Cathepsin inhibitor, VBY-825, attenuates bone cancer induced pain in mice

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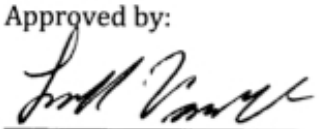

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Abstract

Aims: Cathepsin inhibitors have reduced tumor growth in animal models. In women with breast cancer, cathepsin treatment was able to suppress bone resorption. Studies also show that cathepsin inhibitors reduce nociception in injured animals. Here we test the efficacy of the cathepsin inhibitor, VBY-825, in reducing bone cancer induced pain.

Main methods: We use a murine bone cancer model with breast cancer cells injected into the intramedullary space of the femur. On day 7 post-surgery, animals received VBY-825, zoledronic acid, or vehicle daily via gavage for 14 days. Behavioral testing was performed before start of experimentation, at day 7 and day 14. An ELISA was used to assay presence of the fractalkine protein in plasma samples from animals at day 14, and day 21.

Key findings: Spontaneous bone cancer induced pain was characterized as flinching and guarding. Both behaviors were reduced in animals treated with VBY-825, when compared to those treated with zoledronic acid and vehicle. Evoked pain was tested using von Frey filaments, and pain withdrawal threshold was higher in animals treated with VBY-825 when compared to those treated with vehicle.

Significance: This suggests that cathepsin inhibitors may provide a novel treatment of pain induced by metastatic bone disease.

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Introduction

Cancer is the second leading cause of death in Americans, accounting for almost one in every four deaths. In the year 2010, it was estimated that there were 569,490 deaths due to cancer. In the same year, it was expected that there would be 1,529.560 newly diagnosed cancer cases.[1] The three most common cancers to affect Americans are lung, breast, and prostate.[2] These, along with other epithelial cancers, have been shown to commonly metastasize to the bone.[3,17] Each year, tumor metastases to the bone is thought to affect over 400,000 people.[4] This makes metastatic bone disease and its management an important concern in cancer treatment.

The first symptom of tumor metastasis to the skeleton is often bone pain.[5] As the tumor progresses, additional symptoms such as hypercalecemia, anemia, skeletal fractures, and decreased mobility occur.[4] These consequently affect the patient's quality of life and functional status.[4] Tumor-induced bone pain falls into two categories: ongoing pain and breakthrough pain. Ongoing pain is characterized as a constant, dull pain that increases in intensity over time as the tumor progresses. Breakthrough pain is defined as intermittent bouts of extreme pain that can occur spontaneously or as the affected bone bears weight or strenuous movement.[4] This pain "breaks through" the analgesic therapy that controls ongoing pain. Breakthrough pain appears suddenly and can occur several times each day, making it difficult to predict or control.[4]

Current treatment for metastatic bone cancer uses a combination of radiotherapy, chemotherapy, analgesic therapy and bisphosphonate treatment. Radiotherapy and chemotherapy target the tumor itself and are effective at managing bone pain only when the tumor burden is reduced. Current analgesic treatments include opiates and non-

steroidal inflammatory drugs (NSAIDS). Opiates such as morphine are successful in treating bone pain, but come with many unwanted side effects, such as constipation, respiratory depression, and tolerance.[6,17] Furthermore, it has been shown that murine bone tumor models treated with morphine have increased intensity of bone pain and accelerated bone destruction when compared to vehicle-treated animals after seven days.[7,17] NSAIDS are ineffective at treating the level of bone pain that occurs as the cancer progresses and have also been shown to be detrimental to bone healing.[8,17]

Bisphosphonates are a class of antiresortive compounds being used as a treatment for tumor growth and tumor-induced bone destruction and pain.[9] They are pyrophosphate analogues that have a high affinity for calcium ions, enabling them to rapidly bind to the mineralized bone matrix.[10] The bisphosphonates are then taken up by osteoclasts as they use endocytosis to resorb the bone. Once inside the osteoclast, bisphosphonates induce loss of function and as a result, apoptosis.[9,10] However, bisphosphonates also have unwanted side effects such as arthralgia and osteonecrosis of the jaw.[10] Furthermore, biosphosphonates have not yet been shown to increase the survival of those with metastatic bone disease.[4]

