CONSEQUENCES OF OPIOID ADMINISTRATION IN CANCER-INDUCED BONE PAIN: USING THE PITFALLS OF MORPHINE THERAPY TO DEVELOP TARETED ADJUNCT STRATEGIES

by

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DEDICATION

I dedicate this manuscript to my father, Malcolm Paul Symons (1955-2004), who raised me to pursue my curiosities from an early age. Even in a religious household, he always encouraged me to ask difficult questions and find joy in the pursuit of knowledge. The image of his tenacity, his fervor for life and his compassion continue to inspire and support my endeavors.

"*Why are things as they are and not otherwise*?"
Johannes Kepler (1571-1630)

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ABSTRACT

Many common cancers have a predisposition for bone metastasis. Tumor occupation of bone is both destructive and a source of debilitating pain in cancer patients. As a result, cancer-induced bone pain (CIBP) is the single most common form of clinical cancer pain. Opioids remain the golden standard for the management of CIBP; however, >30% of cancer patients do not experience adequate pain relief with opioids. Furthermore, clinical reports have suggested that opioids can exacerbate bone loss and increase the likelihood of skeletal-related events. To date, there is no known direct mechanism for opioid-induced bone loss (OIBL). We hypothesized that opioid off-target activation of toll-like receptor 4 (TLR4), an innate immune receptor that is expressed in bone, mediates an increase bone loss and associated CIBP. In the 66.1-BALB/cfC3H murine model of breast cancer bone metastasis, TLR4 expression is upregulated in tumor-burdened bone. Chronic morphine treatment exacerbated spontaneous and evoked pain behaviors in a manner paralleled by bone loss: we identified an increase in spontaneous fracture and osteolysis markers including serum collagen-type I (CTX) and intramedullary receptor activator of nuclear κ-B ligand (RANKL). Administration of (+)naloxone, a non-opioid TLR4 antagonist, attenuated both exacerbation of CIBP and morphine-induced osteolytic changes *in vivo*. Morphine did not alter tumor burden *in vivo* or tumor cell growth *in vitro*. Importantly, morphine produced the *in vitro* differentiation and activation of osteoclasts in a dose-dependent manner that was reversible with (+)naloxone, suggesting that morphine may contribute directly to osteolytic activation. To improve opioid management of CIBP, we then posited and evaluated three novel adjunct therapeutic targets: cannabinoid receptor-2,

adenosine 3 receptor and sphingosine-1-phosphate receptor 1. These pharmacological targets were identified as having a multiplicity of anti-cancer, osteoprotective and/or neuroprotective effects in addition to analgesic efficacy in chronic pain. Targets were tested in the 66.1-BALB/cfC3H model of CIBP and demonstrated to have stand-alone efficacy as antinociceptive agents. Taken together, this work provides a cautionary evaluation of opioid therapy in cancer-induced bone pain and seeks to mitigate opioid side effects through the identification of innovative adjunct therapies that can ultimately improve quality of life in patients suffering from cancer pain.

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Prelude on Pain

The French physician Dr. Albert Schweitzer once remarked, “Pain is a more terrible lord of mankind than even death itself.” Chronic pain affects an approximated 100 million Americans—afflicting more individuals than diabetes, heart disease and cancer combined[1] with an economic burden estimated in excess of 600 billion dollars. The International Association for the Study of Pain defines pain as an “unpleasant sensory and emotional experience associated with actual or potential tissue damage.” Physiological pain therefore serves a protective function and promotes survival; however, pathological changes in the processing and perception of pain can lead to the generation of non-productive chronic pain. Non-productive chronic pain serves no protective function and alternatively hampers quality of life: patients who experience chronic pain often suffer comorbid mood disorders (eg. depression, anxiety) and sleep disorders[2] as well as a diminished quality of life[3]. As such, the World Health Organization identifies pain control as the center of palliative care, and improved pain control is ever the heated focus of researchers and physicians alike.

Pain management strategies have been practiced for thousands of years, ranging from the Egyptian use of electric eels on painful wounds to the Greek practice of chewing willow bark—a remedy that would later yield the discovery of aspirin—to the development of the opium tincture “laudanum” in 16th century Germany. Despite decades of technological and research development, pain control remains largely along the lines of these strategies: antipyretics such as aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) remain the first-line therapy for light-to-moderate pain,

whereas opioid pain relievers are the standard of care for a range of moderate-to-severe pain conditions. Innovations in pain control have developed from mechanistic research into the driving factors of abnormal pain states (eg. the use pregabalin to dampen excitatory neurotransmission in fibromyalgia patients), but these targeted therapeutic innovations are often the exception rather than the rule in the clinical approach to pain management. It is the pursuit of a more targeted strategy for cancer pain that drives the following work: that is, this work reflects on the unique state of cancer pain and seeks to recommend tailored adjunct therapies to improve the overall efficacy of palliative care in suffering cancer patients.

