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Targeting cells of the myeloid lineage attenuates pain and disease progression in a prostate model of bone cancer

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Introduction

The vast majority of skeletal cancers are of metastatic origin rather than primary bone tumors. In the United States, it is estimated that there will be 1.6 million new cancer cases in 2015 and 50% of these new cases eventually metastasize to bone^{41,81}. Common tumors that avidly metastasize to bone include lung, breast, prostate, and renal cancer^{1,9}. Currently, bone metastases are a major cause of pain, disability, decreased functional status, and death in patients with metastatic cancer. Due to the limited understanding of the mechanisms that drive bone cancer pain, there have been few therapies developed that could control bone cancer pain without significant unwanted side effects, and few, if any, therapies that improve patient survival once the tumor metastasizes to bone.

The present study was initially focused solely on whether the multi-targeted tyrosine kinase inhibitor PX3397 could attenuate cancer-induced bone pain (CIBP). PLX3397 binds to and inhibits phosphorylation of colony stimulating factor-1 receptor (CSF1R), the tyrosine-protein kinase c-Kit, and the FMS-like tyrosine kinase 3 (FLT3), all of which regulate the proliferation and function of a subset of the myeloid cells including macrophages, osteoclasts and mast cells^{14,78,88}. The observation that prompted this study was that PLX3397 reduced the second stage inflammatory pain that follows formalin injection into the rat hindpaw (Fig. 1). The likely mechanism behind PLX3397 attenuation of this formalin-induced inflammatory pain was the blockade of monocyte/macrophage function as CSF1R has been shown to be involved in the regulation of the differentiation, proliferation, and activation of macrophages^{6,49,60,96}.

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Conflicts of Interest

The authors report no conflicts of interest.

Since activation and proliferation of macrophage and osteoclasts has been shown to be involved in driving CIBP^{50,54,74}, we used a mouse model of bone cancer where canine prostate tumor cells are injected and confined to one femur in the immune-compromised mouse^{30,38}. While the primary measure we were examining was pain, this model also provides a platform to simultaneously assess disease progression, tumor-induced bone remodeling, and tumor metastasis in the same animal. Using this model, we show that sustained administration of PLX3397 reduced CIBP and was also highly efficacious in reducing tumor cell growth, formation of new tumor colonies in bone, and pathological tumor-induced bone remodeling.

Methods and Materials

Surgical procedures and drug treatment

Rats—Experiments were performed on 12 Sprague-Dawley (SD) rats weighing 130 ± 20 g that were provided by BioLasco Taiwan (under Charles River Laboratories Technology Licensee). All animals were maintained in a controlled temperature (22–23°C) and humidity (50–70%) environment with 12 hours light/dark cycles for at least three days in MDS Pharma Services-Taiwan Laboratory prior to use. Animals had free access to standard lab chow MF-18 (Oriental Yeast Co., Ltd., Japan) and reverse osmosis (RO) water.

Mice—Experiments were performed with 48 adult male athymic nude mice (Jackson Laboratories, Bar Harbor, ME), approximately seven to eight weeks old, weighing 25 to 30 g. The mice were housed in accordance with the National Institutes of Health guidelines under specific pathogen-free conditions in autoclaved cages maintained at 22°C with a 12-hr alternating light/dark cycle and access to food and water *ad libitum*.

All animal procedures adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain⁹⁸ and were approved by the Institutional Animal Care and Use Committee at the University of Arizona (Tucson, AZ).

Cells—Canine ACE-1 prostate cancer cells (a kind gift from Dr. Thomas J. Rosol, Ohio State University) were stably transfected with green fluorescent protein (GFP) (Paragon Bioservices, Baltimore, MD), and were maintained at 37°C in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) (Sigma-Aldrich; St. Louis, MO; 51445C) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich; St. Louis, MO; P4333) and 0.1 mg/mL blasticidin (Invitrogen; Carlsbad, CA; R21001) in a 5% CO₂-humidified chamber. The ACE-1 cell line, derived from a spontaneous dog prostate carcinoma⁴⁷, forms osteoblastic bone metastases in athymic nude mice^{30,90,91}. Prostate cancer cells were passaged every 3–4 days, and were harvested for cancer cell injection between 6 and 10 passages after thaw.

