

**NOVEL MECHANISMS AND THERAPEUTICS IN THE TREATMENT FOR
CANCER-INDUCED BONE PAIN**

by

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ABSTRACT

Many common cancers, including breast, prostate and lung, have a predilection to metastasize to the bone, bringing not only bone destruction but severe pain. One possibility of inhibiting cancer-mediated pain inducing factors includes agonism of the Cannabinoid 2 receptor agonists. Osteolytic sarcoma within the femur produced spontaneous and touch evoked behavioral signs of pain within the tumor-bearing limb. The systemic administration of AM1241 both acutely and for 7 days significantly attenuated spontaneous and evoked pain in the inoculated limb. Sustained AM1241 significantly reduced bone loss. The central and systemic administration of the CB2 agonist JWH015 for seven days significantly attenuates pain. Pharmacological characterization with cannabinoid 1 and 2 antagonists demonstrates that the effects JWH015 on pain were mediated by the CB2 receptor. Systemic administration of JWH015 reduces cancer-induced elevation of cytokines in the tumor microenvironment, suggesting a mechanism by which CB2 agonist is attenuating pain peripherally. Additionally, systemic administration improves bone modification, as demonstrated via micro-computed tomography and bone serum markers while decreasing femoral tumor burden. *In vitro*, JWH015 reduced cancer cell proliferation and other inflammatory mediators shown to promote pain, bone loss and proliferation. These results suggest CB2 agonists as a novel treatment for breast cancer-induced bone pain, where disease modifications include a reduction in bone loss, suppression of cancer growth, attenuation of severe bone-pain and increased survival without the major side effects of current therapeutic options. Another future therapeutic option for metastatic bone cancer pain may include 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. Here we demonstrate the cathepsin inhibitor VBY-825 reduces cancer-induced pain behaviors. Additionally, tumor bearing animals treated with VBY-825 demonstrate a reduction in bone resorption, possibly mediated through a reduction in osteoclast activity. The studies presented herein provide preclinical evidence that warrant the investigation of these compounds in the clinic as treatment for cancer-induced bone pain.

CHAPTER 1: INTRODUCTION

1.1 Current therapeutics for cancer pain

Quality of life is defined as an individual's perception of their culture and values, as well as their goals, morals and concerns [99]. Pain induced by progression of disease and adverse events associated with standard therapies significantly diminishes the quality of life in cancer patients [51, 99]. Recent findings with a B-RAF inhibitor in advanced stages of melanoma have emphasized the need for treatments to reduce pain and increase survival time in terminally ill cancer patients [77]. Reports highlighted the importance in patient quality of life in late stages of cancer and how family and physicians surrounding the patient may be affected [77]. Current therapies for advanced stages of cancer pain include opioids and bisphosphonates. Recent studies have demonstrated that sustained morphine not only intensifies tumor-induced pain but also accelerates tumor-induced bone destruction in a murine bone cancer model [134]. In addition, chronic use of opiates results in several unbearable side effects including analgesic tolerance, constipation, respiratory depression and somnolence [258]. Bisphosphonates lack analgesic efficacy and can include adverse events including osteonecrosis of the jaw and disruption of normal serum calcium levels [243]. Although advances in cancer therapeutics have increased survival times of cancer patients, including those with metastases, treatment in advanced stages of cancer and palliative care has not improved. Thus, the development of therapeutics is needed that not only have anti-tumor effects, but attenuate the pain and/or limit the side effects seen with current patient care.

1.2 Discovery of Cannabinoid Receptors

One such therapeutic may include agonists targeting cannabinoid receptors (CB). Extracts from cannabis sativa including cannabidiol and Delta-tetrahydrocannabinol (THC) have been used for thousands of years by ancient cultures [202,212]. However, it was not until the 1960s that scientific researchers began to investigate the therapeutic potential of this compound [202,212]. In following years, it was discovered that an analog of THC bound to a receptor highly expressed in the rat brain [202]. With the screening of orphan receptors, one particular G-protein-coupled receptor (GPCR) demonstrated affinity for the same THC analogue previously found to bind to the rat brain [202]. This receptor was named Cannabinoid 1 Receptor (CB1) [202]. The CB1 receptor is also expressed in the pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, superior cervical ganglion, heart, blood vessels, lung, bladder and adrenal gland, liver, and adipose tissue [105,222]. This receptor is also expressed on central and peripheral nerve terminals. [222].

Subsequently, a receptor with similar homology to the CB1 receptor was found expressed within the immune system (spleen, tonsils, monocytes, B and T cells,) and distinct areas of the CNS (brain stem (neurons); spinal cord (microglia, astrocytes). This receptor was named Cannabinoid 2 Receptor (CB2) [102,202]. The prominence of the CB2 receptor in the immune system results in CB2 mediated cytokine production and immune cell migration [15]. Both of these receptors are coupled to adenylate cyclase via $G_{i/o}$ proteins [102]. Additionally, cannabinoid receptors have been shown to stimulate mitogen-activated protein kinase pathway [102]. However, only CB1 inhibits the activation of calcium channels, N and P/R type, while activating A-type and inwardly

rectifying potassium channels, providing a possible mechanism for how CB1 activation inhibits neuronal excitability and neurotransmitter release [102, 222].

In 1999, another G-protein coupled receptor named GPR55 was discovered in silico and found to be expressed in caudate and putamen areas of the rat brain, dorsal root ganglion, spleen, intestine, adipose, and fetal tissues, although there is still much discrepancy over this receptor's expression [15,140,226]. The genomic sequence of GPR55 maps to the human chromosome 2q37 and encodes a 319 amino acid protein comprised of consensus sequences for glycosylation and several phosphorylation sites for protein kinase A (PKA) or PKC [226]. In years to follow, two independent patents proposed by GlaxoSmithKline and AstraZeneca suggested that endocannabinoids and some exogenous ligands known to activate CB1/CB2 receptors also activated GPR55 [13]. Furthermore in 2007, Ryberg and colleagues provided convincing evidence that GPR55 is indeed a cannabinoid receptor that binds to and is activated by the cannabinoid ligand CP55940. Endocannabinoids, anandamide and virodhamine demonstrated nanomolar GTP γ S binding potencies via GPR55 [222]. Additionally, cannabidiol and abnormal cannabidiol, lacking CB1 or CB2 activity, showed activity at GPR55 [222]. GPR55 was shown to couple to G α 13 and intermediate activation of RhoA, cdc42 and rac1 [222]. In HEK293 cells expressing GPR55, Lauckner and colleagues showed that GPR55 is activated by THC, AEA, MEA, JWH015, and LPI but not by other cannabinoids including CP55,940, WIN55,212-2, and 2-AG [140]. Activation of this receptor results in intracellular calcium release, involving Gq, G12, RhoA, actin, phospholipase C. Additional data suggested GPR55 activation inhibits M-type potassium current, possibly enhancing neuronal activity [140]. The aforementioned

peer-reviewed data and others provide convincing evidence that GPR55 is indeed another cannabinoid receptor, with distinct signaling from CB1/CB2 receptors [15]. The pharmacology of this receptor is a controversial topic, over which ligands act as agonists, antagonists, or have no activity [15]. Evaluation of the physiological role of GPR55 in vivo is still in its infancy, although preliminary data suggests GPR55 may regulate inflammation, neuropathic pain, mitogenic activity, and proliferative effects of tumors [15]. However, with validation that GPR55 is indeed a cannabinoid receptor, the possible interaction of GPR55 receptor signaling with the CB1 and/or CB2 raises imperative questions as to whether preceding functions ascribed to the CB1 or CB2 receptors are attributable to the altered signaling of GPR55 or other unidentified cannabinoid receptors.

1.3 Endocannabinoids

Cloning of the CB1 and CB2 receptors led to the discovery of endogenous ligands for these receptors [202]. N-arachidonylethanolamine (AEA), also known as anandamide, and 2-arachidonoylglycerol (2-AG) were the first endogenous cannabinoids found [50,194]. AEA is calcium dependent and derived from N-arachidonoyl-phosphatidylethanolamine (NAPE) [212]. 2-AG is synthesized from diacylglycerol lipases, identified as DAGL-a and DAGL-b [102]. When the amide and ester bonds of these compounds are cleaved, AEA is hydrolyzed to arachidonic acid and 2-AG is hydrolyzed to ethanolamine or glycerol [202]. AEA can also bind the vanilloid TRPV1 receptor and GPR5 [102]. AEA and 2-AG are not packaged into vesicles; they are released into the synaptic cleft and bind cannabinoid receptors located presynaptically, which mediate neurotransmitters such as GABA, acetylcholine,

and glutamate [212]. They may also function as retrograde signaling molecules [212]. Other endocannabinoids later found include noladin ether, virodhamine, and *N*-arachidonoyldopamine (NADA) [102]. 2-AG, AEA, and NADA are degraded by Fatty Acid Amide Hydrolase (FAAH) [50]. Compounds such as fatty acid derivatives oleamide, palmitoylethanolamide, and arachidonoyl amino acids are considered cannabinergic because they assist in endocannabinoid function, although they lack affinity for the actual cannabinoid receptors [102].

Endocannabinoids participate in many biological roles. Cannabinoid receptors are highly distributed within the central nervous system [102]. Upon activation with endocannabinoids, they mediate synaptic plasticity, neuronal excitability, and long term potentiation [145]. Additionally, endocannabinoids contribute to the perception of emotion, learning, memory, appetite, and supraspinal nociception [50]. Endocannabinoids are a major contributor to pain signaling pathways, as evidenced by their distribution in neurochemical regions that transduce pain pathways such as the paraventricular grey (PAG), rostral ventromedial medulla (RVM), and dorsal horn of the spinal cord [102]. In the immune system, they are involved with cytokine release, chemotaxis, and leukocyte differentiation. In fact, the cannabinoid receptor is the most abundant GPCR found in the human body and thus has a role in almost every organ system (skeletal, reproductive, digestive, and respiratory) [145].

Endocannabinoids are unique from exogenous ligands in that they are synthesized and released from cells on demand to activate CB1 and/or CB2 receptors for a particular duration of time in a specific targeted area [202]. Exogenous ligands, both synthetic and those derived from plants, activate receptors everywhere until they

are metabolized [202]. For this reason, selective inhibitors of endocannabinoid inactivation would affect only endocannabinoids that are being synthesized and degraded, increasing their effectiveness [202]. Use of a FAAH inhibitor to inactivate AEA, indirectly increases endocannabinoid activity creating “a site and event specific” therapeutic where endocannabinoids are being synthesized [117]. This will eliminate the psychotropic side effects produced when an exogenous CB agonist activates cannabinoid receptors [117].

Another possible manipulation of the endocannabinoid system without using cannabinoid agonists is N-palmitoylethanolamide (PEA). PEA is a saturated N-acylamide AEA congener [117]. This endogenous ligand acts at the orphan receptor GPR55, which is still under debate about whether it is a third CB receptor and does not act at CB1 or CB2 receptors (not to human or rat) [117]. However, PEA shares pharmacological properties of CB2 agonists as it is synthesized upon demand, exhibiting both anti-inflammatory and neuroprotective actions, such as relief of neuropathic pain [117]. Thus, PEA may be used in treating neurodegenerative diseases that result from inflammation such as Alzheimer’s Disease, Huntington’s Disease, Parkinson’s Disease, and Multiple Sclerosis [117].

1.4 Exogenous Cannabinoid Ligands

The identification of the cannabinoid receptors and their endogenous ligands revolutionized the development of exogenous agonists and antagonists with different affinities for either the CB1 or CB2 receptor. Commonly utilized CB1 agonists include: arachidonyl-2'-chloroethylamide (ACEA) (1.4 nM for CB1-receptors and over 3000 nM for CB2-receptors, arachidonylcyclopropylamide (ACPA), and methanandamide. CB1

antagonists include SR141716A (K_i value of 2nM for CB1-receptors and well above 1 mM for CB2-receptors), AM251, and AM281 [143, 201].

Agonists more selective for the CB2 receptor are JWH015 (13.8 and 383 nM for CB2- and CB1-receptors, respectively), JWH133, HU308, AM1241 (K_i 20-30 nM), GW405833, GW842166X, and O-1966 [23, 201]. CB2 antagonist SR144528 (K_i values of 0.3 and 437 nM in cell lines expressing either human CB2- or CB1-receptors, respectively) [143]. Compounds such as delta-tetrahydrocannabinol (THC), HU-210, CP55940, *R*-(+)-WIN55212 NOT SURE WHAT THIS NUMBER ID AND FOR WHAT RECEPTOR?, anandamide, 2-arachidonoyl glycerol non-selectively activated both cannabinoid receptors [201]. CB2 antagonists often used are SR144528 and AM630 [201]. CB2 agonists can be classified as thizoles, dihydropyrazoles, indoles, azetidines, amidosulfones, butyramides and ureas, benzamides, pyridines and furopyridines, trtrahydroimidazoles and imidazopyridines, triaryl derivatives, pyrimidines and pyrazines, thienoderivatives, dihydrobenzofuranes [183]. Several companies have submitted patents on compounds acting as CB2 agonists. Amgen discloses a CB2 agonist, 3-*tert*-butyl-1-methyl-1*H*-pyrazol-5-yl-carboxamide (GTP binding; EC₅₀hCB2 = 26 nM) [183]. Pharmacokinetic *in vitro* assays for this compound shows a low intrinsic clearance, in human (14 μl/min/mg) and rat (20 μl/min/mg) liver microsomes, and low plasma protein binding across several species (roughly 94%) [183]. The *i.v.* administration to Sprague-Dawley rats resulted in a moderate clearance (CL = 1.2 L/h/kg), a short half-life (t_{1/2} = 1.9 h) and a small distribution volume (V_{ss} = 2.0 L/Kg) [183]. Oral administration in rats, show this absorbs well (oral bioavailability= of 43%) [183]. The pharmacokinetics implies this compound may have therapeutic potential in

humans. Furthermore, these ligands are used as research tools to help understand the mechanism of cannabinoid receptors and explore their therapeutic potential.

Currently, dronabinol and nabilone, non-selective partial agonists of both CB1 and CB2 receptors, have been approved by the Food and Drug Administration (FDA) to assuage adverse effects of nausea, vomiting, and lack of appetite, associated with chemotherapy [250].

1.5 Cannabinoids Effect on the Central Nervous System

The high expression of cannabinoid receptors within the immune system enables them to exhibit immunomodulatory and anti-inflammatory activity. For example, dendritic cells and macrophages express cannabinoid receptors and can produce anandamide, 2-AG, and FAAH in response to inflammatory conditions [50]. Many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Multiple Sclerosis (MS) cause inflammation and an upregulation of cytokines within the CNS [50]. Normally, glial cells, microglia, and astrocytes in the brain mobilize immune responses and comprise the blood-brain-barrier [50]. In contrast, in certain pathological conditions that involve inflammation, the blood brain barrier loses its selectivity for immune cells [50]. While anti-inflammatory diseases upregulate inducible nitric oxide synthase (iNOS) in glial cells or macrophages that penetrated the BBB, cannabinoids have shown a suppressive effect in the generation of excess nitric oxide (NO) [50]. Nitric oxide contributes to oligodendrocyte injury, demyelination and axonal degeneration [50]. Glial cells such as astrocytes, astrocytome cells, microglial cells and oligodendrocytes have cannabinoid receptors [50]. Astrocytes and microglia are important to neurodegenerative diseases because they produce many chemokines and

cytokines [50]. Astrocytes and microglia can both produce anandamide and 2-AG [50]. Astrocytes, antigen-presenting-cells, dendritic cells, and macrophages can express FAAH [50]. Thus cannabinoids mediate the inflammatory responses in normal and pathological conditions.

Many studies have indicated that cannabinoids may serve as a possible therapeutic for multiple sclerosis (MS). Animal models of MS also show an improvement of the disease's debilitating symptoms [50]. For example in Theiler's murine encephalomyelitis virus (TMEV) infection of the CNS, an accepted MS model, cannabinoid treatment improved motor function and decreased microglial reactivity, MHC class II antigen expression, CD4 T cell infiltrates, and restored spinal cord myelination [50]. Endocannabinoid levels in the brain of multiple sclerosis animal models are decreased compared to sham animals, implicating the cannabinoid receptors as protective against excitotoxicity [145]. This correlated with MS patients who have decreased endocannabinoid levels. Early clinical trials have shown a decrease in pain and an increase in mobility [50].

In another anti-inflammatory disease of the CNS, Autoimmune encephalomyelitis (EAE), the cannabinoid receptor contributes to the protection against neurodegeneration. In a mouse model of the disease, CB1 receptor-deficient mice were compared to wild type (WT) [50]. The CB1 KO showed delayed remission, more axonal lost, decreased expression of neurofilament and increased caspase 3 activities, compared to WT [50]. This implies CB1 receptors and by association endocannabinoids participate in neuroprotection during autoimmune attack.

Cannabinoids may improve many symptoms of AD pathogenesis. In AD, it is believed that excitotoxic damage to neurons in the brain is the result of excessive activation of the NMDA receptor by glutamate [201]. Cannabinoids have been shown to inhibit glutamate release in vivo and in vitro and some may even inhibit NMDA receptors directly [201]. Glial cells, in particular microglia, upregulate the production of reactive oxygen species and thus a desired treatment for AD is antioxidative molecules [201]. Several cannabinoids have antioxidant properties (it is dependent on whether it has a phenolic structure) [201]. Inflammation of the cerebral parenchyma caused by glia cells and their production of cytokines and other inflammatory mediators occurs in AD [201]. Upon activation the CB1 receptor mediates through glial cells the production of inflammatory cytokines: IL-1, TNF-alpha, IL-6 and IL-12, which are associated with the development inflammation in neurodegenerative disorders [201]. CB1 activation also stimulates the production of anti-inflammatory molecules, trophic factors and neurotrophins [201]. Interestingly, CB2 activation is downregulated in neuroinflammatory states [201]. In a model of Alzheimer disease (barryoid peptide-treated mice), on day 12 following treatment, 2-AG and AEA levels are increased and on day 20 following treatment the levels are decreased, suggesting a neuroprotective role in the early AD, and/or promotes the development of later symptoms arising by downregulation of cannabinoid receptors [145]. THC and Dronabinol, synthetic THC, have been shown to improve symptoms of AD patients, such as nocturnal motor activity, emesis, and loss of appetite, agitation, and aggressiveness without unwanted side effects [201].

Cannabinoid signaling also plays a role in Parkinson's Disease (PD). In knockout models of PD (either PARK1, PARK2, or PARK6 gene), the CB1 receptor is downregulated in early stages of the disease [201]. However, in later stages of the same PD model, Cannabinoid receptor signaling becomes upregulated [201]. This observation has been confirmed clinically by observing the number of CB1 receptors in post-mortem basal ganglia and by measuring the concentration of endocannabinoid in the basal ganglia [201]. The upregulation of CB1 receptors can be reversed with levodopa, suggesting that loss of nigral neurons and dopaminergic denervation of the striatum is inversely correlated with the upregulation of cannabinoid signaling [201]. Thus, inhibition of the cannabinoid receptor may help treat PD bradykinesia. In another PD model, where animals received a nigro-striatal lesion, which resulted in side effects seen with dopamine signaling impairment, endocannabinoid levels were found to be both increased and decreased [145]. This may be because CB1 activation in this region can be involved in direct or indirect pathways of locomotor control [145]. It has not yet been shown how CB2 receptors are changed in the disease. In PD patients, increased AEA levels have been measured in the cerebrospinal fluid [145]. Clinically, synthetic cannabinoid agonists have been ineffective. In an anecdotal study, patients reported improvement in levodopa induced dyskinesia with cannabis use (inhalant). A pilot study involving treatment with Nabilone, a synthetic THC, reported a decrease in levodopa-induced dyskinesia compared to placebo, without affecting the therapeutic properties of levodopa. The improvement in dyskinesia with nabilone was reversed by rimonabant, demonstrating a CB1 receptor mediated effect.

Stress and fear can alter endocannabinoids concentrations in various areas of the brain. In a conditioned fear aversion model, AEA and 2-AG levels are increased, suggesting that endocannabinoids serve as a natural mechanism to “inhibit extinction of aversive memories” [102]. Additionally, endocannabinoids and CB1 receptors mediate aversive memory extinction in the amygdala through long-term depression of GABAergic inhibitory currents [102]. When rats are acutely restrained, 2-AG levels in the hypothalamus decrease [145]. Constant restraint (chronic) caused an increase of 2-AG, suggesting an attempt to reverse the effect of corticosterone levels, known to be released during chronic stress [145]. When rats are exposed to a non-noxious electrical shock, stress-induced analgesia is produced and endocannabinoid levels are elevated in the periaqueductal grey [145]. This data indicates that endocannabinoids may also be used to treat anxiety, depression, and post-traumatic stress disorders. Inhibitors of endocannabinoids and CB1 agonists have aided in the elimination of traumatic memories and conditioned fear [145]. Hohmann and colleagues found that in a model of Stress Induced Analgesia (animals exposed to a 3 minute continuous foot shock) resulted in elevated levels of 2-AG and AEA in dorsal midbrain, including PAG. This effect was enhanced by FAAH inhibitors, URB597 and arachidonoylserotonin, that normally would rapidly degrade 2-AG and AEA [102]. With the microinjection of monoacylglycerol lipase (MGL), which typically hydrolyzes 2-AG in vivo, into the PAG, SIA was enhanced and elevated levels of 2-AG were observed [102]. They also found that inhibition of endocannabinoid deactivation enhanced stress antinociception, demonstrated by treatment with FAAH inhibitor prior to the tail-flick assay. These data suggest an endogenous mechanism of pain modulation in the PAG via 2-AG [102]. Although stress

stimulates the release of 2-AG and anandamide, they are released at different time points, which implies the two endocannabinoids may serve different biological functions [102]. MGL and FAAH inhibitors may serve as useful targets for treatment of conditions with symptoms of pain, stress, and anxiety [102]. Inhibition of FAAH, URB597 and URB532, in Wistar rats reduced anxiety symptoms; whereas anxiety was restored by the CB1 antagonist, SR141716 [272]. Varvel and Lichtman demonstrate in their experiments the role of endocannabinoids in memory extinction, where CB1 knockouts were unable to relearn a new platform using a water maze. This notion is further supported by Marsicano and colleagues utilizing CB1 knockout mice where they showed impairment in auditory-cued fear memory [170]. This effect was also confirmed by treating wild-type mice with the CB1 antagonist, SR141716, where the mice demonstrated memory extinction if given 30 minutes prior to test and no effect if given 2 minutes before the test [170]. The mechanisms by which endocannabinoids contribute to early memory consolidation and memory extinction remain to be elucidated. It possibly involves the activation of selected kinases that participate in both consolidation and extinction of aversive memories [272]. Because similar behavior is evoked with inactivation of CB1 and inhibition of phosphatases in the extinction of aversive memories, the endocannabinoid system and phosphatases may interact in this process [272].

Additionally, a plethora of data indicates cannabinoid receptors play an important role in pain. Through the use of various animal models, non-selective cannabinoid agonists prove to have antinociceptive and antihyperalgesic effects [183]. Interestingly, in neuropathic animal models of pain, both the expression of CB1 and CB2 receptors

becomes up-regulated in peripheral and central sensory pathways [146, 272, 283]. In the case of CB1R, this up-regulation augments analgesic response to exogenous cannabinoids in an animal model of neuropathic pain [146]. Because of the high expression of the CB1 receptor in the human body, especially within the central nervous system, dose limiting side effects hinder their analgesic potential in humans [164]. Since the CB2 receptor is localized in the immune system and seem to be up-regulated only in pathological conditions, the CB2 receptor may prove to be a more promising target to treat pain. [164] The CB2 agonist, AM1241 is antinociceptive in response to thermal stimuli [110, 163]. CB2- selective agonist AM1241 suppresses hyperalgesia in several inflammatory models of pain through the use of carrageenan, capsaicin and formalin models [275]. Increasing evidence in the ever growing literature show that the CB2 receptor can modulate acute pain, chronic inflammatory pain, postsurgical pain, cancer pain and pain associated with nerve injury [156, 157, 275].

1.6 Cathepsins are up-regulated in cancer

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.

1.7 Cathepsin S

Cathepsin S (CTSS) has a critical role in antigen presentation, participating in the degradation of a polypeptide that would prevent antigen loading onto major histocompatibility 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 collagenolytic 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, CatS expressing cells in the dorsolateral horn co-label with microglial activation, suggesting the constitutive expression of CatS through spinal microglia promotes nociception [43]. Additionally, animals that received a partial ligation of the left sciatic nerve (PNL), showed increased expression of CatS colocalizing with microglial activation compared to naïve animals in the ipsilateral dorsal horn, indicating that CatS 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].

1.8 Cathepsins B and L

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. Cath-B plays a major role in extracellular protein catabolism, while Cath-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 Cath-B and Cath-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.

1.9 Cathepsin V

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 Cath-S [34]. It is weakly collagenolytic, 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.

1.10 Cathepsin K

Cathepsin K is enzymatically critical for bone remodeling and resorption and is selectively expressed in osteoclasts [34, 252]. A Cath-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.

1.11 Cathepsin inhibitors

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, CatK 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, B, V, L, K showed a significant reduction in tumor incidence and growth [73]. Animal studies presented herein provide evidence that VBY-825 may effectively attenuate pain and decrease bone resorption. Clinical trials of this multivalent inhibitor must be conducted to further validate its therapeutic efficacy.

1.12 Conclusion:

Currently there is little known about cancers that have metastasized to bone and the pursuing production of chronic and breakthrough pain. Ironically, when a primary tumor such as breast cancer or prostate cancer in their tissue of origin results in very little or no pain whatsoever but when metastasized to bone results in excruciating pain. Preclinical studies have just begun to scratch the surface on how such cancers may interact with the bone microenvironment to result in pain. Ideas, of course, include growth factors, cytokines, chemokines, acidic environments, enzymes, excessive glutamate and oxidative species yet, the only pharmacological therapy that is being used to treat the pain are opioids and compounds that inhibit osteoclast activity such as bisphosphonates and denosumab. Further studies are desperately needed at both the preclinical and clinical level to determine whether the mentioned targets, including

cannabinoid 2 receptor agonists and cathepsin inhibitors, are viable and feasible for patient populations. Cancer patients are living longer while being treated with chemotherapeutic agents; however their quality of life is severely diminished due to the pain or side effects of current opioid pain medication.

CHAPTER 2: METHODOLOGY

2.1 In vitro

2.1.1 Cell culture:

Murine CCL-11 (NCTC clone 2472) sarcoma cells were maintained in NCTC media containing 10% fetal bovine serum and 1% penicillin, passaged every 4 days and harvested between 2 and 12 passages (American Type Culture Collection (ATCC), Rockville, MD). Murine mammary tumor line 66.1 cells were cultured in Minimum Essential Medium eagle with 10% fetal bovine serum (FBS), 100 IU⁻¹penicillin and 100 µg/ml streptomycin (P/S), and harvested between 12-24 passages. All cells were plated in 10 cm tissue culture dishes, allowed to grow exponentially and housed in an incubator at 37°C in 95 % O₂, 5% CO₂. For all assays, cells were centrifuged and counted using a gridded hemacytometer (Hausser Scientific).

2.1.2 Drugs:

The CB₂ agonists, AM1241 and JWH015, were purchased from Tocris. The CB₁ antagonist/inverse agonist, SR141716 and CB₂ antagonist/inverse agonist, SR144528 were provided by NIDA. All drugs were dissolved initially with DMSO to make a 10 mM stock solution. Additional dilutions were made Minimum Essential Medium eagle with 10% fetal bovine serum (FBS), 100 IU⁻¹penicillin and 100 µg/ml streptomycin (P/S). Cells were treated with CB₂ agonist for 24 hours or pre-treatment of CB₁ antagonist for 1 hour followed by CB₂ agonist for 24 hours.

2.1.3 Immunoblotting:

The 66.1 murine breast cancer cells were lysed, protein extracts separated electrophoretically and transferred to a PVDF membrane as previously described [249]. Additionally, tumor cells were extruded from the femoral intramedullary space after 14 days implantation, protein extracts were subsequently separated electrophoretically and transferred to a PVDF membrane. The membrane was incubated with polyclonal primary anti-CB₂ antibody (Cell Signaling Technology) followed by horseradish peroxidase-conjugated secondary IgG antibody (Cell Signaling Technology) and developed using a chemiluminescent system (Amersham Biosciences). GAPDH or α -tubulin (Cell Signaling Technology) was used as loading control.

2.1.4 Sulforhodamine B (SRB) assay:

Cell proliferation in response to different treatments was measured as previously described [239]. Briefly, approximately 1.6×10^4 66.1 cells per well were plated on six well plates and allowed to grow overnight. On day 2, complete MEM medium was replaced with Optimem (Invitrogen) serum free medium and incubated for 16 hours. Cells were then stimulated with AM1241 or JWH015 on the morning of day three in varying concentrations. Cells exposed to agonist and antagonist were pre-treated with antagonist 1 hour prior to agonist treatment. Cells were fixed 48 hours later with ice cold 50% trichloroacetic acid (TCA) (500 μ L/well) for one hour at 4 °C. Cells were then washed with deionized water and stained with SRB dye (2 mL/well) for 10 min at room temperature. Cells were washed with 1% acetic acid and the bound SRB dye was

solubilized with 1M unbuffered tris for ten minutes on a plate shaker. Optical density of each well was read at 540 nm using a plate reader (Biomek).

2.1.5 Bromodeoxyuridine (BrdU) Assay:

BrdU cell proliferation assay (Millipore) was performed according to manufacturer instruction after seeding approximately 10,000 66.1 cells per well in a 96 well plate. Cells were treated for 48 hours with vehicle, CB2 agonist, CB₁ antagonist/inverse agonist, SR141716 or CB2 antagonist/ inverse agonists, AM630 and SR144528. Wells treated with both antagonist and agonist were pre-treated with antagonist one hour prior to agonist treatment. After treatment, cells were incubated for 20 hours with BrdU for incorporation and fixed to the plate. Plates were washed to remove excess BrdU. Plates were incubated with a peroxidase conjugated antibody against BrdU and read at 450nm [156].

2.1.6 Cytokine and chemokine secretion:

To evaluate secretion of inflammatory mediators by 66.1 cells, cells were plated at a density of 10^4 cells per well on a 96 well plate and grown overnight in serum-containing media. Three hours prior to assay, cells were switched to serum-free Opti-MEM (Invitrogen) containing drug or vehicle treatments. Cells receiving antagonist were pre-treated for 1 hour prior to a 3 hour incubation with agonist. Supernatant was collected following the 3 hour treatment incubation and centrifuged at 1000 rcf for 5 min to remove cell debris. Concentrations of TNF α , MCP-1 and IL-6 were determined using commercially available ELISA kits according to manufacturer specifications (Invitrogen,

SABiosciences, eBiosciences) with detection limits of 8.0, 25.5 and 0.21 pg/mL respectively [156].

2.1.7 Cytokine proliferation response:

In order to evaluate the proliferative response of cells to cytokine stimulation, cells were plated at a density of 10^4 cells per well on a 96 well plate and grown overnight in serum-containing media. On day 2, cells were switched to serum-free Opti-MEM (Invitrogen) containing cytokine and drug or vehicle treatments. On day 3, cell proliferation was measured using sulforhodamine B (SRB) as previously described (Skehan et al., 1990). Briefly, cells were fixed with cold 10% TCA for one hour at 4 °C. Cells were then washed with deionized water and stained with SRB dye for 10 min at room temperature. Cells were washed with 1% acetic acid to remove excess dye and the bound SRB dye was solubilized with 1M unbuffered tris for 10 min on a plate shaker. Optical density in wells was read at 540 nm.

2.2 In vivo

2.2.1 Animals:

Male C3H/HeJ mice weighed 20-25 grams at the time of surgical inoculation with NCTC 2472 cells (Jackson Laboratories, Bar Harbor, ME). Female BALB/c/c3H (Harlan, Laboratories) were 15-18 grams prior to implantation of 66.1 cells. Mice were maintained in a climate-controlled room on a 12-hour light/dark cycle and allowed food and water ad libitum. Animals were weighed on day of surgery (baseline), days 7, 14, and 21. Animals were monitored for clinical signs of morbidity including paralysis and rapid weight loss (>20% in 1 week). Survival was expressed as a percentage of the

number of animals remaining at day 28 to the number of total animals in each group on day 7.