1.2 Cancer pain

Cancer pain is a debilitating condition that is experienced by one-third of all cancer patients, and 60-80% of patients with advanced disease[4]. The term “cancer pain” encompasses any pain arising from tumor burden or the treatment thereof. As such, cancer pain can have a complex and multifaceted etiology, including but not limited to pain from tumor occupation of soft tissue, tumor occupation of bone and neuropathies induced by common taxane and vinca alkaloid chemotherapeutics[5]. In a 1999 study, a population of 1095 patients in 24 countries was surveyed in order to identify prevalent cancer pain characteristics: 90% of patients experienced pain directly related to tumor burden, and of this population nearly half of patients reported pain due to bone or joint lesions[6]. Indeed, bone pain is experienced and reported by 30-50% of all cancer patients, and by 75-90% of late-stage patients[7]. The prevalence of bone pain is largely due to the propensity of many common cancers, including breast, prostate, thyroid, kidney and lung cancers, to metastasize and colonize the heavily

vascularized areas of the skeleton, such as the red marrow of the long bones, sternum, pelvis, ribs and vertebrae[8]. Breast cancer contributes to the greatest number of bone metastases of all cancer types, where bone metastasis occurs in 70% of advanced cases[9] and is the most common metastasis site for secondary tumor relapse[10].

Cancer-induced bone pain (CIBP) can be divided into two temporal designations: ongoing pain and breakthrough pain. Ongoing pain is the most prevalent form of CIBP and is characterized by a constant dull ache that progressively increases over time. It is ongoing pain that is primarily addressed by palliative care. Breakthrough pain is distinct from ongoing pain and presents as a transitory, acute flare or sharp pain that "breaks through" efforts of pain management[11, 12]; as such, breakthrough pain is difficult to manage with orally administered analgesics. Episodes of breakthrough pain are characterized by a rapid onset (within 3 minutes) and a moderate-to-severe intensity of pain that lasts between 30-60 minutes[11, 13]. Cancer-induced bone pain has a profound negative effect on the functional status, quality of life and survival of cancer patients[12] and is therefore of principal concern. Accordingly, the therapeutic strategy for cancer pain has evolved frantically alongside changes in opioid availability, oncological development and cancer prevalence.

1.3 Therapeutic Approach

The World Health Organization (WHO) published its first clinical standard for the management of cancer-pain in 1986[14]. The first edition, "Cancer pain relief," took into account the few inexpensive, short-acting drugs available for the treatment of cancer pain including morphine, but was published on the verge of pharmacological innovations that would increase the availability, diversity and duration of action of clinical opioids: in

1987, an extended-release morphine pill MS Contin would be approved by the FDA, shortly followed by the Duragesic fentanyl patch in 1990, and approvals thereafter for extended-release oxycodone (OxyContin) and a transmucosal immediate relief form (TIRF) fentanyl for breakthrough cancer pain (Actiq)[15]. In response, the WHO called for an Expert Committee on Cancer Pain Relief and Active Supportive Care in 1989 to consider a revision of the cancer pain standard and by 1996, a second edition “Cancer pain relief, with a guide to opioid availability.” was published[16]. The second edition purported the now well-known 3-step analgesic ladder for cancer pain (Figure 1.1). In this ladder, opioid therapies are employed in the second and third steps following the failure of non-opioid analgesics (eg. NSAIDs, acetaminophen) and supporting adjunct therapies. In step 2, a distinction is made between weak opioids (eg. codeine, tramadol) and the stronger opioids (eg. morphine, fentanyl, oxycodone) that are recommended in step 3. The new 3-step ladder also placed an emphasis on the number of increasingly-available adjunct therapy options: while there existed a continued emphasis on supporting bone integrity through the pharmacological use of bisphosphonates or calcitonin, the ladder left openings for up-and-coming research such the availability of denosumab (Prolia, Xgeva), a RANK ligand sequestering agent, in 2010 and ExAblate ultrasound therapy in 2012. The appropriate use of adjunct therapeutics remains vital to patient comfort and is in direct relationship to the goal of comprehensive palliative care for cancer pain.

