$ vs. sham vehicle).

Sustained blockade of IL-6 signaling blocks tumor-induced bone remodeling

To determine whether the sustained delivery of the TB-2-081 alters disease progression, radiographs were taken following behavioral testing at day 12 post-testing. Representative radiographs indicate that the sustained administration of TB-2-081 significantly blocked tumor-induced bone loss (Fig. 4A). Ratings of the radiographs by an experimenter blinded to the treatment conditions of the bones support the observation that tumor-induced bone loss is minimal in the TB-2-081 treated rats at day 12 (Fig. 4B, *** $p<0.001$ vs. vehicle).

To gain higher resolution and quantitative analysis of tumor-induced bone remodeling, tibias from each treatment group underwent microCT (μ CT) analysis. Representative images demonstrate that there is some bone remodeling of the trabecular bone from the surgery (Fig. 5 Sham/TB-2-081 vs. contralateral). Growth of the MAT B III cells within the tibia induced osteolytic and osteoblastic bone remodeling, with cortical lesions apparent (red arrow) along with signs of osteoblastic remodeling (yellow arrows) (Fig. 5, Cancer/vehicle). Sustained administration of the TB-2-081 blocked tumor-induced bone remodeling, with diminished cortical lesions and osteoblastic growth on the outer cortical bone as well as altered trabecular bone (Fig. 5, Cancer/TB-2-081).

Quantitative analysis of μ CT demonstrates that tumor growth increased trabecular number and decreased trabecular spacing in tumor-bearing bones of vehicle treated rats, indicating

tumor-induced osteoblastic bone remodeling (Fig 6. A&B, respectively; * $p<0.05$; ** $p<0.01$ vs. sham vehicle). These changes were normalized TB-2-081, with trabecular number and spacing in the tumor bearing TB-2-081 treated rats similar to sham treated controls (Fig. 6 A&B, respectively). Analysis of tumor bone volume demonstrates that treatment with TB-2-081 significantly elevated trabecular bone volume selectively in tumor bearing rats (Fig. 6C, ** $p<0.01$ vs. sham vehicle). No differences were observed between tumor-bearing vehicle treated rats and sham controls. (Fig. 6C). TB-2-081 treatment also significantly increased trabecular thickness (Fig. 6D, ** $p<0.01$ vs. sham vehicle). No differences in trabecular thickness was observed between tumor bearing vehicle treated rats and sham controls (Fig. 6D). Although pitting was observed in the x-ray images, and full cortical bone lesions in μ CT images, no significant differences in cortical bone volume or thickness was observed between the treatment groups (Fig. 6 E&F, respectively).

TB-2-081 does not alter MAT B III viability in vitro or growth in vivo

Administration of IL-6 to cultured MAT B III cells increased cell viability at 1 hr post-administration at concentrations of 0.5 ng/ml and higher (Fig. 7A, *** $p<0.001$ vs. 12 hr). Concentrations of 10 and 50 ng/ml significantly increased cell viability at both 1 and 2 hours post administration (** $p<0.01$ vs. 12 hr). IL-6 failed to alter cell viability at 12 hr post-administration at any concentration tested ($p>0.05$ vs 0.1 ng/ml). XTT analysis of MAT B III revealed no change in cell viability in response to treatment with TB-2-081 (Fig. 7B) indicating that blocking autocrine IL-6 signaling from MAT-B-III cells does not alter cell viability. To determine whether sustained delivery of TB-2-081 alters tumor growth in vivo, we performed histological analysis of tumor within the tibia collected 12 days following injection of MAT B III cells. Analysis of tumor growth in tibia sections stained with Hematoxylin and Eosin (H&E) demonstrated equivalent tumor growth 12 days post inoculation (Fig. 7C). Representative images of the H&E staining demonstrated that both vehicle and TB-2-081 treated tibias showed tumor throughout the bone (Fig. 7D). Inset images demonstrate osteoblastic bone remodeling of trabecular bone as well as on the outside of the cortical bone of vehicle treated rats (Fig. 7D).

TB-2-081 reduces expression of RANKL in osteoblast cultures

Administration of IL-6 increased RANKL positive osteoblasts (Fig. 8A, * $p<0.05$ vs. untreated). TB-2-081 blocked the IL-6 induced increase in RANKL positive osteoblasts (Fig. 8B, # $p<0.05$ vs. IL-6; $p>0.05$ vs. untreated).