2.2.2 Surgery:

Animals were anesthetized with ketamine (80mg/kg)/ xylazine (12mg/kg) i.p. An arthrotomy was performed as previous described (Luger et al. 2005; King et al. 2007). The condyles of the right distal femoris were exposed and a hole was drilled to create a space for injection of cancer cells. Injections were made with an injection cannula affixed via plastic tubing to a 10 uL Hamilton syringe. Male mice utilized for sarcoma studies were inoculated with 25,000 CCL-11 murine sarcoma cells in 5 uL of alpha minimal essential medium containing 1% bovine serum albumin or 5 uL of alpha minimal essential medium alone (control) within the intramedullary space of the mouse femur. To look at effect of breast cancer on cancer-induced bone pain, female mice were injected with 1×10^5 66.1 cells (murine mammary adenocarcinoma) in 5 uL complete MEM or 5 uL complete MEM without cells in control animals within the intramedullary space of the mouse femoris. Proper placement of needle was confirmed through use of Faxitron x-ray images. The drilled hole was sealed with bone cement. (Schwei et al. 1999)

2.2.3 Drug Treatment:

Administration of drug for acute studies included one intraperitoneal injection on Day 7. For chronic studies, starting on day 7 after inoculation of the femur with cancer cells, mice were treated for seven days. Cannabinoids utilized included: AM1241 (Tocris), JWH015 (Tocris), SR144628 (NIDA), or SR141716 (NIDA). All cannabinoids were

dissolved with 10% dimethyl sulphoxide, 10% Tween 80, and 80% saline. Cathepsin inhibitor investigated included VBY-825 (Virobay) dissolved in 5% dextrose, 95% H₂O. Control groups were administered dissolving solvent alone. Doses and administration method are specified in figure legends. 2.1.4

2.2.4 Behavioral testing for analysis of chronic pain:

Animals were tested for movement-evoked pain, spontaneous pain, and tactile allodynia. Testing was performed baseline (prior to surgery), post-surgery baseline (day 7 after surgery, before drug treatment), and day 14 (after seven day dosing regimen).

Movement evoked pain evaluated the severity of pain the mouse experienced during normal ambulation. The mouse was placed in an empty mouse pan and limping and guarding behavior of the right leg was observed for two minutes. As the mouse walked across the empty pan, the use of the afflicted hind limb was rated with the following scale: 0 = no use of hind limb at all, 1 = partial non-use, 2 = limp and guard, 3 = limp, and 4 = normal use [158]. Observer of movement evoked pain was blinded to the treatment conditions.

Another evoked pain employs the von Frey test, which assesses tactile hypersensitivity of the right hind limb in which cancer had been induced with the slight touch of calibrated filaments that would not evoke pain in healthy, uninjured animals. This is also called tactile allodynia. Animals were placed in raised plexiglass chambers with wire grid floors. Animals acclimated to the environment for 30 minutes before testing was implemented. The targeted hind limb was probed with von Frey filaments with logarithmically incremental stiffness that were equivalent to weights ranging from

0.03 to 2.34 grams. Starting with the 3.61, the filament was applied perpendicularly to plantar surface of the targeted hind limb while the animal was sitting still with his foot on the floor for duration of 3 seconds. If the mouse began to walk around while being probed, the same filament was reapplied for another 3 seconds. If the mouse did not respond to the touch of the filament, testing proceeded with the next higher filament until the movement of hind limb occurred or cutoff (highest filament used was 4.56) was reached. The 4.56 filament is chosen as the cut-off due to larger filaments ability to push the paw up with out a response. Upon paw withdrawal, the next lighter filament was applied until the mouse did not withdraw his paw or cutoff was reached (lowest filament used was 2.44). Once the mouse responded, he was tested with four more filaments [39]. The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon et. al [66]. Tester of von Frey evoked pain was blinded to the treatment conditions.

In contrast, spontaneous pain is intended to quantify the painful behaviors during a resting state. Flinching and guarding were observed for 2 minute durations. Flinching was characterized by the mouse's lifting of his right foot (ipsilateral to the inoculation of sarcoma cells) off the floor when not associated with walking or movement. However, if a mouse shook its foot while walking, the behavior was counted as a flinch. The number of flinches was recorded on a five-channel counter. Guarding was characterized by holding of the mouse's right hind limb (ipsilateral to inoculation with cancer) up off the floor. Guarding behavior was recorded over a 2 minute period [134]. Observer of spontaneous pain was blinded to the treatment conditions.

2.2.5 Acute analysis of pain:

After a single dose of investigated drug, flinching, guarding and tactile allodynia were performed as described above. Cancer-induced hypersensitivity was tested 2 hours after drug administration for return to baseline levels.

2.2.6 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 [158].

2.3 Ex Vivo

2.3.1 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 μ m sections and stained with hematoxylin and eosin (H&E) to visualize normal marrow

elements and cancer cells under bright field microscopy on a Nikon E800 at 4X magnification. Tumor or marrow areas within the femur (6 bones per treatment) were measured in mm^2 between the epiphyseal plates using Metamorph imaging software by a blinded observer with the aid of a pathologist.

2.3.2 Micro-computed tomography:

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 stored in PBS until scanned. To characterize changes in breast cancer-induced bone remodeling at day 14 post-cell injection in animals treated with a cannabinoid 2 agonist (JWH015) or vehicle (10% dimethyl sulphoxide, 10% Tween 80, and 80% saline) and compared to control animals, femurs were analyzed with an eXplore Locus SP micro-computed tomographer (μCT , GE Healthcare, Schenectady, NY, USA) to visualize densitometric and architectural parameters. This conebeam μCT scanner uses a 2300×2300 CCD detector with current and voltage set at 80 μA and 80 KVp, respectively. Specimens were scanned in 810 views through 270° with a 2100 ms integration time. Scans were then reconstructed at $16\text{-}\mu\text{m}^3$ resolution using Reconstruction Utility software (GE Healthcare). All 3D image manipulations and analyses were performed with the system's accompanying analysis software (Microview, Version 2.1, London, ON, Canada). The femoral reconstructions were reoriented along anatomical axes and the regions of interest (ROIs) selected for quantification were chosen in the following manner. For cortical analysis, a standard ROI measuring 2.5mm wide x 2.5mm long x 1mm tall was generated and positioned

such that the entire region of bone immediately 1mm proximal of the distal growth plate was included in the ROI. Importantly, this area of bone, known as the distal metaphysis, was chosen for quantification because the majority of bone destruction observed in this model of breast cancer-induced bone remodeling occurred in this region. Using a threshold of 2222, the following parameters were gathered using the Cortical Analysis tool: Cortical Thickness (Ct. Th), Total Area (Tt. Ar), Cortical Area (Ct. Ar), / Tt. Ar, Tissue Mineral Density (TMD), and Periosteal Perimeter (Ps. Pm). For trabecular analysis, an irregular ROI was generated 1mm proximal of the distal growth plate using the spline tool to draw endocortical contours in 5 axial planes through 1mm above the distal growth plate. The contours were then integrated and used to generate a 3D ROI that filled the intramedullary space of the distal metaphysis. μ CT parameters used to assess breast cancer-induced changes in trabecular bone micro-architecture were gathered at a threshold of 1337 mg HA/cm³ and included: Trabecular Number (Tb. N), Trabecular Thickness (Tb. Th), Trabecular Spacing (Tb Sp), and Bone Volume / Total Volume (BV/TV) [136].

Contralateral, non-tumor-bearing femurs were scanned with the above μ CT protocol to determine whether the JWH015 compound was having osteogenic effects on naïve bone. Centered along the midpoint of the femoral diaphysis, a rectangular ROI (2.5mm long x 2.5mm wide x 1mm high) was used to evaluate μ CT parameters for cortical bone (detailed above). Similar to the methods listed above, an irregular ROI was generated in the distal metaphysis for analysis of trabecular bone microarchitecture. In all cases, the μ CT operator was blind to the experimental conditions of the specimens.

2.3.3 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 -80°C until utilized for assays. Enzyme immunoassays were used to measure the serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAP5b) for osteoclast number (Immunodiagnostic Systems, Fountain Hills, AZ, USA) and C-terminal telopeptide α 1 chain of type I collagen (CTX) (Immunodiagnostic Systems, Fountain Hills, AZ, USA) for bone loss and osteocalcin (Biomedical technologies, Stoughton, MA). Assays were conducted according to the manufacturers' instructions[156].

2.3.4 Bone marrow extrudate immunoassay:

Animals were sacrificed on day 14 post-innoculation with cell-free media or 66.1 cells and seven day's drug treatment and the ipsilateral and contralateral femurs were removed. For each femur, the proximal and distal ends were clipped and the intermedullary extrudate was rinsed into a vial. Each femur was rinsed 4 times with phosphate-buffered saline containing protease inhibitor cocktail and EDTA (Pierce). Four femurs from four animals were pooled per sample. Semi-quantitative cytokine and chemokine array was utilized to determine the relative change of IL-1 β , IL-4, IL-6, IL-12, IL-17a, IFN γ , TNF α , MCP-1, MIP-1a, MIP-1b due to cancer progression and effects of CB₂ agonist, JWH015 (SABiosciences). From this array, MCP-1, MIP-1a, IL-6 and TNF-a were selected and quantitatively assessed using commercially available ELISA

kits according to manufacturer specifications (SABiosciences, eBiosciences, Invitrogen) with detection limits of 25.5, 26.3, 58.8, and 8.0 pg/mL respectively[156].

2.3.5 Immunoblotting:

Spinal cords were homogenized using a sonicator, centrifuged and the supernatant collected. Lysis buffer (RIPA/PIC) was added to the sample and then placed on ice for 30 minutes. The sample was centrifuged and the supernatant collected. A BCA protein assay kit was used to estimate the amount of protein present. Samples were prepared in Laemmli sample buffer/*β-mercaptoethanol* buffer and denatured for 5 minutes at 95°C. A Tris/glycine running buffer (25M Tris-HCl, 190mM glycine, and 1% SDS, pH 8.3) was added to a Mini-Protean Tetra system (Bio-Rad, Hercules, CA). The samples (40 ug) were loaded onto the gel and the empty wells were filled with loading buffer. To separate the protein electrophoretically, the gel ran for one hour at 140 volts. Transfer buffer (3.03g Tris HCL, 14.4 g glycine, 200 mL methanol, 800mL sterile water at a pH of 8.3) was prepared and chilled. The gel was equilibrated for 15 minutes in the transfer buffer. The polyvinylidene fluoride (PVDF) membranes were hydrated in 100% methanol for 10 seconds and placed in transfer buffer. Gel was placed in transfer cell and ran for 30 volts overnight. After the protein was transferred, the membrane was dried, rehydrated in MeOH, washed in TBS-T (24.23g Trizma, 80.06g NaCl, pH at 7.6), blocked with 5% milk for one hour, then re-washed with TBS-T. Samples were incubated with primary antibodies (as noted above) overnight. PDVF was washed in TBS-T and incubated with the secondary antibody (horseradish peroxidase-conjugated secondary IgG antibody; Cell Signaling Technology, Billerica, MA) and developed using a chemiluminescent system (Amersham Biosciences). GAPDH (Cell Signaling

Technology) was used as loading control. Quantification of western blot was performed using gel analysis in ImageJ [249].

2.3.6 Spinal cytokine enzyme-linked immunosorbent assay:

Spinal cord samples were frozen in -80°C liquid nitrogen until the assay was performed. ELISAs were used to detect and measure IL6-TNF α and IL1 β (EBioscience, San Diego, CA). The assays were performed following the instructions from the manufacturer.

2.4 Statistical analysis:

Statistical comparisons between treatment groups were calculated using ANOVA. Pairwise comparisons were made with Student's t-test while multiple comparisons between groups used Bonferroni's Multiple Comparison Test. Dose-response effects used linear regression analysis of the linear portion of the log dose-response curve. Survival studies utilized the Kaplan Meier estimator with a Log-rank Mantel-Cox test and Gehan-Breslow Wilcoxon test. Significance was set at $p < 0.05$. Survival studies utilized the Kaplan Meir estimator with a Log-rank Mantel-Cox and Gehan-Breslow Wilcoxon test. All data are presented as mean \pm SEM and GraphPad Prism 5.0 (Graph Pad Inc., San Diego, CA, USA) was used to plot data.

CHAPTER 3: A CANNABINOID 2 RECEPTOR AGONIST AM1241 ATTENUATES BONE CANCER-INDUCED PAIN AND BONE LOSS

3.1 Introduction:

Many prevalent forms of cancer including lung, breast, prostate and sarcoma metastasize to bone [45, 158]. Bone metastasis is commonly characterized in cancer patients by bone pain [159] [173]. Destruction of the bone causes chronic pain, which often leads to pathological fractures and/or hypercalcemia. The bone destruction induces an “ongoing” pain arising from the tumor bearing bone that significantly compromises the quality of life and functional status of the patient [119]. With the progression of tumor-induced bone destruction, breakthrough pain , an intermittent occurrence of severe pain, manifests itself either spontaneously or following weight bearing or strenuous movement of the affected bone [159].

Treatment for bone cancer involves multidisciplinary therapies that include a combination of radiotherapy, hormone or chemotherapy, bisphosphonates (carbon-substituted analogs that inhibit osteoclast function), and analgesic therapy [174]. Analgesic therapy can include treatment with opiates and non-steroidal anti-inflammatory drugs. The use of NSAIDS is limited to the alleviation of mild to moderate pain and has been recently reported to delay bone healing following fracture [187]. Chronic use of opiates results in several unwanted side effects including analgesic tolerance, somnolence, constipation, respiratory depression and paradoxical states of hyperalgesia [258]. Recently, we demonstrated that murine bone cancer models

treated with sustained morphine not only intensifies pain after a week of treatment but also accelerates bone destruction when compared to vehicle treated animals [134].

Cannabinoid Receptor-2 (CB2) agonists have been shown to act as an analgesic in acute, chronic, and neuropathic pain [108, 109, 164, 266]. CB2 receptors are expressed in the spleen, tonsils, monocytes, B-cells, and T-cells and are therefore associated with the immune responses and the peripheral nervous system [56, 135, 217]. Although CB2 receptors are considered peripheral receptors, they have been found in distinct areas of the CNS such as the spinal cord, dorsal root ganglia, and microglia [200].

CB2 agonists not only produce antinociceptive and anti-inflammatory effects, but also have been shown to increase bone density [127, 190]. CB2 agonists increase the number of osteoblasts (bone forming cells) and inhibit the production of osteoclasts (bone destruction cells) resulting in an overall increase in bone integrity [190]. CB2 knockout mice experience accelerated trabecular bone loss and cortical expansion further demonstrating the importance of the endogenous CB2 system in the mediation of skeletal maintenance [190]. Mice that undergo an ovariectomy exhibit accelerated bone loss. These ovariectomized mice, when treated with a sustained CB2 agonist, have suppressed osteoclastogenesis and increased osteoblast activity with an overall increase in bone integrity [190].

In this study we will investigate the CB2 selective agonist AM1241. In animal pain models, AM1241 is consistently reported as a CB2 agonist, as effects are blocked by CB2, but not CB1, selective antagonists and not seen in CB2^{-/-} mice. [108, 109,

163]. In contrast to results seen in vivo studies, functional assays attempting to characterize the pharmacological properties of AM1241 have yielded inconsistent results, with activity ranging from agonist, antagonist, or inverse agonist depending on the assay and enantiomer utilized [276] [23, 166]. Differences of pharmacological properties observed in vivo and in vitro could be the result of differences in native versus recombinant receptors. Thus, in vitro assays do not necessarily predict in vivo efficacies. Furthermore, AM1241 was chosen due to its consistency and effectiveness as a CB₂ selective agonist across multiple animal pain models published in the literature.

Based on the antihyperalgesic effects of CB₂ agonists, the lack of potential CNS-induced side effects and their propensity to stimulate bone growth, we addressed whether the sustained selective CB₂ agonist AM1241 has the potential to alleviate bone cancer-induced pain while maintaining bone integrity in a murine model of bone cancer.

3.2 The CB₂ agonist AM1241 attenuated bone cancer-induced spontaneous pain:

In animals injected with media, flinching and guarding behaviors were not observed. By days seven and ten following arthrotomy surgery and femur inoculation with sarcoma, spontaneous pain was elicited. Mice that received sarcoma cells displayed spontaneous flinching and guarding starting at day 7 with continued behavior until day fourteen as compared to control, media only animals (Figure 1A, B). The sustained systemic (i.p.) treatment of AM1241 began on day 7 post surgery and flinching and guarding behaviors were observed on days 10 and 14. At day 10, tumor bearing mice with AM1241 showed a reduction in flinching when compared to tumor bearing treated

mice with vehicle (i.p.), however the effect was not significant until day 14 ($p < 0.001$) (Figure 1A). The sustained systemic (i.p.) treatment of AM1241 resulted in a decrease in guarding by day 14 in sarcoma treated mice when compared to vehicle treated animals ($p < 0.05$) (Figure 1B).

Figure 1

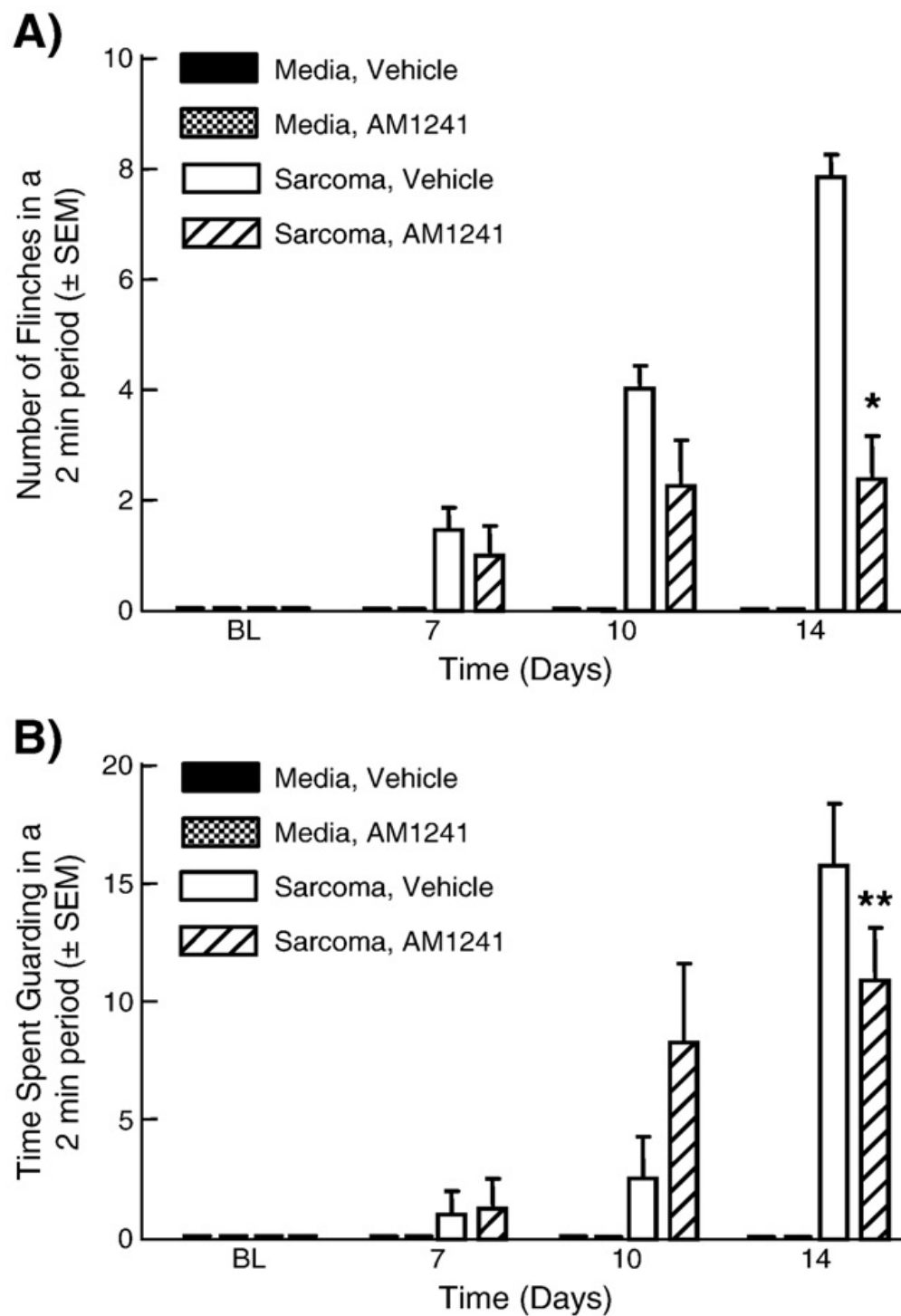


Fig. 1. The CB2 agonist, AM1241, attenuates spontaneous pain behaviors in a murine bone cancer model. Sarcoma cells or cell medium were injected into the intramedullary space of the femur. Beginning on day 7, vehicle or AM1241 (i.p, 3 mg/kg twice daily) was administered to animals. Flinching and guarding behaviors were observed to assess spontaneous pain in mice after surgery. A) The number of flinches was reduced by AM1241 (i.p) treatment in tumor bearing mice compared to mice treated with vehicle (* $p < 0.001$). Flinching was not observed in mice injected with media and treated with vehicle or AM1241. B) AM1241 treatment attenuated guarding behavior in tumor bearing mice compared to mice treated with vehicle (** $p < 0.05$). Guarding was not observed in animals injected with medium and treated with AM1241 or vehicle.

3.3 Treatment with AM1241 reduces sarcoma-induced evoked pain.

Von Frey filaments were used to measure the hindpaw response thresholds of mice to determine the effect of AM1241 treatment on sarcoma-induced tactile hypersensitivity. On day 7 after sarcoma inoculation and prior to either AM1241 or vehicle, animals' mechanical thresholds were not different from baseline values on day 0. However at days 10 and 14 post surgery, animals began to display behavioral signs of tactile sensitivity as compared to animals injected with media (Figure 2A). Beginning on day 10, tumor bearing mice treated with vehicle displayed significantly lower paw withdrawal thresholds compared to sarcoma-induced, AM1241 treated animals ($p < 0.05$) (Figure 2A). On day 14 after surgery animals treated chronically with vehicle demonstrated significant sarcoma-induced mechanical hypersensitivity as compared to the contralateral leg (data not shown). More importantly is that the animals treated with sustained AM1241 demonstrated a significant block of sarcoma-induced mechanical

hypersensitivity ($p < 0.001$) (Figure 2A). In addition to mechanical testing using von Frey filaments, limb use was rated in mice [104] to evaluate the effect of AM1241 on movement-evoked pain. Sarcoma-induced animals treated with both vehicle and AM1241 displayed limping by day 10, yet by day 14, there was a significant difference in movement-evoked pain between AM1241 and vehicle treated groups. Sarcoma-induced mice treated with vehicle alone displayed partial non-use or limping and guarding compared to control (media) treated animals. Sustained administration of AM1241 from day 7 until day 14 significantly reversed the sarcoma-induced loss of limb use by day 14 ($P < 0.001$) (Figure 2B). These data suggest that sustained AM1241 significantly reduces sarcoma-induced evoked pain.

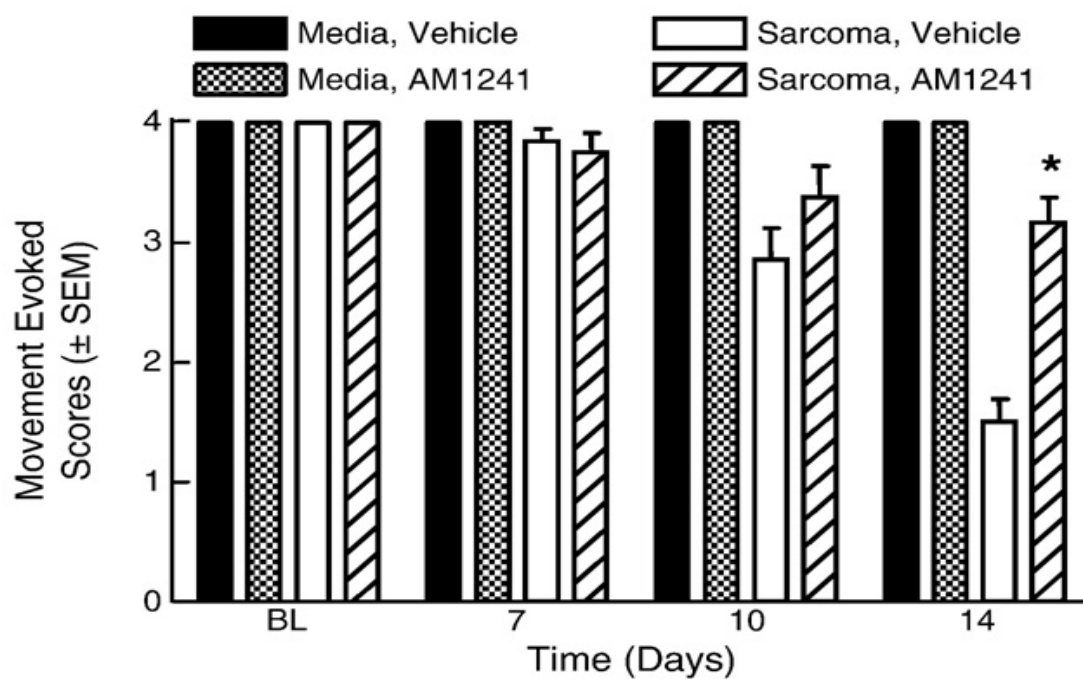
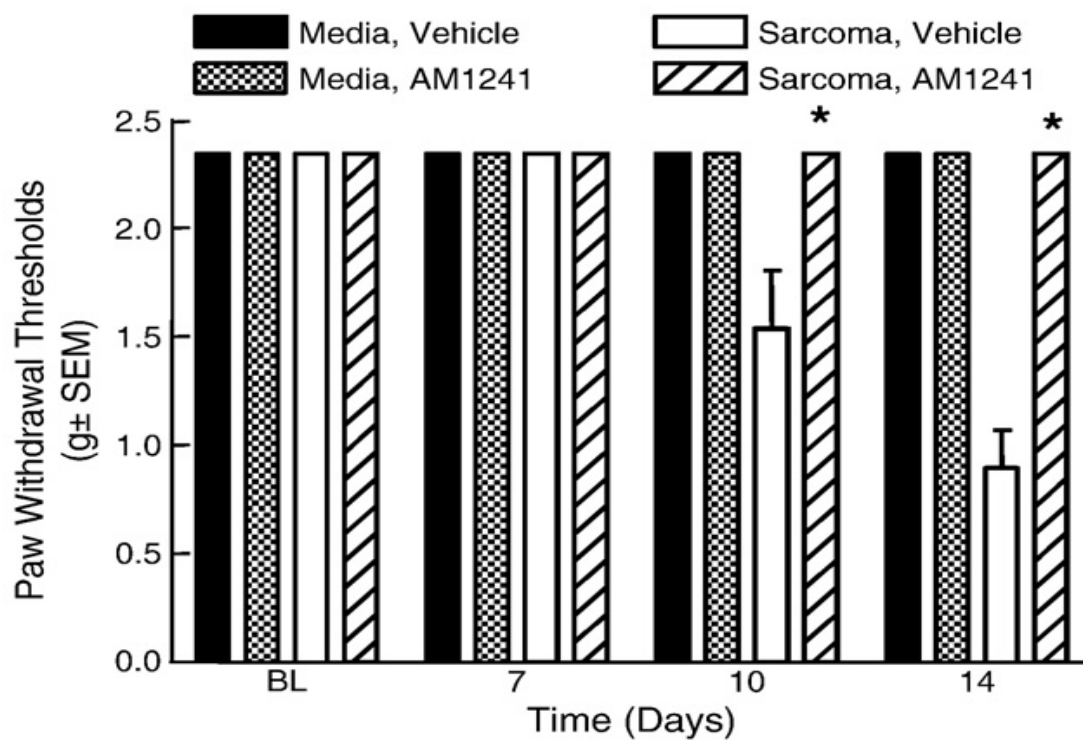
Figure 2

Fig 2. The CB2 agonist, AM1241, attenuates evoked pain behaviors in a murine bone cancer model. Tactile allodynia and movement evoked pain were tested. A) AM1241 (i.p.) treatment blocked tactile allodynia in cancer-induced mice compared to cancer-induced mice treated with vehicle on days 10 and 14 (* $p < 0.001$). Tactile allodynia was not observed in animals injected with media and treated with AM1241 or vehicle. B) AM1241 (i.p.) treatment significantly alleviate movement evoked pain on day 14 (* $p < 0.001$) but not on day 10 in tumor bearing mice treated with AM1241 when compared to cancer-induced mice treated with vehicle. Movement evoked pain was not observed in mice injected with cell medium, treated with AM1241 or vehicle

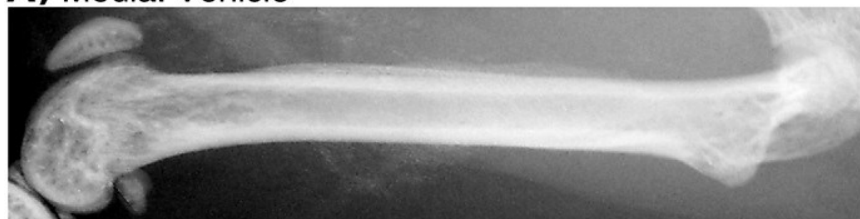
3.4 AM1241 treatment reduces sarcoma-induced bone loss and fracture

Radiographic images were taken following behavioral testing to determine the effect of AM1241 treatment on sarcoma-induced bone loss. Bones were rated with the following scale: 0 = normal, 1 = bone loss observed with no fracture, 2 = unicortical bone loss indicating unicortical bone fracture, 3 = bicortical bone loss indicating bicortical bone fracture [158]. Radiographs were taken prior to surgery eliminating the possibility of baseline group differences. Throughout the time course of the experiment, bone loss was not observed in animals injected with media and treated with vehicle or AM1241. Sarcoma-induced bone loss increased in tumor bearing mice as compared to sham mice. Sarcoma treated animals with vehicle from day 7 to day 14 resulted in a significant amount of bone loss (Figure 3C). Sustained AM1241 from days 7 until day 14 significantly reduced the amount of sarcoma-induced bone loss when compared to the vehicle treated animals ($P < 0.001$) (Figure 3D). Bones were scored by a blind observer with expertise in bone radiology. Animals with sarcoma and vehicle had

severe bone loss with all animals having unicortical fracture (Figure 3E). Sustained AM1241 from day 7 until day 14 significantly reduced bone loss by blind scoring with only 2 out of 10 animals demonstrating unicortical bone loss (Figure 3E).

Figure 3

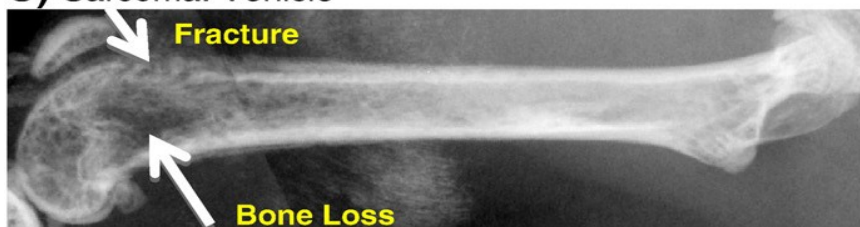
A) Media: Vehicle



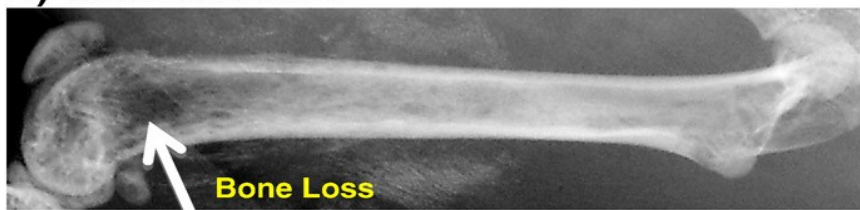
B) Media: AM1241



C) Sarcoma: Vehicle



D) Sarcoma: AM1241



E)

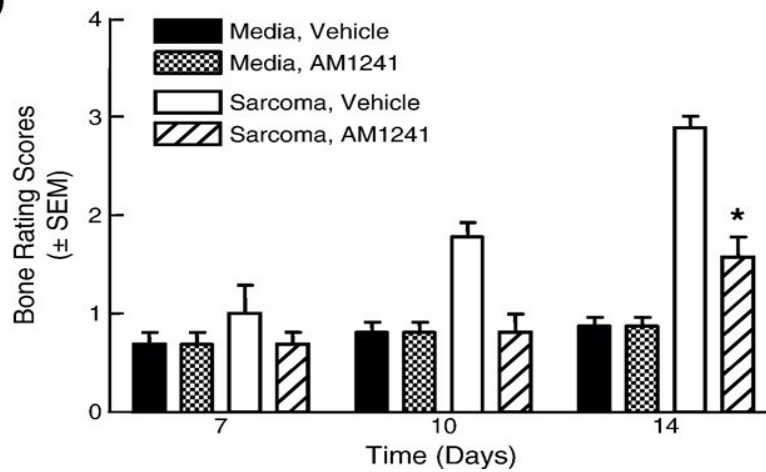


Fig 3. AM1241 reduces sarcoma-induced bone loss. Representative radiographic images of femur injected with medium of sarcoma cells. The femurs were treated with vehicle or AM1241.