Today, the WHO 3-step ladder remains the standard for not only cancer pain but a variety of chronic pain states—both a credit to the scale’s robustness and open perspective for medical development and a quiet reminder of the halting progress that

has been made in the therapeutic management of pain over the past few decades. At present, the most commonly prescribed treatments for skeletal-related events[17] and pain in cancer patients with bone metastases are bisphosphonates or zoledronic acid, radiation, nonsteroidal anti-inflammatory drugs (NSAIDs)[18] and opioids[19]. Although these therapies reduce skeletal related events (SREs) and pain in many patients, the current therapeutic options leave a staggering 30% of patients without sufficient relief from moderate-to-severe cancer pain[20]. Moreover, patients who receive common cancer pain therapeutics suffer unwanted side effects that produce an overall decrease in quality of life without increasing patient survival or slowing disease progression. Bisphosphonates and zoledrenic acid induce serious adverse events including osteonecrosis of the jaw and additional disruption of serum calcium levels[19]. NSAIDs, while effective at reducing inflammatory and musculoskeletal pains[21], have been shown to exacerbate bone loss and interrupt appropriate bone remodeling, resulting in a loss of bone integrity in both animal models[22] and in human studies[23]. The work in this dissertation also offers evidence of a mechanism by which the chronic use of strong opioids such as morphine worsens cancer-related bone loss and promotes pain, corroborating prior work in both animals[24] and human patients[25]. Chronic opiate use also results in a multitude of poorly-tolerated side effects including somnolence, constipation, respiratory depression and, in rare cases, paradoxical hyperalgesia[26]. Side effects can be worsened by the onset of analgesic tolerance and the requirement of dose escalation or opioid switching, as is common in patients experiencing moderate-to-severe cancer pain.

With the failure of “step 3” opioid therapeutics, physicians and patients face a dearth of alternatives. A recent study of pain management in cancer patients emphasized the often devastating lack of treatment options for cancer pain[27]. In recent years, the FDA has been forced to allow fast track reviews of several drugs for cancer pain including denosumab (Prolia, Xgeva) and nabiximols (Sativex). The dire need of patients suffering from cancer pain obligates preclinical research to gain a better understanding of CIBP mechanisms and the pitfalls of current therapeutics in order to develop effective therapeutic adjuncts and alternatives.

Figure 1.1

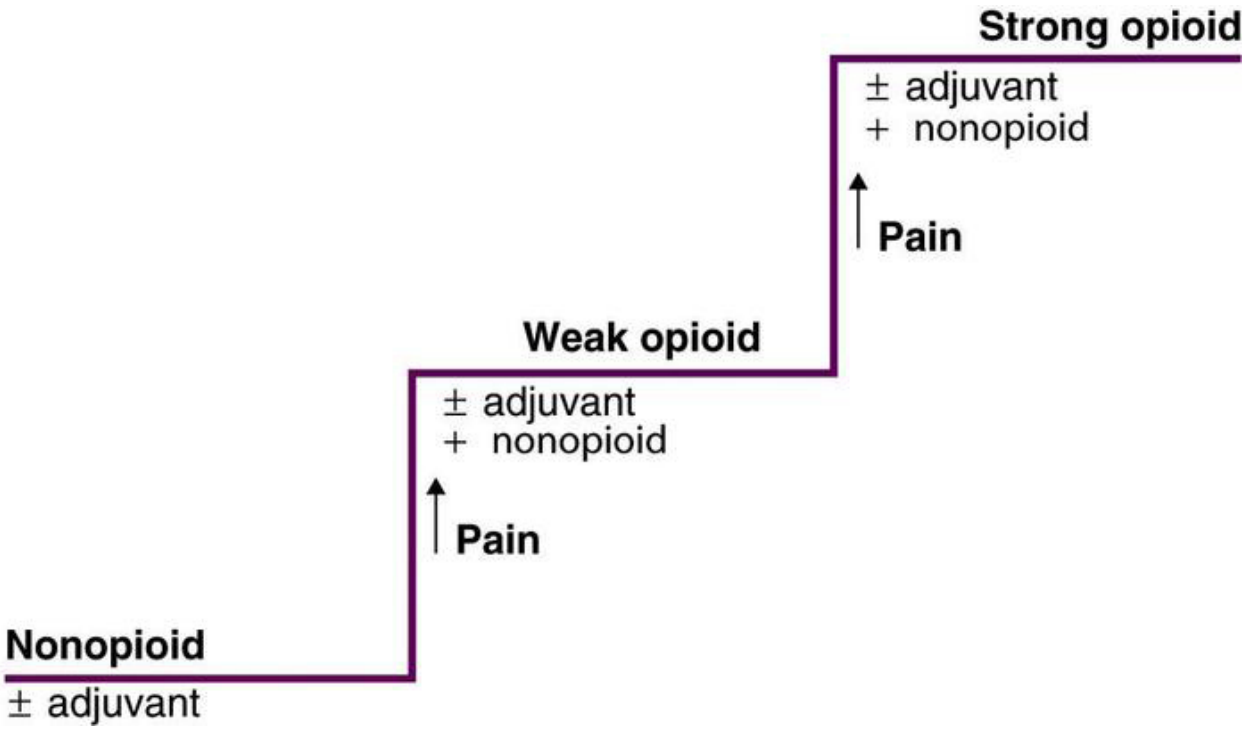

Strong opioid
± adjuvant
+ nonopioid
Pain
Weak opioid
± adjuvant
+ nonopioid
Pain
Nonopioid
± adjuvant