Cancer Cell Injection Surgery

Two sets of experiments were performed with these mice. The first set of experiments defined the time course, extent, and techniques by which to quantify the pain, the pattern of tumor growth, and tumor-induced bone remodeling in sham injected animals vs. animals

that received injection of prostate cancer cells. In the second set of experiments we defined the effect that PLX3397 had on the pain, the extent and pattern of tumor growth, and tumor-induced bone remodeling in animals that received injection of prostate cancer cells. Prostate cancer cells (10^5 cells) were injected directly into the intramedullary space of the mouse femur as previously described^{30,39}. Sham surgery animals underwent similar procedures of needle placement except each ipsilateral femur was injected with 20 μ L of HBSS (1X) (Life Technologies Corp; Grand Island, NY; 14170-112). Naïve animals were age matched and received no surgery or therapy. Following surgery, mice were grouped housed in the animal care facility. Wound clips were removed five days following surgery, and after 70 days in the exploratory experiments and 65 days post-surgery in the therapy group, the animals were euthanized. Surgeries were performed by the same surgeon so that sterile operating conditions would be maintained at all times. The pre-op, X-ray, and post-op tasks were carried out by assistants.

Drug treatment

Rats—PLX3397 (30 mg/kg; Plexxikon; Berkley, CA) and vehicle (0.5% HPMC/1% Tween 80/2.5% DMSO) were orally administered two hours before subplantar injection of formalin (0.05 mL, 1% solution; Wako, Japan)³⁶. The analgesic efficacy (reduction in hind paw licking) was an average of two independent experiments with six SD rats per treatment group per trial.

Mice—Animals were randomly divided into treatment groups (prostate cancer + vehicle; prostate cancer + PLX3397, naïve, and sham). Mice were administered PLX3397 that had been formulated in mouse chow so that the average dose per animal was 30 mg/kg per day as this dose had been defined by pharmacokinetic data, kinase screening (Supplemental Table 1), and efficacy in other models of disease^{13,16,42}. Both PLX3397 and control chow were administered *ad libitum*, beginning on day 14-post cancer cell injection and continuing to the end of the study. In the animals in the therapy experiments there were eight animals per group in both the prostate cancer + vehicle group and the prostate cancer + PLX3397 group. Values for naïve and sham animals in terms of pain and bone remodeling were essentially identical by 14 days post-sham injection in both the exploratory and therapy groups of animals.

Behavioral measures of pain

Rat Formalin Test—The two phases of nocifensive behavior following injection of formalin into the rat hindpaw were the acute phase, which lasts for approximately five minutes, followed by a longer-lasting, inflammatory phase (approximately 30–40 minutes). Both phases are characterized by the extent of licking of the hindpaw. In the experiments reported here hindpaw licking was recorded at 5-minute intervals for 30 minutes following formalin injection.

Mice Spontaneous guarding and flinching behavior—Animals were assessed for both spontaneous guarding and flinching to measure pain behaviors, as previously reported⁵¹. This assay attempts to reflect the clinical condition, as patients with metastatic prostate cancer can experience spontaneous bone pain (flinching) and will protect (guarding)

the tumor-bearing limb⁶⁵. Behavioral testing was performed on the same day as radiological assessment to enable comparison between pain behavior and bone destruction/tumor burden. The same experimenter who was blinded to the drug treatments performed each method of behavioral measure assessment.

Mice were placed in small raised Plexiglass chambers (11.5 × 6.8 × 7.5 cm) with a wire grid floor, and were allowed to acclimate for 30 min (until cage exploration and major grooming activities ceased), following which, their movements were videotaped from below and time spent in spontaneous guarding and flinching was assessed during a 2-min (120 sec) observation period. The number of spontaneous flinches of the ipsilateral hind limb was defined as the number of times the animal raised its hindpaw. The spontaneous guarding of the ipsilateral hind limb was defined as the amount of time the animals held the hindpaw aloft while stationary^{30,73}.

Measures of bone remodeling and tumor burden

Radiography—High resolution X-ray images of the mediolateral plane of the ipsilateral (cancer or vehicle-injected) femur were obtained following short term anesthesia of mice with ketamine/xylazine (0.005 ml/g, 50 mg/10 kg, s.c.; Western Medical; Arcadia, CA; 121127A; Sigma-Aldrich; St. Louis, MO; 534056) several days before surgery (baseline), and immediately following behavioral assessments, using a Faxitron MX-20 digital cabinet X-ray system (Faxitron/Bioptics; Tucson, AZ). Images were saved as dicom files. The extent of bone remodeling and disease progression in the tumor-bearing femur was assessed by quantifying the extent of aberrant bone formation outside the periosteum, the number of individual prostate cancer cell colonies, and the overall tumor burden. All radiographic image quantifications were obtained in a blinded fashion.

Extra-periosteal bone formation—With time, prostate cancer cell colonies destroy the cortical wall of mineralized bone and begin to form woven bone well outside the normal limits of the periosteum. To quantify this extra-periosteal bone formation, the following procedure was performed. The original radiographical dicom images were converted to TIFF file format and opened in Image Pro Plus v 6.0 (Media cybernetics; Bethesda, MD) and magnified 10–100X using the zoom tool. This allows the observer to have a magnified and clear view of the extent and morphology of the extra-periosteal bone. The area of interest (AOI) tool allows the user to manually define a portion of the image in free form. This measure tool was calibrated to millimeters and the aberrant formation of bone outside the normal diameter of the normal mouse femur (as defined in naïve or sham injected mice) was then outlined, the value captured, and exported to Excel, where the average area extra-periosteal bone formation for each treatment group (Prostate cancer + vehicle vs. Prostate cancer + PLX3397) was calculated.

