MULTIVALENT CATHEPSIN INHIBITOR, VBY-825, ATTENUATES BREAST-INDUCED BONE CANCER REMODELLING AND PAIN

By

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A Thesis Submitted to the Honors College

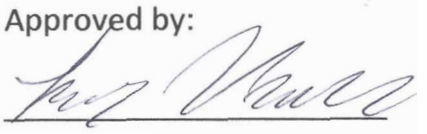
In Partial Fulfillment of the Bachelor's Degree
With Honors in

Physiology

THE UNIVERSITY OF ARIZONA

MAY 2013

Approved by:



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Physiology

Date thesis submitted to Honors College:

Title of Honors thesis:

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Abstract

Metastatic bone cancer originates from breast malignancies causing severe pain and bone destruction in patients. Amongst the novel therapies under clinical development for the treatment of bone metastases are cathepsin inhibitors. Cysteine cathepsins (B, C, F, H, K, L, O, L2/V, W, X/Z) are highly expressed in many human cancers and have been associated with poor patient prognosis. In the RIP1-Tag2 transgenic model of pancreatic cancer, mice treated with VBY-825, reversible inhibitor of cathepsins S, B, V, L, K showed a significant reduction in tumor incidence and growth. In this study, we evaluate the efficacy of the cathepsin inhibitor, VBY-825 as treatment for cancer-induced bone pain. Breast cancer cells, 66.1, were injected within the intramedullary space of the femurs of female mice. After seven days of inoculation, the animals were treated with VBY-825 or vehicle (5% dextrose) subcutaneously for seven days. Spontaneous pain behaviors were significantly attenuated in cancer-induced mice treated with VBY-825, compared to vehicle treated animals. Additionally, cancer-induced animals treated with VBY-825 demonstrated both an improvement in bone integrity and reduction of tumor burden. These results indicate that a cathepsin inhibitor targeting multiple cathepsins, such as VBY-825, could be a novel therapeutic for bone metastases.

Introduction

Cathepsins, which belong to a class of proteolytic enzymes, are ubiquitous to all animals. There are 11 cysteine cathepsins (B, C, F, H, K, L, O, L2/V, W, X/Z), which share a conserved active site formed by cysteine and histidine residues, but are distinguishable from each other by their structure, catalytic mechanism and protein preference [255]. Many cysteine cathepsins are upregulated in various human cancers, active in specific tumorigenic processes, and positively associated with poor patient prognosis [124]. Cathepsins are key players in cancer metastasis, initiating the breakdown of connective barriers of the extracellular matrix and basement membrane, thereby allowing cancerous cells to invade new tissues and organ sites by entering the bloodstream [149, 270]. This process occurs due to abnormal cathepsin localization during cancer development; in malignant cells, cathepsins are translocated to the surface of the cell or secreted, when in normal cells they would be localized to lysosomes [124, 125]. Recent studies have also shown that the deletion of cathepsin B, S, or L -encoding genes leads to a significant decrease in tumor invasion, as well as a change towards more benign lesions [85]. Certain cathepsins have been implicated in chronic pain signaling in neurons, as well as in bone resorption through collagen degradation [112] [42, 71]. The cathepsins mentioned below are those inhibited by the compound, VBY-825, a cathepsin inhibitor investigated in Chapter 6.

Cathepsin S (CTSS) has a critical role in antigen presentation, participating in the degradation of a polypeptide that would prevent antigen loading onto major histocompatability complex class II [42]. As such, antigen-presenting cells such as dendritic cells, macrophages, B-lymphocytes, and microglia express CTSS, which unlike many other cathepsins, is stable outside the lysosome. Cathepsin S is catalytically active at neutral pH and has an optimum pH range between 6.0 and 7.5 [42].

With regards to its role in cancer metastasis, CTSS has been found to cleave ECM proteins laminin, fibronectin, elastin, osteocalcin and some collagens [42]. The enzyme's elastolytic and collegenolytic properties contribute to its role in angiogenesis because cleavage of certain ECM proteins triggers the generation of proangiogenic peptides. The

tumor cells secrete proinflammatory factors, which have been found to trigger cathepsin S, correlates with cathepsin activation being one method by which metastasis occurs.

Cathepsin S has recently become associated with nociception. In naïve rats, Cat S expressing cells in the dorsolateral horn co-label with microglial activation, suggesting the constitutive expression of Cat S through spinal microglia promotes nociception [43]. Additionally, animals that received a partial ligation of the left sciatic nerve (PNL), showed increased expression of Cat S co-localizing with microglial activation compared to naïve animals in the ipsilateral dorsal horn, indicating that Cat S expressing microglia increases after peripheral nerve damage [43]. Additionally, Barclay and colleagues found the coding mRNA of cathepsin S is upregulated in rat dorsal root ganglia following peripheral nerve injury [17]. Recent evidence suggests CTSS expressed in microglia signals pain by liberating soluble fractalkine from the spinal cord [42].