Figure 1.2: The WHO 3-step ladder for relief of cancer pain. At step 1, recommended care includes the use of a non-opioid agent such as a non-steroidal anti-inflammatory drug (NSAID) or corticosteroid. Step 2 recruits the use of a weak opioid such as codeine or tramadol in addition to a non-opioid agent. At step 3, strong opioids such as morphine, oxycodone, buprenorphine and fentanyl are employed in tandem with a non-opioid. Adjuvant or adjunct therapies are recommended at all steps in order tailor pain management strategies for more efficacious relief. Adapted from [28].

1.4 Mechanisms of Cancer Pain

It is often the case that a non-noxious tumor becomes painful upon metastasis to the skeletal system. While metastatic bone pain remains the chief cause of cancer pain, the mechanism by which tumors become painful upon metastasis to the bone is poorly understood. The bone itself is densely innervated by both sympathetic and nociceptive sensory Aβ, Aδ, and C fibers at the level of the periosteum, compact bone, trabecular bone and bone marrow[29]. The periosteum is a highly innervated structure comprised of fibrous connective tissue that envelopes the outer surface of mineralized bone, save at points of ligament and tendon insertion[30]. Compact bone is accessible to periosteal innervation due to its organization as an aggregate of parallel-aligned cylindrical structures known as osteons; the central canal of an osteon, the Haversion canal, hosts vascular, lymphatic and neuronal processes and conveys these processes into the trabecular and marrow compartments. Analyses of sensory innervation the mouse femur suggests that while not all osteons are innervated, osteons near the proximal and distal head of the femur are likely to have peptidergic calcitonin gene-related peptide (CGRP)-positive innervation associated with blood vessels[29]; these CGRP-positive fibers represent nociceptors. Accordingly, it is logical that protective nociceptive sensory innervation is greatest at the proximal and distal ends of the femur, which experiences the highest degree of mechanical stress and bone turnover[31]. In the marrow, sensory innervation is conveyed both via Haversion canals and through the nutrient foramen, where CGRP-positive fibers enter as bundles that later associate with vascular beds in the marrow and trabecular bone[32]. Importantly, the density of innervation at the level of the periosteum is significantly greater than that of the compact

bone or marrow space[29], such that the periosteum is often the focus of skeletal pain studies.

Interestingly, tumors arising in human bone often lack detectable nerve fibers within the tumor mass or bone in immediate proximity[33]. Rather, pro-nociceptive mediators derivative from the bone-tumor microenvironment are reported to stimulate nearby primary afferent nociceptors[34, 35] and induce bone pain. Systems which are tightly regulated by osteoblasts (bone building cells), osteoclasts (bone resorbing cells) and osteocytes including local acidity, glutamate homeostasis, inflammatory signaling and oxidation potential become chronically dysregulated by tumor occupation of bone (Figure 1.1). Together with mechanical compression of bone by tumor mass and painful changes in bone remodeling, these mechanisms make CIBP a complex and multifaceted pain state.

Skeletal pathology. At face value, the mechanical effects of tumor burden on bone and changes in skeletal integrity are important contributors to cancer-induced bone pain. Physical tumor occupation of bone produces compression and the activation of integrin-mediated mechanotransduction[36]. While there exists little preclinical work on mechanoreceptors in cancer-induced bone pain, the relevance of integrin activation has already been demonstrated at the clinical level: studies utilizing a humanized antibody against αv-integrins as an anticancer tool produced significant clinical relief from bone pain associated with prostate cancer bone metastasis[37]. The dependence of clinical CIBP on integrin function suggests that mechanotransduction is an important basic contributor to CIBP and should be addressed in preclinical mechanistic studies.

In addition to physical tumor occupation, tumor-bearing bone is vulnerable to dysregulation of bone homeostasis subsequent losses in integrity that make CIBP patients susceptible to painful skeletal related events (SREs)[38]. While it was originally hypothesized that bone degradation might result from bone compression by metastatic tumors, it is now understood that osteoclast activation is largely responsible for tumor-associated bone loss[39]. The most common paradigm for tumor-induced osteolysis is termed the “vicious cycle of bone metastasis[40]:” in this model, tumor-derived growth factors elicit the upregulation of osteoblast RANKL expression—a stimulator of osteoclastogenesis and osteolysis—and coincident downregulation of osteoprotegerin (OPG), the endogenous “decoy” for the RANK receptor[41]. The net result of this effect is to shift bone homeostasis to favor osteolysis and the liberation of additional growth factors and positive osteoclast regulators stored in bone matrix, which can include insulin-like growth factors (IGF-1, IGF-2), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), bone morphogenic proteins and calcium[42]. Growth factors released from the bone matrix serve to nourish the viability of malignant cells as well as reinforce osteoclastogenesis and resorption. Therefore, a “vicious cycle” of tumor-associated osteoclast activation and subsequent osteolysis-associated tumor progression results in a pathological state of bone loss. In reality, the “vicious cycle” paradigm is a simplification of a much more complex state of bone-tumor signaling: independent osteoclast activation by tumor-derived cytokines[43] and chemokines[44], tumor-related osteoblast inflammatory stress response[45] and tumor acidity all serve to encumber osteoblast differentiation and bone mineralization.