Bone is reduced in tumor bearing animals treated with vehicle as compared to animals treated with AM1241. A) Bone injected with media and treated with vehicle. B) Bone injected with media and treated with AM1241. C) Bone injected with sarcoma cells and treated with vehicle. D) Bone injected with sarcoma cells and treated with AM1241. E) Bone rating scores demonstrating AM1241 treatment reduced the occurrence of unicortical bone fractures in sarcoma-induced mice compared to sarcoma-induced mice treated with vehicle. Set to scale 1.5 mm.

3.5 Acute treatment of the CB2 agonist AM1241 attenuated bone cancer-induced spontaneous pain and evoked pain; blocked by the CB2 antagonist SR144528

Flinching and guarding behaviors were observed to determine the acute effects of AM1241 on sarcoma-induced spontaneous pain. Animals were observed for behavioral baselines 10 days following surgeries and given a single injection of AM1241 (6mg/kg, i.p.) or vehicle. Behavioral measurements of sarcoma-induced flinching and guarding were taken 30 and 60 minutes after injection in a blinded fashion (observer blinded to treatment groups AM1241 or vehicle). Baselines resulted in significant sarcoma-induced flinching and guarding (Figure 4A and 4B). However, 30 minutes and 60 minutes following injection with AM1241 animals showed a significant reduction in flinching ($p < 0.001$) and guarding (30 min, $p < 0.05$) (60 min, $p < 0.001$) when compared to vehicle treated mice (Figure 4A and 4B). The pre-administration (8-10 min prior to

AM1241) of the CB2 antagonist SR144528 (1 mg/kg, i.p.) resulted in a significant attenuation of the AM1241 effects ($p < 0.001$) in both flinching and guarding (Figure 4A and 4B) demonstrating that the reduction of sarcoma-induced spontaneous pain by AM1241 is CB2 receptor mediated. The antagonist alone had no significant effect on sarcoma-induced flinching and guarding. (Figure 4A and 4B). All behavioral studies were carried out in a blinded fashion.

Von Frey filaments were used to measure the hindpaw response thresholds of mice to determine the acute effect of AM1241 treatment on sarcoma-induced touch evoked hypersensitivity. Animals were tested 10 days following sarcoma inoculation and given a single injection of AM1241 (6mg/kg, i.p.) or vehicle (control). Behavioral measurements were taken before injection, 30 and 60 minutes after injection. Animals treated with acute AM1241 demonstrated a significant attenuation of sarcoma-induced touch evoked hypersensitivity compared to control (vehicle) (Figure 4C). Although 30 minutes following AM1241 injection no significant attenuation of evoked responses was observed, the 60 minute time point resulted in a significant attenuation of evoked responses ($p < 0.05$) when compared to vehicle treated animals and/or baseline thresholds (Figure 4C). The pre-administration (8-10 min prior to AM1241) of the CB₂ antagonist, SR144528 (1 mg/kg, i.p.) resulted in a significant attenuation of the AM1241 effects ($p < 0.001$) in evoked responses (Figure 4C) demonstrating that the reduction of sarcoma-induced evoked pain by AM1241 is CB2 receptor mediated. The antagonist alone had no significant effect on sarcoma-induced touch evoked hypersensitivity. (Figure 4C). All behavioral studies were carried out in a blinded fashion.

Figure 4

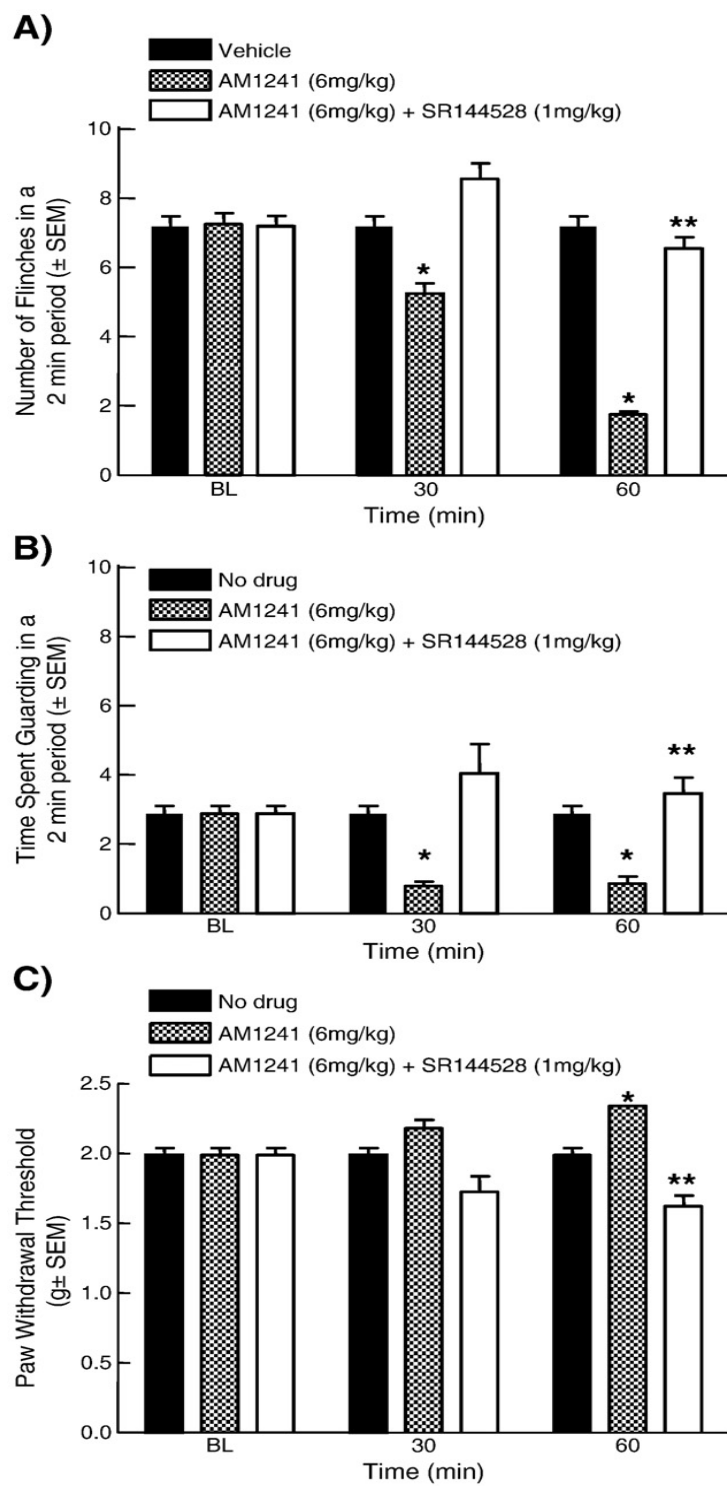


Fig 4. Acute treatment of AM1241 reduced sarcoma-induced spontaneous and evoked pain. These effects were blocked by a single pretreatment of the CB2 antagonist SR144528. The number of sarcoma-induced flinches (A) and guarding (B) on day 10 was significantly reduced by AM1241 (6 mg/kg, i.p) treatment compared to vehicle treated mice with a significant difference observed at 30 and 60 minutes (* $p < 0.001$). This effect was blocked in animals pretreated (8-10 min) with SR144528 (1 mg/kg, i.p.), at the 60 minute time point (** $p < 0.001$). C) AM1241 (6mg/kg, i.p) treatment attenuated sarcoma-induced evoked responses compared to vehicle treated mice at 60 minutes (* $p < 0.05$). Treatment of SR144528 (i.p.) prior to AM1241 blocked the antiallodynic effects of AM1241 (** $p < 0.001$). Vehicle or antagonist alone had no significant effects.

3.6 Conclusion:

Many epithelial-derived cancers including sarcoma, breast, prostate and lung commonly metastasize to bone [45]. Once cancer metastasis to bone occurs, bone pain can significantly impact the quality of life and functional status of the patient [221, 241]. In advanced stages, skeletal metastasis is associated with bone remodeling and eventual bone fracture that contributes to severe and difficult to control pain with limited or total loss of mobility. Here we utilized an animal model of bone cancer metastases using sarcoma cells that results in behavioral signs of spontaneous and evoked pain. Similar to what was reported by Schwei et al., we found that the animals developed severe bone loss by day 14 after inoculation with the sarcoma cells [230].

Here we demonstrated the acute effects of a CB2 agonist as well as how sustained administration of a CB2 agonist for seven days attenuates both spontaneous

and evoked pain behaviors. The antinociceptive effect of sustained administration slightly decreased when compared to the acute treatment, suggesting tolerance. However, in the CB2 sustained studies the CB2 agonist was tested after 14 days as compared to after 10 days in the acute study suggesting an escalation in pain behavior from day 10 to day 14. This effect is most likely due to a decrease in the potency of CB2 antinociception rather than a decrease in tolerance. Compound administration was by the systemic route suggesting that the effects may have acted both locally as well as in the central nervous system. CB2 receptors are found in the spleen, tonsils, monocytes, osteoclasts, macrophages, B-cells and T-cells and are therefore associated with the immune responses, as well as the peripheral nervous system but not directly with the central nervous system [56]. Recent studies have identified an increase in mRNA for CB2 receptors in the CNS after nerve injury with upregulation in the CNS associated with microglia after inflammation, yet their receptor activation in the CNS lacks unwanted psychoactive effects [219, 260, 283] [257]. Cancer metastases to bone results in the activation of the immune response within the bone and within the central nervous system. The activation of CB2 receptors on immune cells results in the attenuation of inflammatory factors including cytokines [135, 171, 211]. Studies from our group along with others have demonstrated that the activation of CB₂ receptors by specific agonists will inhibit inflammatory, acute and chronic pain without the psychoactive effects demonstrated by activation of CB1 receptors or opiates [109, 164, 266, 274]. A recent study by Romero-Sandoval and colleagues has shown that CB2 receptor activation within the spinal cord after L5 nerve injury resulted in an increase in CB2 receptor expression on microglia and perivascular cells with a reduction in

hypersensitivity using the CB2 selective agonist JWH015, a compound lacking unwanted CNS side effects [219]. They concluded that CB2 agonists may offer pain relief by modulating the immune response and microglia function under chronic pain conditions without inducing tolerance or CNS side effects.

Due to the fact that the CB2 receptors are located on immune cells including macrophages [135], we believe that the significant reduction in pain behaviors is due to a reduction in the many inflammatory mediators released when cancer invades the bone. Metastases to the bone results in the accumulation of macrophages termed tumor-associated macrophages (TAMs) which have been found to enhance angiogenic programming by producing pro-angiogenic factors such as cytokines, chemokines, VEGF and proteases [48, 54, 147, 247]. Cancer metastases to bone results in a significant inflammatory/immune response including a significant increase in macrophages, monocytes, dendritic cells, leukocytes and neutrophils [54, 63]. The number of macrophages present in tumor stroma correlates with increased microvessel density, tumor size, tumor proliferation and decreased survival in cancer patients [27, 142, 188, 192]. It is well known that certain cytokines can enhance and even cause nociception [259]. Recent studies have demonstrated that the cytokines IL-1 β , TNF α and IL-6 are released from macrophages, monocytes and glial cells to promote nociception indirectly via increasing prostanoids and sympathetic amines, as well as by direct activation of receptors on nociceptive fibers [259]. Recent studies by Li and colleagues have shown that peripheral nerve stimulation, similar to that which would be seen in bone cancer, results in the increase expression of IL-6, TNF α and IL-1 β in the dorsal horn of the spinal cord leading to intracellular changes on secondary neurons

that may lead to central sensitization [144]. In the end, these pronociceptive cytokines are released from cancer-induced infiltrating immune cells as well as from the tumor cells promoting pain and continual tumor proliferation, creating a painful and destructive "feed-forward" process that may be inhibited by CB2 receptor activation.

Studies here demonstrate that sustained CB2 agonists maintain bone integrity when compared to vehicle treated animals. There was a significant reduction in sarcoma-induced bone loss and a reduction in the number of unicortical fractures due to the administration of the AM1241. Bone integrity is maintained by osteogenic cells found on the surface of the bone and in the lacunae of the bone matrix including osteoblasts and osteoclasts [210]. Osteoblasts are found along the bone surface where they synthesize the organic matrix and regulate mineralization of bone resulting in bone-building [210]. Osteoblast activity is regulated by CB2 agonists. The selective CB2 agonist HU-308 enhanced osteoblast number and bone building activity [190]. Bone marrow-derived primary monocytic cultures showed a dramatic (205%) increase in the expression of osteoblast-like cells following application of a selective CB2 agonist [231]. Osteoblasts, in part, control the cells that breakdown bone called osteoclasts by releasing RANKL, a member of the TNF cytokine superfamily, osteopontin and IL-6. Osteoblasts themselves can be suppressed either directly or indirectly by cytokines including IL-1 β and TNF α ([21, 144, 242]. Osteoblasts are influenced by cancer cells to release cytokines that enhance osteoclast activity [133]. Osteoclasts are cells that are derived from the monocyte-macrophage lineage and have high levels of CB2 receptors [30] [12, 190, 231]. Osteoclasts resorb bone by creating a local acidic microenvironment to dissolve bone and activate proteases to break down bone [210]. Osteoclast function

is regulated by a number of mediators including endogenous cannabinoids and cytokines (TNF α , IL-6) [285]. For example, CB2 receptor activation on osteoclasts and osteocytes by the selective CB2 agonist HU-308 significantly suppressed osteoclast activity and osteoclastogenesis (production of osteoclasts) considerably reducing the activity of osteoclasts in trabecular and cortical bone [231] [190]. Bone density in CB2 knockout mice was significantly lower when compared to wild type littermates [127]. In addition, CB2 knockout mice displayed a markedly accelerated age-related trabecular and cortical bone remodeling [190].

The CB2 agonists may also act by decreasing the activation of microglia in the central nervous system [219]. Sustained administration of CB2 agonists may result in changes in expression of CB2 receptor on glial cells or intracellular regulation. Future studies will investigate endogenous cytokine levels, immunohistochemistry for activated microglia, and changes in receptor number.

Here we have demonstrated that a CB2 agonist administered acutely or chronically for 7 days significantly attenuates both spontaneous and evoked pain behaviors. Unlike results with sustained morphine in the sarcoma cancer model [134], the sustained administration of the CB2 agonist resulted in the inhibition of bone loss. In addition, CB2 agonist do not result in the many unwanted side effects of current analgesic therapies due to the lack of direct activity on neuronal pathways within the reward and respiratory pathways of the CNS suggesting that CB2 agonists may be an ideal treatment for bone cancer pain.

Chapter 4: DISEASE MODIFYING EFFECTS OF JW015 AND AM1241 IN CANCER-INDUCED BONE PAIN

4.1 Introduction:

The World Health Organization [62] estimates that the number of women living with breast cancer globally will rise to 15 million by the year 2020 [240] with a significant portion of these patients in advanced stages of the disease. Breast cancer is the second leading cause of death in women [240] with nearly 200,000 diagnoses each year in the U.S. alone. In this cancer which most commonly metastasizes to bone [45], breast cancer cells in the bone microenvironment cause bone loss, fractures, anemia and severe pain [63]. Despite marked advances in chemotherapeutics for early stage breast cancers, few new therapies are effective in slowing disease progression and increasing survival in advanced disease states.

The most commonly prescribed treatments for skeletal-related events [251] in advanced-stage breast cancer patients with bone metastasis and pain are bisphosphonates, radiation, nonsteroidal anti-inflammatory drugs (NSAIDs) [53] and opioids [243]. Although these therapies reduce skeletal related events (SREs) and/or pain, they bring about unwanted side effects and an overall decrease in quality of life without increasing patient survival or slowing disease progression [243]. The FDA recently allowed a fast track review of denosumab (Xgeva™), a human monoclonal antibody targeting receptor activator of nuclear factor- κ ligand (RANKL), due to the inadequacy of current therapies for treating advanced stage breast cancer patients. Denosumab demonstrated a greater efficacy in preventing SREs in breast cancer patients with bone metastasis than the bisphosphonate zoledronic acid, however neither

bisphosphonates nor denosumab have shown a survival benefit [243]. Additionally, both treatments induce a small but significant subset of patients that develop serious adverse events including osteonecrosis of the jaw and disruption of normal serum calcium levels [243]. Cancer pain caused by bone metastases is inadequately managed by a combination of opiates and NSAIDs, as defined by the guidelines set by the WHO Ladder Approach for Relief of Cancer Pain. NSAIDs, while effective at reducing inflammatory and musculoskeletal pains [19], have been shown to aid in bone destruction and prevention of proper bone remodeling, thus decreasing bone strength in both animal models [6] and in human studies [268]. Recently, we demonstrated that sustained morphine not only intensifies tumor-induced pain but also accelerates tumor-induced bone destruction in a murine model of bone cancer [134]. Chronic opiate use results in a multitude of unbearable side effects including analgesic tolerance, somnolence, constipation, respiratory depression and paradoxical states of hyperalgesia [258]. Neither palliative care nor therapeutics approved for use in advanced stages of breast cancer have improved in the last 30 years despite the persistence of the disease; for these reasons, alternative therapies must be developed.

One possible alternative includes agonist activity at the cannabinoid receptor 2 (CB2), which has previously shown analgesic activity in acute, chronic, inflammatory and neuropathic pain without producing psychoactive or rewarding behavior [108, 164]. Additionally, CB2 receptors are integral components of normal bone metabolism [127, 189, 190]. Activation of CB2 receptors improves bone integrity by stimulating the proliferation of osteoblasts and inhibiting the proliferation and activation of osteoclasts [157, 189, 190]. Recently, CB2 agonists have also been shown to have anti-tumor

potential in the MMTV-neu mouse, ErbB2-driven breast cancer model in an AKT pathway-dependent fashion (17). The specific mechanism(s) of antiproliferation by CB2 agonists is under-studied and unclear. However, it is evident that CB2 agonists suppress the release of cytokines and chemokines from immune cells and some types of cancer cells [135, 171, 211], molecules that are well known to promote breast cancer proliferation, migration, pain, and bone resorption [162, 236],36-39,55).

The present work evaluates the efficacy and mechanism of CB2 agonists in attenuating bone loss, pain, and cancer proliferation in a murine model of breast-associated bone cancer and pain. Our data suggest that CB2 agonists may provide cancer patients with bone metastases a superior alternative to current available therapeutics.

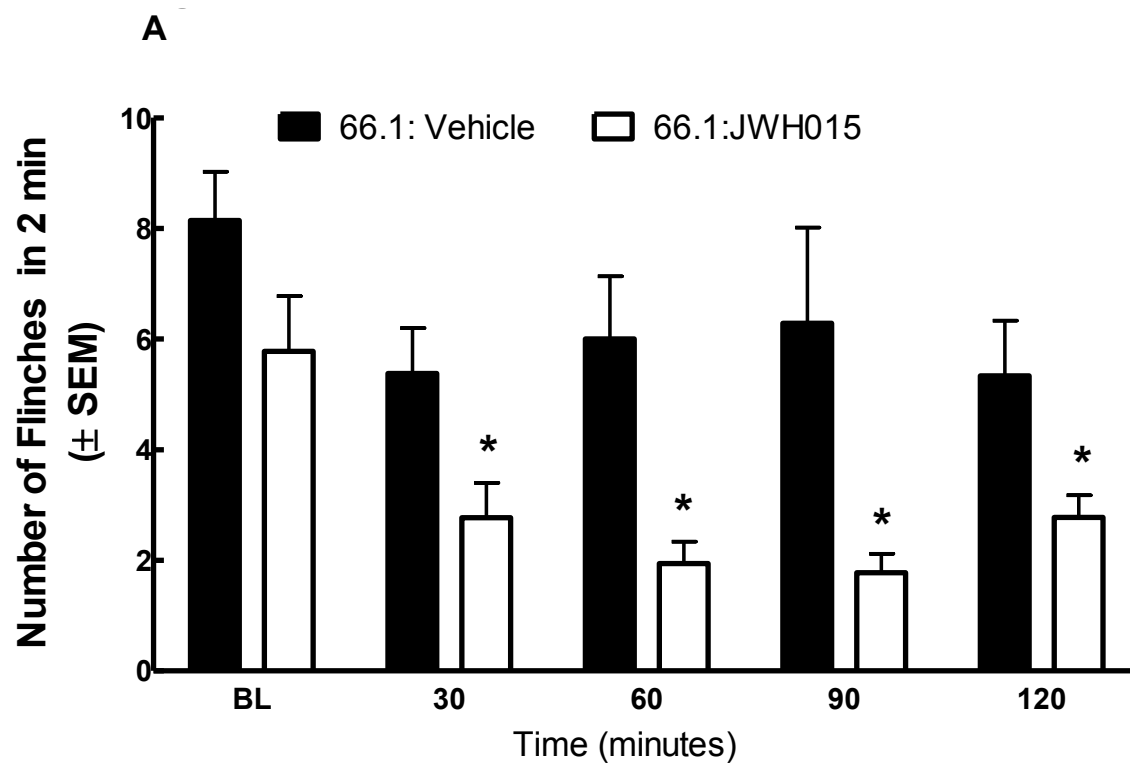
4.2 Acute or sustained CB2 agonist treatment attenuates breast cancer-induced bone pain:

Flinching and guarding behaviors were observed to determine the acute effects of JWH015 on bone cancer-induced spontaneous pain. Von Frey filaments were used to determine the withdrawal thresholds of the ipsilateral hind paw. Fourteen days after intra-femoral cancer (66.1) inoculation, mice displayed significant bone cancer-induced flinching and guarding and decreases in hind paw withdrawal thresholds (Figure 5 A, B, C) indicating behavioral signs of pain. JWH015 resulted in a significant, time-related reduction in flinching and guarding with an increase in paw withdrawal thresholds when compared to vehicle treated mice (Figure 5A, B, C). Pain behaviors returned to a hypersensitive state in cancer treated animals 240-320 minutes after JWH015

administration. Control animals inoculated with media showed no significant flinching, guarding or mechanical hypersensitivity when administered JWH015 or vehicle (data not shown).

Mice receiving intra-femoral 66.1 cells and treated with vehicle displayed spontaneous flinching and guarding beginning at day 7 and increasing through day 14 (Figure 6A, B). Sustained treatment of JWH015 or AM1241 (6mg/kg, i.p. q.d., from day 7-14) in cancer-inoculated mice resulted in decreased guarding and flinching by day 14 in comparison to cancer-inoculated vehicle treated animals (Figure 2A, B).

Pre-administration of the CB₂ antagonist/inverse agonist, SR144528, but not the CB₁ antagonist/inverse agonist, SR141716, inhibited the antinociceptive effect produced by the CB₂ agonist (Figure 6C, D). The treatment with either antagonist/inverse agonist alone had no effect on flinching or guarding. By day 7 post-surgery, cancer-inoculated animals began to display behavioral signs of tactile sensitivity (Figure 7), and media treated mice displayed minor tactile sensitivity resultant of invasive surgery. On day 14 however, media control animals' mechanical thresholds returned to baseline, whereas cancer-inoculated animals demonstrated significant mechanical hypersensitivity. Animals treated with sustained JWH015 or AM1241 demonstrated a significant attenuation of cancer-induced mechanical hypersensitivity compared to vehicle-treated controls (Figure 7).

Figure 5

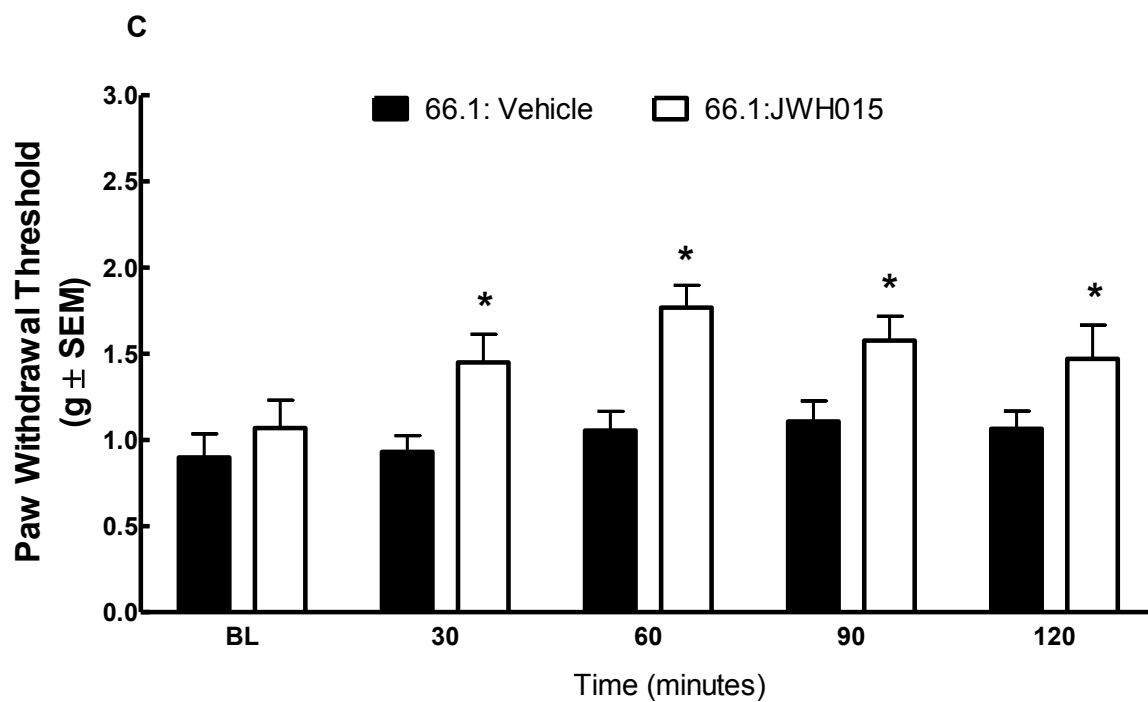
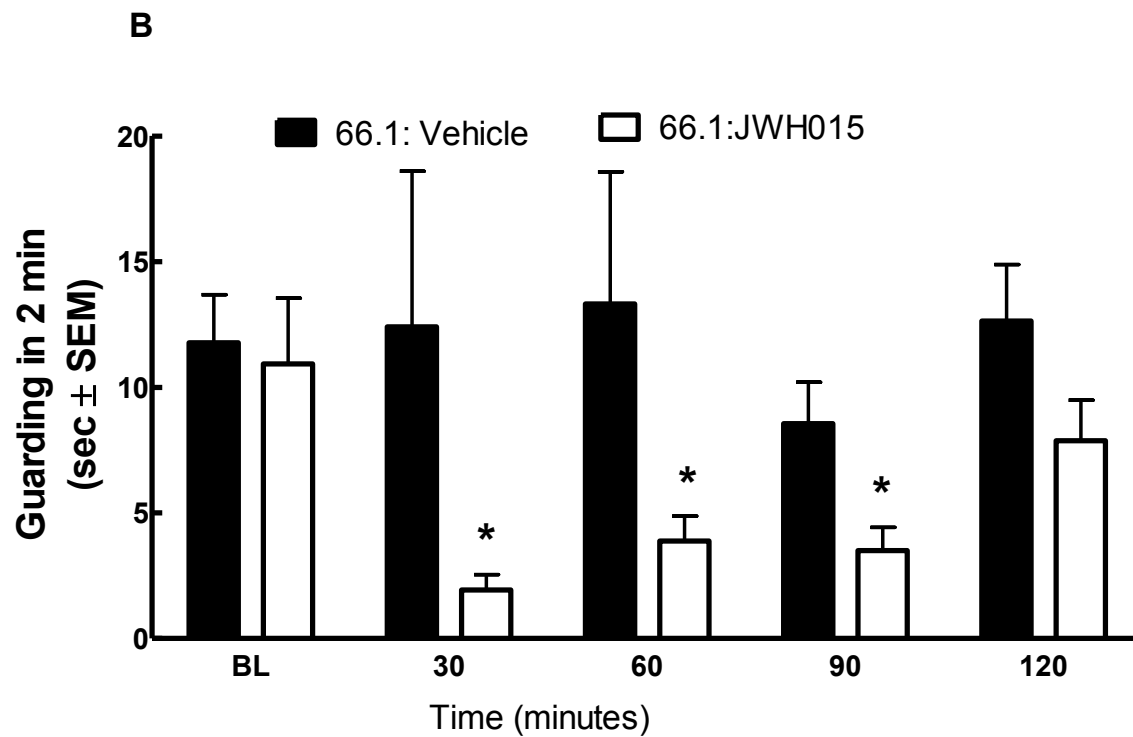
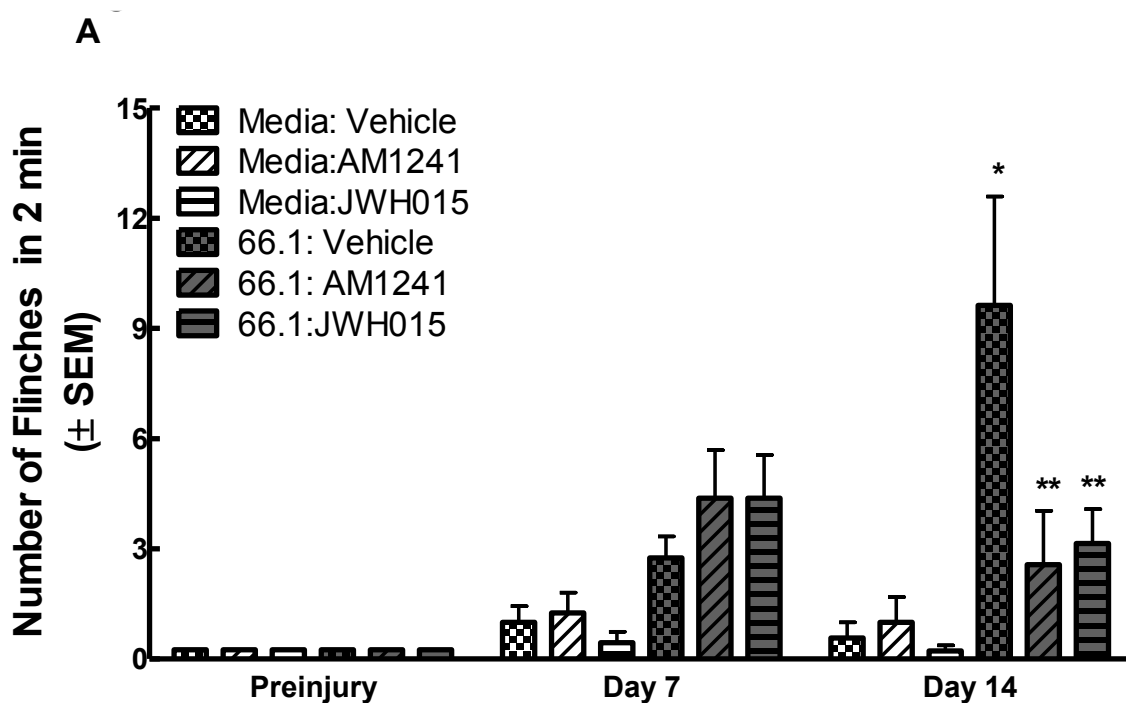
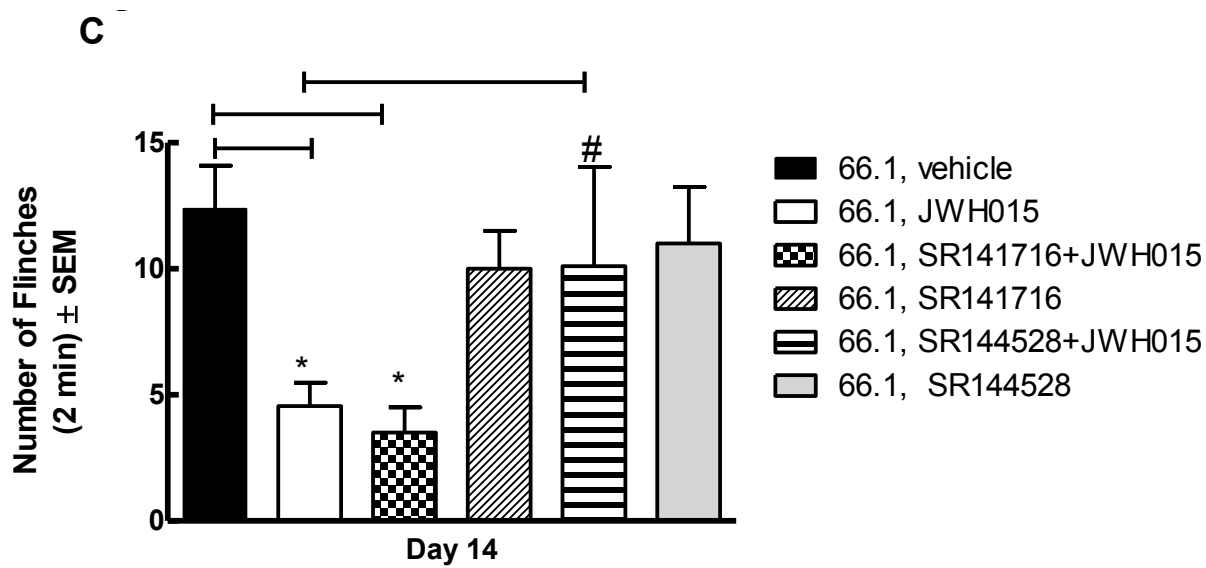
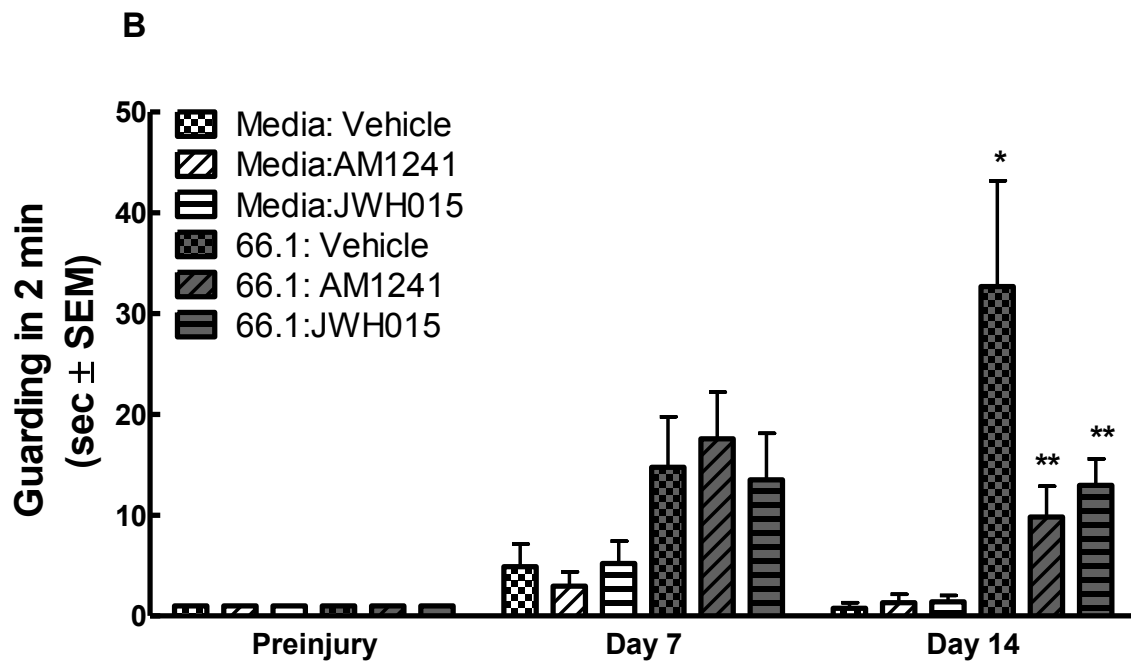


Fig. 5 Acute systemic administration of CB₂ receptor agonist JWH015 attenuates breast (66.1) cancer-induced spontaneous and evoked pain on day 14 after femoral inoculation (time 0). Bone cancer-induced spontaneous (A) flinches (B) guarding and (C) evoked paw withdrawal were all significantly attenuated in a time related fashion in animals administered JWH015 (i.p.) compared to vehicle treated animals ($p \leq 0.05$; $n = 15$ mice per group).