Cathepsins B and L are expressed in almost all mammalian cells. Studies have demonstrated that cathepsin B is protective against Alzheimer's disease due to its breakdown of β-amyloid precursor proteins that can turn into plaque [175]. Cathepsin L has been implicated in multiple pathological processes of the heart and kidney, including myofibril necrosis in myopathies and in myocardial ischemia, and in the renal tubular response to proteinuria [175].

Cathepsins B & L are both translocated in lysosomal vesicles following post-translational modification and, in cancers, become associated with the plasma membrane or secreted, resulting in ECM degradation during tumor progression. Cat B plays a major role in extracellular protein catabolism, while Cat L degrades proteins intracellularly with high effectiveness. Both enzymes are regulated by cystatins A, B and C, and clinicians use an imbalance between the inhibitors and proteases to determine whether a cancer is malignant [185]. The activity of Cat B and Cat L has been shown to increase in breast, prostate and gastric cancers, and enzyme levels are useful prognostic factors for chances of relapse due to their correlation with invasiveness of breast and prostate carcinomas.

Highly expressed in the thymus, testes, and corneal epithelium, cathepsin V is autocatalytically activated at acidic pHs, is more stable than Cath-L but less so than Cat S

[34]. It is weakly collegenolytic, cleaving fibronectin peptide bonds. It is believed that cathepsin V is involved in positive T-cell selection due to its specific expression in the thymic cortex and its homology to cathepsin L (which aids in mounting the immune response) [234]. Cathepsin V plays an important role in corneal diseases, but little is specifically known about its mechanism of action.

Cathepsin K is enzymatically critical for bone remodeling and resorption and is selectively expressed in osteoclasts [34, 252]. A Cat K deficiency causes bone dysplasia pycnodysostosis [252], and knockout mice develop osteopetrosis from impaired resorption of bone matrix [124, 125]. A cytokine RANKL, and transcription factors NFAT, Mitf, and various components of AP-1 all enhance osteoclast formation and bone resorption acting to stimulate cathepsin K gene expression, whereas IFN-γ, calcitonin, estradiol and calcium inhibit cathepsin K gene expression and inhibit osteoclast formation [252]. The catabolic ability of cathepsin K to break down bone and cartilage is partially responsible for the loss of lung recoil and elasticity found in patients presenting with emphysema. A significant fraction of human breast cancers express cathepsin K, where it could conceivably contribute to tumor progression, invasiveness and metastasis.

Amongst the novel therapies under clinical development for bone modifying diseases, including but not limited to bone metastases, are the cathepsin inhibitors. Several clinical trials are currently evaluating the efficacy of cat k. Most anti-resorptive drugs stimulate osteoclast apoptosis. However, Cat K inhibitors suppress the function of osteoclasts, while maintaining their viability, whereas other anti-resorptive drugs enhance osteoclast apoptosis [28]. In a study of postmenopausal women with low bone mineral density (BMD), participants were treated weekly with the Cat k inhibitor, odanacatib (10, 25, or 50 mg or placebo) for two years, which dose dependently increased BMD and decreased bone resorption markers, C-terminal telopeptide of type I collagen (CTX) and N-terminal telopeptide of type I collagen (NTX) [28]. These same subjects participated in a one-year extension of the original study, where they received the most efficacious dose of odanacatib (50 mg) or placebo [72]. After three years of odanacatib treatment, BMD continued to increase and levels of bone-resorption markers remained suppressed [72]. In patients that began taking placebo after 24 months, bone resorption markers increased

above baseline levels and BMD decreased, indicating that the effects of odanacatib are readily reversible [72]. Additionally, a four-week clinical trial of women with breast cancer and metastatic bone disease found that odanacatib suppressed the bone resorption marker, urinary N-telopeptide of type I collagen [118].

Besides targeting Cat K, a multivalent inhibitor of many cathepsins could be a possible therapeutic for cancer patients. In the RIP1-Tag2 transgenic model of pancreatic cancer, mice treated with VBY-825, reversible inhibitor of cathepsins S (Ki=130 pM), B (Ki=330 pM), V (Ki=250 pM), L (Ki= 250 pM), K (Ki=2.3 nM) showed a significant reduction in tumor incidence and growth (Elie 2012). In this study, we additionally investigated the cathepsin inhibitor, VBY-825. A murine breast-induced bone cancer model was used to evaluate the efficacy of VBY-825 in attenuating bone cancer pain behaviors, improving bone integrity, and decreasing tumor burden. Clinical trials of this multivalent inhibitor must be conducted to further validate its therapeutic efficacy.

Methods

Cell culture

Murine 66.1 breast cancer cells were maintained in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS), 100 IU^{-1}penicillin and 100 µg/ml streptomycin (P/S). Cells were plated on 10cm tissue culture dishes and passed every four to five days. Cells were kept in 37°C and 5% CO_2 conditions.