Inflammatory pathology. Inflammation plays a vital role in several aspects of cancer-induced bone pain, including the activation of osteoclasts, tumor self-propagation and activation of innervating nociceptors[46]. Inflammatory mediators in the bone-tumor microenvironment are known as cytokines, a class of small intracellular signaling proteins that facilitate cell-cell signaling between immune cells and cells in the local environment[47]. Within the cytokine family is a subgroup of chemoattractant molecules known as chemokines (chemotactic cytokines), which elicit GPCR-mediated cell chemotaxis in the immune response[48]. Tumor-derived inflammatory mediators in bone metastases are commonly of the pro-inflammatory Th1/Th17 phenotype[49].

Parallel to the “vicious cycle” hypothesis, tumor cytokine production is a mechanism of self-propagation[46, 50] for malignant cells. To this end, cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) play an important role in the growth and progression of several cancer types[51, 52] including breast cancer[53]. Cytokines elicit receptor-mediated events at the level of local nociceptors and in the spinal cord to contribute to CIBP: studies utilizing a transgenic mouse lacking TNF receptors (TNFR1-/-, TNFR2-/-) in a model of bone cancer implicate TNF signaling in cancer pain and spinal astrogliosis[54, 55]. Unsurprisingly, TNF-α also plays a profound role in neuropathic pain[56]. IL-6 production from reactive monocytes including tumor-associated macrophages (TAMs) and spinal glia also propagates cancer pain in preclinical models of bone cancer[56, 57] and neuropathic pain[58]. Accordingly, in this dissertation we will discuss anti-inflammatory strategies that decrease the availability of cytokines in the bone-tumor microenvironment as a mechanism of pain relief for CIBP.

Chemokines contribute to cancer progression through the recruitment of quiescent macrophages as tumor-activated macrophages (TAMs)[59], which can comprise up to 50% of the mass in tumors of epithelial origin[60]. Chemokines also play a provocative role in pain[61] and are thought to initiate nociceptive sensitization at the level of the primary afferent neuron and the spinal cord. Monocyte chemoattractant protein-1 (MCP-1) is one such mediator that has been shown to contribute to tumor-associated peripheral[62] and central[63] mechanisms of nociceptive sensitization. Along with MCP-1, chemokines macrophage inflammatory protein 1a and 1b (MIP-1a, MIP-1b) have been posited as therapeutic targets for the treatment of metastatic bone cancer due to elevations of these chemokines in bone[64]. MIP-1a has a demonstrated role in the sensitization of TRPV1 on nociceptors[65] and similarly, MIP-1b has been implicated in the etiology of neuropathic pain following nerve injury[66]. Accordingly, changes in circulating concentrations of chemokines including MCP-1 and MIP-1b are purported as bio-indicators for response to cancer pain relief[67]. Strategies to target chemokine signaling provide both immediate relief of suffering and prevent the development of neuropathic pathology in CIBP[63, 68, 69].

Neuropathic pathology. Neuropathic features of CIBP add an additional layer of complexity to mechanistic comprehension and pain management efforts. We have briefly discussed the ability of chronic tumor-associated inflammation to produce both peripheral and central nociceptive sensitization. However, the interaction between nociceptors and proximate tumors has also been demonstrated to produce pathological sprouting of nociceptors in the periosteum [70-73], specifically in models of breast cancer metastasis to bone from primary breast[74] and prostate[75] tumors. Whereas

physiological sensory innervation of the periosteum assumes a striated pattern, studies of metastatic bone tumors have observed the formation of irregular neuroma-like structures associated with a severe pain state[76]. Tumoral release of growth factors including nerve growth factor (NGF) and brain derived neruotrophic factor (BDNF) play a role in the induction of neuropathic changes via their cognate receptors, tropomyosin receptor kinases A and B (TrkA, TrkB)[77]. In preclinical models of CIBP, NGF is enhanced in tumor-bearing bone marrow[78] and blockade of NGF/TrkA signaling attenuates the formation of pathological neuroma-like structures in the periosteum and results in a decrease in cancer pain behaviors[76, 79]. BDNF signaling in cancer-induced bone pain appears to be most relevant at the site of the dorsal root ganglion (DRG) and spinal cord: CIBP elicits pathological increases in BDNF at the level of the DRG that are associated with the cancer pain state[78]. In the spinal cord, CIBP-associated BDNF contributes to central sensitization through suppression of proteinase-activated receptor 2 (PAR2) signaling and enhancement of glutamatergic synapse strength[80]. Neuropathic mechanisms of CIBP are not well understood to date, and are likely diverse in origin and function; as such, greater investigative efforts are required to appreciate the clinical utility of neuroprotective agents in CIBP.