Number of individual cancer cell colonies and overall tumor burden—

Exploratory studies established that by 65–70 days following the initial injection of prostate tumor cells into the mouse femur, less than 5% of the entire area of bone contains normal hematopoietic bone marrow. Following injection and confinement of prostate cancer cells to the marrow space of the femur, prostate cells colonize the marrow and mineralized bone by

forming highly individual and radiologically evident prostate cancer colonies. Previous data has shown that each cell colony is composed of viable tumor and tumor associated stromal cells (which are radiologically translucent) surrounded by dense newly formed woven bone (which is radiologically opaque)^{38,39}. Using Image Pro Plus, radiographic images of the tumor-bearing femur were divided into two halves, the distal half and the proximal half. The radiographs were enlarged as described above so that each individual cancer cell colony was easily visualized, assigned a number, traced, the location and the area of the viable cancer cell colony (the translucent area) captured and transferred to Image Pro Plus. The number of cancer colonies in each region (distal and proximal) was calculated as was the total tumor area in each tumor-bearing femur. These measurements were exported to Excel where the average area and average number of cell colonies of each treatment group was then determined (Prostate cancer + vehicle vs. Prostate cancer + PLX3397).

Immunohistochemical analysis

At Day 65 or 70 days post-cancer cell injection, mice were deeply anesthetized with ketamine/xylazine (0.01 ml/g, 100 mg/10 kg, s.c.) and perfused intracardially as previously described^{38,39,55}. After perfusion, the hind limbs were removed and placed in the same perfusion fixative solution for 24 hours. After separation of the femur and tibia, the tissue was placed in PBS (pH 7.4) solution for 48 hours. The femurs were decalcified in 10% ethylenediaminetetracetic acid (EDTA; Sigma-Aldrich, St. Louis, MO; E5134) at 4°C. EDTA was changed after seven days and decalcification was monitored radiographically with a Faxitron MX-20 digital cabinet X-ray system at day 7 post-EDTA immersion and then every 1–3 days until complete decalcification was observed (after approximately two weeks). Following complete decalcification, each femur was cryoprotected in 30% sucrose (Fisher Scientific, Waltham, MA, S5-3) at 4°C for at least 48 hrs before sectioning of tissue.

Cryoprotection and sectioning of tissue—Serial bone sections were cut using a Bright OTF5000 cryostat (A-M Systems; Sequim, WA) at 20µm and thaw mounted with two sections of bone being on each slide. The slides were then placed in the –20°C freezer until ready for staining.

Immunohistochemistry—The decalcified frozen bone sections (20 µm thick) were used to perform immunofluorescent staining as previously described^{38,39,55}. The 20 µm thick section slides were removed from the –20°C freezer and were allowed to thaw and dry at room temperature (RT) for 30 min. Slides were washed in 0.1M PBS 3X for 10 min each, blocked with 3% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA; Cat# 017-11-121) in PBS with 0.3% Triton-X 100 (Sigma Chemical Co., St. Louis, MO; Cat# X100) for 60 min and then incubated overnight with primary antibodies made in 1% NDS and 0.1% Triton-X 100 in 0.1M PBS at RT.

After primary antibody incubation, each preparation was washed 3X10 min in PBS and incubated for three hrs at RT with Cy3-conjugated secondary antibodies (1:600, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Preparations were then washed 3X10 min each in PBS, dehydrated through an alcohol gradient (Sigma-Aldrich; St. Louis, MO, 459844) gradient (70, 80, 90 and 100%) for two minutes each wash, cleared in xylene

(Sigma Chemical Co.; St. Louis, MO; 534053) for two minutes (2X), and cover slipped with di-n-butylphthalate-polystyrene-xylene (Sigma Chemical Co., St. Louis, MO, 44581). Final preparations were allowed to dry at RT (covered due to light sensitivity of secondary antibodies) for at least 12 hrs before imaging.

To quantify the number of macrophages/osteoclasts per μm^3 of tumor, a primary antibody (CD68+) was used that was originally generated against a single chain glycoprotein of 110kDa that is expressed predominantly on the lysosomal membrane of myeloid cells (rat anti-mouse CD68; 1:2,000, AbD Serotec, Oxford, UK, Cat# MCA1957). Since the ACE-1 prostate cells have been transfected with green fluorescent protein (GFP), endogenous GFP signal from ACE-1 prostate cancer cells can be visualized in the green channel (band-pass emission filter from 500 to 530 nm) and no amplification of the GFP signal was needed for analysis. Sections were also stained for DAPI, which stains the nucleus of all cells.