Discussion

Interleukin-6 is thought to play a causal role in chronic inflammatory and immune diseases, including rheumatoid arthritis, Castleman's disease, and pancreatitis [12; 13; 32; 59; 62]. Increasing evidence now points to a role of IL-6 in cancer [1; 3; 6; 44; 58] where increased levels serve as a negative prognostic indicator of advanced stage disease, including breast cancer patients with skeletal metastases [4; 5; 9; 19; 20; 28; 33; 38; 41; 54]. Consistent with these observations, we demonstrate increased IL-6 levels in both the local bone microenvironment and the blood serum of tumor-bearing rats. Sustained blockade of IL-6 signaling with the novel small molecule antagonist, TB-2-081 modified disease progression,

including prevention of tumor-induced osteoclastogenesis, bone loss and fracture, and blockade of ongoing pain. Notably, acute administration of the TB-2-081 at a time that tumor-induced bone loss and pain are well established failed to block tumor-induced ongoing pain at a dose that fully reversed hindpaw tactile hypersensitivity. These observations indicate that blockade of IL-6 signaling early in metastatic progression would yield the most beneficial effects in patient populations.

Interleukin-6 signals through both a classical pathway involving transmembrane bound glycoprotein 80 (gp80) and subsequent dimerization to glycoprotein 130 (gp130), and a trans-signaling pathway through a soluble IL-6/gp80 complex binding to gp130 [27; 54; 57]. Both pathways activate the Jak/STAT cascade allowing for transcription of pro-survival, growth, and cellular proliferation genes. TB-2-081 is a small molecule receptor antagonist that has been demonstrated to be effective at inhibiting IL-6 binding and reducing Jak/STAT3 signaling [37]. We confirmed TB-2-081 as an IL-6 receptor antagonist by demonstrating that blockade of IL-6-induced increased phosphorylation of the signal transducer and activator of transcription 3 (pSTAT3) in MAT B III cells and total STAT. IL-6 increased viability of cultured MAT B III cells. However, TB-2-081 failed to alter cell viability suggesting that the effects observed in vivo were not the result of direct action upon tumor progression. Consistent with this interpretation, histological analysis of tumor within the tibia collected 12 days following injection of MAT B III cells showed equivalent tumor growth in both groups treated with vehicle and TB-2-081 infusion starting at time of surgery.

Interleukin-6 is a pro-inflammatory cytokine that has been shown to promote pain by sensitizing nociceptors and amplifying signaling at the site of injury or disease [2; 11; 26; 45]. Under normal conditions, peripheral nociceptors fail to express detectable levels of IL-6, IL-6 mRNA, or IL-6 receptors (gp80), yet have high levels of gp130 expression [8; 39]. Injection of IL-6 with soluble IL-6 receptor (sIL-6R) into a rat hindpaw induces thermal and mechanical hypersensitivity. Subsequent deletion of gp130 in peripheral nociceptive fibers blocks thermal hyperalgesia induced by IL-6/sIL-6R injection [2]. Additionally, thermal hypersensitivity was restored in mice lacking peripheral gp130 by administration of a sIL-6R/gp130 complex. Several studies have demonstrated that blockade of IL-6 signaling decreases responses to evoked noxious or normally non-noxious stimuli (i.e., hypersensitivity to thermal and/or mechanical stimulation) across a variety of preclinical models [25; 34; 40; 42; 60]. Subcutaneous and oral administration of TB-2-081 was demonstrated to successfully reverse abdominal hypersensitivity in a rat model of pancreatitis [60]. In the present studies, acute administration of the TB-2-081 blocked the tumor-induced tactile hypersensitivity. Moreover, continuous infusion of TB-2-081 blocked development of tumor-induced tactile hypersensitivity suggesting that blockade of IL-6 signaling can both prevent and reverse tumor-induced tactile hypersensitivity.

We have previously demonstrated that animals will learn to associate contexts with treatments that produce relief of ongoing pain [36; 47; 48], and have shown conditioned place preference (CPP) via a saphenous nerve block selectively in rats with bone cancer [52]. We used this paradigm to determine if blockade of IL-6 signaling will eliminate the motivational drive to seek pain relief in tumor-bearing rats. Acute pretreatment with TB-2-081 30 min prior to saphenous nerve block failed to block lidocaine-induced CPP in

tumor-bearing rats. As the nerve block was given at a time-point associated with peak alleviation of tumor-induced tactile hypersensitivity, these data indicate that acute blockade of IL-6 signaling is insufficient to block ongoing pain once tumor growth and bone remodeling have developed. In contrast, we found that sustained treatment of TB-2-081 for 12 days prevented CPP to saphenous nerve block, signifying that continuous blockade of IL-6 signaling starting simultaneously at tumor implantation is sufficient to block tumor-induced ongoing pain. Together, our behavioral observations indicate that blockade of IL-6 signaling at time of tumor implantation blocked tumor-induced ongoing pain, but acute treatment once bone loss and tumor-induced bone pain are established, is insufficient to block ongoing pain.