In Vitro: Cell Culture. Murine mammary tumor line 66.1 cells were cultured in Minimum Essential Medium eagle with 10% fetal bovine serum (FBS), 100 IU^{-1}penicillin and 100 µg/ml streptomycin (P/S). The cell line was plated in 10 cm tissue culture dishes, allowed to grow exponentially, and housed in an incubator at 37ºC and 5% CO2. Cells were centrifuged and counted using a gridded hemacytometer (Hausser Scientific).

In Vivo: Animals. All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the Guidelines by the National Institutes of Health and the International Association for the Study of Pain. Female BALB/cfC3H mice (Harlan, IN) were 15-18 grams prior to initiation of study (N=10-12 animals per treatment group). Mice were maintained in a climate-controlled room on a 12-hour light/dark cycle and

allowed food and water ad libitum. 66.1 breast cancer cells were initially isolated from this strain of mice, thus creating a syngeneic model of metastatic bone disease.
Intramedullary Implantation of 66.1 Cells. Mice were anesthetized with ketamine/xylazine (10 mg/kg, i.p). An arthrotomy was performed. The condyles of the right distal femoris were exposed and a hole was drilled to create a space for injection of 1 x 10^5 66.1 cells in 5 uL complete MEM or 5 uL complete MEM without cells in control animals within the intramedullary space of the mouse femoris. Injections were made with an injection cannula affixed via plastic tubing to a 10 uL Hamilton syringe. Proper placement of the injector was confirmed through use of Faxitron x-ray imaging. Hole was sealed with bone cement.

Drug Treatment. Drug administration began on day 7 following surgery and occurred daily for 7 days. Cancer and control animals were divided into three treatment groups Animals received VBY-825 (10 mg/kg, s.c.), zoledronic acid (100 ug/kg, s.c), or vehicle (5% dextrose, s.c).

Analysis of Chronic Pain. Animals were tested for evoked and spontaneous pain (flinching and guarding) before surgery (baseline) and at days 7, and 14 following surgery. All testing was performed during the day portion of the circadian cycle in a blinded fashion.

Spontaneous Pain. Flinching and guarding were observed for duration of two minutes during a resting state. Flinching was characterized by the lifting and rapid flexing of the right hind paw when not associated with walking or movement. Flinches were recorded on a five-channel counter. Guarding was characterized by the lifting the right hind limb into a fully retracted position under the torso. Time spent guarding over the duration of two minutes was recorded and measurements were performed in blinded fashion.

Tactile Allodynia. The assessment of tactile allodynia consisted of measuring the withdrawal threshold of the paw ipsilateral to the site of tumor inoculation in response to probing with a series of calibrated von Frey filaments using the Chaplan up-down method with the experimenter blinded to treatment groups. The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon.

Radiography. A digital Faxitron machine was used to acquire live radiographs on days 0, 7, and 14 of the intramedullary inoculation model. Bone loss was rated by a blinded third party expert in animal radiographs according to the following scale: 0 = normal, 1 = bone loss observed with no fracture, 2 = full thickness unicortical bone loss indicating unicortical bone fracture, 3 = full thickness bicortical bone loss indicating bicortical bone fracture. From this rating, the incidence of fractures was reported and used to calculate the percent of animals with fractures. Before capturing images, mice were anesthetized with ketamine/xylazine.

Ex Vivo: Bone Histology. Immediately following behavioral testing on day 14, mice were anesthetized (ketamine/xylazine, 100mg/kg i.p.) and perfused transcardially with 0.1 M PBS followed by 10% neutral buffered formalin (Sigma, St Louis, MO, USA). Femurs were collected and post-fixed in picric acid with 4% formalin at 8°C overnight and decalcified in 10% EDTA (RDO-Apex, Aurora, IL) for 14 days. Femora were cut in the frontal plane into 5 um sections and stained with tartrate resistant alkaline phosphatase kit (Sigma) according to manufacturer's instructions to visualize osteoclasts. Femoras were visualized/analyzed using Image J.

Serum Biochemical Assays. Animals were deeply anesthetized and whole blood was collected by transcardial puncture. Blood coagulated at room temperature for 1 hour, and was centrifuged to isolate serum. Serum was stored at -80ºC until utilized for assays. Enzyme immunoassays were used to measure the serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAP5b) as a marker of bone resorption (Immunodiagnostic Systems, Fountain Hills, AZ, USA). Assays were conducted according to the manufacturers' instructions.