Laser confocal microscopy—Confocal images were acquired with an Olympus Fluoview FV1000 system (Olympus, Melville, NY) equipped with LD (405, 440, 473, 559, 635 nm), Multiline Argon (457, 488, 515 nm), and HeNe(G) (534 nm) lasers. Sequential acquisition mode was used to reduce bleed-through from fluorophores. Images were obtained using UPlanFL N 40x/1.30 and PlanApo N 60x/1.42 (FV1200) oil objectives.

Average number of CD68+ cells in tumor-bearing bone sections—Images were acquired with an Olympus Fluoview FV1000 confocal microscope. Two confocal images (400X magnification) from three different bone sections per animal were captured. Acquired images of bone were separated by at least 0.1mm (5 sections) to minimize duplication of quantification measurements. Images of CD68+ cells were acquired in areas where GFP+ cancer cells were present. CD68-positive staining located at the interface of tumor and woven bone were considered osteoclasts, while those CD68-positive staining cells in the bone marrow space were considered macrophages. The average volume analyzed of bone with tumor cells was 310 μM (length), 310 μM (width), and 20 μM (depth). Confocal images were opened in Imaris Pro-Software v. 6.0 (Bitplane AG; South Windsor, CT) and quantification of CD68+ cells was performed in a blinded fashion by at least two individuals. Data from three bone sections per mouse were acquired and averaged. The results were expressed as the average number of CD68+ cells per tissue volume (μm^3) in each treatment group (Prostate cancer + vehicle; Prostate cancer + PLX3397).

Prostate Cancer Cells In Vitro Growth Assay—Cells were plated at a density of 3000 cells/well in 96-well white TC-Treated microplates (Corning® catalog #3610) and allowed to adhere overnight at 37°C in 5% CO_2 . Compounds (PLX3397 and staurosporine) were diluted in DMSO at a maximum concentration of 10 mM, and 3-fold serial dilutions were made in DMSO to prepare an 8-point titration. A 1 μL volume of DMSO vehicle or compound was further diluted 250-fold in growth media, and this was added to the cells at an equivalent volume, so the DMSO and compounds were diluted 500-fold from the stock solutions. The cells were incubated with compound for 3 days, and cell growth was quantified using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Compound inhibition was analyzed by dividing the raw signal by the average DMSO signal

(% Control). The % Control was plotted against the compound concentration, and the IC50 was calculated using Assay Explorer (Accelrys).

Osteoclast Differentiation Assay—Human osteoclast precursors (Lonza Walkersville, Inc.) were incubated at 37°C in 5% CO₂ with CSF-1 (33 ng/mL) and RANKL (66 ng/mL) in the presence of either DMSO (0.2%) or PLX3397 (0.2% to maximum of 10 µM) for 6 days. The cultures were given fresh growth media and further incubated for 1 day. The cell supernatants were analyzed using the Acid Phosphatase Assay (Cayman Chemical) per manufacturer's recommended procedure³⁵. Absorbance was read at 405 nm on a Tecan Safire plate reader.

Biochemical Kinase Assays—To assess the affinity and specificity of PLX3397 in binding to diverse kinases, PLX3397 was tested against a panel of 200 kinases that represent members of all major branches of the kinome phylogenetic tree. PLX3397 was tested at concentrations of 0.03 or 1 µM in duplicate. An average of 20% kinase inhibition was followed by IC50 determination. PLX3397 IC50 activities for KIT, CSF1R_(FMS), FLT3 and KDR_(VEGFR2) were determined in a reaction buffer of 25 mM Hepes (pH 7.5), 2 mM MgCl₂, 2 mM MnCl₂, 0.01% BSA, 0.01% Tween-20, 1 mM DTT, 5% DMSO, and 100 µM ATP (10 µM ATP for KDR) (all reagents from Sigma). The final enzyme concentration was ~1 µg/ml. A biotinylated substrate peptide poly-(Glu4-Tyr) (EMD Millipore #12-440) was used at a final concentration of 30 nM. Twenty-microliter reactions at room temperature were initiated by ATP addition. Eight three-fold dilutions of PLX3397 were added as a DMSO solution. After 30 min, the reactions were stopped by adding 5 µl per well of buffer including 100 mM EDTA. The extent of substrate phosphorylation was measured by using AlphaScreen PY20 kits (PerkinElmer, #6760601M). IC50s values are averages of at least 5 independent determinations (See Supplemental Table 1).