Cancer-induced bone pain is a state cluttered by pathological nociceptive changes that occur at the level of the periphery in bone and centrally in the spinal cord. These changes can be resultant from dysregulated skeletal homeostasis, inflammatory status and tumor-derived growth factors. Importantly, few of these mechanisms are addressed in the clinical treatment of CIBP. Specific strategies targeting the progression of pro-nociceptive mechanisms in CIBP will not only provide improved pain

relief in patients, but also have the potential to delay time-to-opioid-failure and produce feed-forward protection against bone pain. The first half of this dissertation delves into the unique consequences of chronic morphine administration in a preclinical model of CIBP to provide a final aspect of clinical shortcomings that can be targeted by future therapies. The second half of this dissertation will explore a number of targeted adjunct options that provide much-needed osteoprotective and neuroprotective support against cancer pain mechanisms.

Figure 1.2

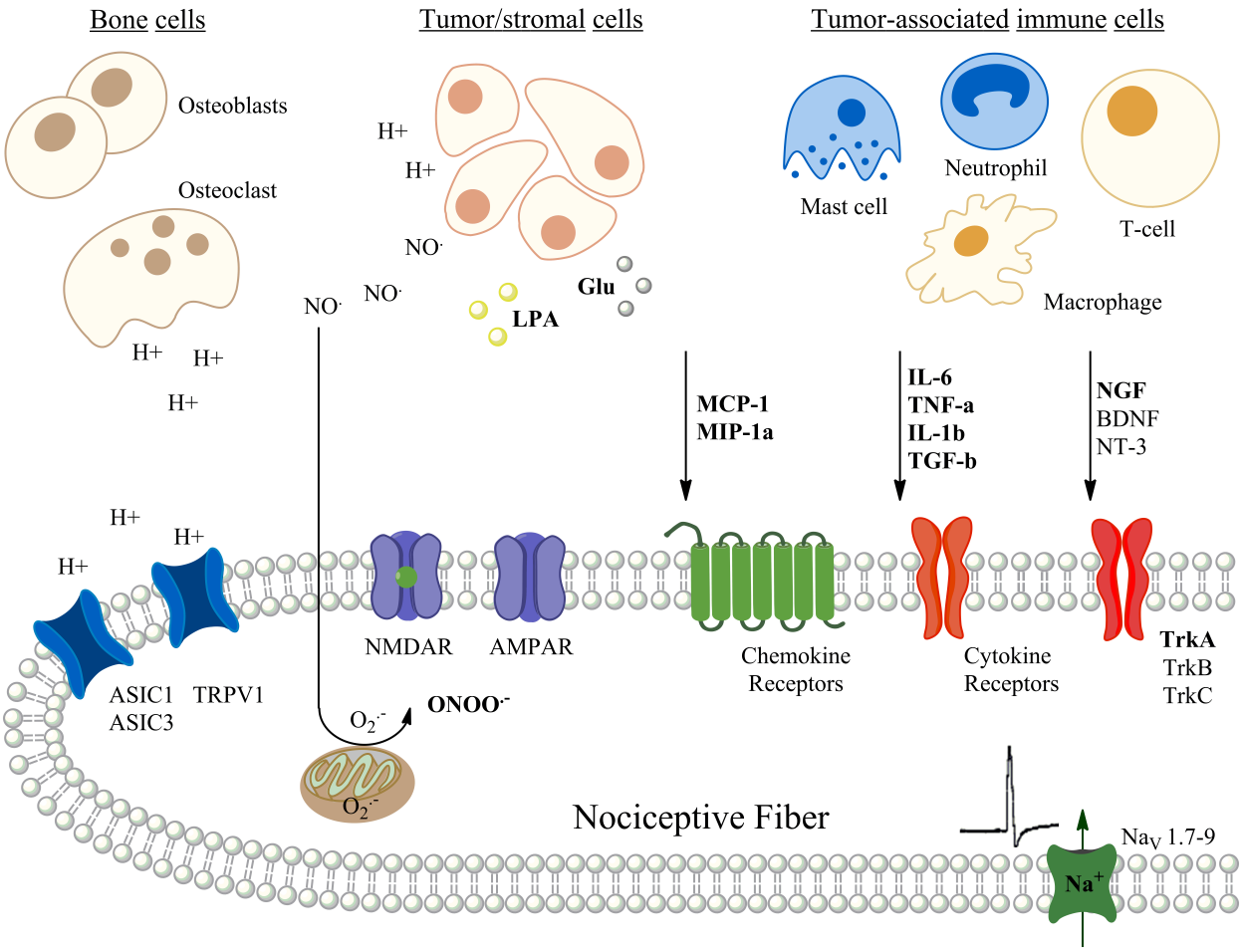

Bone cells
Tumor/stromal cells
Tumor-associated immune cells
Osteoblasts
Osteoclast
H+
NO·
Glu
LPA
Mast cell
Neutrophil
T-cell
Macrophage
MCP-1
MIP-1a
IL-6
TNF-a
IL-1b
TGF-b
NGF
BDNF
NT-3
NMDAR
AMPAR
ASIC1
ASIC3
TRPV1
Chemokine Receptors
Cytokine Receptors
TrkA
TrkB
TrkC
ONOO-
O2·-
Nociceptive Fiber
NaV 1.7-9
Na+

Figure 2.1: Nociceptive mechanisms in the bone-tumor microenvironment. Preclinical models of CIBP have demonstrated that nociceptive stimuli are released from cancer cells and a number of different tumor-associated cells. Cancer cells can enhance and dysregulate osteoclast-generated acidity that may act on ion channels expressed on innervating nociceptive fibers. The generation of tumor-associated inflammatory mediators and growth factors contribute to the excitability and activation of local nociceptors. (H^+ = proton; NO = nitric oxide; O_2^- = superoxide; ONOO = peroxynitrate, LPA = lysophosphatidic acid; Glu = glutamate; MCP-1 = monocyte chemoattractant protein-1; MIP-1a = macrophage inflammatory protein-1α; IL-6 = interleukin-6; TNF α = tumor necrosis factor-α; IL-1β = interleukin-1β; TGF-β = transforming growth factor-β; NGF = nerve growth factor; BDNF = brain-derived neurotrophic growth factor; NT-3 = neurotrophin-3; ASIC1 = acid sensing ion channel-1; ASIC3 = acid sensing ion channel-3; TRPV1 = transient receptor potential cation channel-subfamilyV1; NMDAR = N-methyl-d-aspartate receptor; AMPAR = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; TrkA, B, & C = tyrosine kinase receptors A, B & C). Figure by Ashley Symons-Liguori.