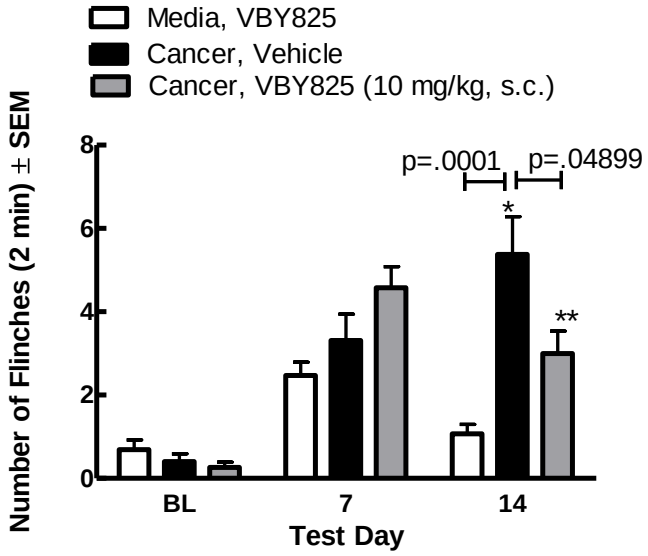
Statistical Analysis. Statistical comparisons between treatment groups were done using ANOVA. Pairwise comparisons were made with Student's t-test, multiple comparisons between groups were done using Bonferroni's Multiple Comparison Test. Significance was set at $p<0.05$. All data are presented as mean ± SEM and GraphPad Prism 5.0 (Graph Pad Inc., San Diego, CA, USA) used to plot data.

Results

The cathepsin inhibitor, VBY-825, attenuates bone cancer induced spontaneous pain behaviors.

Flinching and guarding behaviors were observed to determine the effects of VBY-825 on bone cancer-induced spontaneous pain. By day 14 post-surgery, control animals inoculated with media showed no significant flinching or guarding when administered VBY-825 (Fig.1A,B). However, animals inoculated with 66.1 cells displayed significant bone cancer-induced flinching and guarding beginning at day 7 and increasing through day 14. Continuous treatment with VBY-825 (10 mg/kg, s.c, from day 7-14) in cancer-inoculated mice resulted in decreased guarding and flinching by day 14 compared to cancer-inoculated vehicle treated animals. (Fig.1A,B)

Figure 1A



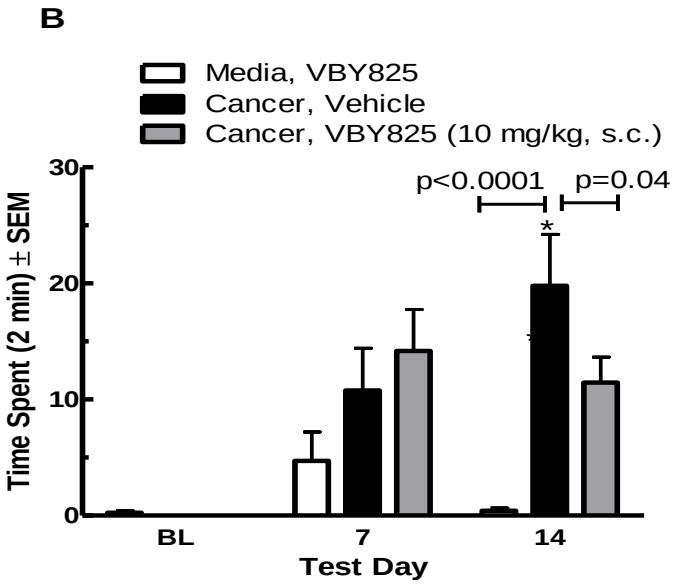


Figure 1: Chronic subcutaneous administration of the cathepsin inhibitor VBY-825 reduces cancer-induced spontaneous pain behaviors. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral inoculation, animals demonstrated bone cancer-induced (A) flinching and (B) guarding. VBY-825 (10 mg/kg, s.c.) was administered after behavioral measurements and continued for 7 days. On day 14, spontaneous (A) flinching and (B) guarding in cancer bearing animals was significantly reduced by VBY-825 compared to animals that received vehicle ($p<0.05$; n = 10-12 animals per group). Spontaneous pain behaviors were not seen in control animals inoculated with cell-free media and treated with VBY-825 (n = 10-12 animals per group).

VBY-825 reduces bone cancer induced evoked pain

Von Frey filaments were used to determine the withdrawal thresholds of the ipsilateral hind paw. By day 14, cancer inoculated animals treated with vehicle exhibit a significant decrease in paw withdrawal threshold compared to control animals (Fig1C). Cancer-inoculated animals treated with VBY-825 for seven days show a significant increase in withdrawal threshold compared to vehicle treated animals (Fig1C).