Statistical analysis

A one-way ANOVA was used to compare behavioral results and immunohistochemical measures between the experimental groups. For multiple comparisons, the Fisher's PLSD (Protected Least Significant Difference) post hoc test was used. For the formalin rat model, one-way ANOVA followed by Dunnett's test was applied for comparison between the PLX3397 and vehicle control groups. Significance level was set at $p < 0.05$. In all cases, the investigator responsible for behavioral testing, plotting, measuring, and data analysis was blinded to the experimental grouping of the animals.

Results

PLX3397 reduces nociceptive behaviors in the rat formalin test and in the prostate model of bone cancer

The effects of PLX3397 were initially evaluated in the rat formalin model and analgesic efficacy was compared to vehicle treated animals. The hind paw licking time was measured at 5-minute intervals for 30 minutes following subplantar injection of formalin. PLX3397 had both analgesic efficacy activities in both the acute (early) and inflammatory (late) phases of this test (Fig. 1).

To determine if PLX3397 had an effect on prostate-induced bone cancer pain, pain behaviors of tumor-bearing mice were monitored when there was significant tumor growth in the intramedullary space of the femur and bone remodeling was apparent. Spontaneous guarding and flinching of the tumor-bearing limb were monitored over a 2-minute period. Sham animals (needle placement + injection of culture medium) exhibited pain behaviors that were indistinguishable from naïve animals over the entire evaluation period. In contrast, tumor-bearing mice with vehicle treatment showed pain-related behaviors that gradually increased with time (Fig. 2).

Administration of PLX3397, beginning 14 days post prostate cancer injection, significantly reduced spontaneous flinching behaviors at later time points of disease progression (days 49, 56, and 63) when compared to vehicle treated mice (Fig. 2A). In addition, administration of PLX3397 reduced the time animals spent guarding behaviors (days 56 and 63) when compared to vehicle treated mice (Fig. 2B).

PLX3397 attenuates prostate cancer-induced bone remodeling

Femurs from prostate cancer mice treated with vehicle showed cancer cell-induced sclerotic areas that are nodular, rounded, and well-circumscribed. These sclerotic regions are caused by the production of woven bone and are observed throughout the bone that surround the tumor and associated stromal cells. PLX3397 treatment in the tumor-bearing mice resulted in an increase in the overall radio-opacity in contrast to prostate cancer-injected vehicle animals, presumably due to the bone protecting effects of PLX3397 treatment. Radiographs of all the animals treated with PLX3397 illustrate the significant attenuating effects PLX3397 has on cancer-induced bone destruction (Fig. 3).

Radiographic analysis indicated that bones from sham mice were nearly identical to naïve mice (Supplemental Fig. 1A, B). Prostate tumor cells cause massive bone remodeling and can be quantified by measuring the aberrant bone formation outside the cortical wall of the normal femur. In prostate cancer + vehicle-treated animals, this feature is very prominent (Fig. 4A), beginning as early as day 35 post prostate cancer cell injection. This phenotype is significantly attenuated in prostate cancer animals that received sustained administration of PLX3397 (Fig. 4A). Histogram shows that over time, the aberrant extra-periosteal bone formation was significantly reduced with this therapy (Fig. 4B).

Reduction in the number of individual cancer cell colonies and overall tumor burden

To determine if PLX3397 had a direct anti-proliferative effect on the prostate cancer cells used in this study, a three day growth assay was performed *in vitro* using both the parental ACE1 cells and the GFP-transfected cells. Neither cell line appeared to be sensitive to PLX3397 up to 10 μ M. As a positive control, both cell lines were sensitive to two controls: BEZ-235 (PI3K/mTOR inhibitor; data not shown) staurosporine (protein kinase inhibitor) (Supplemental Figure 2).

Following injection of prostate cancer cells into the marrow space, the tumor cells colonized nearly the entire marrow space and mineralized bone by forming highly individual and radiologically evident prostate cancer colonies (Fig. 5B,D). Each prostate cancer cell colony

is composed of viable tumor and tumor associated stromal cells (which are radiologically translucent) surrounded by dense newly formed woven bone (which is radiologically opaque). Sustained treatment with PLX3397 not only reduced the number of cancer cell colonies but also reduced the overall tumor burden in the femur (Fig. 5F, G).

PLX3397 reduces the number of CD68+ cells in vivo and inhibits osteoclast differentiation in vitro

Immunohistochemical analysis was used to determine whether administration of PLX3397 influenced CD68-positive macrophage infiltration in bones injected with prostate cancer cells. CD68 is a well-established marker for the myeloid lineage, which includes macrophages and osteoclasts ^{4,15} and confocal analysis showed high association with the prostate cancer cells (Fig. 6A), which were unique in morphology and location. Quantification of the average number of CD68-positive cells per tissue volume within the bone of tumor-bearing mice at day 65 post-tumor cell injection revealed that chronic treatment of PLX3397 significantly reduced the number CD68-positive cells as compared to vehicle-treated animals (Fig. 6B,C).