CHAPTER 2: MATERIALS AND METHODS

2.1 In vitro

2.1.1 Cell culture

The murine mammary adenocarcinoma line 66.1 was a generous gift from Dr. Fulton at the University of Maryland. 66.1 cells were maintained in minimum essential medium (MEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (10% FBS), 100 IU^{-1} penicillin and 100 µg/mL streptomycin (1% P/S). RAW264.7 murine monocyte cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified eagle medium (ATCC, Manassas, VA) supplemented with 10% FBS and 1% P/S. Osteoclasts generated from RAW264.7 were cultured in phenol red-free minimum essential medium α (MEM-α) supplemented with 10% FBS and 1% P/S. Cell lines were plated in 75cm^2 filter top flasks and housed in an incubator at 37°C, 5% CO_2, 95% relative humidity. Cells were allowed to grow exponentially to 70% confluence before passage. Experimental studies were performed in Opti-MEM reduced serum media (Life Technologies, Grand Island, NY) supplemented with 1% P/S unless otherwise specified. All studies used cells in the range of 10-20 passages.

2.1.2 Drug treatments

SR144528, SR141716, morphine sulfate pentahydrate and (+)naloxone HCl were generously supplied by the National Institute on Drug Abuse (NIDA). JWH015 was purchased from Tocris Biosciences (Ellisville, MO). (-)Naloxone HCl, mouse MCP-1 and mouse TNF-α were purchased from Sigma Aldrich (St Louis, MO). Mouse IL-6 was purchased from Genscript (Picastaway, NJ). Recombinant human sRANK ligand was purchased from PeproTech (Rocky Hill, NJ). Recombinant human M-CSF was

purchased from RD Systems (Minneapolis, MN). Drugs soluble in aqueous solution were prepared directly in Opti-MEM; otherwise, drugs were solubilized in 100% dimethyl sulfoxide (DMSO) solution to make a 40mM stock solution and diluted accordingly into Opti-MEM. Final solutions were <0.1% DMSO.

2.1.3 Osteoclast differentiation assay

To study osteoclast differentiation, RAW264.7 cells were seeded on a 96-well plate at a density of 10^4 cells per well in phenol red-free MEM-α containing 10% FBS and 1% P/S. Cells were immediately treated with RANKL (1-100ng/mL) and/or drug treatment for 5-7 days with media changes on every third day. Osteoclastogenesis was measured by observing the formation of multinucleated cells, or by quantifying tartrate-resistant acid phosphatase (TRAP) in cell supernatant or on fixed cells using the K-assay TRAP staining kit (Kamiya Biomedical Company, Seattle, WA) according to manufacturer specifications.