Figure 6





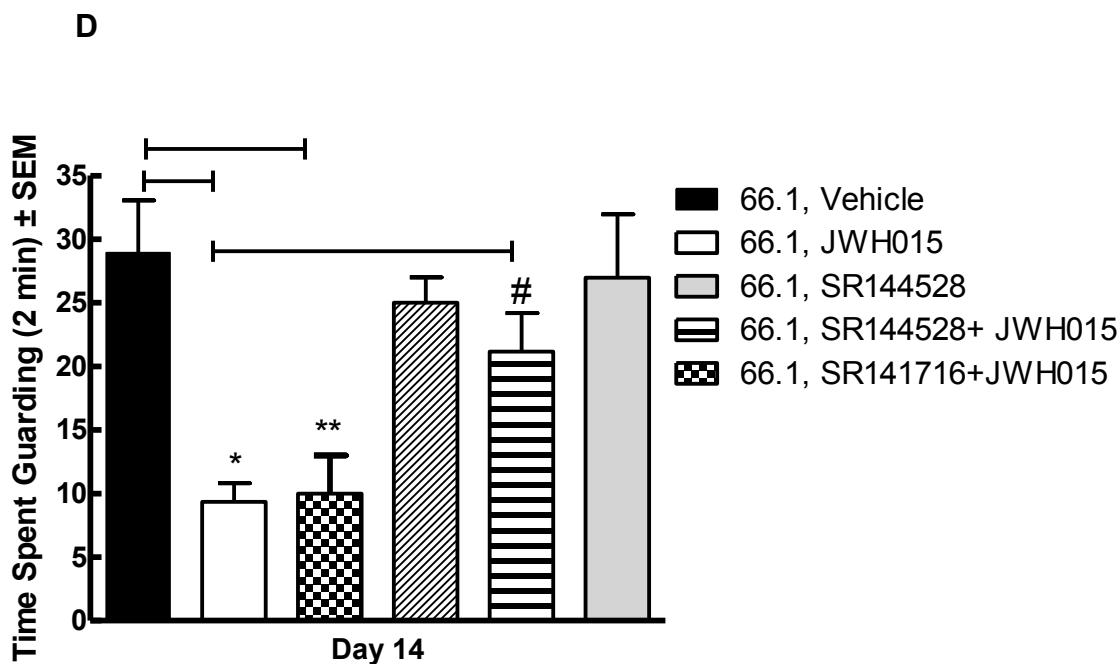


Fig. 6 Chronic systemic administration of CB2 receptor agonist JWH015 and AM1241 attenuates spontaneous pain which is blocked by a CB2 antagonist/inverse agonist but not by a CB₁ antagonist/inverse agonist. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral inoculation animals demonstrated bone cancer-induced (A) flinching and (B) guarding. CB2 agonists, JWH015 (6 mg/kg, i.p., q.d.) or AM1241 (6 mg/kg, i.p., q.d.) were administered after behavioral measurements and continued for 7 days. Spontaneous (A) flinching and (B) guarding in cancer bearing animals was significantly reduced by JWH015 ($p=0.0005$; $n = 16$) and AM1241 ($p=0.0001$; $n = 12$) compared to animals that received vehicle on day 14 ($n = 20$). No significant difference was observed between media-only control animals and vehicle treated ($n = 15$), JWH015 treated ($n = 12$), or AM1241 treated ($n = 12$) animals. The attenuation of bone cancer-induced (C) flinching and (D) guarding by JWH015 ($n = 15$)

on day 14 was blocked by the pre-treatment with the CB2 antagonist SR144528 (3 mg/kg, i.p., q.d. 30 min prior to agonist; n = 10) but not by the CB1 antagonist SR141716 (3 mg/kg, i.p., q.d. 30 min prior to agonist; p=0.003; n = 15). Either CB2 antagonist/inverse agonist (n = 10) or CB1 antagonist/inverse agonist (n = 15) alone had no significant effect on the bone cancer-induced pain as compared to vehicle (n = 12).

Figure 7

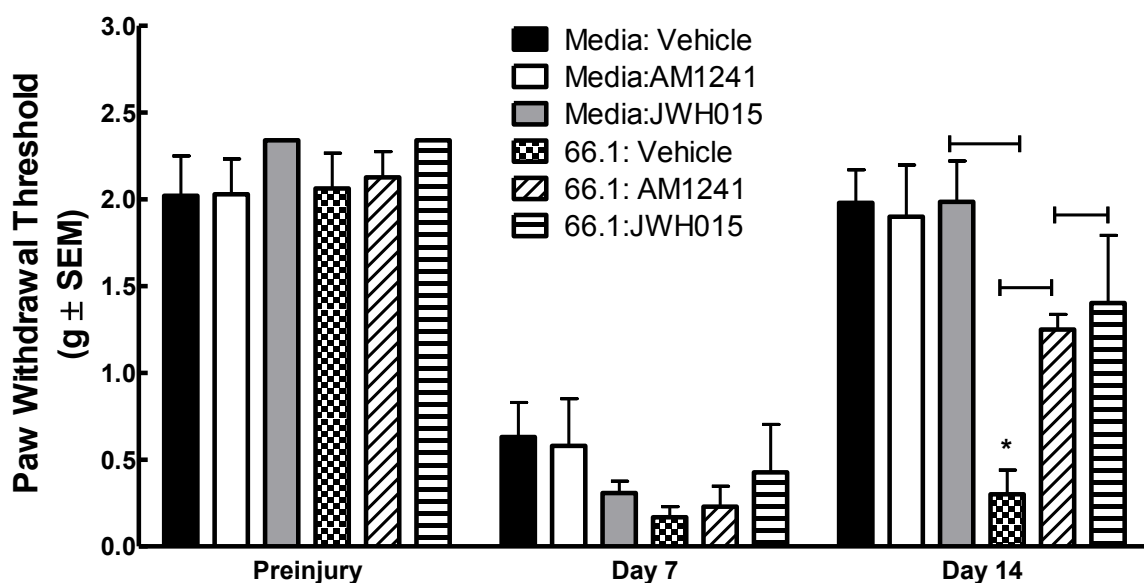


Fig. 7 (A) On day seven after femoral inoculation of 66.1 cells, animals demonstrated bone cancer-induced tactile allodynia. CB2 agonists, JWH015 (6 mg/kg, i.p., q.d.) or AM1241 (6 mg/kg, i.p., q.d.) were administered after behavioral measurements and continued for 7 days. Tactile allodynia in cancer bearing animals was significantly reduced by JWH015 ($p < 0.0001$; n = 16) and AM1241 ($p = 0.006$; n = 16) compared to

animals that received vehicle on day 14 (n = 16). No significant difference was observed in media only control animals between vehicle treated (n = 17), JWH015 treated (n = 15), or AM1241 treated (n = 15) animals.

4.3 CB2 agonist treatment reduces breast cancer-induced bone loss

Radiographic images were taken following behavioral testing to determine the effect of sustained JWH015 on cancer-induced bone degradation. No bone loss or fractures were observed in animals injected with media and treated with vehicle (Figure 8A). 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. At day 14 post-cell injection, 68% of tumor-bearing mice treated with vehicle displayed femoral fractures, while no mice injected with media displayed femoral fractures (Figure 8A, 9A). Sustained JWH015 treatment significantly reduced the amount of cancer-induced bone loss with a 40% reduction in the incidence of fractures (Figures 8A, B, 9A). Animals inoculated with media and treated with JWH015 had no observable changes in bone structure. The pre-administration of the CB2 antagonist/inverse agonist significantly attenuated the ability of JWH015 to decrease cancer-induced fracture (Figure 8A, B, 9A). Similar findings were seen with AM1241 (Figure 9A). Antagonist/inverse agonist alone in either cancer or media-inoculated animals produced no significant change from controls (Figure 8A, 9A).

At day 14, femora were analyzed using micro-CT (Figure 8B, C). In this cancer model, we observe osteolytic and osteoblastic characteristics, resulting in ectopic periosteal bone remodeling. This is consistent with human breast cancers where 70% of breast lesions in bone are osteolytic, 15% are osteoblastic, and 15% take on a mixed

growth pattern [65]. In cancer-inoculated (66.1) vehicle-treated animals, vehicle treatment (Table 1) there is a significant increase in trabecular bone parameters of BV/TV, Tb. N, Tb. Sp compared to control animals. Cancer-inoculated animals treated with JWH-015 showed a significant increase of BV/TV, Tb. N, and Tb. Sp compared to cancer- inoculated vehicle treated animals (Table 1). Additionally, cancer-inoculated animals show a significant change in cortical bone parameters, Ct. Ar, Ct. Ar/ Tt. Ar, TMD, and PsPm compared to control animals (Table 3). Cancer-inoculated animals treated with JWH015 show a significant change in TMD and PsPm compared to cancer-inoculated animals treated with cancer (Table 3). For the contralateral femurs in which surgery was not performed, significant changes were not observed across treatment groups (Table 2 and Table 4). These data indicate that in this breast cancer model, abnormal bone remodeling occurs locally and is attenuated by administration with the CB2 agonist JWH015.

To confirm CB₂ agonist attenuation of cancer-induced bone remodeling, the bone resorption markers, TRACP 5b and CTX, and bone formation marker, osteocalcin, were 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 9B). Sustained JWH015 treatment attenuated cancer-induced elevations in serum TRACP 5b (Figure 9B). CTX concentration in cancer-inoculated mice treated with vehicle was significantly increased compared to control animals injected with media (Figure 9C). Cancer-inoculated mice treated with JWH015 showed attenuation of CTX elevations in serum (Figure 9C). Osteocalcin concentration in cancer-inoculated mice treated with vehicle was significantly decreased compared to

control animals injected with media (Figure 9D). Cancer-inoculated mice treated with JWH015 showed significant increase in osteocalcin levels compared to cancer-inoculated vehicle treated animals' attenuation of CTX elevations in serum (Figure 9D).

Figure 8

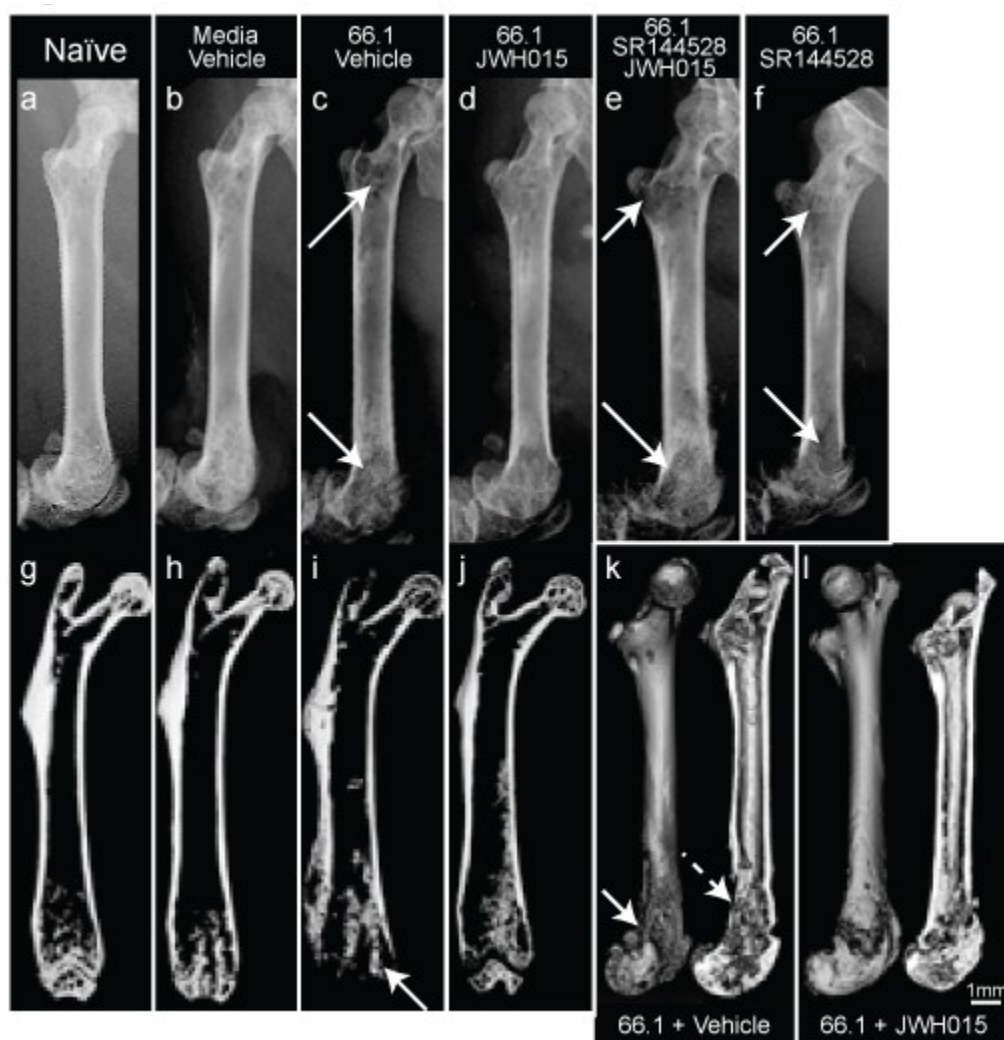
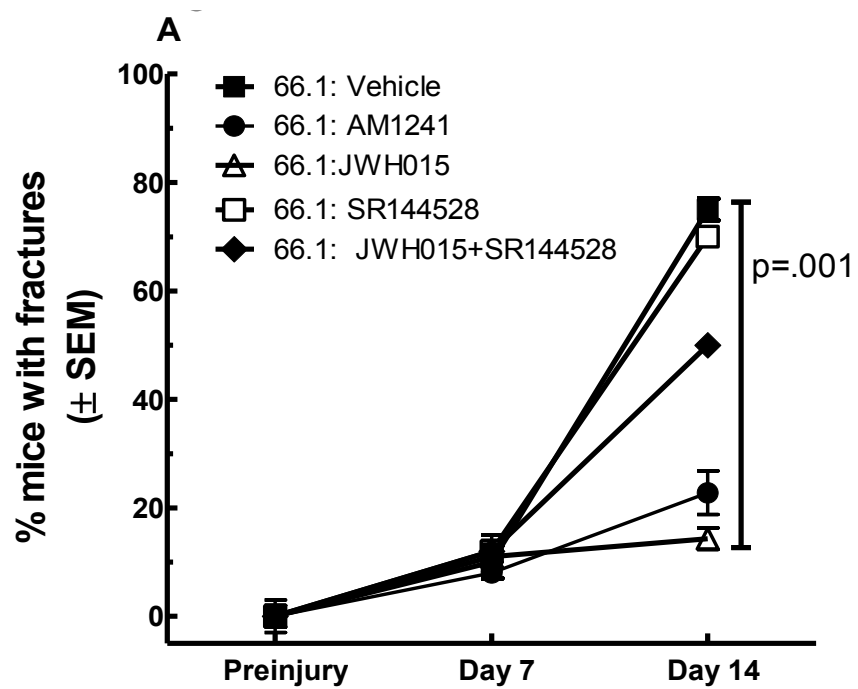
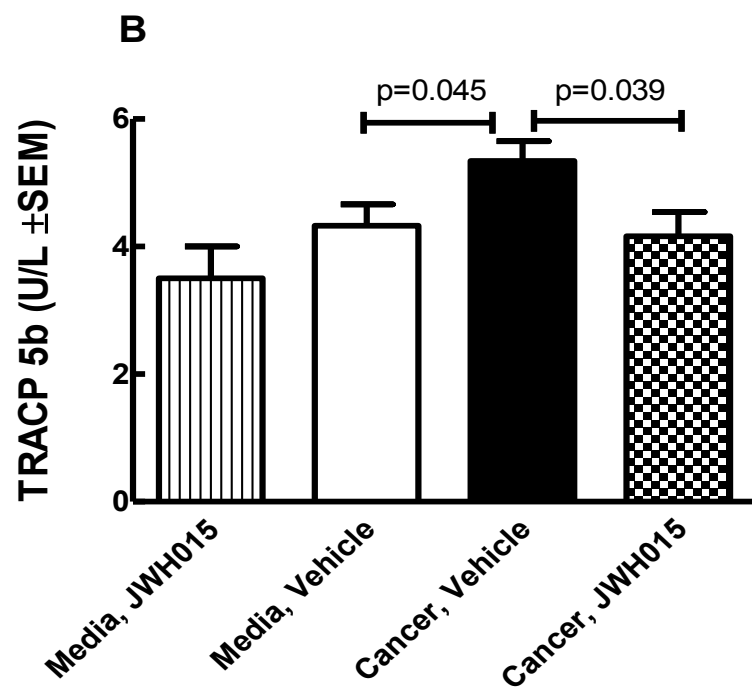


Fig 8. (A) Radiographs of the femora in the presence of either media (control) or breast cancer cells (66.1) on day 14 after inoculation. Mice received either vehicle or a CB2 agonist (JWH015, 6 mg/kg, i.p., q.d.) in the absence or presence of the CB2 antagonist/inverse agonist (SR144528, 3 mg/kg, i.p., q.d. 30 min prior to agonist) 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 as compared to media only (control) animals. JWH015 (6 mg/kg, i.p., q.d. days 7-14) attenuates breast cancer-induced bone loss compared to (B) cancer inoculated, vehicle administration (10-10-80%DMSO, Tween 80, saline). The attenuation of bone cancer-induced bone loss by JWH015 on day 14 was inhibited by the pre-treatment with the CB2 antagonist/inverse agonist SR144528 (3 mg/kg, i.p., q.d. 30 min prior to agonist). SR144528 (3 mg/kg, i.p., q.d. 30 min prior to agonist) alone did not result in any differences. (B, C) Micro-computed tomographic analysis of tumor-induced bone indicated destruction in the absence or presence of JWH015. Cortical bone loss is demonstrated in breast cancer (66.1) inoculated animals as compared to control, naïve or media treated animals. Radiographs in all panels are representative of images obtained of femurs obtained from each animal in figure 2.

Figure 9





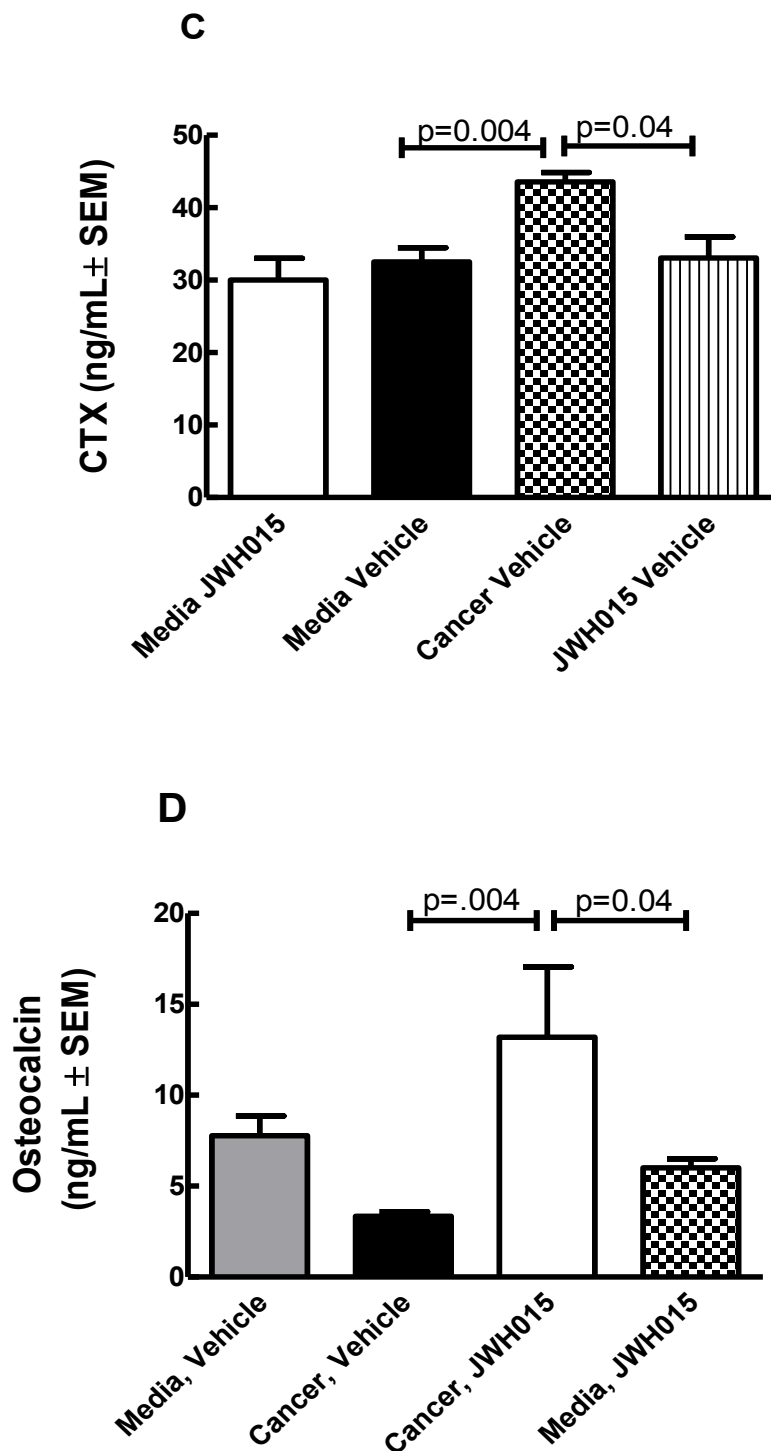


Fig 9. Sustained CB₂ agonist attenuates breast cancer-induced bone remodeling. (A) Based on radiographic images, the number of animals with a clear cortical bone fracture was counted from the different groups of mice at day 0, 7 and 14. Either CB₂ agonists,

AM1241 (n = 15) or JWH015 (n = 30) significantly reduced the number of animals with cancer-induced cortical fracture of the femoris by day 14 ($p=0.001$) as compared to vehicle (n = 30) and this was blocked by the pre-administration of the CB2 antagonist, SR144528 ($p=0.001$; n = 10). Antagonist alone (n = 10) had no significant effect. (B) Using micro-CT, bone mineral density (BMD) was calculated indicating a significant decrease in intra-femoral cancer (66.1) treated animals administered vehicle from day 7 to day 14 ($p=0.047$; n = 9,). Intra-femoral cancer (66.1) treated animals that received CB2 agonist JWH015 (n = 9, pooled from 3 experiments) from day 7 to day 14 demonstrated a significant reversal from the intra-femoral cancer vehicle treated animals ($p=0.039$; n = 9, pooled from 3 experiments). No difference was observed between JWH015 (n = 6, pooled from 2 experiments) and vehicle treated (n = 6, pooled from 2 experiments) of non-tumor bearing mice. (C) TRAP5b was measured in animals on day 14 as a marker of osteoclast activity. 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 JWH015 (6mg/kg, i.p., q.d. from day7 to 14) treated animals ($p=0.039$; n=8). (D) CTX was measured in animals on day 14 as a marker of bone resorption. The amount of CTX was significantly higher in animals that received intra-femoral breast cancer cells (66.1) ($p=0.006$; n=5) compared to intra-femoral media treated animals. This increase in cancer-induced CTX levels was significantly reduced in JWH015 (6mg/kg, i.p., q.d. from day7 to 14) treated animals ($p=0.01$; n=5).

Table 1

Ipsilateral Femur	Media, Vehicle	Cancer, Vehicle	Cancer, JWH015
BV / TV (% \pm SEM)	0.17 \pm 0.02	*0.32 \pm 0.03	**0.24 \pm 0.01
Tb. N (1/mm \pm SEM)	4.16 \pm 0.4	*5.6 \pm 0.16	**5.06 \pm 0.38
Tb. Th (mm \pm SEM)	0.04 \pm 0.002	*0.05 \pm 0.003	**0.04 \pm 0.001
Tb. Sp (mm \pm SEM)	0.16 \pm 0.01	0.16 \pm 0.02	0.14 \pm 0.01

Table 2

Contralateral Femur	Media, Vehicle	Cancer, Vehicle	Cancer, JWH015
BV / TV (% \pm SEM)	0.13 \pm 0.02	0.10 \pm 0.01	0.13 \pm 0.02
Tb. N (1/mm \pm SEM)	3.63 \pm 0.41	2.99 \pm 0.38	3.59 \pm 0.41
Tb. Th (mm \pm SEM)	0.03 \pm 0.001	0.03 \pm 0.001	0.03 \pm 0.002
Tb. Sp (mm \pm SEM)	0.22 \pm 0.02	0.28 \pm 0.04	0.24 \pm 0.03

Table 3

Ipsilateral Femur	Media, Vehicle	Cancer, Vehicle	Cancer, JWH015
Tt. Ar (mm ² \pm SEM)	1.29 \pm 0.09	1.51 \pm 0.02	1.4 \pm 0.06
Ct. Ar (mm ² \pm SEM)	0.68 \pm 0.06	*0.80 \pm 0.03	*0.85 \pm 0.06
Ct.Ar / Tt. Ar (% \pm SEM)	0.19 \pm 0.02	*0.53 \pm 0.02	*0.61 \pm 0.01
Ct. Th (mm \pm SEM)	0.17 \pm 0.01	*0.14 \pm 0.01	**0.16 \pm 0.01

TMD (mg/cm ³ ± SEM)	1136.72±18.12	*1081.23±2.65	**1096.17±7.89
PsPm (mm ± SEM)	4.03±0.25	*4.97±0.12	**4.51±0.04

Table 4

Contralateral Femur	Media, Vehicle	Cancer, Vehicle	Cancer, JWH015
Tt. Ar (mm ² ± SEM)	1.43±0.03	1.3±0.03	1.18±0.08
Ct. Ar (mm ² ± SEM)	0.76±0.02	0.66±0.02	0.64±0.05
Ct.Ar / Tt. Ar (% ± SEM)	0.53±0.01	0.51±0.01	0.54±0.01
Ct. Th (mm ± SEM)	0.21±0.004	0.19±0.01	0.19±0.01
TMD (mg/cm ³ ± SEM)	1190.72±4.03	1129.79±12.36	1132.47±81.8
PsPm (mm ± SEM)	4.37±0.05	4.97±0.12	3.74±0.36

*p<0.05; indicates significant change from control animals (media, vehicle)

**p<0.05; indicates significant change from cancer, vehicle animals

Trabecular parameters:

BV/TV= bone volume fraction, Tb. N= trabecular number, Tb Th= Trabecular thickness, Tb. Sp =Trabecular separation

Cortical bone:

Tt. Ar= total cross sectional area inside the periosteal envelope, Ct. Ar= Cortical bone area (cortical volume/ number of slices X slice thickness), Ct. Ar/ Tt Ar= cortical area fraction, Ct. Th= average cortical thickness, Ps Pm= periosteal perimeter, TMD= tissue mineral density

4.4 CB₂ agonists inhibit cancer cell growth *in vivo* and *in vitro*

The murine breast cancer cells used in this study, 66.1, were found to express the CB₂ receptor (Fig 10). Breast cancer cells were treated *in vitro* with varying concentrations of JWH015 or AM1241. In a concentration-dependent manner, both JWH015 and AM1241 (Figure 11 A,C) significantly decreased percent cell viability compared to vehicle treated cells. Additionally, JWH015 significantly decreased cellular incorporation of BrdU as a direct measure of proliferation (Figure 11D). Pre-incubation of JWH015-treated cells with the CB₂ antagonist/inverse agonists SR144528 and AM630 reversed the antiproliferative effect of JWH015; pre-incubation of JWH015 with the CB₁ antagonist/inverse agonist SR141716 had no effect on JWH015-mediated antiproliferation. SR144528, AM630 and SR141716 had no independent effect on BrdU incorporation at the concentrations used (Figure 11D). These data suggest that JWH015 exerts a CB₂-mediated suppression of cell proliferation in the 66.1 breast cancer cells.