Figure 2A

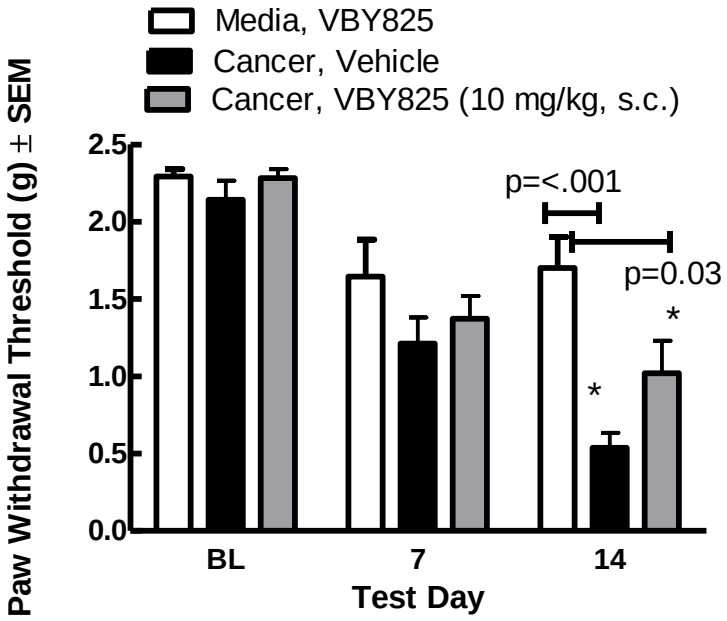


Figure 2: Chronic subcutaneous administration of the cathepsin inhibitor VBY-825 reduces cancer-induced evoked pain behaviors. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral inoculation, animals demonstrated decreases in bone cancer-induced paw withdrawal threshold. VBY-825 (10 mg/kg, s.c.) was administered after behavioral measurements and continued for 7 days. On day 14, paw withdrawal thresholds significantly increased in VBY-825 treated animals compared to animals that received vehicle ($p<0.05$; n = 10-12 animals per group). Spontaneous pain

behaviors were not seen in control animals inoculated with cell-free media and treated with VBY-825 (n = 10-12 animals per group).

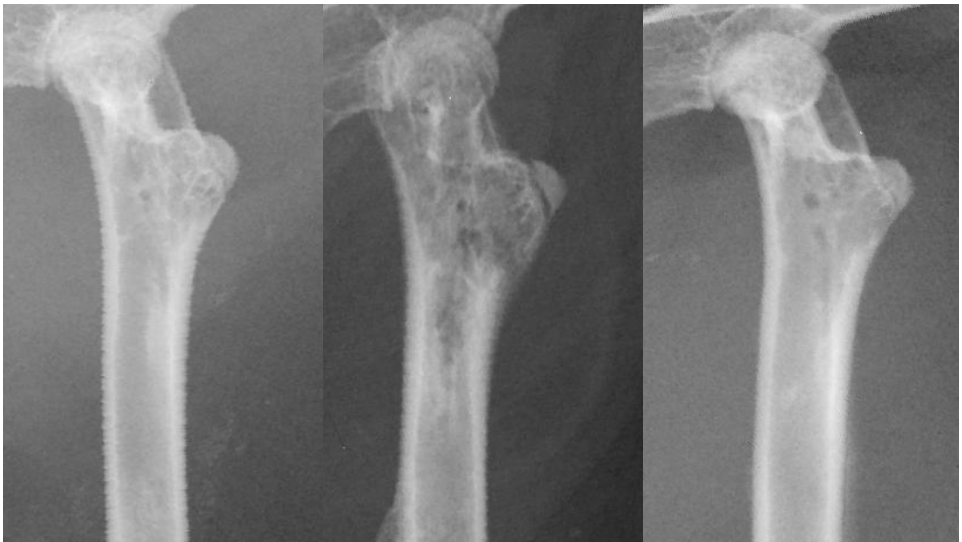
VBY-825 significantly decreases bone resorption

Radiographic images were taken following behavioral testing to determine the effect of sustained VBY-825 on cancer-induced bone degradation. No bone loss or fractures were observed in animals injected with media and treated with vehicle (Figure 3A). Cancer-induced bone loss (evidenced by the presence of radiolucent areas in the proximal and distal femoral heads) increased in tumor-bearing mice treated with vehicle compared to control animals (Figure 3B). Cancer-induced animals treated with VBY-825 appeared to have less bone loss (Figure 3C).

To confirm VBY-825 attenuation of cancer-induced bone remodeling, the bone resorption marker TRACP5b was measured in serum. Additionally, the levels of TRACP 5b were significantly increased in cancer-inoculated animals treated with vehicle when compared to media-inoculated control animals (Figure 3D). Sustained JWH015 treatment attenuated cancer-induced elevations in serum TRACP 5b (Figure 3D).