Recent studies have advocated the potential of cathepsin inhibitors as a novel therapeutic approach to cancer treatment. Cysteine cathepsins belong to a protease family that has been shown to be upregulated in many cancers, including lung and breast.[11] Additionally, increased levels of cathepsin have been correlated to poor patient prognosis.[11,7] This may be due to the fact that proteolytic activity have several tumor developing and tumor promoting functions. Proteases play a role in the degradation of the basement membrane and extracellular matrix allowing angiogenesis and the loss of cell to

cell adhesion, facilitating metastasis.[11] Cathepsin inhibitors in transgenic mouse pancreatic tumor models have significantly reduced tumor growth and invasion.[11] A four-week clinical trial of women with breast cancer and metastatic bone disease shows that cathepsin inhibitors were able to successfully suppress markers of bone resorption.[12] Cathepsin inhibitors have also been shown to reduce nociception in guinea pig models of spontaneous osteoarthritis[13] and rat contusion models[14]. Together this data makes cathepsin inhibitors a viable target for further study as a potential treatment for metastatic bone disease and pain.

In this study we will investigate the cathepsin inhibitor, VBY-825. This compound acts as a reversible covalent inhibitor and is thought to exhibit inhibitory effects on several members of the cathepsin family.[11] Previous studies have shown that VBY-825 results in a significant decrease in tumor growth and tumor incidence in a mouse model of pancreatic islet cancer.[11] A murine metastatic bone cancer model will be used to compare the efficacy of compound VBY-825 in attenuating bone cancer pain related behavior with zoledronic acid, bisphosphonate commonly used in bone cancer treatment.

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.

Animals

All procedures were approved by the University of Arizona Animal Care and Use Committee. Procedures follow the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and the guidelines of the International Association for the Study of Pain. Female Balb/C mice were 20-30 grams at the time of testing. Mice were maintained in a climate-controlled environment with a 12h light/dark cycle and allowed food and water ad libitum. 66.1 breast cancer cells were initially isolated from this strain of mice, thus using this strain creates a syngeneic model of metastatic bone disease.

Surgery

Mice were anesthetized with ketamine/xylazine prior to performing the arthrotomy. The condyles of the right distal femur were exposed and a hole was drilled to create a space for a needle injection into the intramedullary space of the murine femur.[7,15,17] Proper needle placement was verified by Faxitron x-ray imaging. Cancer treated animals were given 5uL of 100,000 66.1 breast cancer cells in minimum essential medium, while control animals were given 5uL of only minimum essential medium. The drilled hole was then sealed with bone cement.[16]

Drug treatment

Drug administration began on day 7 following surgery and occurred daily for 14 days. Cancer and control animals were divided into three treatment groups. One group was given the cathepsin inhibitor, VBY-825/k, dissolved in 5% dextrose (10mg/kg). The second group was given zoledronic acid (1mg/mL). The third group was given 5% dextrose as a vehicle treatment. Drugs were given via gavage.

Analysis of Chronic Pain

Animals were tested for spontaneous and evoked pain before surgery to get a baseline, and at day 7, 10, and 14 after surgery. Testing was performed during the day portion of the circadian cycle.

Spontaneous Pain

Flinching and guarding were characterized as painful behaviors during a resting state. Flinching was defined as the mouse lifting the affected foot off of the floor when not walking or moving. A flinch was also defined as the mouse shaking her foot while walking. Guarding was defined as lifting mouse's affected hind limb off of the floor. Both behaviors were observed for two minutes and recorded on a five-channel counter.[7]