To determine the relevance of *in vitro* findings, bone marrow extrudates were collected from experimental day 14 animals and analyzed for tumor burden. Intramedullary tumors were shown to express the CB₂ receptor (Fig 10). Morphological analysis demonstrated that breast cancer cells occupied 70% of the medullary cavity on day 14 in animals treated with vehicle, whereas breast cancer cells occupied 40% of the medullary cavity on day 14 after JWH015 treatment (Figure 11B).

Figure 10

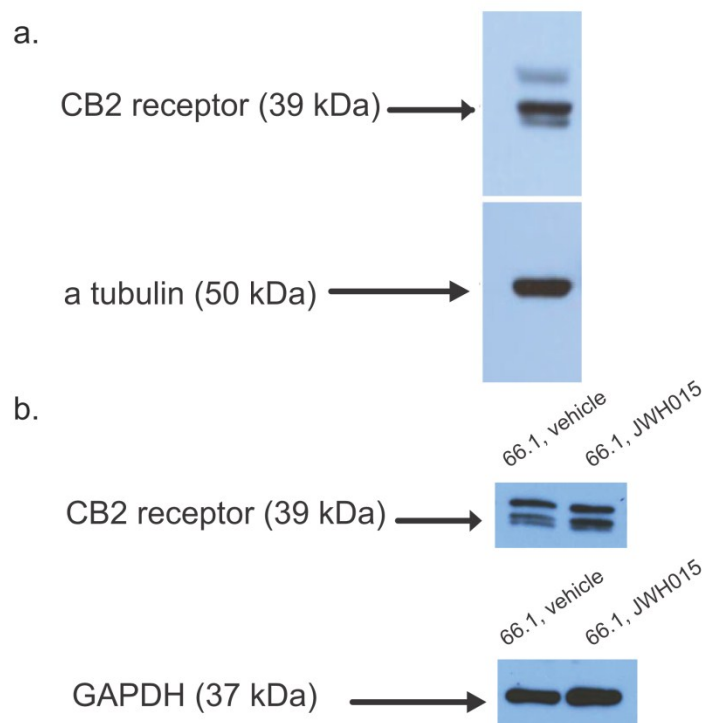
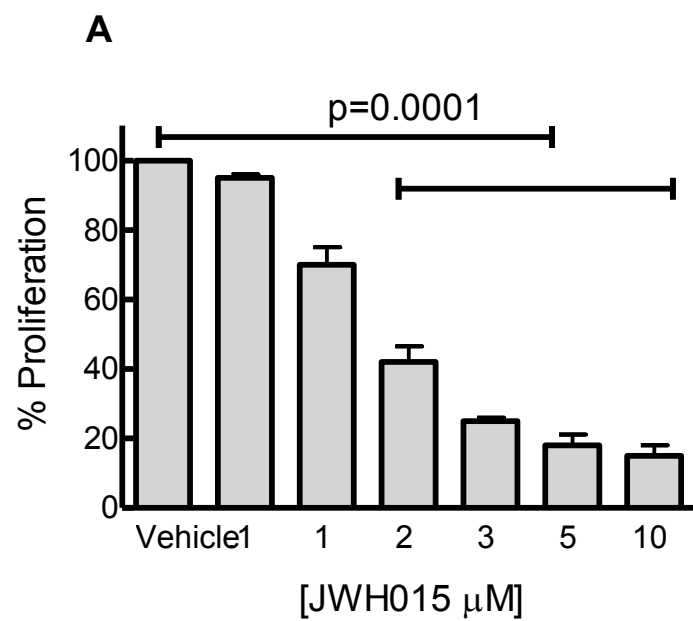
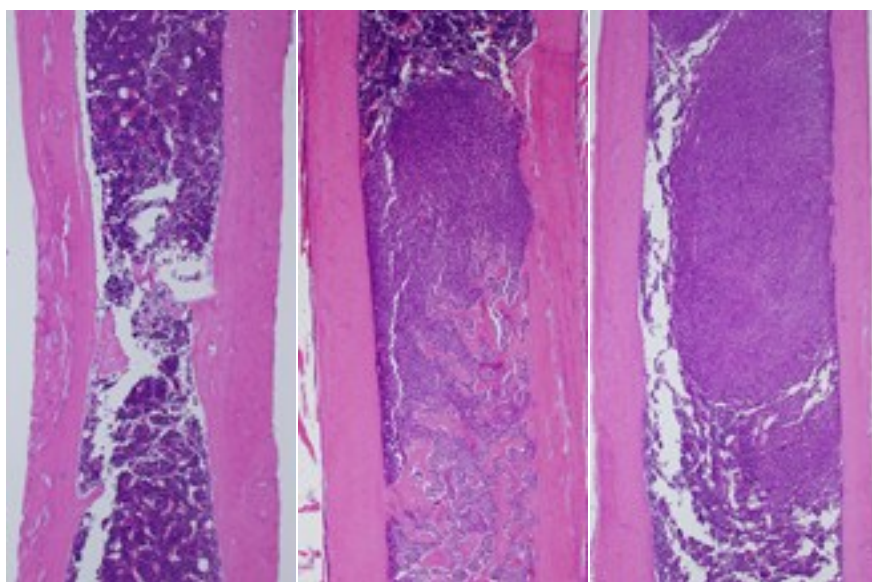


Fig 10 (A) Western blot confirming that 66.1 breast cancer cells express the CB2 receptor with a molecular weight of 39kDa. The membrane was stripped and re-probed with mouse anti α -tubulin as a loading control. Blot displayed is representative of four western blots run from separate cell pellets on each run (B). Western blot confirming that 66.1 breast cancer cells express the CB2 receptor after 14 day surgical implantation with a molecular weight of 39kDa. The membrane was stripped and re-probed with rabbit anti GAPDH as a loading control. Blot displayed is representative of four western blots run from 4 pooled samples in four independent experiments.

Figure 11



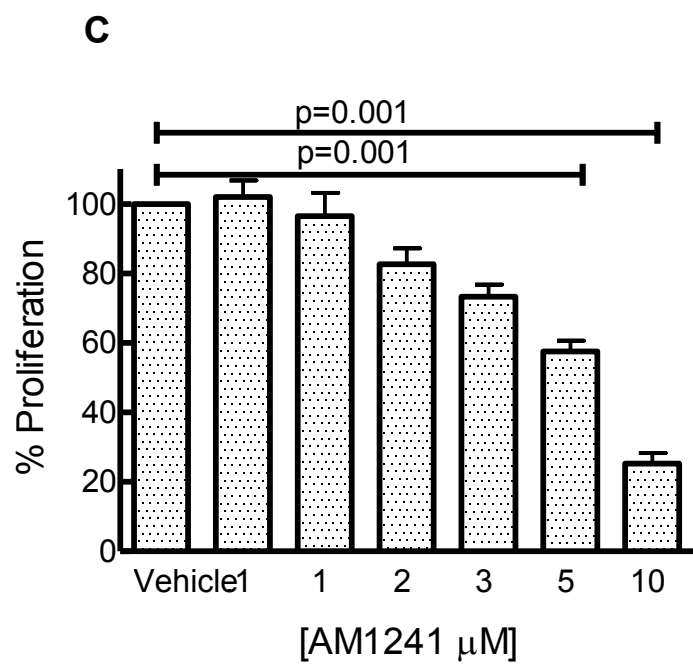
B



Media, Vehicle

66.1, Vehicle

66.1, JWH015



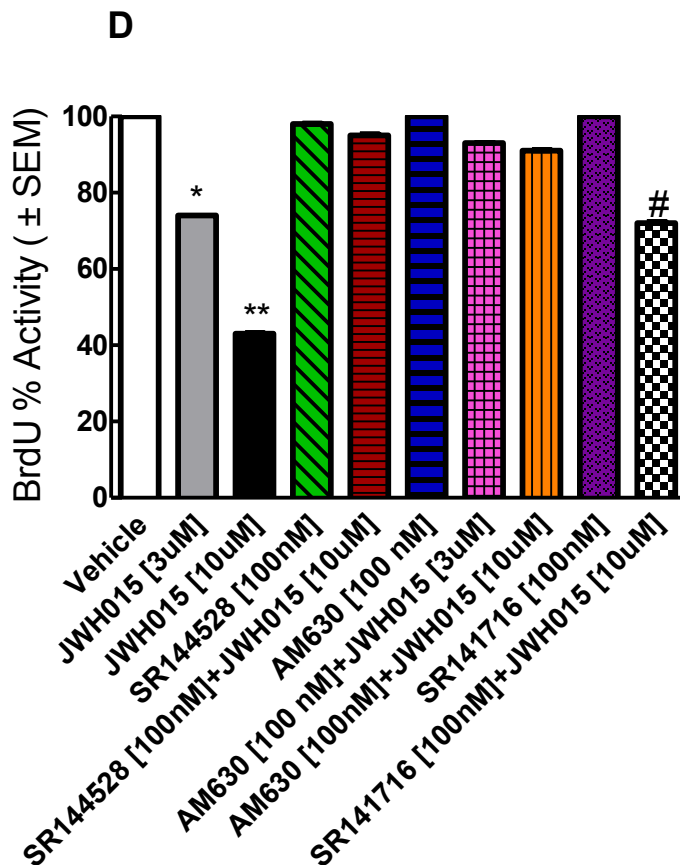


Fig 11 CB2 Agonist decreases 66.1 breast cancer proliferation in vitro and in vivo. (A,C) SRB assay indicates breast cancer cells (66.1) treated with JWH015 or AM1241 demonstrate a concentration related decrease in their viability over a 48 hour period in a 6-well plate compared to vehicle treated cells ($p \leq 0.001$; $n = 18$ per group). (B) Tumor burden using H&E staining of a femoris from intra-femoral cancer animals treated with either vehicle or JWH015. JWH015 reduces the number of 66.1 cells within the intramedullary space compared to vehicle treated animals ($p = 0.01$; $n = 4$ per group). (D) BrDU assay demonstrates breast cancer cells (66.1) treated with JWH015 decrease their ability to proliferate in a 96-well plate compared to vehicle treated cells; this effect can be reversed by the CB2 antagonist/inverse agonist SR144528 but not CB1 antagonist/inverse agonist SR141716 ($p = 0.0001$; $n = 6$ per group).

4.5 Antinociceptive effect of CB2 agonists in breast-induced bone cancer in vivo is associated with suppression of select cytokines and chemokines:

Intramedullary bone extrudates were collected from breast cancer- or media-inoculated mice treated with either vehicle or JWH015 for 7 days. A multi-antibody ELISA array was used to semi-quantitatively assess alterations in inflammatory mediators that occurred with cancer progression and determine whether changes were sensitive to JWH015 treatment. Inflammatory mediators (TNF α , IL6, MIP-1A, and MCP-1) showed the most appreciable alterations from control and sensitivity to JWH015 treatment and were therefore selected for further quantitative ELISA analysis (Fig 12). Concentrations of TNF α , IL6, MIP-1A, and MCP-1 in bone marrow extrudate were elevated in cancer-inoculated animals as compared to media-inoculated controls on day 14 (Fig 13A,B,C,D). Cancer-inoculated animals that received chronic CB₂ agonist (6mg/kg, i.p. q.d., from day 7-14) showed a significant reduction in intramedullary TNF α , IL-6, MIP-1A and MCP-1 as compared to vehicle treated, cancer-inoculated animals (Figure 13A,B,C,D).

To determine whether inflammatory mediators could be secreted and affected at the level of the 66.1 tumor, cell supernatant from treated or non-treated 66.1 cells was assayed via ELISA for secreted levels of MCP-1, TNF α and IL-6. Cultured 66.1 cells alone secreted appreciable levels of the cytokines and chemokines assayed (Figure 13A-C). JWH015 treatment (1 μ M, 3hr) significantly decreased supernatant values for MCP-1 (Figure 7A), IL6 (Figure 14B) and TNF α (Figure 14C), and suppression was partially reversed by pretreatment with the CB₂ inverse agonist, SR144528 (100nM, 1 hr). SR144528 (100nM) alone had no effect on the release of

MCP-1, TNF α or IL-6. These data suggest that JWH015 suppresses secretion of inflammatory mediators from 66.1 cells in a CB₂-dependent manner.

To demonstrate that cytokines elicited from 66.1 tumors could contribute to proliferation, 66.1 cells were treated with log concentrations of either IL-6 or TNF α . At 1 ng/mL, both IL-6 and TNF α promoted cell proliferation as measured by SRB assay (Fig. 15). Additionally, JWH015 (1 μ M) attenuated IL-6-induced proliferation in a manner that was partially reversible by SR144528 pre-treatment (100nM, 1hr) (Figure 6D) but not by SR141716 (100nM, 1hr) (data not shown).

Figure 12

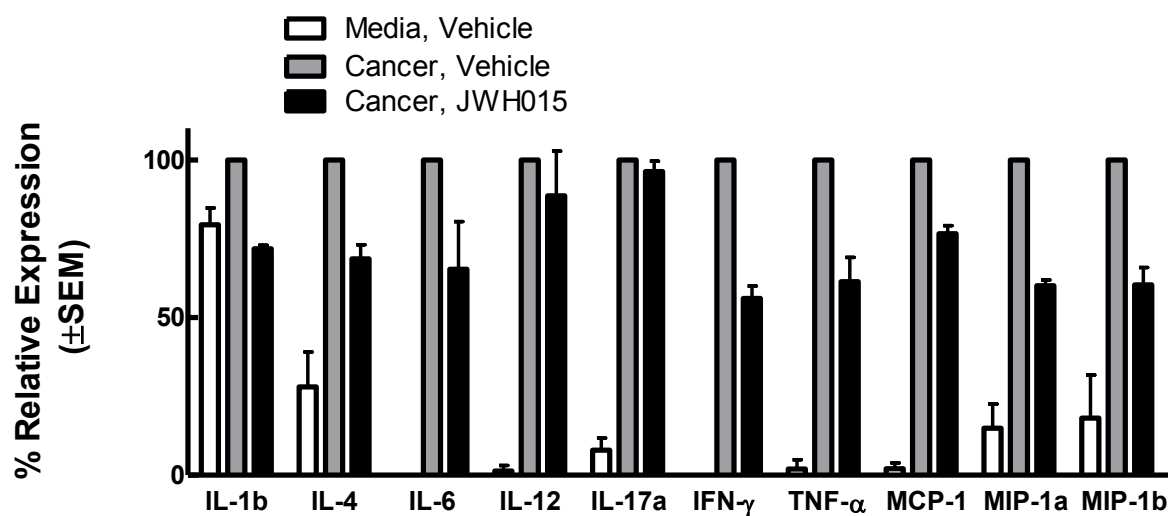
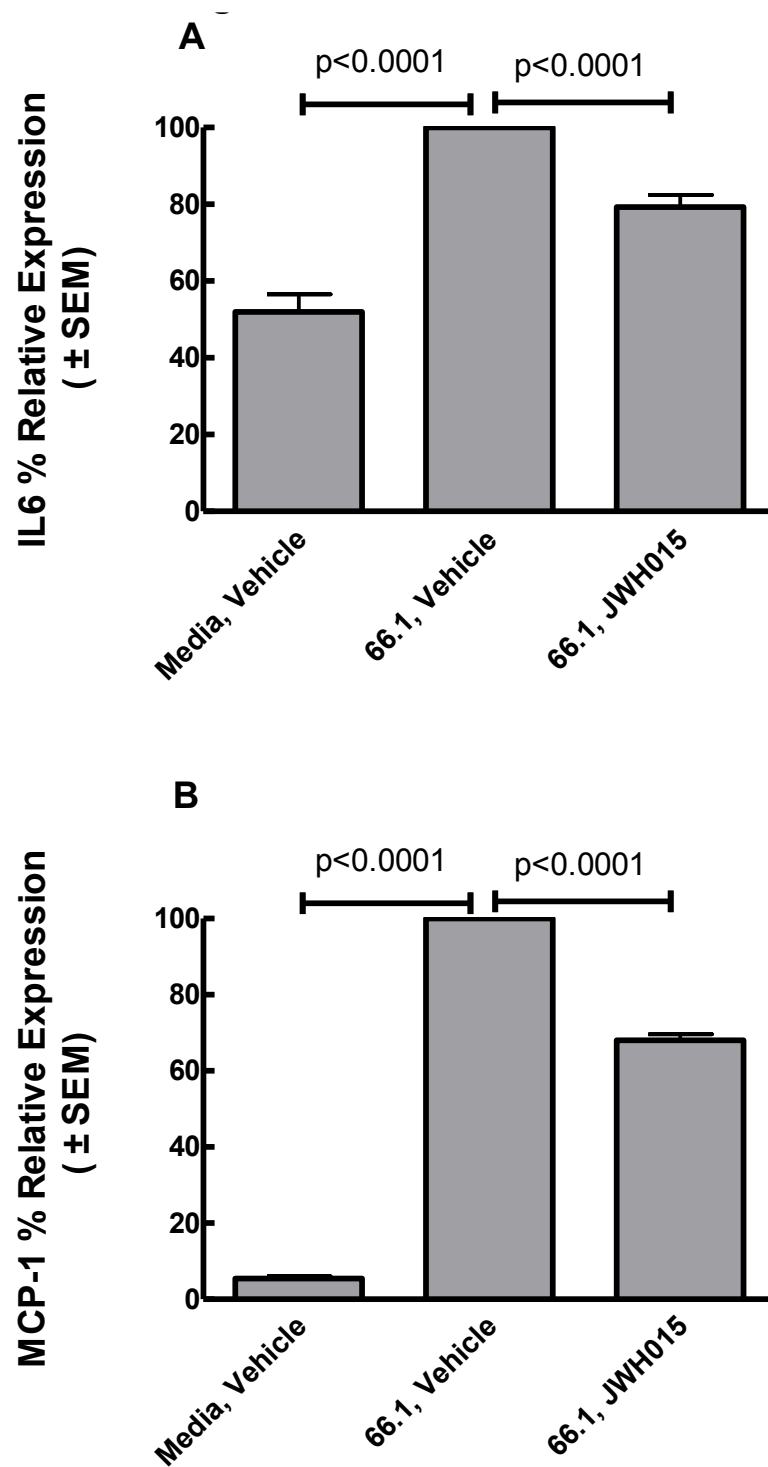


Fig 12 Qualitative ELISA used to determine cancer-induced changes in the expression of cytokines and chemokines from bone marrow extrudates.

Figure 13

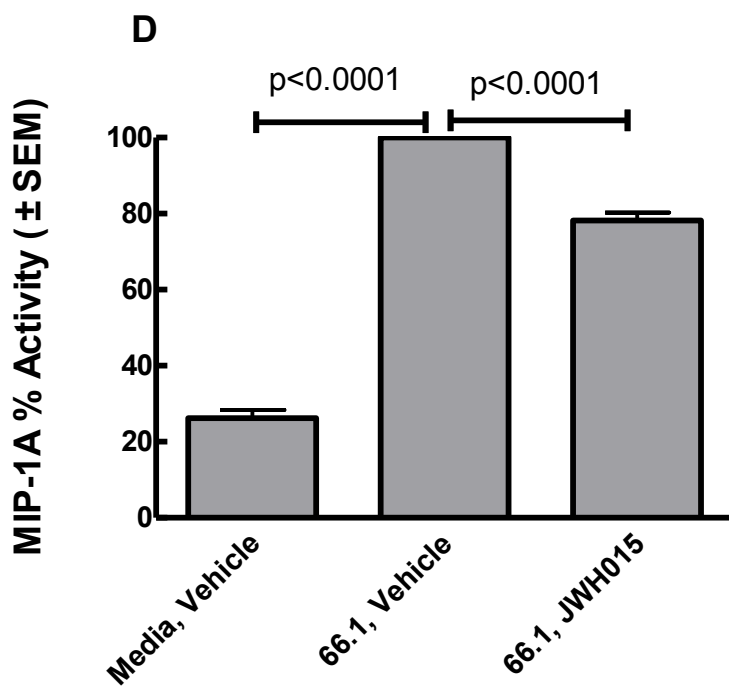
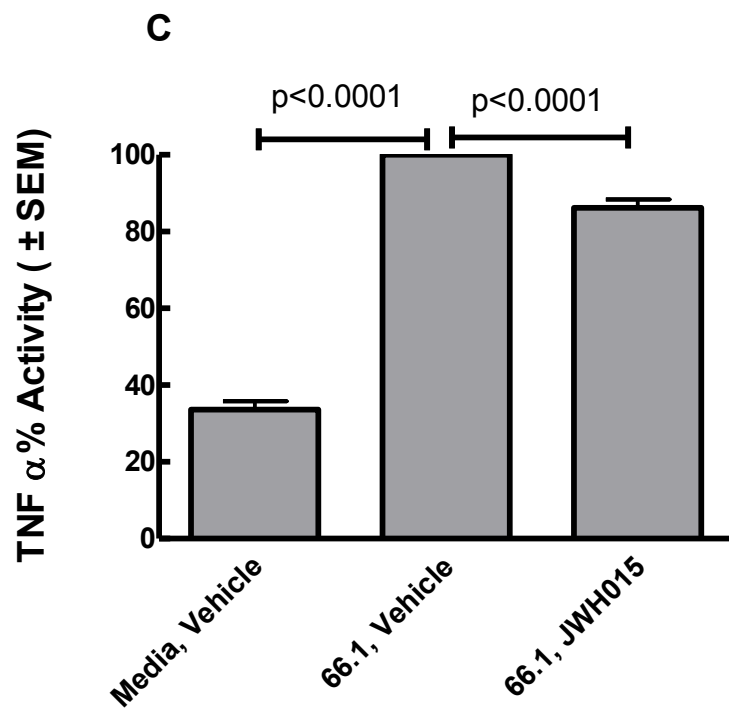
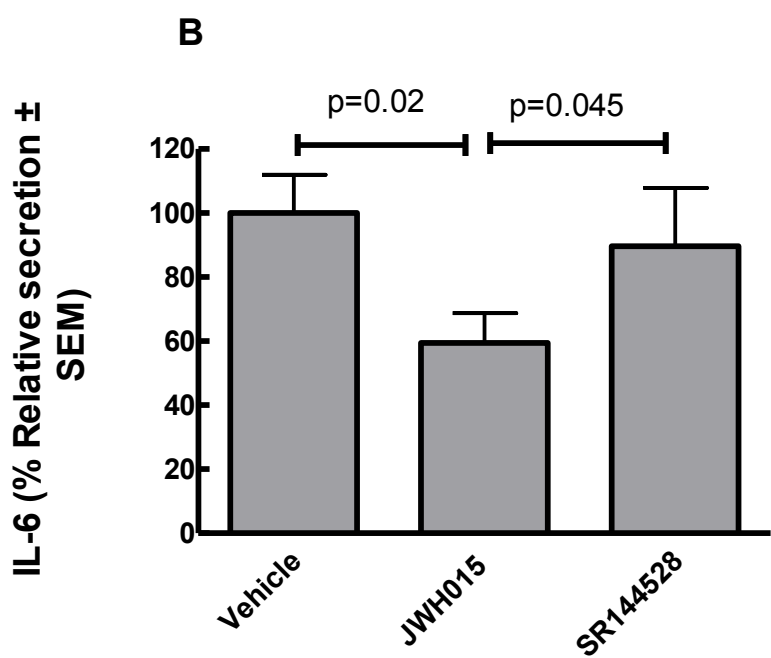
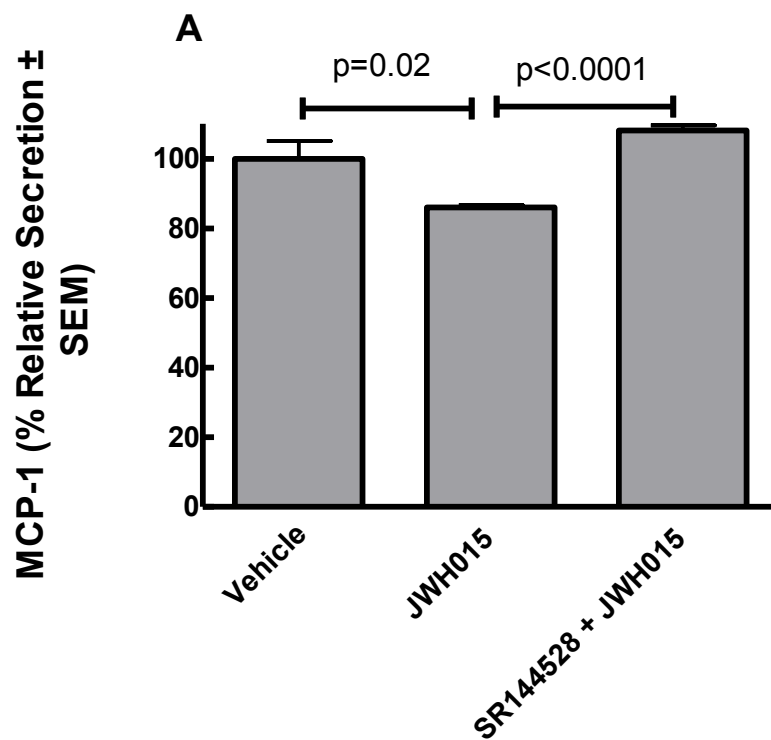


Fig 13 Quantitative ELISA was used to measure amounts of each inflammatory mediator, reported as relative expression compared to control (animals inoculated with cell-free media and treated with vehicle). (A) Increased levels of IL-6 from extrudates from intramedullary femoral space of animals inoculated with 66.1 breast cancer cells and treated with vehicle (day 14; $p < 0.0001$; $n = 4$) as compared to animals inoculated with media. Cancer inoculated animals treated with JWH015 exhibited significantly decreased 66.1-induced levels of IL-6 ($p < 0.0001$; $n = 4$). (B) Increased levels of MCP-1 from extrudates from intramedullary space of femur of animals inoculated with 66.1 breast cancer cells and treated with vehicle (day 14; $p < 0.0001$; $n = 4$) as compared to animals inoculated with media. Cancer inoculated animals treated with JWH015 exhibited significantly decreased 66.1-induced levels of IL-6 ($p < 0.0001$; $n = 4$). (C) Increased levels of TNF α from extrudates from intramedullary femoral space of animals inoculated with 66.1 breast cancer cells and treated with vehicle (day 14; $p < 0.05$; $n = 4$) as compared to animals inoculated with media. Cancer inoculated animals treated with AM1241 or JWH015 exhibited significantly decreased 66.1-induced levels of TNF α ($p < 0.05$; $n = 4$). (D) Increased levels of MIP-1A from extrudates from intramedullary femoral space of animals inoculated with 66.1 breast cancer cells and treated with vehicle (day 14; $p < 0.0001$; $n = 4$) as compared to animals inoculated with media. Cancer inoculated animals treated with JWH015 exhibited significantly decreased 66.1-induced levels of MIP-1A ($p < 0.0001$; $n = 4$).

Figure 14



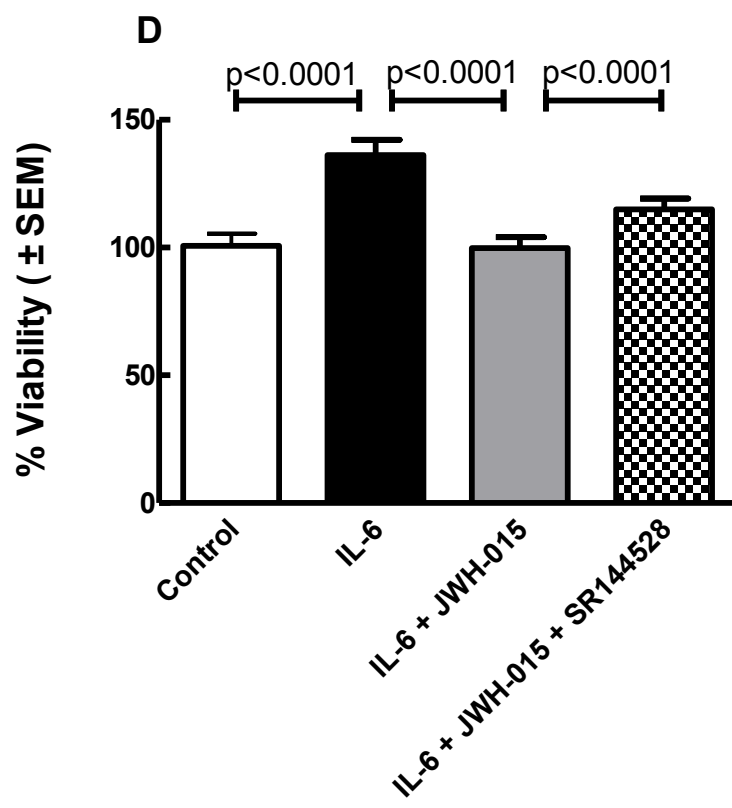
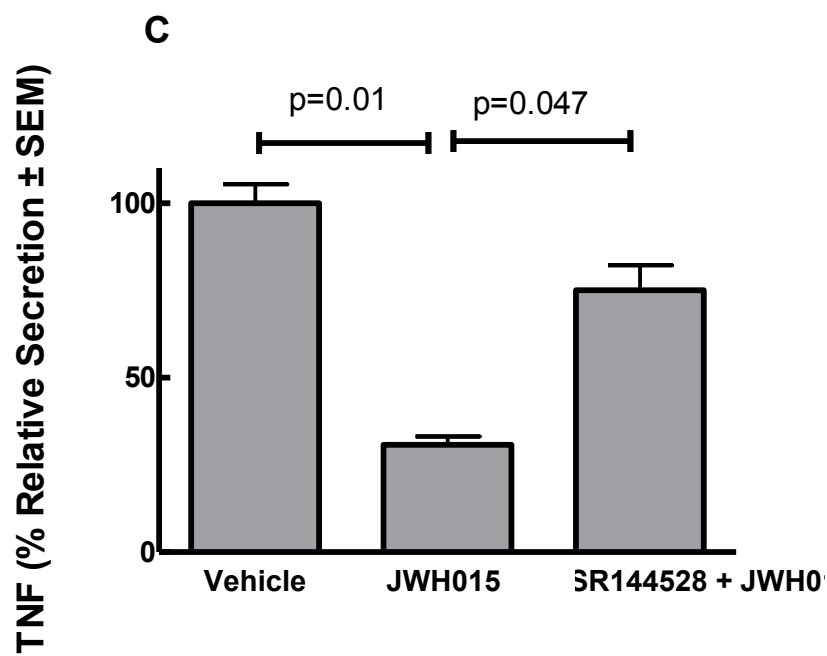


Fig 14. 66.1 cells were treated acutely for 3 hours with JWH015 (1000 ng) and/or SR144528 (100 ng) (n=6 wells per treatment group) Quantitative ELISA was used to measure amount of each inflammatory mediator secreted by cells, reported as % relative expression of secretion compared to control (vehicle treated cells). (A) JWH015 treatment alone significantly reduces expression of MCP-1 ($p=0.02$) but not in the presence of SR144528 ($p<0.0001$). (B) JWH015 treatment alone significantly reduces expression of IL6 ($p=0.02$) but this effect is not seen with SR144528 treatment. (C) JWH015 treatment significantly reduces expression of TNF α but not in the presence of SR144528. (D) 24 hour IL6 treatment (1 ng) significantly increases viability ($p<0.00001$). 24 hour JWH015 treatment attenuates IL6 induced effect on viability ($P<0.00001$). 1 hour SR144528 pre-treatment inhibits 24 hour JWH015 attenuation of IL6 induced increase in viability ($p<0.00001$), as evidenced by SBR assay.