Figure 3

A. Media, VBY-825 B. Cancer, Vehicle C. Cancer, VBY-825



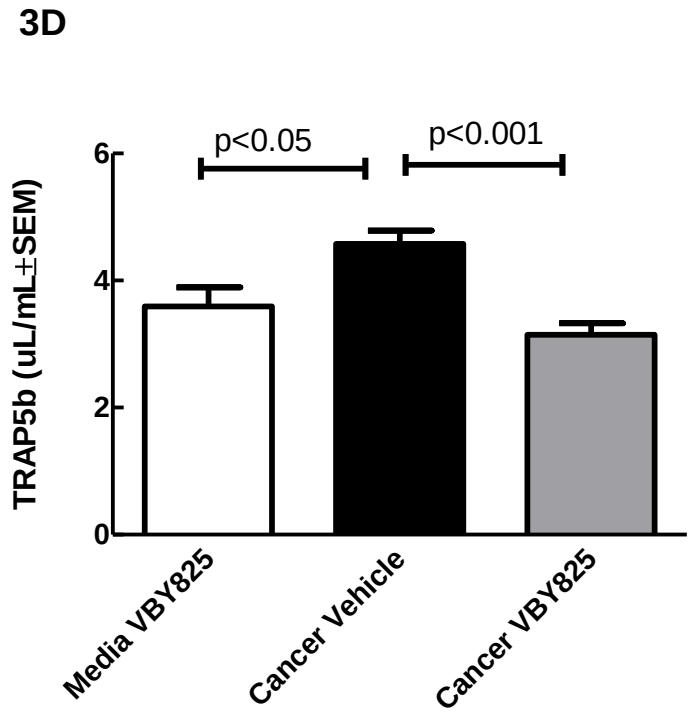


Figure 3: (A) Radiographs of the femora in the presence of either media (control) or breast cancer cells (66.1) on day 14 after inoculation. Mice received either vehicle or a cathepsin inhibitor, VBY-825 (10 mg/kg, s.c) from days 7 to day 14 after femoral inoculation. Bone

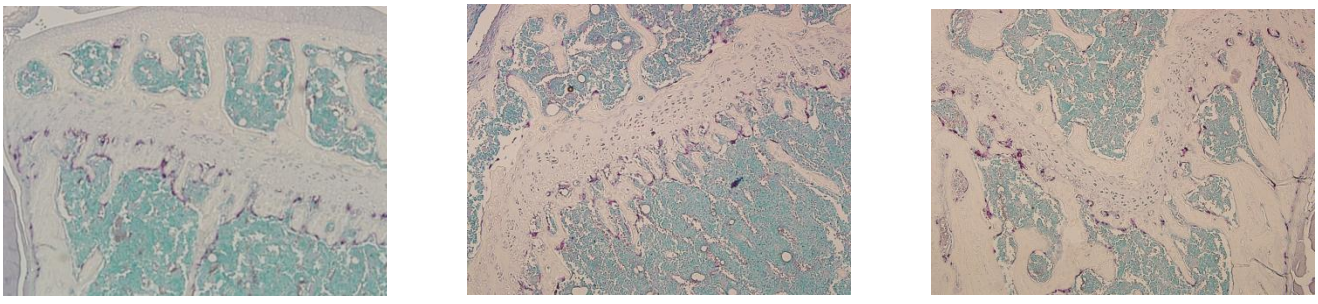
loss (hypodense at proximal and distal ends) was identified in cancer (66.1) treated animals in comparison to media only (control) animals. (B) VBY-825 (10 mg/kg, s.c., days 7-14) attenuates breast cancer-induced bone loss compared to (C) cancer inoculated mice with vehicle administration. Radiographs in all panels are representative of images obtained of femurs obtained from each animal in figure 1. (D) On day 14, serum was withdrawn from animals. TRAP5b was measured in animals on day 14 as a marker of bone resorption. TRAP5b levels were significantly higher in animals that received intra-femoral breast cancer cells (66.1) ($p=0.045$) compared to intra-femoral media treated animals. This increase in cancer-induced TRAP5b levels was significantly reduced in VBY-825 (10mg/kg, s.c., from day7 to 14) treated animals ($p<0.001$; $n=6$ per group).

VBY-825 significantly reduces osteoclast number

Decalcified bones were sectioned and stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts. Cancer-induced animals treated with vehicle showed a significant increase ($p<0.05$) in osteoclast number compared to animals injected with cell-free media (Fig. 4A,B,D). A significant increase in number of osteoclasts was observed in cancer-induced animals treated with VBY-825 compared to vehicle treated animals (Fig 4B, C, D).