To determine if PLX3397 had direct action against osteoclasts, osteoclast precursors were incubated with either vehicle or PLX3397 for six days in the presence of CSF-1 and RANKL. The cell supernatants were assayed for acid phosphatase activity to quantify the TRAP5b produced by mature osteoclast and results showed that PLX3397 inhibited osteoclast differentiation (Supplemental Figure 3).

Discussion

Mechanisms by which blockade of a subset of cells in the myeloid lineage attenuates cancer-induced bone pain

In the present report, sustained administration of PLX3397 had a significant effect on attenuating spontaneous bone cancer pain. There are at least four major mechanisms by which PLX3397 may produce this analgesic effect. First, previous studies have shown that osteoclasts participate in driving bone cancer pain by generating a highly acidic environment that not only induces resorption of mineralized bone but activates TRPV1 and ASIC channels that can be expressed by primary afferent sensory nerve fibers that innervate the bone ^{26,59}. Treatment with PLX3397 markedly reduced CD68-positive cells in the prostate tumor-bearing bone. Given that PLX3397 targets the myeloid lineage, including osteoclasts and macrophages, this would greatly reduce the osteoclast-induced acidosis that activates TRPV1 and ASIC channels. Second, reduced tumor-associated macrophages in the tumor bearing bone should diminish the release of macrophage derived pro-algesic molecules such as proteases ^{20,63,83}, bradykinin ⁵, prostaglandins ^{66,68}, tyrosine kinase activators ^{75,77}, and other colony stimulating factors ⁷⁶ that have previously been shown to drive CIBP. The overall reduction in tumor-associated macrophages (TAMs) should reduce the sensitization and activation of sensory nerve fibers by macrophage-released products ⁴⁴. Third, there is evidence that CSF1 signaling activates microglial in the spinal cord. PLX3397 does cross the blood brain barrier and has been shown to inhibit microglia activation ^{4,16,19}. As

microglial activation in the spinal cord has been shown to be involved in CIBP, part of the potential analgesic actions of PLX3397 could be occur this this pathway

Lastly, and probably most importantly, sustained administration of PLX3397 significantly reduced disease progression (as measured by formation of new tumor cell colonies, overall tumor burden, and tumor-induced bone destruction) which may itself result in significant reduction in CIBP. Reducing tumor burden by radiotherapy has been use for decades to control CIBP ^{2,92}. Ionizing radiation administered by either external beam and/or gamma emitting radionucleotide therapy results in tumor shrinkage which is usually accompanied by a concomitant decrease (~50%) in pain relief that usually occurs 1–2 weeks post therapy ^{61,92,93} and this pain relief can last for months. As sustained administration of PLX3397 also reduces several aspects of disease progression, a significant portion of the “analgesic” actions of PLX3397 may in fact be due to reduction in disease progression vs. direct inhibition of nerve fibers that innervate the prostate tumor bearing bone.

Inhibition of the myeloid lineage reduces prostate tumor-induced bone remodeling and tumor growth in vivo

In the present investigation it was surprising to see the size of the effect that PLX3397 had in terms of reducing prostate-induced bone remodeling, especially as prostate tumors on bone have traditionally been thought of as driving osteoblast-induced bone formation vs osteoclast-induced bone destruction. However, previous data has shown that human patients with prostate metastasis to bone can have remarkably high levels of plasma markers of osteoclast-induced bone resorption ^{58,72}.

In the present study we also observed a marked reduction in the number and size of individual tumor colonies in the tumor-bearing femur. Once a tumor metastasizes to bone the expression of a wide variety of factors including CSF-1 and c-Kit, are thought to promote tumor growth and metastasis to other sites. Previous data has also shown that TAMs play a pivotal role in promoting tumor invasion, vascularization, tumor growth, immune cell response, and metastasis ²⁸ as high levels of TAMs are correlated with poor prognosis in patients with breast, prostate, ovarian, cervical, stomach, and lung cancers ⁶⁷.

Given that at least *in vitro*, PLX3397 did not affect prostate cancer cell growth in the prostate cell lines ACE1, LNCaP and PC-3 (data not shown). Although several reports have demonstrated the expression of CSF1R and c-Kit on prostate cancer cells ^{37,43,94}, our results suggests that the disease modifying effects of PLX3397 observed in the present study are most likely due to the inhibition of tumor-associated stromal cells (osteoclasts, macrophage, and mast cells) rather than direct inhibition of the cancer cells. This data fits with previous studies that have shown that PLX3397 can have an inhibitory effect not only on osteoclasts ³³ but blocking CSF1R also inhibit disease progression where there are no osteoclasts present ^{18,69,70,84}.