Figure 15

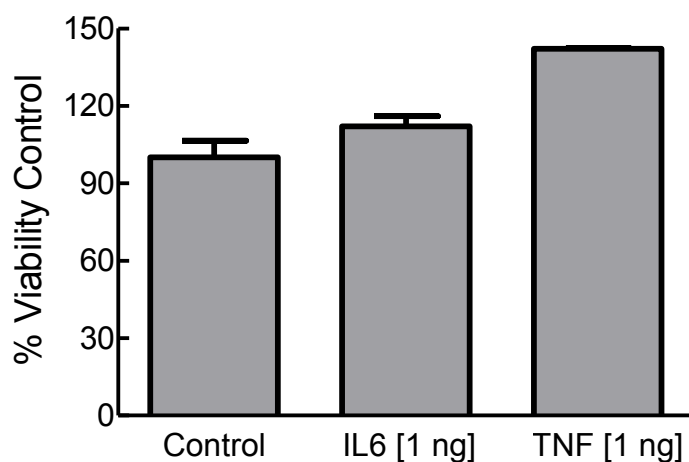


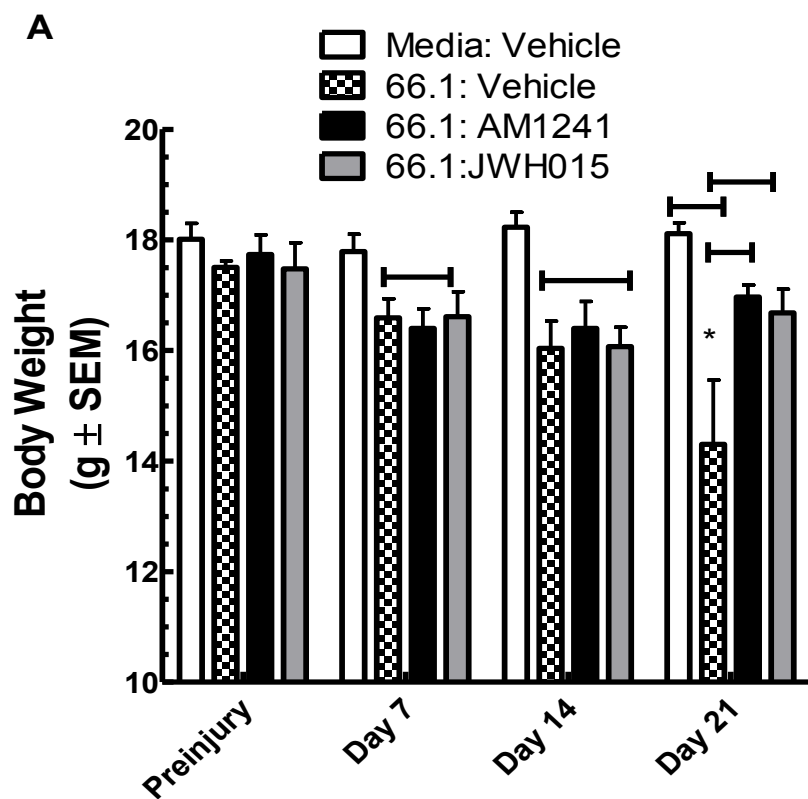
Figure 15: 66.1 cells were treated with increasing doses of TNF α or IL6 for 24 hours to determine change in proliferation (N=5 wells per treatment group). (A) 66.1 cells treated with 1 ng of TNF α significantly increases ($p=0.03$) and 1 ng of IL6 significantly increases ($p<0.0001$) cell viability.

4.6 CB2 agonists help maintain body weight and increase survival:

Animal weights were significantly decreased from baseline ($17.21 \text{ g} \pm 0.25$) by day 21 in cancer-inoculated, vehicle treated animals ($14.30 \text{ g} \pm 1.17$) compared to cancer treated animals that received sustained JWH015 or AM1241 ($16.87 \text{ g} \pm 0.43$; $17.03 \text{ g} \pm 0.15$, respectively) (Fig. 16A). Animals with cancer-inoculated femora and sustained vehicle demonstrated a significant decrease in survival by day 21 compared

to a non-significant change in survival from control in the JWH015 or AM1241 treated group (Fig 16B).

Figure 16



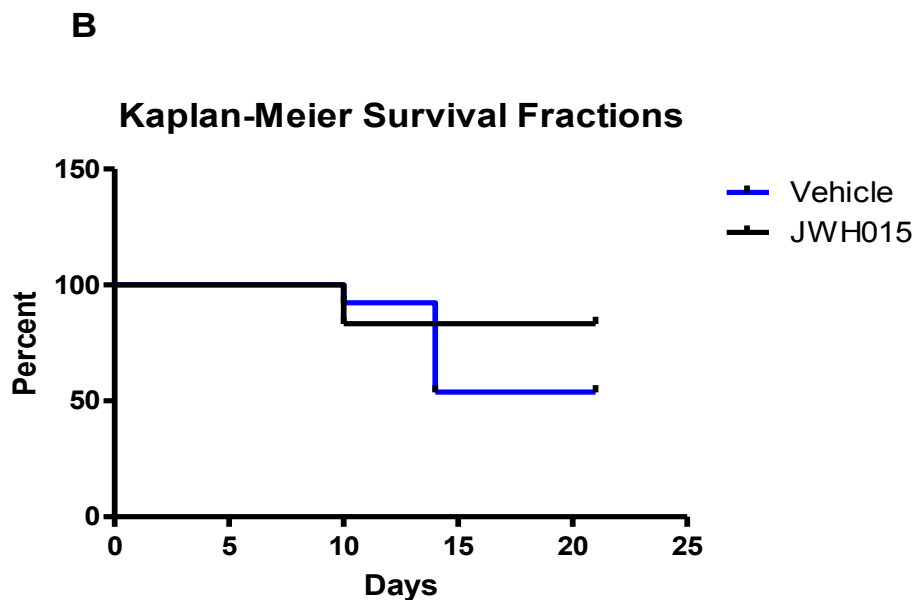


Figure 16 (A) On day 7 animals are equally divided by weight and post-surgery behavioral baselines. Cancer-inoculated animals treated with vehicle weigh significantly less on day 21 compared to control animals and cancer-inoculated animals treated with JWH015 ($p=0.001$, $n=8$ per group) (B) Survival studies utilized the Kaplan-Meier estimator. The percent of mice surviving to day 21 was significantly reduced in intra-femoral cancer treated (66.1) vehicle (i.p., q.d. from day7 to 21) animals ($p=0.032$) whereas animals administered JWH015 or AM1241 were not statistically different from control ($n = 8$ per group).

4.7 Conclusion

Epidemiological studies show that 1 out of 7 women in the U.S. will develop breast cancer in their life-time [240]. The National Institutes of Health estimates overall costs of cancer in the U.S. at \$206 billion with breast cancer being the most frequent malignant tumor [240]. Up to 80% of patients with advanced breast cancer develop bone metastases associated with bone loss and fracture which contribute to

incapacitating pain and limited or total loss of mobility [51, 52, 203, 207]. Pain, the first symptom in many cancer patients, substantially decreases the quality of life [51, 52, 203, 207] and is poorly addressed by the current first-line therapies (7). Skeletal related events (SREs) and pain due to breast cancer metastases are treated using radiation therapy, opiates and bisphosphonates [2,46,150]. Bisphosphonate treatment is associated with an array of off-target effects including nephrotoxicity, osteonecrosis of the jaw, hypocalcemia and flu-like symptoms [38, 191]; furthermore, the unwanted side effects of opioids and NSAIDs are well-documented (11-13). Chronic pain treatments for advanced stage breast cancer have not been developed, and the failure of current pain therapies has generated a need for innovative and tailored approaches to managing cancer-pain.

Here we show that either a single injection of a CB₂ agonist or sustained administration significantly attenuates spontaneous and evoked pain behaviors in an animal model of breast-induced bone cancer pain. Inhibition of pain behaviors by the CB₂ agonist is similar in efficacy to a single injection of morphine [159], suggesting that CB₂ agonists have the ability to directly inhibit pain activity. Our antagonist studies support the assertion that CB₂ agonist effects occur via CB₂ receptors and not off-target CB₁ receptors. Based on studies both *in vivo* and *in vitro*, we speculate that antinociceptive effects by CB₂ agonists in our bone cancer model occur via anti-cytokine/anti-inflammatory activity localized to the tumor-bone microenvironment. Cancer metastasis to the bone initiates an immune response within the bone and the nervous system [63]. This immune response activates nociceptors and subsequently generates pain [259]. A mechanism of pain inhibition by CB₂ agonists is proposed by

decreasing pronociceptive cytokines such as IL-6, TNF α , and IL-1 β released from infiltrating immune cells [135, 171, 211] and from the cancer cells themselves [16, 54]; we have further validated the ability of cancer cells to release pronociceptive cytokines in our cancer model. Studies have demonstrated that IL-1 β , TNF α and IL-6 are released from macrophages, monocytes, breast cancer and glial cells to promote nociception [58, 59, 101]. IL-1 β and IL-6 have been shown to increase the expression of other pronociceptive factors such as prostaglandins via upregulation of COX-2 [60, 225] and nerve growth factor (NGF) [271] while increasing the production and release of other cytokines to promote nociception. TNF α acts directly at its receptor, TNFR1, to produce nociception and indirectly via increasing prostanoids and sympathetic amines [193, 259]. Activation of CB₂ receptors on immune cells has been shown to inhibit the release of a number of cytokines from monocytes and macrophages in animal [56, 135, 160, 219] and human studies [84, 211]. In addition to cytokine activity, it is well known that certain chemokines can result in nociception (for review, see Verri Jr. WA et al., 2006). The chemokine, macrophage inflammatory protein-1 (MCP-1, also known as CCL2) and macrophage inflammatory protein-1a (MIP-1A, also known as CCL3), in mice have been reported as producing time- and dose-related hypernociception via their receptors CCR2 and CCR5 (GPCRs) on nociceptive fibers [8, 216]. A significant decrease in neuropathic pain response was demonstrated in CCR2^{-/-} mice [269]. In addition to directly stimulating nociceptive fibers, it has been shown that MCP-1/CCL2 can increase pronociceptive sympathetic amines and IL-1 β production [114]. MIP-1A/CCL3 has been shown to sensitize primary afferents to capsaicin while the *in vivo* administration of MIP-1A/CCL3 resulted in thermal and mechanical hypernociception

[18, 259]. In the end, these pronociceptive cytokines and chemokines are released from cancer-related infiltrating immune cells as well as from the tumor cells to promote pain and continual tumor proliferation, resulting in a "feed-forward" process that we demonstrate to be inhibited by CB₂ receptor activation. Additional explanations of pain inhibition by CB₂ agonists may include the inhibition of cancer-induced activation of spinal microglia that contribute to central sensitization [219], as well as their effects on bone remodeling.

Another major component of bone cancer pain that results in decreased quality of life and survival is osteolysis and bone fracture [180]. When metastatic breast cancer invades the bone, balance between bone building and bone destruction (normal bone metabolism) is disrupted in favor of net bone loss (60). Tumor-induced bone remodeling is associated with an acidic environment created by the tumor itself, local acidosis associated with tissue injury and bone resorption by osteoclasts that directly sensitize and excite local primary afferent fibers within the bone[176]. Both osteolytic activity and osteoblastic activity result in weakening of the normal healthy bone and predispose patients to skeletal complications including bone pain from loading stress as well as from damage to sensory nerve endings in the intramedullary space, impaired mobility, pathological fracture, spinal cord compression and symptomatic hypercalcemia. [159] CB₂ receptors are expressed on osteoclasts and have the ability to affect bone homeostasis and structure [190]. CB₂ agonists attenuate ovariectomy-induced bone loss in mice [190]. Our radiographic analysis indicated that implanted breast cancer cells induced significant bone remodeling and that 7-day treatment with JWH015 in cancer-bearing mice reduced the incidence of fractures. Further μ CT

analysis indicated a cancer-induced modification of both cortical and trabecular bone parameters which were attenuated by JWH015 treatment. Finally, JWH015 treatment also significantly reduced serum markers of bone degradation in cancer animals, including CTX and TRACP 5b, and increased the serum bone formation marker osteocalcin. Our studies suggest that treatment with a CB₂ agonist attenuates breast cancer-induced bone loss and helps stabilize cancer-inflicted bone activity. Recent studies demonstrating that a selective CB₂ agonist significantly inhibits osteoclast activity and osteoclastogenesis [81, 190], combined with our findings of a CB₂-dependent reversal of cancer-related bone loss evidenced via markers relating to bone resorption, we believe that CB₂ agonists in breast cancer-induced bone malignancy are reducing osteoclast activity and number, therefore decreasing bone loss and related pain.

Recent studies (17, 63, 22), as well as findings reported here suggest that CB₂ agonists may have antiproliferative effects yet further explanation of how such compounds may alter proliferation are needed. Bone cancer including breast metastasis results in a marked influx of hematological and inflammatory cells into the medullary portion of the bone, resulting in not only activation of nociceptors[100, 205, 227], but also secretion of RANKL and initiation of NF-κB signaling which stimulates osteoclastogenesis. Osteolytic processes further promote the proliferation of the metastases [63] resulting in further invasion of the bone, advanced bone degradation and pursuant pain. The cancer-driven influx of hematological and inflammatory cells increases the production of cytokines and chemokines [273] including IL-6, TNFα, MCP-1 and MIP-1A that accelerate cancer proliferation [63]. IL-6 levels are significantly

elevated in breast cancer patients and IL-6 via its major intracellular effector, STAT3, has been reported as protumorigenic in breast cancer cells [182]. Immune and inflammatory cells in close proximity to breast cancer cells are capable of producing prodigious amounts of “start-up” IL-6, required for early tumor promotion [254]. Furthermore, IL-6 has been shown to be released from breast cancer cells and act in an autocrine and paracrine fashion through IL-6R/gp130 receptors expressed by the breast cancer cells contributing to cellular transformation and growth [87]. TNF α , released from macrophages as well as from primary breast cancer cell lines [101], regulates epithelial invasion through activation of downstream signaling cascades including JNK and NF κ B [177]. Breast cancer cell release of TNF α [177] demonstrates an autocrine and/or a paracrine secretion that results in breast cancer self-proliferation and invasion [181]. Likewise, chemokines such as MCP-1/CCL2 and MIP-1A/CCL3 were recently shown to have trophic qualities, known to attract inflammatory cells to tumors and to inhibit the generation of tumor reactive T-cells and natural killer cells [169, 178].

Here we demonstrate that 66.1 breast cancer cells express the CB₂ receptor and release cytokines and chemokines that promote self-proliferation, which can be inhibited by a CB₂ agonist and reversed by the CB₂ antagonist/inverse agonist. The application of cytokines IL-6 and TNF directly onto 66.1 cells results in a significant increase in proliferation that was attenuated by a CB₂ agonist, suggesting that CB₂ agonists act by inhibiting cytokines that promote proliferation. Using our murine model, we demonstrated a significant cancer-induced increase in IL-6, TNF α , MCP-1 and MIP-1A within the intramedullary space that was significantly attenuated by the administration of a CB₂ agonist. These data support a mechanism of CB₂ receptor-mediated inhibition of

factors that have been shown both *in vitro* and *in vivo* to promote the proliferation and invasion of breast cancer cells. Using H&E staining, we found that sustained CB₂ agonist treatment in breast cancer-inoculated animals significantly reduced the percentage of tumor burden within the intramedullary cavity of the femoris, supporting the presence of an antiproliferative effect *in vivo*. Further studies will highlight the importance of each of the cytokines and chemokines using antagonists or antibodies towards the cytokines and chemokines to determine whether the inhibition of one individual factor has similar antiproliferative properties to the the broad inhibition seen by the CB₂ agonist. In addition, further studies are needed to elucidate the molecular intracellular pathway of CB₂ receptor coupling and cytokine/chemokine release in breast cancer cell lines. Previous studies in MMTV-neu mice with ErbB2-driven metastatic breast cancer demonstrate the expression of the CB₂ receptor and the selective CB₂ agonist JWH133 reduced tumor growth, tumor number, and lung metastases by inhibiting active Akt [35]. Some groups have postulated that cannabinoid-mediated inhibition of tumor growth may induce apoptosis through modulation of the Ras-MAPK/ERK and PI3K-AKT pathways [35, 74]. These observations may be in line with our data, where we have already observed the antitumoral effects of other selective CB₂ compounds, JWH015 and AM1241, *in vivo* and *in vitro*. However, recent findings also suggest alternative receptor coupling of the CB₂ receptor or unidentified cannabinoid receptors and splice variants (80). The difficulty of determining that effects are in fact cannabinoid receptor-mediated, specifically, is complicated by the constitutive activity of both cannabinoid receptors that may sustain biological processes, and a lack of neutral antagonists for both receptors. Future studies are likely to determine the molecular

pathway in which CB₂ agonists successfully inhibit breast cancer proliferation and cytokine-chemokine secretion.

Unlike existing treatments for advanced-stage cancers, we found that sustained treatment of breast cancer-induced bone malignancy with CB₂ agonists significantly increased subject survival and helped maintain animal body weight. Patients who develop metastatic breast cancer have an average survival of 1.5 to 3 years [4]. A predictor of a poor prognosis is linked to the lack of treatment available once tumor cells have migrated to the bone [173]. Although advances have been made in breast cancer detection and early stage treatment, there have been relatively few advances in late stage drug development for cancer proliferation, SREs and pain ([173]. Recent preclinical trials with a B-RAF inhibitor in advanced skin cancer patients resulted in an average increase in survival rate of 8-12 months with a significant reduction in tumors and pain. Although the cancer returned, the extended months without pain were beneficial and meaningful to both the patient and family members [97]. Here we have shown by using an established, advanced cancer model that CB₂ agonists, unlike drugs approved for late stage breast cancer, may yield increased patient survival times.

In conclusion, advanced stage cancer demands novel drugs for the treatment of bone metastasis. Our most recent data suggests that nonpsychotropic CB₂ agonists may serve as a disease-modifying treatment for metastatic breast cancer patients with the potential to increase the survival rate, relieve pain, improve bone structure and inhibit tumor cell growth. As demonstrated by the recent discovery of the B-RAF inhibitor for advanced melanoma, drugs that increase the survival time and quality of life have a great benefit for patients, family, doctors and the community.

CHAPTER 5: CB2 AGONISTS ATTENUATE PAIN IN THE CENTRAL NERVOUS SYSTEM VIA INHIBITION OF PRO-INFLAMMATORY CYTOKINES

5.1 Introduction

Historically, chronic pain has been believed to be induced by aberrant neuronal modulation along the nociceptive pathways, mediated through peripheral and/or central factors [284]. Glial cells are up to 50 times more abundant in the CNS and, unsurprisingly, the emerging literature indicates glial cells, particularly microglia and astrocytes, are important in the development of chronic pain. Across neuropathic, inflammatory, and cancer pain models in rodents, glial activity is modulated through early and transient microglial responses and subsequent morphological changes in astrocytes [219, 230].

Glial cells have a neuroprotective role in the CNS, acting as the resident innate immune system. In response to changes in physiological conditions, glial cells can be modified from a quiescent to activated state. Activation can be in the “acute” or “chronic phase”. In the acute activation phase, microglia detect the bacterial cell wall toxin lipopolysaccharide (LPS) and respond in a restorative manner, as in the case of systemic infection or repairable injury. With the elimination of LPS, microglia revert back to a quiescent state. In contrast, persistent noxious stimuli induce sustained central sensitization. In these conditions, neuropeptides and neurotransmitters activate intracellular signaling cascades in glia including p38 mitogen-activated protein kinase (p-p38 MAPK) and C-Jun N-terminal kinase (JNK) resulting in production of inflammatory mediators [267]. Additionally, activated microglia produce inducible nitric oxide synthase (iNOS) while astrocytes produce nitric oxide, further perpetuating JNK

MAPK cellular pathways and production of cytokines and chemokines [267]. Activated microglia up-regulate ionized calcium binding adaptor molecule-1 (Iba1) and CD11b/c. When activated, astrocytes, express high levels of vimentin and glial fibrillary acidic protein (GFAP) and develop thickened processes [267]. In animal models of bone and paclitaxel induced pain, astrocyte and microglia expression were unregulated in the spinal cord suggesting involvement of glial cells in maintaining chronic pain in these conditions [104] [282]. Research establishing the roles of glial cells in pain is in its infancy. Thus, when looking at compounds targeting glial cells in pain, it is important to look at changes in neuroinflammation rather than designate a particular cell type as the main contributor to chronic pain.

Interestingly, microglia and astrocytes have been shown to produce the endocannabinoids, AEA, 2AG, DEA, and HEA [261]. Several groups have demonstrated that microglia and astrocytes express CB2 receptors [20, 219, 261]. Activation of the CB2 receptor in neuropathic, inflammatory, and postoperative pain attenuates behavioral hypersensitivity correlated with reduced glial activity [218, 219]. Romero-Sandoval presents evidence indicating CB2 activation inhibits the ERK pathway, reducing TNF expression [220]. While it has been shown that cancer-induced bone pain results in distinct neurochemical changes in spinal cord from other chronic pain, such as astrocyte gliosis, the downstream effects of persistent glial activation remain unclear. Furthermore, herein we investigated the role of CB2 agonists in the modulation of microglia and astrocytes of the CNS in a breast-induced cancer model. We hypothesize that sustained CB2 receptor activation, relieves pain centrally by modulating the activation of glial cells and inhibiting their release of pro-inflammatory

cytokines. Identifying the mechanism of CB2 activation within the central nervous system may offer novel therapeutic strategies for patients who suffer from bone cancer pain.

5.2 Spinal administration of a CB2 agonist reduces cancer-induced bone pain

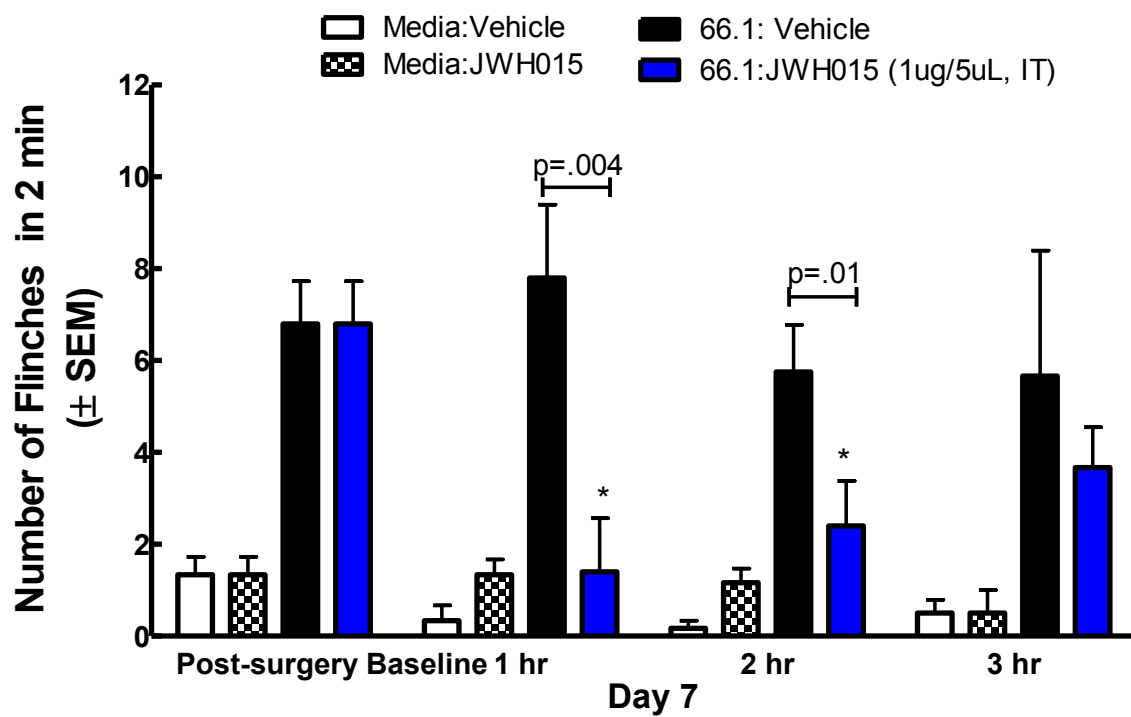
To determine the effects of the CB2 agonist JWH015 on bone cancer-induced spontaneous pain, flinching and guarding behaviors were observed. Animals were observed for two minutes to determine the number of flinches and the duration of time spent guarding. Evoked pain was quantified by using von Frey filaments to determine the withdrawal threshold of the ipsilateral hind paw. Beginning 7 days after cancer inoculation, mice displayed cancer-induced flinching and guarding and decreased paw withdrawal thresholds demonstrating behavioral signs of pain. Control animals injected with media did not show significant guarding, flinching, or tactile allodynia when treated with vehicle or JWH015 (Fig. 17A, 1B, 1C). Acute administration of JWH015 (10 ug/5 uL, i.t) reduced evoked pain behaviors in tumor-bearing mice compared to vehicle treated animals (Fig. 17A, 1B). Additionally, this one time dose of JWH015 effectively reduces cancer-induced spontaneous flinching and guarding compared to control animals (Fig. 17C).

Mice injected with cancer cells and treated with vehicle displayed increasing flinching and guarding behaviors beginning on day 7 through day 14. To determine whether sustained treatment would attenuate pain behaviors, JWH015 was given daily for 7 consecutive days. Daily treatment with JWH015 in cancer-inoculated mice led to significant decreases in flinching and guarding on day 14 compared to the cancer

animals treated with vehicle (Figures 18A, B). Sustained treatment of JWH015 also decreased hypersensitivity as measured by von Frey filaments (18C).

Figure 17

A



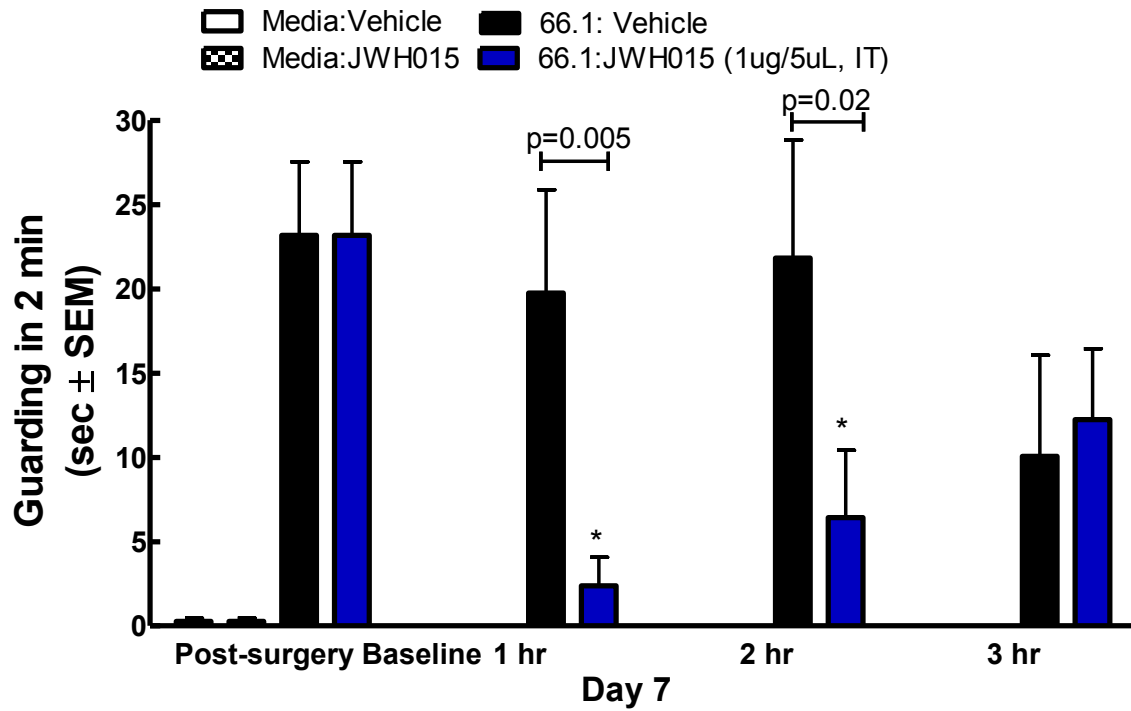
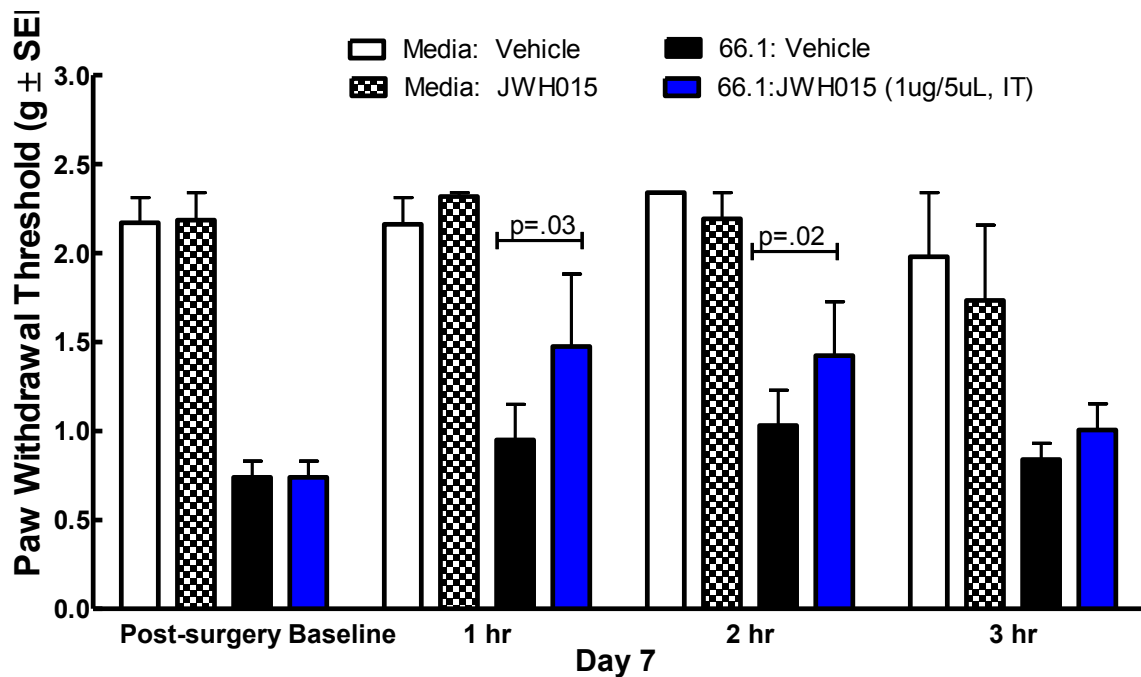
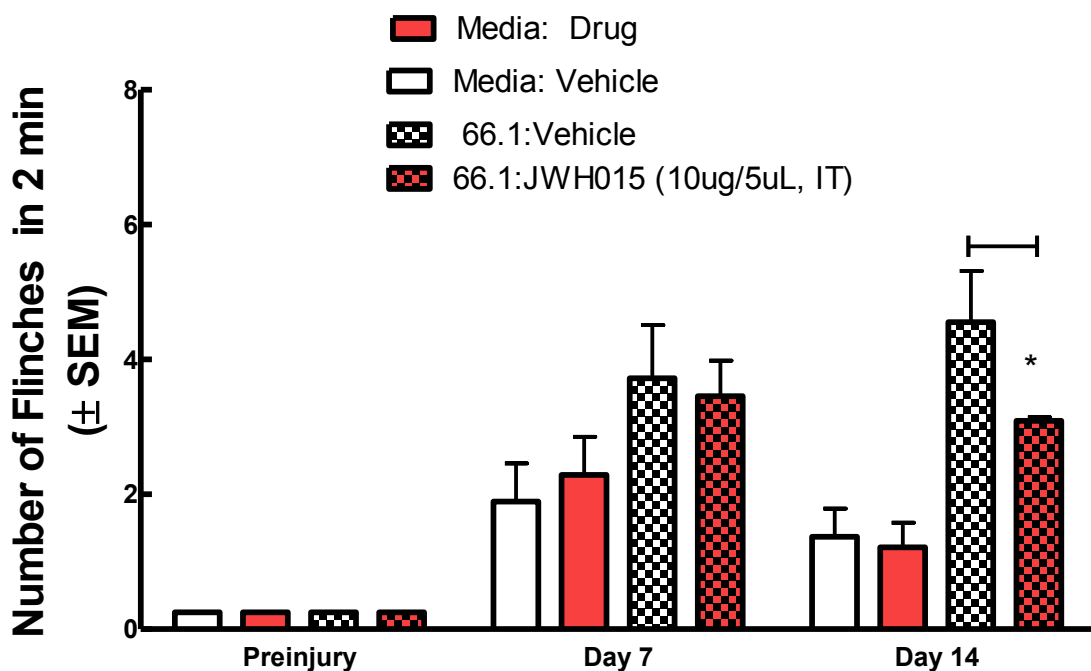
B**C**

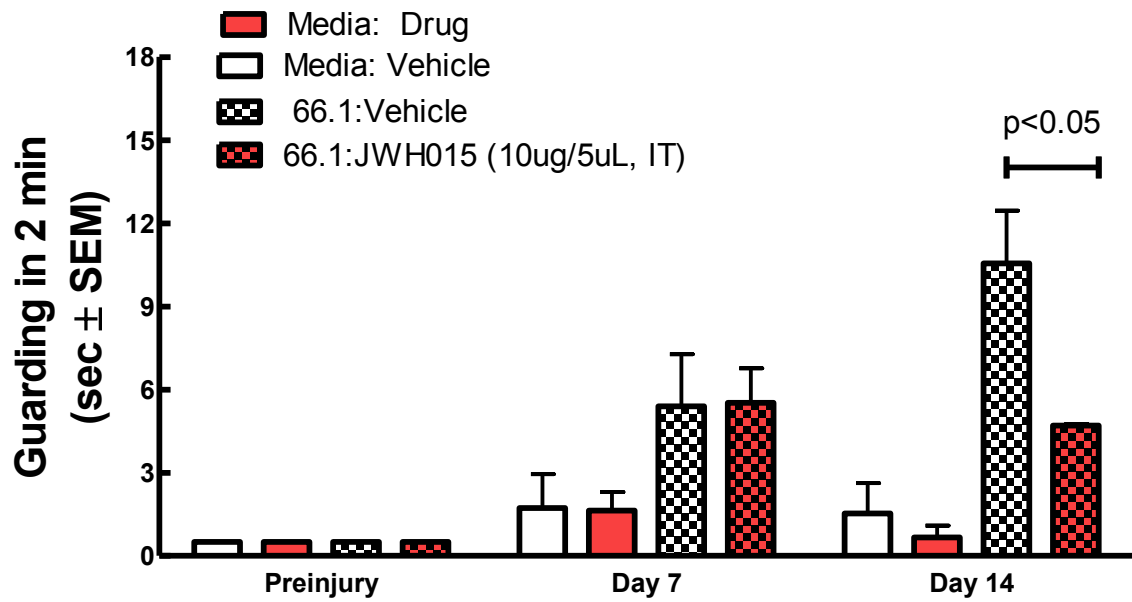
Fig. 17 Acute spinal administration of CB₂ receptor agonist JWH015 attenuates breast (66.1) cancer-induced spontaneous and evoked pain on day 7 after femoral inoculation (Day 0). Bone cancer-induced (A) spontaneous flinches (B) guarding and (C) evoked paw withdrawal were all significantly attenuated over time in animals administered JWH015 (i.t.) compared to vehicle treated animals ($p \leq 0.05$; $n = 10$ mice per group).