Based upon these observations, we investigated the effects of sustained TB-2-081 treatment on tumor-induced bone remodeling and tumor growth within the bone. Radiograph images of the tumor-bearing tibias of vehicle-treated rats had noticeable pitting and fractures along the cortical shaft of the bone, while rats treated with TB-2-081 showed reduced bone loss. Imaging analysis with μ CT confirmed that TB-2-081 treatment significantly reduced bone remodeling in tumor-bearing rats, while histological analysis revealed no discernable changes in tumor volume within the intramedullary space of the tibias of either treatment group. These results suggest that IL-6 signaling is a key mediator of tumor-induced bone remodeling, but not necessary to tumor growth.

Osteoclastogenesis has been proposed to be a key component in osteolytic bone loss as a result of bone metastases [31; 55] and is a key target for therapies such as bisphosphonates or RANKL sequestering antibodies such as Denosumab [15; 31; 55; 64]. Supporting the role of IL-6 in tumor-induced bone remodeling, transgenic mice overexpressing IL-6 show impaired skeletal development, reduced osteoblasts and increased osteoclasts [21]. We demonstrate that TB-2-081 blocks IL-6 induced receptor activator of NF- κ B ligand (RANKL) expression in cultured osteoblasts, consistent with observations that IL-6 signaling production and release of RANKL from osteoblasts [6]. These observations suggest that the observed TB-2-081 blockade of tumor-induced bone remodeling in our study is likely through blockade of IL-6 receptors on osteoblasts resulting in diminishing RANKL production in turn reducing osteoclastogenesis and maintaining bone integrity.

Interleukin-6 signaling has been implicated in a multitude of events that likely contribute to disease progression in breast cancer bone metastasis, including osteolysis, tumor cell proliferation, and additional cytokine production. IL-6 regulates multiple components of immune function including regulation of macrophages and bone marrow mesenchymal cells (BMMCs) that are the source of growth factors, chemokines and cytokines and that play a role in tumor cell proliferation and survival [3,13]. Moreover, IL-6 has been implicated in increased release of pro-inflammatory cytokines that may contribute to multiple aspects of cancer-induced bone pain including tumor-induced bone loss and tumor-induced pain [3,6,13,26]. Furthermore, IL-6 is frequently upregulated in pathophysiological conditions where patients' display evoked and spontaneous pain symptoms. However, the potential role of blockade of IL-6 signaling in tumor-induced bone remodeling, tumor growth, or bone pain has not previously been explored. In this study, we show that sustained blockade of IL-6 signaling during disease progression blocks tumor-induced bone remodeling and

ongoing bone pain without altering tumor growth within the bone microenvironment. These data suggest that inhibiting IL-6 signaling may be a viable therapeutic option to reduce osteolytic bone loss and ongoing bone pain in cancer patients with breast cancer skeletal metastases. Blockade of IL-6 signaling could potentially eliminate, or reduce, reliance on opioids for pain relief, or delay the implementation of opioid therapy. Such an outcome could diminish tolerance and other adverse side effects such as nausea, constipation, somnolence, dizziness and mental clouding resulting in improved quality of life of these patients. Reducing opioids may have additional beneficial effects as studies have indicated potential effects of prolonged opioid treatment on bone loss and risk of fracture [35]. In fact, the stabilizing effect on the bone, preventing bone loss and diminishing risk of fracture, would further improve these patients' mobility. As reduced mobility associated with bone fragility and fracture are associated with great increases in morbidity and mortality in these patients [18; 43], such effects would be highly desirable in these patients.