Tactile Allodynia

The von Frey test was used to assess tactile hypersensitivity of the affected hind limb as described in Lozano-Ondua et. al.[17] The test uses a slight touch of a calibrated filament that would not evoke pain in healthy, uninjured animals. Animals were placed in raised plexiglass chambers and allowed to acclimate to the new environment for 30 minutes prior to testing. The affected hind limb was touched with von Frey filaments in logarithmically increasing stiffness that corresponded to weights ranging from 0.03 to 2.34

grams. First the 3.61 filament was applied to the plantar surface of the affected hind limb perpendicularly for 3 seconds. If the mouse began to move during the application of the filament, the same filament was applied again for another 3 seconds. If the mouse was unresponsive to the filament, the next higher filament was used until the mouse either moved her hind limb or the cutoff filament of 4.56 was reached. If the mouse withdrew her paw, the next lighter filament was used until the mouse no longer withdrew her paw or the lowest filament of 2.44 was reached. After a mouse was responsive, she was tested with four additional filaments as outlined in Chaplan.[19] The 50% pain withdrawal threshold was determined by Dixon's non-parametric method.[20]

Plasma Preparation

Plasma was collected using EDTA as an anticoagulant. Samples were centrifuged for 30 minutes at 2000x g within 30 minute of collection. Aliquotted samples were stored at -20°C.

Fractalkine ELISA

Quantikine kit for Mouse CX_3/CL1/Fractalkine was purchased from R&D Systems. Reagents, standard dilutions, and controls were prepared as indicated. 50uL of Assay Diluent RD1W was added to each well. 50uL of standard, control, or sample were added to each well. Samples were plated in triplicate. Plate was mixed by gentle tapping the plate frame for one minute. Plate was incubated for two hours at room temperature. Wells were aspirated and washed 5 times using the given Wash Buffer. 100uL of Mouse Fractalkine Conjugate was added to each well and the plate was incubated for two hours at room temperature. The wells were aspirated and washed 5 times. 100uL of the Substrate Solution was added to each well and incubated in complete darkness for 30 minutes at

room temperature. 100uL of Stop Solution was added to each well and the plate was tapped gently to mix. A microplate reader was used to determine the optical density of each well at wavelengths 540nm and 450nm. Readings at 540nm were subtracted from reading at 450nm to correct for optical imperfections.

Results

The cathepsin inhibitor, VBY-825, reduced bon cancer induced spontaneous pain behavior

(Fig. 1) Spontaneous pain behavior induced by bone cancer was measured on day 7 and day 14 after the arthrotomy surgery. Daily drug administration via gavage began on day 7 and animals were given vehicle (5% dextrose), zoledronic acid, or the cathepsin inhibitor, VBY-825. Animals that were given media injections during the arthrotomy did not display any spontaneous pain behavior. On day 14, animals treated with VBY-825 showed a significant reduction in flinching when compared to vehicle treated animals. Though the flinching was reduced when compared to animals treated with zoledronic acid, the results were not significant. However, guarding on day 14 was significantly reduced in VBY-825 treated animals when compared to both vehicle treated animals and zoledronic acid treated animals.

Treatment with VBY-825 reduced bone cancer induced evoked pain

(Fig. 2) Von Frey filaments were used to determine the affected hind paw pain withdrawal threshold of mice to measure the effectiveness of VBY-825 treatment in alleviating cancer-induced hypersensitivity. Animals were tested both 7 and 14 days after surgery. Animals were given the VBY-825 compound, zoledronic acid, or vehicle daily via gavage beginning on day 7. By day 14, the pain withdrawal threshold of animals treated with VBY-825 was significantly higher than those treated with vehicle.

Treatment with VBY-825 did not have significant effect on fractalkine levels in plasma

(Fig. 3) A fractalkine ELISA was used an indirect measure of the cathepsin present in the animals. Cathepsin induces nociception by cleaving fractalkine from the surface of sensory neurons.[19] Amount of fractalkine present with respect to total protein in the plasma of samples 14 days after surgery and 21 days after surgery was measured. Though VBY-825 treated animals had reduced levels of fractalkine present in the plasma after day 7 and day 14, the results were not statistically significant.