Analgesics and their potential effects on cancer disease progression and overall survival

Clinical and epidemiological studies from the past 30 years have repeatedly shown that stress can promote tumor cell growth and decrease survival time in both humans and

experimental animals with cancer^{12,27,95}. As severe pain can induce significant stress response, an obvious question is whether all analgesics used to treat cancer pain have clear and positive disease modifying effects as measured by overall tumor burden and survival? Here the answer appears to be no, with opiates being the most studied group of agents in both preclinical and human studies on cancer pain. Thus, while opiates are one of the most frequently used class of agents to control moderate to severe cancer pain, the disease modifying actions of this set of agents is controversial with some studies indicating that opiates promote^{29,46,80}, inhibit^{23,25,31,32,40,53,56,82,85}, or have no effect^{7,24,57,71,89} on disease progression and/or patient survival. Interestingly, even in light of the controversy surrounding the effectiveness of opiates, recent studies have demonstrated that cancer patients that received early and aggressive treatment of cancer symptoms including pain did show an increase in overall survival^{17,86}.

A second question is whether non-opiate analgesics that are commonly used to relieve cancer pain can have clear disease modifying actions in terms of cancer disease progression and overall patient survival? Here the answer in terms of bone cancer is an unequivocal yes, with bisphosphonate and the alpha emitting bone homing radionucleotide radium-223 being the best examples in CIBP⁶⁴.

Bisphosphonates and Denosumab (both which target osteoclasts) have been shown to reduce both tumor-induced bone destruction, increase the time to the first bone fracture, and at least with nitrogen containing bisphosphonates, a reduction in tumor burden^{8,10,34,62,87}. Interestingly, even with these significant disease modifying effects, definitive evidence of an increase in overall patient survival with either bisphosphonate or Denosumab therapy has been elusive. Whether PLX3397 which reduces osteoclasts, macrophages, and mast cells in bone is superior in analgesic efficacy to bisphosphonates or Denosumab remains an important but unanswered question.

While external beam radiation and gamma emitting radionuclides clearly can attenuate CIBP, there is little evidence for increased patient survival in patients treated with these therapies⁶⁴. However, in a recent study using the alpha emitter Radium-223 in patients CIBP, there was a significant reduction in time to the first symptomatic skeletal event, reductions in plasma markers of bone remodeling, and a clear increase in overall survival⁶⁴.

Other non-opiate therapies that have been shown to reduce CIBP and disease progression in preclinical models of bone cancer are NSAIDs and inhibitors of COX-2^{3,11,21,22,45,48}. Both these therapies have also been shown to slow the growth of a wide variety of cancers and are commonly used as a first line therapy to treat CIBP^{3,11,21,22,45,48}. Additionally, at least in preclinical models, anti-NGF therapy has also been shown to reduce the growth of head and neck cancer as well as reducing tumor-induced bone remodeling and time to fracture in a mouse model of bone cancer pain^{30,52,79,97}.

Conclusions

The major finding in the present study is that in a mouse model of bone cancer, a high affinity, small molecular antagonist that inhibits CSF1R, c-Kit, and FLT3 simultaneously attenuates skeletal pain, disease progression, and tumor-induced bone remodeling by

approximately 50%. While it is still unclear the extent to which the “analgesic” actions of PLX3397 are due to reduction in disease progression, the present results do emphasize the importance of simultaneously assessing pain and disease modification when assessing novel therapies in animal models of cancer pain.

There were several limitations to the present study. First, although PLX3397 significantly reduced CIBP by 50% what remains unclear are the mechanism(s) that generate the remaining 50% of the cancer pain. Possibilities include that cancer or non-myeloid stromal cells that are not targeted by PLX3397 continue to release pro-algesic molecules such as NGF, BDNF, or prostaglandins as well as unimpeded, ectopic nerve sprouting that induces a neuropathic pain state and continue to drive CIBP. Second, future experiments need to more clearly define and disambiguate what specific myeloid cells targeted by PLX3397 (osteoclasts, macrophages and mast cells) individually contribute to the reduction in pain, bone remodeling and tumor growth observed in the present study. Lastly, although PLX3397 reduced pain and disease progression in the present prostate model of CIBP, it will be important to define whether similar effects will be seen in other cancers such as breast, lung, and renal that also frequently metastasize to bone and generate CIBP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary statement

Inhibition of myeloid lineage attenuates bone cancer pain and disease progression.