Figure 18

A



B



C

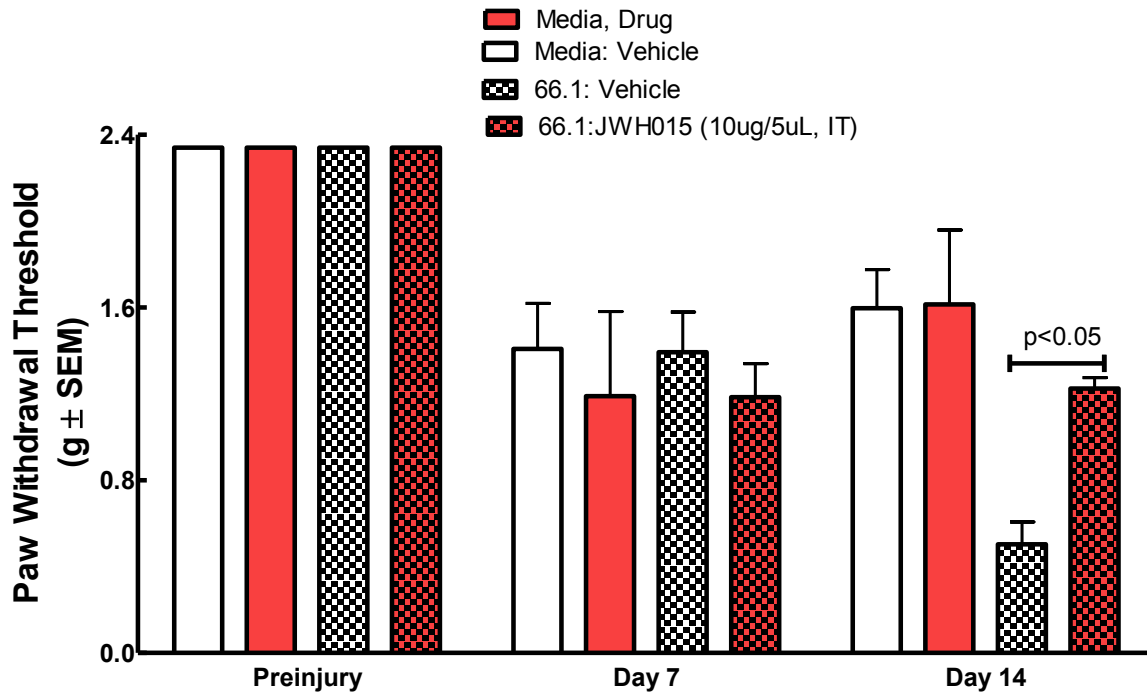


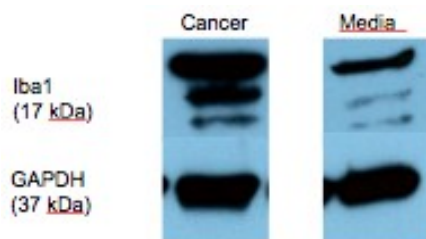
Fig. 18 Chronic spinal administration of the CB₂ receptor agonist JWH015 reduces cancer-induced pain behaviors. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral inoculation, animals demonstrated bone cancer-induced (A) flinching and (B) guarding. The CB₂ agonist JWH015 (10 ug/5uL, i.t.) 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 JWH015 ($p < 0.05$; $n = 10$) compared to animals that received vehicle ($n = 10$). No significant difference was observed between media-only control animals and vehicle treated ($n = 10$) or JWH015 treated ($n = 10$) animals.

5.3 Cancer induces up-regulation of glial markers.

To determine the mechanism by which JWH015 reduces cancer-induced bone pain on day 14, spinal cords were extracted for gel electrophoresis and western blots used to determine the protein expression of the Iba1 and GFAP markers. Cancer up-regulates microglial and astrocytes markers, Iba1 and GFAP, respectively compared to media (Figures 19A, 3B).

Figure 19

A



B

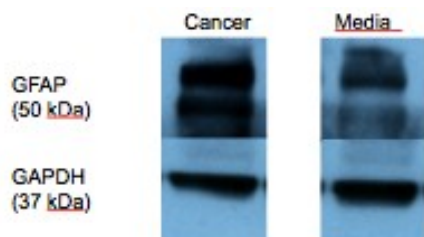


Fig. 19: Spinal cord expression of the glial markers, Iba1 and GFAP, after chronic spinal cord administration of JWH015 or vehicle. Breast cancer increases expression of Iba1 and GFAP compared to control animals as demonstrated via Western blot (n=3 animals per group).

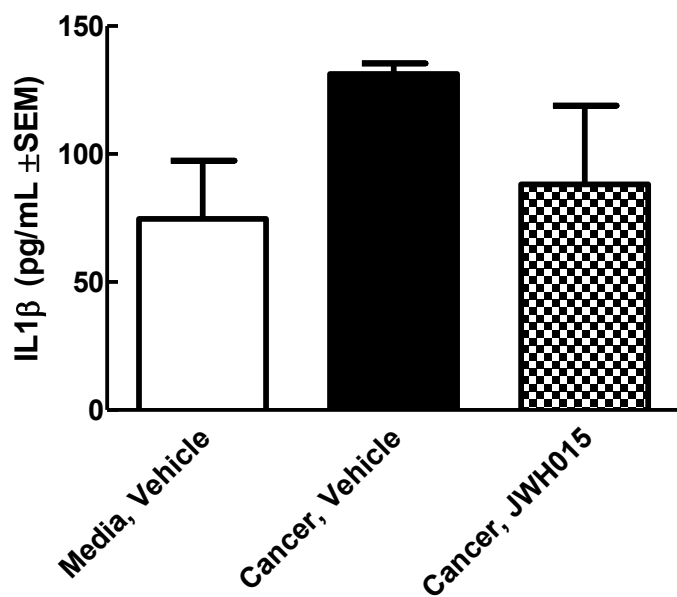
5.4 A CB2 agonist reduces spinal release of pro-inflammatory cytokines:

To determine the mechanism by which the CB2 agonist inhibits pain, on day 14 spinal cords were extracted and examined by an ELISA for the expression of the pro-inflammatory cytokines TNF α and IL-6. Cancer-induced animals treated with vehicle showed significant increases in the amount of TNF α and IL-6 present in the spinal cord compared to control animals (Fig. 20A, B). JWH015 reduced the cancer-induced release of pro-inflammatory cytokines TNF α and IL-6 in the central nervous system over

the amounts of TNF α and IL-6 released in cancer animals treated with vehicle. (Figures 20A, B).

Figure 20

A



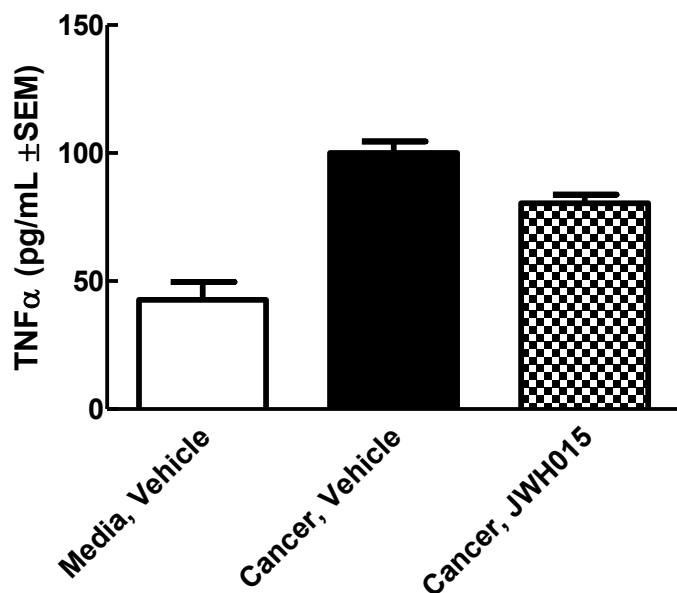
B

Figure 20: Cancer induces up-regulation of spinal cytokines, TNF α and IL1 β . Chronic central administration of JWH015 (10 μ g/5 μ L, i.t.) reduces amount of TNF α and IL1 β released in the spinal cord (n=3 animals per group).

5.5 Conclusion

Cancer metastasis to bone leads to serious pain that often decreases the quality of life for patients. Here we demonstrated that a CB2 agonist administered both acutely and for seven consecutive days reduces both spontaneous and evoked pain behaviors. We administered the drug within the spinal cord to determine the effects of a central CB2 agonist on bone cancer pain. The flinching and guarding data from this study demonstrated that after seven days of administration of the CB2 agonist, the number of flinches decreased and a decreased duration of time spent guarding compared to the animals treated only with vehicle. Tactile allodynia data showed that animals receiving the CB2 agonist had significantly higher paw withdrawal thresholds than animals

receiving vehicle. Similar studies utilizing other chronic pain models demonstrate JWH-015 is effective in reducing behavioral hypersensitivity and glial activation [219, 220].

Additionally, in several cancer pain models, direct spinal activation of CB2 receptors attenuates pain after acute administration of CB2 agonists [61, 88]. These studies fail to evaluate the efficacy of sustained administration of JWH15 or AM1241. Gu and colleagues suggest the antihyperalgesic action of cannabinoid 2 agonists is due to reduced NMDA expression and the ensuing spinal neural activity [88]. Curto-Reyes et al. imply reduction of cancer-induced bone pain may be the result of synergistic activation of opioid receptors [61]. However, both of these studies overlook the involvement of glial activity and only investigate the efficacy of acute administration of cannabinoid 2 agonists. On the other hand, we also found that cancer increases the expression of astrocytes (GFAP) and microglia (Iba1) in the spinal cord compared to control animals consistent with other groups looking at the neurochemical changes in the spinal cord [104, 230].

Results from our experiments also show a diminished release of the pro-inflammatory cytokines IL-6 and TNF α into the spinal cord in animals treated with a CB2 agonist. Other studies showed that the CB2 agonist JWH-015 led to a reduction in TNF α , microglial migration and glial modulator effects [220]. Bone cancer causes an increase in spinal astrocytes and microglia activation, an increase in spinal pro-inflammatory mediators that are inhibited by a CB2 agonist, hence decreasing behavioral signs of pain. Future research should examine whether a CB2 agonist will decrease cancer-induced up-regulation of microglia and astrocytes markers, as well as the effect of CB2 agonists on the release of other cytokines and chemokines.

CHAPTER 6: THE CATHEPSIN INHIBITOR VBY-825 ATTENUATES BREAST CANCER- INDUCED PAIN AND BONE REMODELING

6.1 Introduction

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 hypercalcemia, 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 hypocalcemia, 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 overexpression 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 (CatK) and cathepsin L (CatL), both expressed by osteoclasts, degrade collagen type I [128]. In addition, exogenous intrathecal rat recombinant CatS, but not rat recombinant cathepsin B or L, induced hyperalgesia suggesting that CatS but not other related cathepsins have a pronociceptive effect in the spinal cord [43].

Furthermore, in the RIP1-Tag2 transgenic model of pancreatic cancer, mice treated with VBY-825, reversible inhibitor of cathepsins S ($K_i=130$ pM), B ($K_i=330$ pM), V ($K_i=250$ pM), L ($K_i=250$ pM), K ($K_i=2.3$ nM), showed a significant reduction in tumor incidence and growth [73]. In this study, we additionally investigated the cathepsin inhibitor, VBY-825. A murine breast-induced bone cancer model was used to compare the efficacy of VBY-825 in attenuating bone cancer pain behaviors, improving bone integrity and decreasing tumor burden to zoledronic acid, a bisphosphonate commonly used in bone cancer treatment.

6.2 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.21A,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.21A, B)

Figure 21

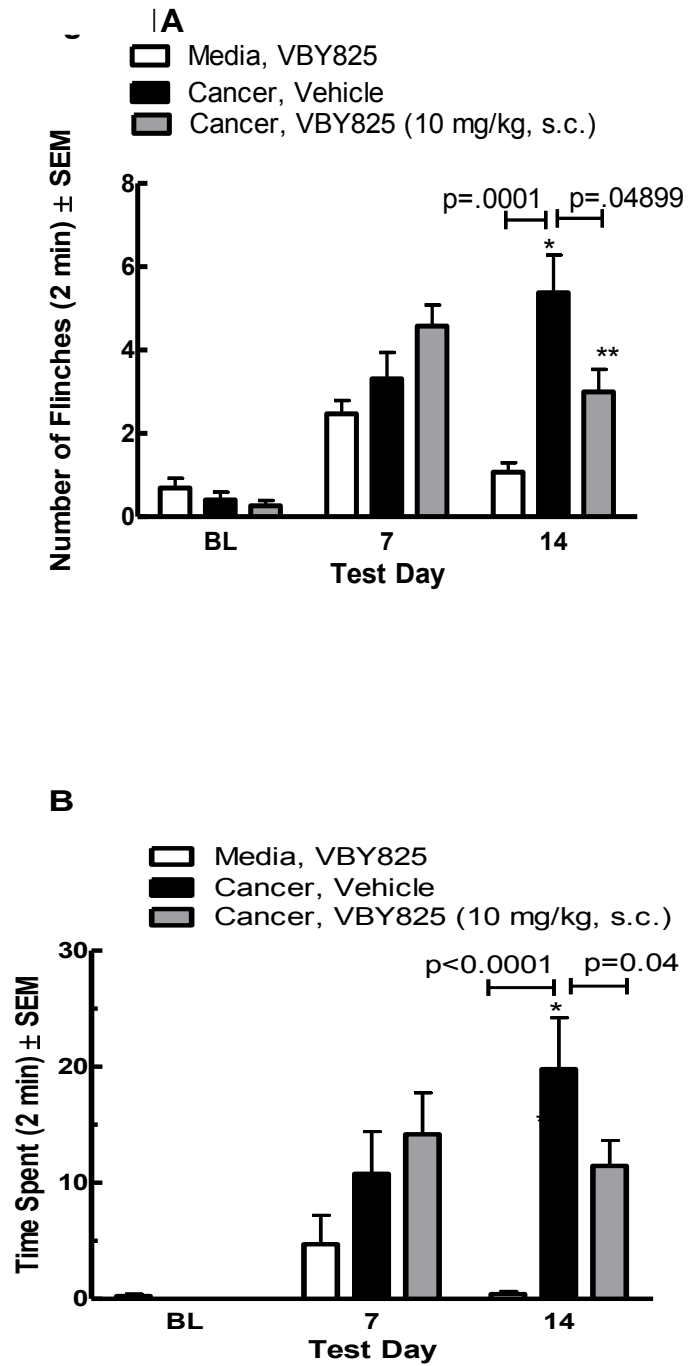


Fig. 21 Chronic subcutaneous administration of the cathepsin inhibitor VBY-825 reduces cancer-induced spontaneous pain behaviors. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral 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).

6.3 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 (Fig 221). Cancer-inoculated animals treated with VBY-825 for seven days show a significant increase in withdrawal threshold compared to vehicle treated animals (Fig 22C).

Figure 22

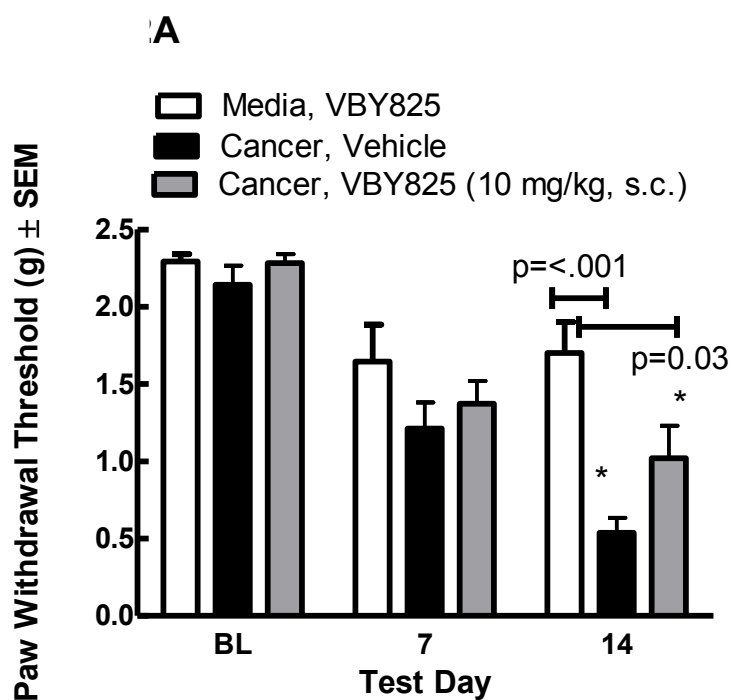


Figure 22: Chronic subcutaneous administration of the cathepsin inhibitor VBY-825 reduces cancer-induced evoked pain behaviors. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral 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).

6.4 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 23A). 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 23B). Cancer-induced animals treated with VBY-825 appeared to have less bone loss (Figure 23C).

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 23D). Sustained JWH015 treatment attenuated cancer-induced elevations in serum TRACP 5b (Figure 23D).

Figure 23

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



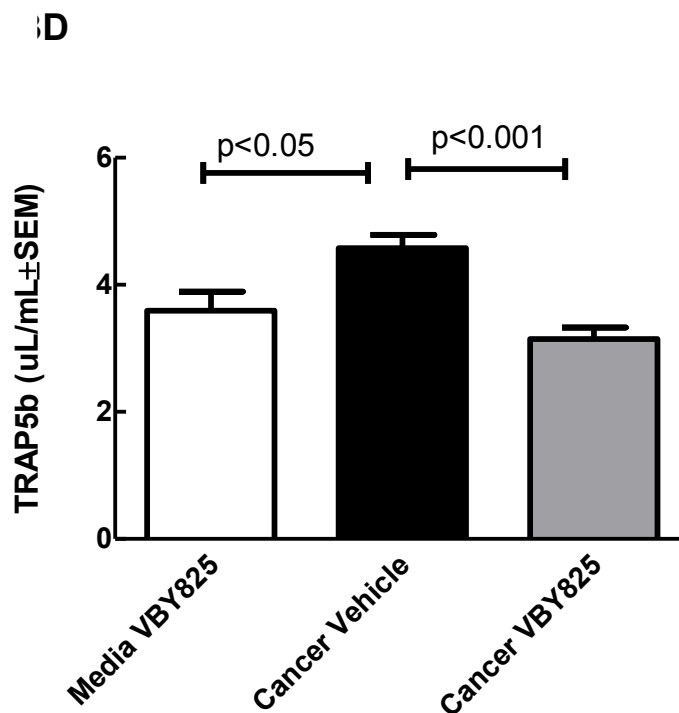


Fig 23 (A) Radiographs of the femora in the presence of either media (control) or breast cancer cells (66.1) on day 14 after inoculation. Mice received either vehicle or a cathepsin inhibitor, VBY-825 (10 mg/kg, s.c) from days 7 to day 14 after femoral inoculation. Bone loss (hypodense at proximal and 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

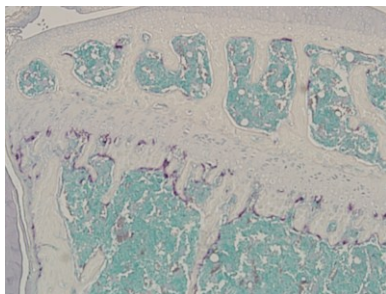
intra-femoral media treated animals. This increase in cancer-induced TRAP5b levels was significantly reduced in VBY-825 (10mg/kg, s.c., from day7 to 14) treated animals ($p < 0.001$; $n = 6$ per group).

6.5 VBY-825 significantly reduces osteoclast number

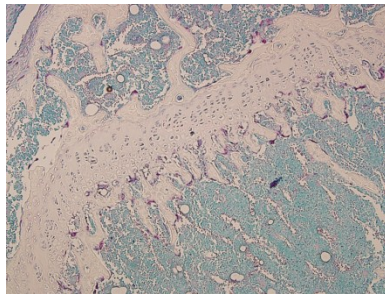
Decalcified bones were sectioned and stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts. Cancer-induced animals treated with vehicle showed a significant increase ($p < 0.05$) in osteoclast number compared to animals injected with cell-free media (Fig. 24A,B,D). A significant increase in number of osteoclasts was observed in cancer-induced animals treated with VBY-825 compared to vehicle treated animals (Fig 24B,C,D).

Figure 24

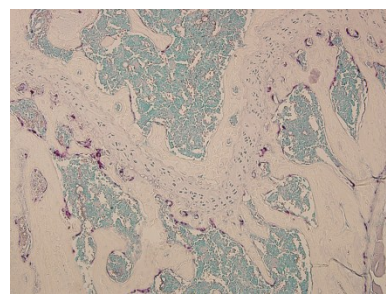
A. Media, VBY-825



B. Cancer, Vehicle



C. Cancer, VBY-825



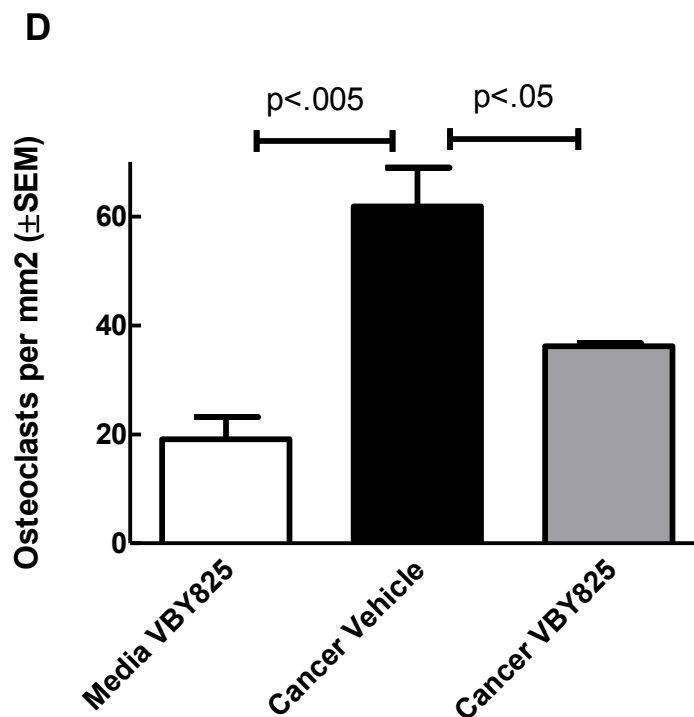


Figure 24 (A-C) On day 14, femurs were extracted from animals, decalcified, paraffin embedded, and stained for tartrate resistant alkaline phosphatase to visualize osteoclasts. Osteoclasts were counted at the distal end of femur. (D) A significant increase in osteoclasts was found in vehicle treated 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).

6.6 Conclusion:

Cathepsins are intracellular proteases overexpressed in human cancers and many chronic pain states. There are 11 different subtypes, each expressed by different

tissues and cell types. Cathepsins are found in the lysosomes of cells with roles in antigen presentation, apoptosis, autophagy and cellular homeostasis. These proteases contribute to tumor cell invasion, angiogenesis and metastasis. Thus, cathepsins may not only contribute to the development of cancer, but the accompanying cancer-induced pain as well.

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 CatS 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.

CHAPTER 7: DISCUSSION AND FUTURE DIRECTIONS

7.1 Cancer-induced bone pain

The most common cancer types, including breast cancer, prostate cancer, and lung cancer, have a propensity to metastasize to bone. Metastasis to bone subsequently induces cancer-induced bone pain (CIBP) [173, 207] and can be characterized as ongoing or breakthrough pain. Ongoing pain is described as dull in character, persistent and increasing over time [129, 206]. Breakthrough pain can be further characterized as “spontaneous pain,” ensuing without an apparent triggering event, as well as a “movement-evoked pain,” initiated by movement of the tumor-bearing bone [173, 207]. Breakthrough pain is often more intense and irregular than ongoing cancer pain, and can severely compromise a patient’s quality of life [207].

Cancer-induced bone pain depicts unique neurochemical changes from other chronic pain states including inflammatory or neuropathic pain. The bone itself is innervated with A β , A δ and C fibers [161]. Interestingly, the majority of human tumors arising in bone lack detectable nerve fibers within the tumor mass or peripheral bone in close proximity [186]. Instead, the acidic environment and secretion of substances, such as growth factors, cytokines, chemokines or from tumor cells themselves stimulate nearby primary afferent nociceptors inducing pain [179, 230, 265]. The principal challenge in understanding the mechanism of cancer pain was the development of an animal model of pain displaying similar characteristics to human cancer-induced bone pain. The first in vivo model of cancer-induced bone destruction entailed a tail vein injection of human plasma leukemia cells in severe combined immunodeficiency (SCID) mice resulting in the development of bone disease similar to

the clinical profile of multiple myeloma as assessed by blood ionized calcium levels, x-ray, and histology [5]. However, myeloma cells infiltrated vital organs such as liver and spleen in addition to vertebra and long bones [5]. Tumor burden within the spinal cord caused hind leg paralysis [5]. The degree of metastasis in this model does not allow for a localized assessment of nociceptive behaviors.

Another cancer model resulting in observable bone destruction involved inoculation of sarcoma cells inside the intramedullary space of a mouse's femur, sealed with dental amalgam, which allowed cancer cells to remain localized [230]. This model allows researchers to correlate cancer-induced bone remodeling, behavioral signs of pain and neurochemical changes in the spinal cord and primary afferent neurons. Following cancer-induced bone destruction in this model, increases in spontaneous and evoked pain behaviors over time can be correlated with primary afferent neurons sensitization, as evidenced by substance P receptor internalization and C-Fos expression [230]. In the tumor bearing femur, sarcoma also induces peripheral changes, such as up-regulation of activating transcription factor-3 (ATF-3), a marker for injured neurons, and macrophage infiltration in dorsal root ganglion (DRG) [230, 233]. Additionally, the spinal cord is reorganized in a manner similar to the central sensitization observed in other chronic pain states, including dynorphin up-regulation and astrocyte hypertrophy [104, 230, 233]. These data suggest changes in astrocyte activity in spinal cord are unique characteristics of CIBP.

Consistent with human tumors, mouse neoplasms contain few nerve fibers, particularly those expressing calcitonin gene related peptide (CGRP) [186] [233]. However, human studies have indicated that the tumor microenvironment causes an

abnormal remodeling of nearby sensory nerve fibers perceived as painful by patients [148], [24] [37]. Similarly, in this established mouse model, the tumor does not become innervated [230]. Rather, changes occur in the periosteum surrounding the bone extracellular matrix. The periosteum expresses CGRP and substance P (SP), neuropeptides expressed by a subgroup of small neurons in DRG, trigeminal, and vagal ganglia. These small neurons expressing CGRP and SP respond to noxious, thermal, or visceral input and are described as peptidergic [244]. In naïve animals, CGRP and SP fibers are found in mineralized bone, marrow, and periosteum enveloping bone extracellular matrix. Of these three tissues, the periosteum contains the highest amounts of CGRP and SP [233]. However, unlike skin innervation, bone does not have significant Mrgprd(+) and P2X3(+) C-fiber innervation suggesting that unique therapies are needed to target skeletal pain versus skin pain [122]. Interestingly, sarcoma, prostate and breast cancer induce ectopic sprouting of sensory nerve fibers and the formation of neuroma like-features in the periosteum [26, 82, 120, 121, 168]. These nociceptive sensory nerve fibers innervating the bone appeared reorganized and more dense than control animals. [26, 82, 120, 121, 168].

Furthermore, the model developed by Schwei et al. [230] allows for development of pain behaviors similar to cancer-induced bone pain exhibited in humans. It can be modified to include inoculation into other bone types (i.e. tibia), model host variation (i.e. mice or rats), and investigation of different tumor types in bone microenvironment. Utilization of this model in the last 14 years has provided significant insight to the etiology of cancer pain and the possibility of more efficacious therapeutics for this distinct pain profile.

7.2 Mechanisms of cancer pain

7.2.1 Neurotrophins

Nerve growth factor (NGF), brain-derived neurotrophic factor, (BDNF) and neurotrophin-3 (NT-3) are a family of molecules whose cognate receptors are tropomyosin-related kinase (Trk) A, B and C, respectively [238]. Neurotrophins also bind the p75 receptor. These proteins regulate survival, development and function of subsets of sensory and sympathetic neurons, essential to the maintenance and generation of pain [209, 167]. Neurotrophins are normally expressed in low levels in the majority of adult tissues, becoming up-regulated with inflammatory or injurious states, particularly NGF.

ARRY-470 (Array BioPharma, Boulder, CO) is a selective inhibitor of neurotrophin receptors, exhibiting nanomolar cellular inhibition of TrkA (6.5 nM), TrkB, (8.1 nM), and TrkC (10.6 nM) with a high level of selectivity over a panel of kinases and non-kinase receptors [82]. Doses between 10-100 mg/kg ARRY-470 can obtain high concentrations in plasma and peripheral tissues, while the brain concentrations remain negligible, implying limited crossing of the blood brain barrier [82]. In a mouse model of CIBP, ARRY-470 attenuates spontaneous and evoked pain behaviors compared to untreated animals [82]. ARRY-470 can also effectively diminish nerve fiber sprouting and neuroma formation in the periosteum [82]. Although these data suggests neurotrophins can reduce pain, it does not allow distinguishing between receptors.