Discussion

Epithelial cancers include the three most common cancers to affect Americans today: lung, breast, and prostate.[2] The predisposition of these cancers to metastasize to the bone[4] thus makes bone cancer a concern in the therapeutic regimen of these cancers. Current treatment for metastatic bone disease includes radiotherapy, chemotherapy, analgesic therapy, and bisphosphonates such as zoledronic acid.[4,17] Analgesic therapy includes opiates and NSAIDs, both of which present unwanted side effects. NSAIDS are unable to control the level of pain present in bone cancer, and opiates build tolerance.[4,17] Bisphosphonates are a relatively new class of compounds that induce osteoclast apoptosis and prevent bone degradation. However, bisphosphonates also present unwanted side effects such as osteonecrosis.[4] This leads us to the most novel discovery in bone cancer treatment – cathepsin inhibitors.

Cathepsins are a class of proteases that have been found to be upregulated in cancers and chronic pain states. Increased cathepsin levels have also been linked to poor patient prognosis.[11] The reason for this may be that cathepsins are necessary for tumor development and metastasis, by causing basement membrane and extracellular matrix

breakdown and enabling angiogenesis to occur.[11] Recent studies have focused on cathepsin inhibitors as a novel approach to cancer treatment.[12,13] 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. We also compare it to the leading bisphosphonate drug, zoledronic acid.

Arthrotomies were conducted on female Balb/C mice to inject either 66.1 breast cancer cells or medium into the intramedullary space of the femur bone. On day 7 post surgery, daily drug administration via gavage of VBY-825, zoledronic acid, or vehicle began for 14 days.

Spontaneous pain behavior characterized by flinching and guarding were measured on day 7 and day 14. By day 14, VBY-825 treated cancer animals showed a significant reduction in flinching when compared to vehicle treated cancer animals. Though the flinching of VBY-825 treated cancer animals was lower than the zoledronic acid treated cancer animals, the results were not significant. By day 14, guarding was significantly reduced in VBY-825 treated cancer animals when compared to both vehicle and zoledronic acid treated animals. Spontaneous pain behavior often evades current analgesic therapy for bone cancer because it occurs suddenly and more severely than ongoing pain. It cannot be predicted and thus cannot be treated in advance. Thus the ability of VBY-825 to decrease the frequency of spontaneous pain thus has clinical implications in the treatment of the breakthrough pain associated with bone cancer.

Bone cancer induced evoked pain was tested by observing tactile allodynia using von Frey filaments that do not trigger reaction in naïve animals. In cancer treated animals, however, hypersensitivity to the von Frey filaments has been observed in the affected limb.

By day 14, VBY-825 treated cancer animals showed a significantly increased pain withdrawal threshold than the vehicle treated cancer animals, indicating lessened hypersensitivity. This again has many clinical implications because cathepsin inhibitors have the potential to treat bone cancer related pain behaviors, possibly without the severe side effects present with current analgesic therapy.

Plasma samples were harvested from animals at day 7, day 14, and day 21. A fractalkine ELISA was performed on all the samples to measure levels of fractalkine present in the plasma. Fractalkine served as an indirect measure of cathepsin because cathepsin cleaves fractalkine from sensory neurons. Though levels of fractalkine were reduced in the plasma samples of cancer animals treated with the VBY-825 compound, the results were not significant.

Though the fractalkine assay did not provide significant results, the assay should be repeated with a greater sample number to determine if significance does exist. The behavioral data suggests that the VBY-825 compound is effective at reducing pain related behavior and this should be evidenced in the fractalkine assay. Repeating this experiment with greater sample numbers in each group may yield better results.

In this study we show that the cathepsin inhibitor, VBY-825, was able to successfully reduce bone cancer induced pain behavior in a murine model. This warrants further testing because current analgesic therapies are only mildly effective and have many side effects. Metastatic bone disease affects thousands of cancer patients each year and can be debilitating to the patients' quality of life. Finding a better therapeutic agent for bone cancer pain would serve to better the lives of many patients.