Figure 4

A. Media, VBY-825 **B. Cancer, Vehicle** **C. Cancer, VBY-825**



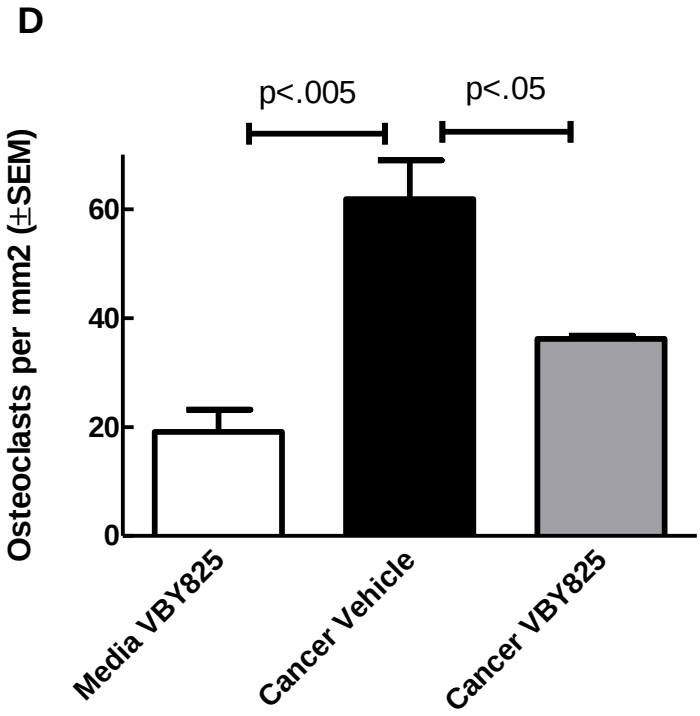


Figure 4 (A-C) On day 14, femurs were extracted from animals, decalcified, paraffin embedded, and stained for tartrate resistant alkaline phosphatase to visualize osteoclasts. Osteoclasts were counted at the distal end of femur. (D) A significant increase in osteoclasts was found in vehicle treated cancer-animals ($p<0.005$, n=8 animals per group). VBY-825 (10 mg/kg, s.c. for 7 days) brought a significant decrease in osteoclast count ($p<0.05$, N=8 per group).

Conclusion

Cancer continues to be the second leading cause of death in Americans. The high mortality rate of cancer is typically associated with primary tumor metastasis to the bone. Many adenocarcinomas, including those arising from the breast, prostate, and lung, have a predisposition for bone metastases [123]. The first symptom of tumor metastasis to the skeleton is often bone pain [173]. As the tumor progresses, additional symptoms such as hypercalecemia, anemia, skeletal fractures, and decreased mobility occur [123]. Not only is

pain inadequately managed with palliative care, most notably opiates and NSAIDS, but these analgesics also have been shown to exacerbate bone destruction [6, 19, 134].

Cancer-induced osteolysis and fractures are typically treated with bisphosphonates or denosumab [45, 86]. Bisphosphonates are pyrophosphate analogues with a high affinity for calcium ions, enabling them to rapidly bind to the mineralized bone matrix [68]. The bisphosphonates are then taken up by osteoclasts via endocytosis to resorb the bone. Once inside the osteoclast, bisphosphonates induce loss of function, and as a result, apoptosis [68]. However, bisphosphonates can induce adverse events such as hypocalcaemia, arthralgia and osteonecrosis of the jaw [68]. Denosumab is a monoclonal antibody that binds RANKL and approved by the FDA for use in patients with osteoporosis or metastatic bone cancer arising from solid tumors. In a randomized, double blind study, denosumab was compared to the front-line bisphosphonate therapy, zoledronic acid, in patients with advanced breast cancer and radiographic evidence of bone metastasis [243]. Denosumab delayed the time to on-study pathological fracture and reduced levels of bone resorption markers with minimal adverse events compared to zoledronic acid [243]. In a phase III, randomized double-blind study, men with prostate cancer, clinical signs of bone metastases and failure of a minimum of one hormonal therapy, when treated with denosumab significantly increased the time before incidence of on-study skeletal related event [76]. Unlike zoledronic acid, denosumab can be administered subcutaneously and kidney function does not need to be monitored, making denosumab a more convenient therapeutic option for patients [86]. Furthermore, clinical trials with denosumab are still ongoing to determine the long-term safety, efficacy and potential use as a prophylactic therapy for bone metastases.

Recent studies have advocated the potential of cathepsin inhibitors as a novel therapeutic approach to cancer treatment. Cysteine cathepsins are proteases classified as B, C, F, H, K, L, O, L2/V, W, X/Z, each specific to the tissue it is expressed by. Several transgenic mouse models of cancer have shown upregulation of multiple cathepsins. [72]. This is consistent with human cancers, in which over expression of cathepsins is associated with poor patient prognosis [72]. Proteolytic activity of cathepsins S, L, B and K may play a role in degradation of the basement membrane and extracellular matrix allowing tumor cell invasion and facilitating tumor metastasis [73]. Cathepsin K (Cat K) and cathepsin L