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Figure 1. PLX3397 reduces nociceptive behavior in rat formalin test

The effects of PLX3397 (30 mg/kg) were evaluated by oral (PO) administration in analgesic activity in the rat formalin test and compared to vehicle (0.5% HPMC/1% Tween80/2.5% DMSO, PO). The hind paw licking time was measured at 5-minute intervals for 30 minutes following subplantar injection of formalin (0.05 mL, 1% solution). Note that PLX3397 has analgesic effects at both the acute (0–5 minutes) and inflammatory (10–30 minutes) phases. Error bars represent SEM; $p < 0.05$ PLX3397 vs. vehicle.

Figure 2. Sustained administration of PLX3397 attenuates prostate cancer-induced skeletal pain-related behaviors

Animals with prostate cancer cells injected and confined to one femur displayed progressively increased nocifensive behaviors with time. The number of spontaneous flinches (**A**) and the time spent guarding (**B**) of the tumor-bearing limb were measured over a 2-minute observation period. Sustained administration of PLX3397 in chow (30mg/kg/day) was begun 14 days post prostate cancer cell injection when prostate cancer-induced bone remodeling was first evident. PLX3397 significantly reduced cancer pain behaviors

especially at later time points in the disease progression. Error bars represent S.E.M.; *
 $p < 0.05$ Prostate cancer + PLX3397 vs. Prostate cancer + vehicle.

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Figure 3. Attenuation of disease progression in a prostate tumor model of bone cancer
High resolution radiographs of tumor-bearing femurs in mice treated with vehicle or PLX3397 at day 65 post prostate cancer cell injection illustrate the overall effect in attenuating bone remodeling, formation of tumor cell colonies and preservation of normal radio-opacity of bone. Note that with PLX3397 therapy, there was a significant reduction in the number of cancer cell colonies (radiolucent lesions demarcated by sclerotic areas due to the production of woven bone) and areas of extra-periosteal bone formation (radio-opaque regions outside the defined cortical wall). Numbers above each radiograph represent the animal number assigned prior to the beginning of the study.

Figure 4. Inhibition of prostate cancer-induced pathological extra-periosteal bone formation

Representative radiographs from prostate cancer cell injected mice treated with vehicle and PLX3397 (A) at all time-points post cancer cell injection, starting at day 14 and repeated every seven days. At the beginning of the tumor cell progression, individual prostate cancer cell colonies begin to form throughout the medullary space. These colonies are surrounded by newly formed bone (woven bone) that is mechanically weak. During disease progression these prostate cancer cell colonies extend outside the normal limits of the cortical wall and periosteum, termed extra-periosteal bone formation. The tumor-induced periosteal reaction area (mm^2) was quantified at all time points (Days 14–63) post cancer cell injection and PLX3397 significantly reduced the periosteal reaction area (B). Note that the overall radio-opacity of the PLX3397-treated animals was also preserved when compared to vehicle treated prostate cancer mice. Error bars represent SEM; * $p < 0.05$, Prostate cancer + PLX3397 vs Prostate cancer + vehicle.

Figure 5. Chronic administration of PLX3397 significantly reduces the number of individual cancer cell colonies and overall tumor burden

In prostate cancer + vehicle treated mice (B, D), there was extensive tumor burden (determined by the area of the tumor colonies delineated with yellow lines) in both the distal and proximal ends of the femur as compared to sham animals (A). Prostate colonies were defined by radio-translucent areas, indicative of soft prostate tumor mass, surrounded by radio-opaque woven bone. High magnification radiographs illustrate the clearly defined cancer cell colonies of both treatment groups. Treatment of prostate tumor-bearing animals with PLX3397 (C, E) significantly reduced the total tumor burden area (mm²) (F) and number of cancer cell colonies (G) in both the distal and proximal ends of the femur as compared to vehicle-treated animals. Error bars represent SEM; $p < 0.05$ Prostate cancer + PLX3397 vs. Prostate cancer + vehicle.

Figure 6. Reduction in the number of tumor-associated CD68⁺ macrophages and osteoclasts
 Representative confocal images of decalcified frozen bone sections (20 μm thick) from mice at 65 days-post injection of GFP⁺ prostate cancer cells treated with vehicle (**A**) and PLX3397 (**B**). GFP⁺ prostate tumor cells (green) grew and formed cancer cell colonies throughout the femur and these cancer cells are accompanied by increases in both CD68⁺ macrophages and osteoclasts (yellow). The larger CD68-positive cells located at the interface of tumor cells and woven bone are osteoclasts, while the smaller cells located throughout the tumor cells and surrounding tissue are macrophages. This disease progression induced formation of woven bone (gray). A differential interference contrast (DIC) image was taken after acquiring a confocal image of the same section to visualize woven bone. Note that administration of PLX3397 had little effect on the viability of cancer cells but did significantly reduce the number of CD68⁺ cells per tissue volume (μm³) in the tumor cell colony (**C**). Error bars represent SEM; $p < 0.05$ Prostate cancer + PLX3397 vs. Prostate cancer + vehicle.