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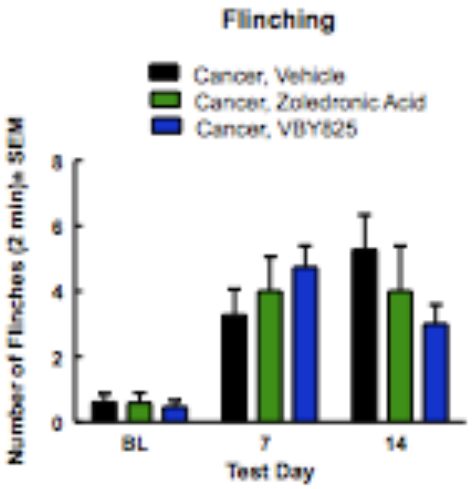
A



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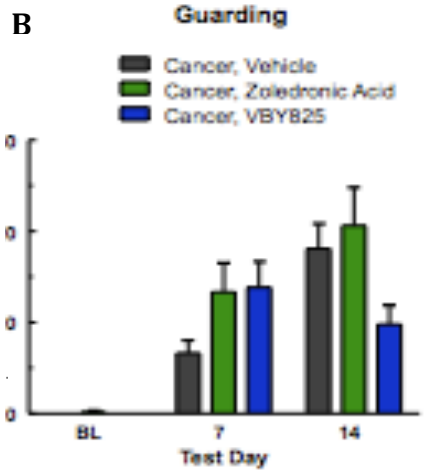

Figure 1

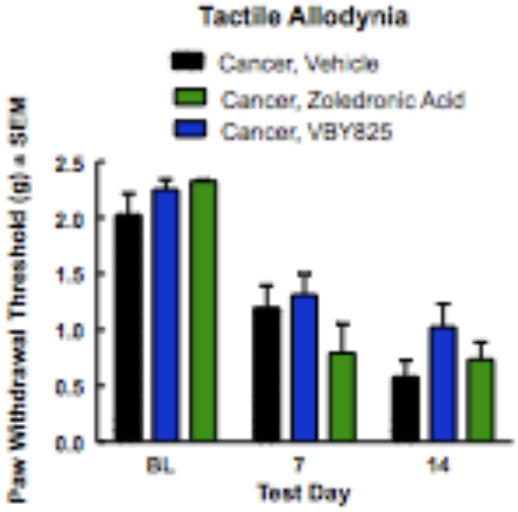

Figure 2

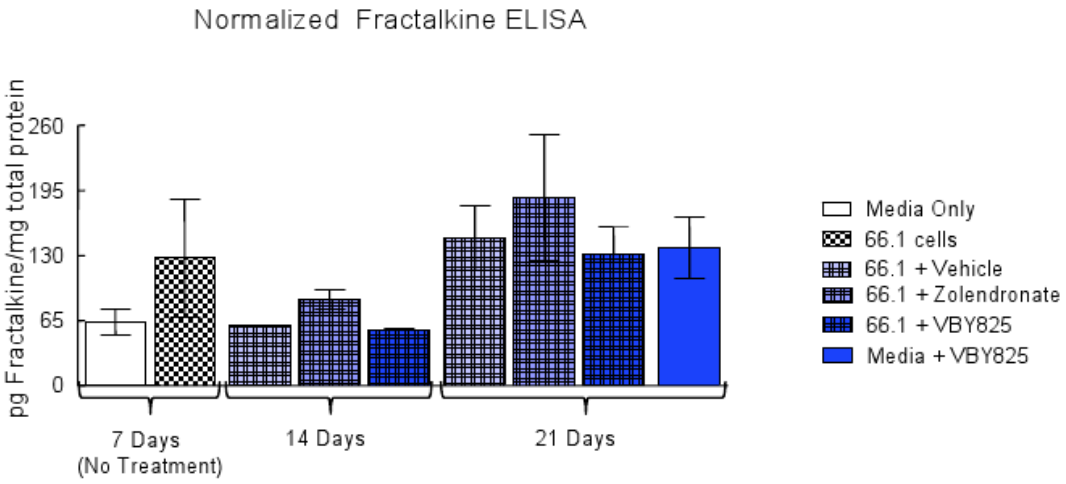
Normalized Fractalkine ELISA
pg Fractalkine/mg total protein
260
195
130
65
0
7 Days
(No Treatment)
14 Days
21 Days
Media Only
66.1 cells
66.1 + Vehicle
66.1 + Zolendronate
66.1 + VBY825
Media + VBY825