Tocilizumab, a humanized anti-IL-6 receptor antibody, is presently approved for human use for rheumatoid arthritis and Castleman's disease [17], and may be a viable candidate for consideration in the treatment of patients with breast cancer bone metastases. Whether IL-6 signaling plays a similar role in propagating bone loss and ongoing pain in other cancers is not yet known and requires further study. While not a clinical candidate, the data with TB-2-081 suggest that the development of a small molecule antagonist to block IL-6 receptor signaling would be desirable in overcoming known risks associated with antibody immunotherapy, including acute anaphylaxis, increased risk of infection, serum sickness, antibody rejection, and potentially life-threatening cytokine release [14; 30]. The results of this preclinical investigation provides a basis for a proof of concept trial to evaluate whether blockade of IL-6 signaling may result in prevention or delay in bone remodeling and fracture, as well as decreased ongoing pain resulting in improved quality of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NCI T32CA009213 (BR) and NIH R01 DA034975 (FP), and support from the University of Arizona Cancer Center grant NCI P30 CA023074. The microCT was performed and analyzed by Lucy Liaw, PhD and Terry Henderson at the Microcomputed Tomography Services at Maine Medical Center Research Institute. This core is supported by the Maine INBRE funded by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health. The H&E images were performed by Peter Caradona within the COBRE Histology and Imaging core at the University of New England that is supported by the National Institute of General Medical Sciences (NIGMS) (P20GM103643). Osteoblast images were analyzed by Naomi Goshima at the University of Arizona. A portion of this work was supported by the NIH Intramural Research Programs of the National Institute on Drug Abuse (NIDA) and the National Institute of Alcohol Abuse and Alcoholism (NIAAA) (KCR).

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Summary

Sustained administration of an IL-6 receptor antagonist prevented cancer-induced bone loss and pain suggesting a disease modifying strategy for patients with cancer-induced bone pain.

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Figure 1. TB-2-081 blocks IL-6-induced up-regulation of the Jak/STAT pathway in cultured MAT B III cells

A. Representative western blot of pSTAT signaling **B.** Quantitative analysis of the Western blot demonstrates that IL-6 significantly increases phosphorylated STAT3 (** $p < 0.01$ vs. untreated). This is blocked by TB-2-081. **C.** Representative Western blot of total STAT. **D.** Quantitative analysis revealed that IL-6 challenge decreased total STAT signal (** $p < 0.01$ vs. untreated). This was decreased by co-administration of TB-2-081. All graphs display mean $\pm$ SEM.

A. IL-6 Levels in Bone Exudates (D12 post-surgery) **B. IL-6 Levels in Plasma (D12 post-surgery)**

Figure 2. Tumor growth increases local and systemic IL-6 levels

A. Tumor growth within the tibia increased IL-6 within the bone microenvironment collected 12 days post tumor implantation, * $p < 0.05$ vs. naive, $n = 4$ naive; 6 cancer. **B.** Tumor growth within the tibia increased serum IL-6 collected 12 days post tumor implantation, ** $p < 0.01$ vs. naive, $n = 3$ naive, 7 cancer.

Figure 3. Sustained blockade of IL-6 signaling blocks tumor-induced pain

A. Tumor-induced tactile hypersensitivity observed day 12 post cancer surgery was fully reversed by administration of TB-2-081 (10 mg/kg, i.p) within 30 min of administration. Hypersensitivity reemerged at 60 min post-TB-2-081 administration, with sensory thresholds returning to pre-drug levels at 90 min post-administration. ($^{\#}p < 0.05$ vs. BL, $^{***}p < 0.001$ vs. D12 pre-drug thresholds) **B.** Saphenous lidocaine significantly increased time spent in the drug-paired chamber in vehicle treated rats ($^{**}p < 0.01$ vs. sham vehicle). A similar increase in time spent in the saphenous lidocaine paired chamber was observed in rats that received acute administration of TB-2-081 (10 mg/kg, i.p.) 30 min prior to saphenous nerve block indicated failure of the antagonist to block the tumor-induced ongoing pain. **C.** Sustained infusion of TB-2-081 (1 mg/kg/day) starting the day of tumor injection partially blocks development of tumor-induced tactile hypersensitivity ($^{**}p < 0.01$, $^{****}p < 0.001$ vs. D7). Between groups analysis confirmed statistical differences between tumor bearing rats treated with TB-2-081 compared to vehicle ($^{****}p < 0.0001$). **D.** Sustained infusion of TB-2-081 (1 mg/kg/day) starting the day of tumor injection into the tibia blocks tumor-induced ongoing pain as measured by CPP to saphenous nerve block. Vehicle treated tumor-bearing rats demonstrated a significant increase in time spent in the saphenous nerve

block paired chamber (** $p < 0.01$ vs. sham vehicle). TB-2-081 treated rats did not demonstrate increased time spent in the saphenous lidocaine paired-chamber, with difference scores equivalent to sham treated rats ($p > 0.05$ vs. sham vehicle). Only cancer treated rats receiving vehicle treatment showed significantly elevated time in the saphenous nerve block paired chamber ($^{##}p < 0.01$ t-test vs. null hypothesis). All graphs represent mean $\pm$ SEM.