(Cat L), both expressed by osteoclasts, degrade collagen type I [128]. In particular, cathepsin S (Cat S) is an important mediator of pain released primarily from immune cells, macrophages, dendritic cells and microglia. Inhibitors targeting cathepsin S have been shown to alleviate neuropathic pain [172]. Intrathecal administration of morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS), an irreversible cathepsin S inhibitor, in neuropathic animals attenuated mechanical allodynia and hyperalgesia [43]. LHVS reduced the expression of activated microglia in the spinal cord, suggesting its mechanism of action. In theory, continuous noxious stimuli cause a local ATP increase, stimulating the microglial receptor P2X7 to release Cat S. Cat S cleaves the cytosine bonds in fractalkine (FKN), a neuronal transmembrane protein, allowing soluble FKN to activate the CX3CRI receptor on microglia. The subsequent release of inflammatory mediators following activation of p38 MAPK can sensitize local neurons and facilitate pain transmission. A administration in the lumbar spinal cord of soluble FKN is pro-nociceptive, whereas administration of uncleaved FRN is not [42]. However, intrathecal sFKN in CX3CR1 knockout mice does not induce mechanical allodynia shown in WT littermates [43]. This would provide additional evidence to the current premise that neural-immune interactions are an essential component in the development of chronic pain states [42]. Studies indicate competitive inhibitors of Cat S can selectively attenuate mechanical allodynia in animal models of collagen-induced arthritis and peripheral nerve injured rats [41, 42].

Currently, anti-resorptive drugs stimulate osteoclast apoptosis; Cat K inhibitors suppress the function of osteoclasts while maintaining their viability [118]. Clinical trials evaluating the efficacy of inhibitors of Cat K have found that these compounds increase bone mineral density and decrease bone resorption markers. Additionally, cathepsin B activates the latent collagenase enzyme procollagenase which is found in bone [71]. The in vivo activation of this enzyme subsequently leads to activation of collagenase and the breakdown of collagen. Evidence also points to the fact that cathepsin B is a crucial enzyme in the activation of pro-caspase-1 which induces the maturation and secretion of IL-1β and IL-18 [245]. These chemokines are strongly linked with the induction of chronic inflammatory pain. Relevant to CIBP, the cascade caused by Cat B activation in bone could

be, in part, responsible for the symptoms of hypercalecemia, anemia, skeletal fractures, and decreased mobility.

Furthermore, the reversible cathepsin inhibitor VBY-825, a potent inhibitor of Cat S, Cat L, Cat V, Cat B, Cat K and Cat F has multiple mechanisms to potentially attenuate CIBP. Inhibition of Cat S reduces microglia activation occurring in chronic pain states such as arthritis and neuropathic pain. Our lab has shown that VBY-825 can also attenuate CIBP, suggesting VBY-825 can ameliorate activation of microglia in the spinal cord. By inhibiting Cat B and Cat K, VBY-825 can reduce bone loss accompanying metastatic cancer to the bone by suppressing osteoclast activity and breakdown of collagen. Because bone degradation and accompanying bone fractures releases acid, nitric acid and other inflammatory mediators cause excruciating pain, inhibition of Cat B and Cat K indirectly attenuate CIBP. Future studies with this compound should determine whether inhibition of select cathepsins by VBY-825 affects the expression of inflammatory mediators, particularly those that promote pain, released from the intracellular signaling cascades that cathepsin activity influence in normal physiological conditions.

While several preclinical models demonstrate that inhibition of cathepsins affects tumor progression, bone resorption or attenuates chronic/neuropathic pain, studies investigating whether inhibition of select cathepsins reduce cancer-induced bone pain have not yet been conducted [42, 43, 73, 115, 124, 125]. In this study, we examine the efficacy of the reversible covalent binding cathepsin inhibitor VBY-825 in the attenuation of pain related behaviors in a murine model of metastatic bone disease. Breast cancer cells, 66.1, were injected within the intramedullary space of the femurs of female mice. After seven days of inoculation, the animals were treated with VBY-825 or vehicle (5% dextrose) subcutaneously for seven days. Similar to results seen with direct intrathecal delivery of Cat S inhibitor, subcutaneous administration of VBY-825 significantly attenuates pain behaviors [42-44].

Additionally, cancer-induced animals treated with VBY-825 demonstrated decreased bone resorption compared to control animals. Previous studies have indicated cathepsin inhibitors such as VBY-825 and JPM-OEet impede tumor progression [73, 125]. Previous pharmacokinetic studies demonstrate that the dose used in this study, 10

mg/kg/day, reaches plasma concentration >200nM, sufficient to inhibit the activity of cathepsins B, F, K, L, S, and V [73]. Because VBY-825 binds reversibly to targeted cathepsins, VBY-825 avoids the possibility of inadvertently affecting the immune system that may be associated with chronic use of irreversible cathepsin inhibitors [73]. Thus, with sustained bioavailability after daily dosing, VBY-825 has the feasibility for self-administration in humans. These results provide preclinical evidence that a cathepsin inhibitor targeting multiple cathepsins, such as VBY-825, could be a novel therapy for bone metastases.

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