Figure 3

References

1. American Cancer Society. *Cancer Facts & Figures 2010*. Atlanta: American Cancer Society; 2010.

2. National Cancer Institute. *Common Cancer Types.* National Institutes of Health, 2010.

3. Coleman, R.E. 2006. Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin. Cancer res.* **12:** 6243s-6249s.

4. Jimenez-Andrade, J.M. *et al.* 2010. Bone cancer pain. *Ann. N.Y. Acad. Sci.* **1198:** 173-181.

5. Mercadante, S. 1997. Malignant bone: pathophysiology and treatment. *Pain.* **69:** 1-18.

6. Vanderah, T.W. *et al*. 2000. Dynorphin promotes abnormal pain and spinal opiod antinociceptive tolerance. *J. Neurosci.* **20 (18):** 7074-7079.

7. King, T. *et al*. 2007. Morphine treatment accelerates sarcoma-induced bone pain, bone loss, and spontaneous fracture in a murine model of bone cancer. Pain. **132 (1-2):** 154-168.

8. O'Connor JP. *et al*. 2008. NSAIDS and the skeleton. *Drugs Today*. **9**: 693-709.

9. Mantyh, P.W. 2006. Cancer pain and its impact on diagnosis, survival and quality of life. *Nat. Rev. Neurosci.* **7:**797-809.

10. Drake, M.T. *et al.* 2008. Bisphosphonates: mechanism of action and role in clinical practice. *Mayo Clin. Proc.* **83:** 1032-1045.

11. Elie, B.T. *et al*. 2010. Identification and pre-clinical testing of a reversible cathepsin protease inhibitor reveals anti-tumor efficacy in a pancreatic cancer model. *Biochimie*. **92**: 1618-1624.

12. Jensen, A.B. *et al.* 2010. The cathepsin K inhibitor odanacatib suppresses bone resorption in women with breast cancer and established bone metastases: results of a 4-week, double-blind, randomized, controlled trial. *Clin. Breast Cancer*. **10(6):** 452-458.

13. McDougall, J.J. *et al*. 2010. Cathepsin K inhibition reduces CTXII levels and joint pain in the guinea pig model of spontaneous osteoarthritis. *Osteoarthritis and Cartilage*. **18(10):** 1355-1357.

14. Clark, A.K. *et al.* 2010. Cathepsin S Inhibition Attenuates Neuropathic Pain and Microglia Response Associated with Spinal Cord Injury. *The Open Pain Journal*. **3:** 117-122.

15. Luger, N.M. *et al*. 2005. Bone cancer pain: from model to mechanism to therapy. *J. of Pain and Symptom Management*. **29**: S32-S46.

16. Schwei, M.A. *et al.* 1999. Neurochemical and cellular reorganization of the spina cord in a murine model of bone cancer pain. *J. Neurosci.* **19**: 10886-10897.

17. Lozano-Ondua, A.N. *et al*. 2010. A cannabinoid 2 receptor agonist attenuates bone cancer-induced pain and bone loss. *Life Sci.* **86**: 646-653

18. Virobay. 2011. About Cathepsin S and Neuropathic Pain. ViroBay Inc. [Online]. Available: http://www.virobayinc.com/neuropathic-pain.php. April, 2011.

19. Chaplan, S.R. *et al*. 1994. Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Met.* **53 (1)**: 55-63.

20. Dixon, W.J. 1980. Efficient analysis of experimental observations. *Annual Review of Pharmacology and Toxicology*. **20:** 441-462.