Figure 4. Sustained blockade of IL-6 signaling blocks tumor-induced bone loss

A. Representative radiographs demonstrates tumor-induced bone loss and cortical lesions in the vehicle treated rats. Sustained administration of the TB-2-081 blocked this tumor-induced bone loss. **B.** Bones were rated using a 3-point scale by an observer blinded to the treatment groups. Ratings indicate that the TB-2-081 blocks radiological evidence of tumor-induced bone loss, (* $p < 0.005$ vs. vehicle, Mann Whitney U nonparametric analysis; $n = 17$ TB-2-081; $n = 26$ vehicle).

Contralateral **Sham** **Cancer/Vehicle** **Cancer/TB-2-081**

Figure 5. Representative μ CT images demonstrate that MAT B III cell growth within the tibia generates osteolytic and osteoblastic bone remodeling

Cortical lesions are apparent (red arrow) as are clear regions of osteoblastic remodeling (yellow arrows). Sustained infusion of IL-6 antagonist starting day of tumor injection into the tibia blocks the apparent tumor-induced osteolytic and osteoblastic bone remodeling.

Figure 6. Quantitative analysis of μ CT revealed an osteoblastic bone remodeling of the trabecular bone that was normalized, in part, by treatment with TB-2-081

A. Cancer treated rats treated with vehicle demonstrated elevated trabecular number compared to sham controls (** $p < 0.01$ vs. sham vehicle). TB-2-081 treated tumor-bearing rats demonstrated equivalent number of trabecular bone compared to sham controls. **B.** Cancer treated rats receiving vehicle treatment demonstrate diminished trabecular spacing (* $p < 0.05$ vs. sham vehicle). TB-2-081 treated tumor-bearing rats demonstrated equivalent trabecular spacing compared to sham controls). **C.** TB-2-081 treated tumor-bearing rats

demonstrated increased overall trabecular bone volume compared to sham controls (** $p < 0.01$ vs. sham vehicle). Tumor-bearing rats failed to demonstrate altered trabecular bone volume. **D.** Tumor-bearing rats treated with TB-2-081 demonstrated elevated trabecular thickness compared to sham controls (** $p < 0.01$ vs. sham vehicle). Tumor-bearing rats failed to demonstrate altered trabecular thickness. No changes in cortical bone volume (**E**) or thickness (**F**) were observed between treatment groups.

Figure 7. TB-2-081 does not alter tumor growth in vitro and in vivo

A. Application of IL-6 increases MAT B III cell viability 1-2 hrs post administration as measured by XTT cell viability analysis. Administration of 0.5 ng/ml and higher concentrations of IL-6 increased cell viability at 1 hr (* $p < 0.05$ vs. 24 hr). Administration of IL-6 at 5 ng/ml and higher concentrations increased cell viability at 2 hrs post administration (# $p > 0.05$ vs. 24 hr post IL-6 administration). IL-6 did not increase cell viability at 12 or 24 hrs irrespective of dose administered. **B.** Application of TB-2-081 in vitro failed to alter MAT B III cell viability at any of the concentrations tested 2 or 24 hrs post administration as

measured by XTT cell viability analysis. **C.** Analysis of tumor growth within the intramedullary space of the tibia revealed equivalent tumor growth by D12 post tumor inoculation in vehicle and tumor bearing tibias. Analysis were performed across 6-8 sections from 3-4 tibias per group. **D.** Representative H&E staining demonstrating tibias from a sham control, vehicle treated tumor bearing bone, and TB-2-081 treated tumor bearing tibia. Call out boxes illustrate different tumor states within the bone as well as osteoblastic bone remodeling outside and inside the bone.

Figure 8. TB-2-081 blocks IL-6 induced RANKL release from cultured osteoblasts

A. Administration of IL-6 increased RANKL expressing cultured osteoblasts. Pretreatment with TB-2-081 (10 mg/ml) for 60 min blocked IL-6 (5 ng/ml, 60 min) induced RANKL expressing osteoblasts. **B.** Diagram of proposed mechanism of TB-2-081 blockade of tumor-induced bone remodeling. Administration of the small molecule sIL-6 antagonist blocks IL-6 induced release of further IL-6 from tumor cells. In addition, the TB-2-081 blocks IL-6 stimulation of RANKL from osteoblasts resulting in reduced osteoclastogenesis and associated reduction in the tumor-induced bone remodeling.