Once bound to its cognate receptor, NGF can modulate the function of proteins expressed by nociceptors, such as neurotransmitters (substance P and CGRP), receptors (bradykinin), channels (P2X3, TRPV1, ASIC-3, and sodium) and structural

molecules (neurofilaments and p11), namely by up-regulating their expression [94] and resulting in increased nociceptive activity. NGF can also be expressed by tumor, inflammatory and immune cells that may also add to CIBP [233]. The monoclonal antibody, MAb 911, (Rinat/Pfizer) is a sequestering antibody that inhibits the selective binding of NGF to TrkA and p75 over TrkB or TrkC receptors in rodents and humans [103]. The antibody also blocks TrkA autophosphorylation [103] and has a half-life of approximately five days in mice [233]. In mouse models of CIBP, nociceptive sensory nerve fibers innervating the bone express TrkA receptors, and treatment with MAb 911 towards NGF in this model attenuates cancer-induced behavioral signs of pain [94, 120, 121, 26, 168]. Treatment with anti-NGF modulates peripheral changes in DRG and central changes in spinal cord [233]. Early and sustained administration of MAb 911 induces marked reduction of sprouting (indicated by CGRP+ expression) in nerve fibers in the tumor-bearing bone and without affecting the density of CGRP+ sensory nerve [26, 94, 120]. This provides evidence that NGF/TrkA signaling over other neurotrophin subtypes may be more important in cancer pain [121, 168]. Recent studies of BDNF have demonstrated a role in the induction and maintenance of behavioral hypersensitivity in a rat model of CIBP by BDNF modulation of the NMDA subunit 1 (NR1) at the level of the spinal cord and DRG [262]. Upregulation of BDNF in descending pain modulating areas of the rostral ventromedial medulla (RVM) have been reported in a rat model of CIBP [153] suggesting that BDNF may also play a role in CIBP, yet clinical evidence of anti-NGF or anti-BDNF attenuation of CIBP is unknown.

7.2.2 Acid sensing ion channels

Neoplasms are comprised of a heterogeneous population of cells essential to tumor microenvironment function. Besides the prototypical cancer cells, containing oncogenic and tumor suppressor mutations characterizing cancer, cancer stem cells, pericytes, cancer-associated fibroblast, stem and progenitor cells of the tumor stroma, and particularly immune pro-inflammatory cells tumor promoting cells that may include macrophage subtypes, mast cells, neutrophils, T and B lymphocytes [96]. Furthermore, they release many different signaling molecules, including protons, which promote an acidic environment. Cancer cells exhibit a reversed pH gradient from normal cells, having a higher intracellular pH (~7.4) and a lower extracellular pH (~6.7-7.2) [96]. Advantages of reversed pH gradient include growth factor independent proliferation, evasion of apoptosis, migration and invasion and reprogramming energy metabolism [31-33, 96]. Excessive cancer cell proliferation and the typically poor vasculature of the central tumor mass results in hypoxia ([31-33]. As an adaptation to hypoxia, cells can up-regulate glucose transporters, such as GLUT1, and enzymes of the glycolytic pathway[31-33, 96] This increase in glycolysis then increases acid production [31-33, 96].

Upregulation of growth factors, cytokines and chemokines in the tumor microenvironment, disrupt normal bone metabolism maintained by osteoclasts, bone resorbing cells, and bone forming osteoblasts. As extracellular pH increases, mineralization of pre-osteoblasts and transcription of the osteoblastic gene, osteopontin increases *in vitro* [116]. The decrease in extracellular pH in metabolic acidosis reduces osteoblast mediated collagen synthesis and alkaline phosphatase activity [137].

Osteoclasts mediate bone resorption through secretion of proteases such as cathepsin K, B-glucuronidase and the generation of protons [47, 137]. Extracellular protons effectively sensitize primary afferent neurons [22, 138, 214]. More specifically, in CIBP, breast cancer upregulates ASIC1a/b expression in the primary sensory neuron which likely contributes to hyperalgesia [184]. In normal and cancer-bearing bone, sensory fibers of mineralized bone and bone marrow express the Transient Receptor Potential Channel, Vanilloid subfamily member 1 (TRPV1) [83]. Thus, persistent activation of acid-sensing channels may not be the only channels that contribute to CIBP.

TRPV1 channel is a member of the six transmembrane domain heterotetramer TRP ion channel super family modulated by not only low pH but also by capsaicin, resiniferatoxin (a capsaicin analogue), noxious heat (>43 °C), voltage, and endovanilloids [36, 248]. TRPV1 is expressed by a variety of cell types including astrocytes, perivascular structures and neurons [198]. TRPV1 has been found in DRG, small C and A δ sensory fibers, and co-localizes with TrKA [198]. In human osteoclasts in vitro, biomolecular and functional experiments showed that resiniferatoxin (RTX), a selective TRPV1 receptor agonist, increased the expression and activity of the osteoclast biomarkers, TRAP and cathepsin K [198]. Capsazepine has been shown to inhibit osteoclastic bone resorption, osteoblast activity and bone formation [111]. Inhibition of TRPV1 also protects against ovariectomy induced bone loss in mice [111]. This implies pharmacological blockade of TRPV1 may interfere with osteoclastogenesis, reducing one source of extracellular proton production and ensuing pain.

In rat and mouse models of squamous cell carcinoma, upregulation of TRPV1-positive large-sized neurons were observed in the dorsal root ganglion [10, 237].

Administration of the competitive TRPV1 antagonist, capsazepine reduced the thermal hyperalgesia and mechanical allodynia induced by the cancer [10, 237]. Utilization of the sarcoma-induced bone cancer model provided evidence that a significant portion of sensory neurons that innervate the tumor-bearing bone express TRPV1 [83]. JNJ-17203212, a selective potent antagonist of both rodent and human TRPV1, with an IC50 value of 38 ± 10 nM, was used in a murine bone cancer model [246]. In TRPV1 wildtype animals (+/+) and TRPV1 heterozygotes (+/-), spontaneous- and movement-evoked pain were inhibited through chronic treatment with the TRPV1 antagonist JNJ-17203212. In contrast, TRPV1 null mice (-/-) exhibited reduction in pain behaviors not further attenuated with JNJ-17203212 treatment, implying attenuation of pain was mediated by TRPV1 [83]. Sarcoma cells in vitro were found to release a lipophilic substance that activates TRPV1 in dorsal root ganglion neurons [141]. Activation of TRPV1 with this releasing substance was blocked by a TRPV1 antagonist [141]. These data suggest that not only is the acidic environment created by cancer promoting sensitization of TRPV1, but that some cancer cells may also be producing endogenous agonists for TRPV1 receptors [141].

One acid particularly important to TRPV1 sensitization may be lysophosphatidic acid (LPA), a lipid metabolite released by cancer cells and blood platelets after tissue injury known to induce proliferation, adhesion, migration, morphogenesis, differentiation and survival [29, 75]. Ascitic fluid and plasma of cancer patients contain high levels of LPA and the ascitic fluids or the LPA have been shown to facilitate bone metastasis by stimulating secretion of IL-6 and IL-8 [29,70, 141]. In naïve animals, TRPV1 and LPA1

receptors in DRG co-localized, enabling possible crosstalk [199]. This was verified by TRPV1 currents being potentiated by LPA and blocked by the TRPV1 antagonist capsazepine and LPA1 antagonist VPC32183 [199]. In a rat model of CIBP, the LPA1 receptor antagonist VPC32183, attenuated mechanical allodynia and thermal hyperalgesia, suggesting release of LPA from cancer cells and cross-talk between LPA1 and TRPV1 in CIBP [199].

ASICs belong to the epithelial sodium channel (ENaC)/degenerin (DEG) superfamily of ion channels [64]. Seven subtypes (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC4 and ASIC5) have been identified in rodents [64]. They can form homotetramers or heterotetramers, each depicting a distinctive functional heterogeneity. These channels are characterized by their sensitivity to an extracellular decrease in pH [260]. In rodents, ASICs are expressed throughout the nervous system [195, 260]. ASIC1b and ASIC3 were identified exclusively in dorsal root ganglion primary afferent neurons [195, 260]. ASIC channels are also expressed by osteoblasts and osteoclasts, and may explain how bone cell function can be modulated by environmental pH under physiological (e.g. bone resorption) and pathological (e.g. bone metastases) conditions [116].

ASIC3 is unique within its family since the channel produces a rapidly inactivating peak current and a sustained current [64, 260]. This sustained current is thought to contribute to non-adapting pain induced by a range of acidities [64]. ASIC3 is important for inflammatory pain in the peripheral nervous system [64]. In DRG neuron primary culture, pro-inflammatory mediators, specifically NGF, serotonin, interleukin-1 and

bradykinin, reportedly up-regulate the expression of ASIC3 and leads to their hyperexcitability. This finding suggests a mechanism by which ASIC3 channels contribute to hyperalgesia in vivo [165].

Investigation of ASIC3 knockout mice in the presence of nociceptive stimuli indicates the ASIC3 channel can have a pro-nociceptive or modulatory role in pain sensation, dependent on location and heterotetramer composition. Furthermore, in a neuropathic pain model sciatic nerve ligation of L5 (SNL) induces hyperalgesia to mechanical and thermal stimuli [132]. In this model, ASIC currents are modulated and the expression of ASIC subunit mRNA is altered in DRGs from injured animals, providing evidence of ASIC channels in neuropathic pain [204]. Omori et al. [196] demonstrated injection of acetic acid to SNL rats evoked an increase in spontaneous pain and mechanical allodynia compared to sham animals, providing evidence that ASIC3 is associated with hyperalgesia in response to an acidic stimulus in this neuropathic pain model. Additionally, application of acetic acid to neuropathic animals increased the total ASIC3-ir neurons in the L4 DRG and modified the cell size of ASIC3-ir neurons in the L4/5 DRGs [196]. These studies suggest ASIC3 modulation contributes to maintenance and generation of acute inflammatory and chronic neuropathic pain. With the unique expression of ASIC channels found solely in the bone and nervous system to date, pharmacological modulation of ASICs warrant attention for bone degenerative diseases, including bone metastases.

7.2.3 Inflammatory mediators

A variety of small proteins produced by immune cells that facilitate intercellular communication can be termed cytokines [9]. Two cytokine subtypes, the pro-inflammatory cytokines and the chemokines, have been shown to contribute to inflammatory and neuropathic pain (for review see [259]). This has been demonstrated through use of cytokine inhibitors, KO mice and direct application of cytokines administered within the central and peripheral nervous system [259, 281]. Osteoclasts are derived from a monocyte/macrophage hematopoietic lineage [30]. Thus, it is unsurprising that osteoclasts have receptors for IL-1 β , TNF α , IL-6 and TGF β and induce osteoclastogenesis [30]. Furthermore, it is well established that cytokines play a role in tumor development and metastasis and their effects vary depending on the type and location of the tumor [69]. With the ability to modulate pain both centrally and peripherally and stimulate bone resorption, up-regulation of cytokines in bone microenvironment may therefore contribute to peripheral and central components of CIBP.

Pro-inflammatory cytokines include IL-1 β , TNF α , IL-6 and TGF β . Baamonde and colleagues demonstrated that in an osteosarcoma model, cancer-bearing animals had a decrease of IL-1 β levels at the site of tumor and in the spinal cord and that an IL-1R-receptor antagonist attenuated cancer-induced thermal and mechanical hyperalgesia [11]. Studies utilizing bone cancer models in wildtype or transgenic animals, lacking TNF α receptors TNFR1 and TNFR2, implicate the involvement of TNF gene in cancer-induced hyperalgesia, spinal astrogliosis, and tumor growth [80, 89]. Studies by Constantine and colleagues suggest that TNF α sensitized TRPV1 channels via

p38/MAP kinase- and PKC-dependent pathways and upregulate TRPV1 in cultured DRG neurons [49]. In a soft tissue cancer/metastasis model, cancer-induced heat hyperalgesia correlates with an increased expression of TRPV1 protein possibly due to cytokines [49].

Evidence supports roles for chemokines in addition to cytokines in CIBP. Development of co-cultures with femur and cancer cells, up-regulates MCP-1 and TGF β expression and secretion in sarcoma, but down-regulates MCP-1 and TGF β expression and secretion in breast cancer cultures [229]. However, recent studies measuring cytokines and chemokines *in vivo* in a murine model of breast cancer demonstrated a significant increase in MCP-1, MIP-1A, IL-6 and TNF α [156]. These cytokines and chemokines were significantly reduced by the systemic administration of a cannabinoid-2 receptor agonist suggesting a novel treatment in bone cancer pain [156]. These results provide further evidence that cancer in the bone environment modifies cytokines and chemokines present. Additionally, intrathecal administration of an anti-MCP-1 neutralizing antibody attenuated the mechanical allodynia established in a rat model carcinoma induced bone pain [107]. Over an 18 day period of carcinoma implanted in the tibia, expression levels in the spinal cord of CX3CR1, the receptor for fractalkine, gradually increased [106,278] suggesting the rise in central chemokine-receptor interactions may promote pain in a tibia model of cancer pain. Hence, intrathecal injection of a neutralizing antibody against CX3CR1 reduced mechanical allodynia in cancer-induced rats with further studies suggesting CX3CR1 mediated pain through microglia and p38 mitogen-activated protein kinase (MAPK) activation in the spinal cord [106].

7.2.4 Oxidative Stress

Oxidative stress is an established hallmark of tumor burden and decidedly plays a role in multiple types of chronic pain, including inflammatory and neuropathic [152, 263, 126, 92, 151, 228]. In murine models of breast cancer and in human cancers, studies detect systemic and tissue-specific elevations of lipid markers of oxidative stress and associated changes in detoxification enzymes including glutathione peroxidase (GPx) and superoxide dismutase (SOD) isoforms [90, 98]. Specific antioxidants including SOD mimetics have been utilized to relieve pain in preclinical models however little work to date has investigated the therapeutic potential of antioxidant therapy for CIBP [92, 131, 67, 277, 224, 213]. Sources of oxidative stress in the tumor-bone microenvironment include cancerous cells and a diverse population of tumor stromal cells (including monocytes and T-lymphocytes) that elicit a stress response from both local cells involved in bone homeostasis and innervating sympathetic and nociceptive fibers. The pro-nociceptive action of oxidative stress in CIBP can be understood as a function of stress-induced production of glutamate as an algogenic substance by cancer cells, and secondly as a result of tumor-derived nitrating species altering the response to glutamate by primary afferent neurons. Glutamatergic transmission plays a key role in several types of pain including cancer pain [197] [40] [256] and is also vital for normal bone homeostasis [55].

In cancer cells, glutamate secretion is tightly coupled to the uptake of antioxidant precursors [235]. Several types of cancer cells have adapted to express the glutamate-cystine antiporter, system x_c , which passively exchanges intracellular glutamate as a byproduct of metabolism for extracellular cystine [155]. In the cell, cystine is cleaved

into two cysteine residues for incorporation into the potent antioxidant glutathione. where h it plays a profound role in resilience against oxidative challenge [113]. Accordingly, elevated cysteine consumption can be detected in chemoresistant subgroups of human MCF-7 breast cancer cells [223]. Tumoral expression of system x_c is thought to confer protection against oxidative stress as an adaptation to a metabolic abnormality known as the Warburg effect, or the observation that cancer cells rely upon anaerobic glycolysis for ATP generation in low-glucose hypoxic environments [264, 139]. The Warburg effect results in the inefficient use of glucose and excessive generation of reactive oxygen species as the normal byproducts of respiration [14]. Therefore, system x_c facilitates the removal of both metabolic waste and harmful oxidants from cancer cells at the expense of extracellular glutamate in the bone-tumor microenvironment. AMPA receptors, NMDA receptors and mGluR1 and 5 are expressed at the peripheral terminals of primary afferent nociceptive neurons [25]. Therefore, glutamate derived from cancer cells may evoke nociceptive signaling and lead to persistent nociceptive states found in CIBP [256]. Furthermore, because glutamate is a vital communicator in bone homeostasis and function, elevated glutamate may lead to further dysregulation of bone and add-on mechanisms of pain. Indeed, extracellular glutamate released from bone tumor cells induces osteoclastogenesis and modulates osteoblast function [232].

Tumor burden also generates oxidative stress in the bone-tumor microenvironment. In pathological states, induction of a constitutively active form of nitric oxide synthase iNOS results in the generation of both nitric oxide (NO) and peroxynitrite (ONOO⁻) as reaction products of NO with byproducts of cellular respiration.

Pathological generation of these radicals results in nitration modification of protein function and stress signaling events. In the context of CIBP, both cancer cells and tumor stromal cells act as a source of NO. The viability of breast cancer lines MCF-7, T47D and 4T1 is promoted by the uptake of the NO precursor L-arginine and its conversion to nitric oxide [1]. Our own laboratory has demonstrated basal release of NO from the murine 4T1 and 66.1 mammary carcinoma cell lines *in vitro* (unpublished). Furthermore, in human breast cancer tumors, aberrant iNOS expression and production of NO is thought to activate oncogenic pathways, select for mutant tumor suppressor loss, induce stem cell-like tumor characteristics and promote tumor viability [7]. Because NO diffuses easily from its site of release, primary afferent neurons innervating the bone-tumor microenvironment are vulnerable to reaction with NO and subsequent nitration events. Exposure to nitrating agents derived from NO increases the sensitivity of the NMDA receptor to glutamate *in vivo* by increasing the receptor affinity for glutamate [279] and calcium conductance. In cortical synaptosomes, it was demonstrated that nitration events may be specific to the NR1, NR2a and NR2b subunits of the NMDAR [280]. Accordingly, NMDA antagonists have demonstrated efficacy in preclinical models of pain associated with nitration events [78] and in a model of CIBP [215]. It has also been posited that nitration of glutamate recycling mechanisms may contribute to enhanced glutamatergic neurotransmission in the bone-tumor microenvironment: the nitration of glutamate transporters including GLT-1 and GLAST reduces uptake of glutamate which may lead prolonged synaptic glutamate concentrations [253]. Understanding mechanisms of nitration-mediated enhancements

in glutamatergic transmission in the context of CIBP models is an on-going effort that requires research attention.

7.2.5 Cathepsins

Cysteine cathepsins (B, C, F, H, K, L, O, L2/V, W, X/Z) are intracellular proteases each expressed by distinct tissues and cell types [73]. In particular, cathepsin S (CatS) is an important mediator of pain released primarily from immune cells, macrophages, dendritic cells and microglia. Inhibitors targeting cathepsin S (CatS) 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 CatS. CatS cleaves the cytosine bonds in fractalkine (FKN), a neuronal transmembrane protein, allowing soluble FKN to activate the CX3CR1 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 CatS

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; CatK inhibitors suppress the function of osteoclasts while maintaining their viability [118]. Clinical trials evaluating the efficacy of inhibitors of CatK 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-1 β and IL-18 [245]. These chemokines are strongly linked with the induction of chronic inflammatory pain. Relevant to CIBP, the cascade caused by CatB activation in bone could be, in part, responsible for the symptoms of hypercalcemia, anemia, skeletal fractures, and decreased mobility.

Furthermore, the reversible cathepsin inhibitor VBY-825, a potent inhibitor of CatS, CatL, CatV, CatB, CatK and CatF has multiple mechanisms to potentially attenuate CIBP. Inhibition of CatS 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 CatB and CatK, 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 CatB

and CatK 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.

7.2.6 Cannabinoid receptors

The cannabinoid receptors most investigated include the CB1 and CB2 receptors. The CB1 receptor is the most highly expressed GPCR in the CNS, while CB2 receptor is highly expressed in the periphery, mainly immune associated. Modulation of the cannabinoids 1 and 2 receptors has been found to elicit analgesic effects in inflammatory, acute and chronic pain. In recent years, increasing evidence implicates endocannabinoids and CB1R and CB2R in CIBP.

Increases in endogenous cannabinoids produce analgesia presumably by inhibiting enzymatic degradation of cellular re-uptake. For example, mechanical hyperalgesia caused by implantation of fibrosarcoma in calcaneus bone was attenuated with an intraplantar injection in tumor-bearing limb of AEA and URB597, a FAAH inhibitor, both leading to an increase of FAAH activity demonstrated by increase of FAAH mRNA [130]. These effects were inhibited with concurrent administration of a CB1 receptor antagonist implicating AEA produces analgesia by acting in concert with the CB1 receptor [130]. In contrast to AEA, 2-AG has been implicated in reduced cancer-induced hyperalgesia via CB2 receptor activation [130]. The authors suggest CB2 receptors on keratinocytes, tumor cells, and immune cells reduce hyperalgesia indirectly by preventing the secretion of algogenic mediators that increase the

excitability of nociceptors [130]. Additionally, CB2 receptors on keratinocytes possibly inhibit ATP release [130]. Notably, antagonism of the P2X receptor improves nocifensive behavior in CIBP [130]. Furthermore, pharmacological manipulation of endogenous levels of both AEA and 2-AG in the periphery may be promising approach to treat CIBP without the side effects implicated with systemic cannabinoid administration.

Agonists of cannabinoid receptors have been shown to attenuate CIBP across several different cancer types. In rat models of CIBP, WIN 55,212-2, a non-selective CB receptor, attenuates mechanical allodynia via CB1 and CB2 receptors [57,208]. Liu et. al present evidence suggesting cannabinoids produce analgesia in CIBP through interacting with ASICs [154]. Another non-selective activator of CB1 and CB2, CP 55,940, reduces tumor-evoked hyperalgesia [95]. Simultaneous administration of a CB1 agonist and a CB2 agonist synergistically improves mechanical hyperalgesia in a model of CIBP [130]. To avoid unwanted CB1-induced CNS effects and the development of tolerance, a low dose of 0.5 mg/kg/day, ineffective at reducing pain with acute administration, with chronic administration (lasting 18 days) reduced pain related behavior and expression of spinal glial fibrillary acidic protein in the CIBP but not in a neuropathic pain model [93]. This implies pain is being attenuated from inflammatory mechanisms and not damaged neurons [93]. Hald et. al also found an increase in the expression of CB2 receptor in the DRG, likely due to an increase of macrophage infiltration [93]. This increased expression of CB2 receptors in the DRG in chronic pain explains why CB treatment is effective in hypersensitive subjects, but not healthy controls [93].

In CIBP, spinal administration of CB1 receptor with CB1 agonist arachidonyl-2-chloroethylamide (ACEA) reduced spontaneous and movement-evoked pain [79]. Additionally, authors show that CB1 receptor expression remains unaltered although mu-opioid receptor 1 expression was reduced in the superficial dorsal horn contributing to the processing of nociceptive transmission from the periphery to the central nervous system [79]. CB1 receptors were localized on axon terminals; however, they were not expressed in the dendritic process in the superficial dorsal horn [79]. Agonism of the CB1 receptor with a CB1 agonist or AEA in combination with an NSAID resulted in antinociception suggesting that CBR activation with current analgesics may be synergistic (Hama 2013; Ruggieri 2010; Guindon 2006). CB1 and MOR colocalize on the same neurons, activating the same brainstem circuitry, and possibly CB2 receptors (Seely 2012). Evidence demonstrates CB1 antagonist binds MOR while naloxone blocks the attenuation of CB2 mediated mechanical allodynia and thermal hyperalgesia. (Seely 2012; Curto-Reyes 2010) However, to delineate the synergistic activity between cannabinoid receptors and MOR, future studies should determine whether opiate or cannabinoid agonists in a cancer model developed by Schwei et. al. result in synergy. Additionally, inoculation of melanoma (within tibia) or osteosarcoma (within tibia or femur) elicited mechanical allodynia and thermal hyperalgesia attenuated with systemic or intrathecal administration of CB2 agonists, blocked by CB2 antagonists but not a CB1 antagonist [61,88]. Gu et. al also find CB2 receptor activation decreases cancer-induced up-regulation of NMDA in the spinal cord providing a mechanism by which CB2 receptors reduce CIBP [88]. Preliminary data suggests that spinal administration may attenuate pain in the CNS via pro-inflammatory mediators released by glial cells. Future

studies to confirm CB2 receptor activation attenuates glial activity include immunohistochemistry analysis of astrocyte marker, GFAP, and the microglia marker IBA1.

Moreover, treatment with cannabinoids in multiple *in vivo* and *in vitro* models has suggested anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effects in many cancers, including lung, glioma, thyroid, lymphoma, skin, pancreas, uterus, breast, and prostate carcinoma [3]. For example, JWH-133, the CB2 receptor-selective agonist reduced tumor growth, incidence and degree of lung metastases in MMTV-neu mice [35]. Histological analyses of the tumors unveiled that cannabinoid 2 agonists inhibit cancer cell proliferation, induce cancer cell apoptosis and impair tumor angiogenesis [35]. In CIBP model investigated herein, CB2 agonists attenuated pain behaviors and bone remodeling elicited by cancer [157].

Currently, dronabinol and nabilone, non-selective partial agonists of both CB1 and CB2 receptors, have been approved by the FDA to assuage adverse effects of the nausea, vomiting and lack of appetite associated with chemotherapy [250]. In a pilot phase I study, patients with glioblastoma multiforme after failing standard therapy, were treated with Δ^9 -tetrahydrocannabinol (THC) to define the safety of THC administration [91]. Doses given to patients did not result in significant psychotropic effects. Additionally, treatment appeared to improve clinical symptoms, such as dysphasia, cranial hypertension, cephalalgia, hallucinations, or motor deficit and to reduce tumor-cell proliferation, although significance was not achieved due to the small cohort size [91]. This study suggests that in addition to improving pain in cancer patients, cannabinoid agonists may have anti-tumor effects in humans. Further clinical studies must be conducted to further validate this hypothesis. The

future of cannabinoid treatment in cancer patients may be in the use of selective cannabinoid 2 agonists to avoid psychotropic effects induced by activation of the CB1 receptors.

7.3 Conclusion

Cancer-induced bone pain has distinctive neurochemical characteristics from other chronic pain conditions such as inflammatory or neuropathic pain. These include astrocyte hypertrophy, c-Fos expression and internalization of substance P receptor, as well as inflammatory components. Unsurprisingly analgesics utilized to treat inflammatory or neuropathic pain ineffectively manage cancer pain. The development of future efficacious analgesics for CIBP should emphasize a compound that attenuates pain via multiple mechanisms. Here, significant evidence of two investigational compounds demonstrates that may be attenuated pain via several independent mechanisms resulting in analgesic efficacy. Pre-clinical evidence suggests VBY-825 can attenuate pain, bone resorption, and reduce tumor growth in CIBP by inhibiting multiple cathepsins expressed by different cell types with nanomolar affinity. Specifically, inhibition of CatS modulates microglia activity to reduce production of inflammatory mediators and transmission of pain in the CNS. Additionally, inhibition of CatK may reduce osteoclast-mediated bone resorption. Additionally, Lozano-Ondoua et al. demonstrates agonism of CB2 receptor attenuates pain, reduces cancer cell proliferation, and modifies bone remodeling in mouse model of CIBP [156,157]. Thus far the mechanism of CB2 agonist inhibition of pain would include the reduction of cytokines and chemokines in both the bone microenvironment and in the spinal cord via CB2 receptors on tumor activated macrophages and microglia, respectively. In addition, CB2 agonists significantly inhibit osteoclast activity and osteoclastogenesis,

reducing the loss of bone and pursuing pain. Finally, there may be a direct CB2 mediated effect on the reduction of some type of cancer proliferation as demonstrated in our murine breast cancer model. CB agonist should be pursued for their effects in bone cancer pain along with current treatments such as opioids. Other groups have demonstrated that agonist activation of CB1 and CB2 receptors can result in synergism, or cannabinoid receptor activation with NSAIDS or opiates having synergistic analgesic capacities likely due to the inhibition of pain via different mechanisms [152, 263, 126, 92, 151, 228]. This would allow for lower doses of CB agonists and opiates achieving analgesic efficacy without unwanted CNS effects. Animal models suggest manipulation of endogenous CBs production and reuptake also has potential to attenuate CIBP. Clinical studies of such compounds are necessary to determine whether these compounds would be safe and effective treatment options in humans.

APPENDIX A: LIST OF PUBLICATIONS

MANUSCRIPTS

- **Lozano-Ondoua AN**, Bui L, De la Torre D, Rodriguez J, Havelin J, Bilsky E, Vanderah TW. "Cathepsin inhibitor VBY-825 attenuates breast-cancer induced bone remodeling and pain." *Manuscript in preparation*
- **Lozano-Ondoua AN**, Symons-Liguori AM, Vanderah TW. "Mechanisms of Cancer Induced Bone Pain." *Neuroscience Letter*, 2013
- **Lozano-Ondoua AN**, Hanlon KE, Largent-Milnes TM, Chandramouli A, Umaretiya PJ, Nelson MA., Mantyh PW and Vanderah TW. "Cannabinoid receptor 2 compounds in the attenuation of breast cancer proliferation: mechanisms of Action," *manuscript in review, Journal of Cancer Review*, 2013.
- **Lozano-Ondoua AN**, Hanlon KE, Largent-Milnes TM, Chandramouli A, Owusu-Ankomah M, Bloom AP, Jimenez-Andrade JM, King T, Porreca F, Malan TP Jr., Nelson MA, Mantyh PW, and Vanderah TW. "Disease Modification of Breast Cancer-Induced Bone Remodeling by Cannabinoid 2 Receptor Agonists," *Journal of Bone and Mineral Research*, 28(1); 92-107, 2013. PMID: 22903605
- **Lozano-Ondoua AN** and Vanderah TW. "Emerging Analgesics and Future Directions: Targeting Neuroplasticity." *Analgesics for Cancer Pain*. Eds. Frank Porreca and Tamara King. London, UK: Future Science Group. February 2012. eBook ISBN: 978-1-78084-074-1
- **Lozano-Ondoua A**, Wright C, **Vardanyan A**, King T, Largent-Milnes TM, Nelson MN, Jimenez-Andrade JM, Mantyh PW, and Vanderah TW. "A CB2 Receptor Agonist Attenuates Bone Cancer-induced Pain and Bone Loss." *Life Science*, 2010 Apr 24; 86(17-18): 646-53. PMID: 20176037

POSTER PRESENTATIONS

- "Cathepsin S Inhibition: Reducing Pain is the Mission." Radler S, Lantzsch M, Finrock J, Hefferan S, Shimmin B, Currier K, Futch L, **Ondoua A**. *Experimental Biology*, San Diego, CA, April 2012.
- "Multivalent Cathepsin Inhibitor, VBY-825, attenuates breast-cancer induced bone remodeling and pain" **Ondoua AL**, Rodriguez J, Hanlon K, Bilsky Ed, Havelin J, Holsinger LJ, Dener J, Green M, Booth R, Vanderah TW. *31th Annual Meeting for the American Pain Society*, Honolulu, Hawaii, May 2012.
- "A CB2 receptor agonist attenuates breast induced bone cancer pain through spinal CB2 receptors." **Ondoua AL** and Vanderah TW. *41st Annual Meeting for the Society for Neuroscience*, Washington D.C., November 2011.
- "CB2 receptor agonist acts as a disease modifying treatment by attenuating breast-cancer induced bone pain and improving bone health in a murine model of breast cancer metastases." **Ondoua AL**, Hanlon KE, Largent-Milnes TM, Bloom,

AP, Jimenez-Andrade JM, Nelson MA, King T, Mantyh PW, and Vanderah TW. *30th Annual Meeting for the American Pain Society*, Austin, Texas, April 2011.

- “Activation of the CB2 receptor attenuates breast cancer induced bone pain and promotes bone health in a murine model of breast cancer metastases.” **Ondoua AL**, Hanlon KE, Largent-Milnes TM, Chandramouli A, Sukhtankar DD, Okun A, Nelson MA, King T, Mantyh PW, and Vanderah TW. *The Ninth International Meeting on Cancer Induced Bone Disease*, Arlington, Virginia, October 2009.
- “Differences in the expression of the ligands and receptors of B7 family of CD4⁺ T regulatory and effector cells between young and old mice.” Duque-Correa MA, **Ondoua A**, Lustgarten J. *Annual Biomedical Research Conference for Minority Students*, Orlando, Florida, November 2008.
- “Cannabinoid 2 Receptors Attenuate Pain and Maintain Bone Structure in a Murine Model of Bone Cancer.” **Lozano A**, Vanderah TW, Ph.D *Annual Biomedical Research Conference for Minority Students*, Austin, Texas, November 2007.

APPENDIX B: HUMAN/ANIMAL SUBJECT APPROVAL



Institutional Animal Care
and Use Committee

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Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

This proposal has been granted **Authorization to Commence** according to the review policies of the IACUC. This approval authorizes only information as submitted on the Protocol Form.

Principal Investigator: **Todd Vanderah**
Department: **Pharmacology**

Protocol Number: **09-071**
Title: **Cannabinoid CB2 Agonists for Treatment of Breast Cancer-Induced Bone Pain**

Approval Date: **9/17/2012**
Expiration Date: **9/17/2015**

Funding Source: **NCI**

Grant to Protocol Review: **No Significant Discrepancies**

Additional Notes: **None**

Institutional Official: Leslie P. Tolbert, PhD
Senior Vice President for Research

Authorization Status for this Project was Confirmed on: 9/17/2012



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