2.1.4 Immunoblotting

Cells were lysed on ice with RIPA buffer and spun at 10000rpm to isolate protein extracts. Protein content in extracts was quantified by BCA assay (Thermo Scientific, Rockford, IL). Total protein (20μg) was separated electrophoretically and transferred to a PVDF membrane as previously described [81]. Membranes were probed with polyclonal primary antibodies anti-CB_2 antibody (Cell Signaling Technology), anti-TLR4 (Abcam), or anti-GAPDH (Cell Signaling Technology).

2.1.5 Immunocytochemistry

RAW264.7 cells were differentiated in the presence of 50ng/mL RANKL for 5 days. Cells were then rinsed with Dulbecco's Phosphate-Buffered Saline (DPBS, Mediatech)

and fixed with cold 70% ethanol for 30 minutes at 4˚C. Following fixation, cells were permeabilized with 0.25% Triton 100x in DPBS for 30 minutes. Slides were blocked in 5% BSA in DPBS for 2 hours before incubation with anti-TLR4 (L-14) polyclonal antibody (Santa Cruz Biotechnology, Inc.). Coverslips were incubated in the presence of primary antibody (1:50 dilution in 5% BSA) for 1 hour at room temperature. Primary incubation was followed by two 5 minute washes in DPBS and incubation with DyLight® 594 donkey anti-goat (2.5 µg/ml) in 5% BSA with 1% normal donkey serum for 40 minutes at room temperature in the dark. Following an additional two rinses at 5 minutes each with DPBS, coverslips were mounted on glass slides with ProLong Gold antifade reagent with DAPI (Molecular Probes). Slides were allowed to cure for 1 hour prior to imaging on a Zeiss Axioskop 40 using a 63x/0.08 numerical aperture Achroplan objective lens. Images were obtained with a Zeiss AxioCam-Cm 1.

2.1.6 Tetrazolium dye (XTT) viability assay

66.1 or RAW264.7 cells were seeded overnight on a 96-well plate at a density of 10^4 cells per well in Opti-MEM containing 1% P/S. Adherent cells were then treated with media containing drug or appropriate vehicle for 24 or 48 hours. At 24 or 48 hours, 50µl of activated tetrazolium dye reagent was added to each well and plates were incubated at 37°C for 2-4 hours until an appropriate colorimetric range was achieved. Optical density in wells was read at 450nm and 660nm.

2.1.7 Cytokine/chemokine secretion assay

To evaluate secretion of inflammatory mediators by 66.1 cells, cells were seeded overnight in growth medium on a 96-well plate at a density of 10^4 cells per well. At 3 hours prior to assay, cells were switched to Opti-MEM containing 1% P/S and drug or

vehicle treatments. Cells receiving antagonist were pre-treated for 1hr prior to 3hr incubation with agonist. Supernatant was collected following the 3 hour treatment incubation and centrifuged at 1000 rcf, 5 min to remove cell debris. Concentrations of TNFα, 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.

2.1.8 Cytokine/chemokine proliferation response

In order to evaluate proliferative response of 66.1 mammary adenocarcinoma cells to cytokine stimulation, 66.1 cells were seeded overnight in growth medium on a 96-well plate at a density of 10^4 cells per well. Cells were then switched to Opti-MEM containing 1% P/S and cytokine, drug or vehicle treatments. On day 3, cell proliferation was measured using sulforhodamine B (SRB) as previously described[82]; briefly, cells were fixed with cold 10% TCA for one hour at 4 °C and 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

Studies utilized naïve female BALB/cfC3H mice weighing 18-20g (Harlan Laboratories, Indianapolis, IN) for immunocompatibility with the 66.1 mammary adenocarcinoma cell line. Animals were housed in a climate-controlled room on a 12-hour light/dark cycle with access to food and water *ad libitum*. Mice were monitored daily for signs of

morbidity including rapid weight loss (>20% in 1 week), signs of stress (self-mutilation, rough hair coat, porphyrin staining around the eyes or nose) and paralysis. Animals were humanely euthanized at 14 days post-surgery to prevent unnecessary suffering.

2.2.2 Surgical Procedures

Arthrotomy surgery. Naïve BALB/cfC3H mice were anesthetized with ketamine/xylazine (80/20 mg/kg, 10 ml/kg) and an arthrotomy was performed as previously described[83, 84]. Briefly, the condyles of the right distal femur were exposed. The femur was arthrotomized and a placement needle was inserted to verify access to the intramedullary space via radiographic imaging (Faxitron, Lincolnshire, IL, USA). An optimized suspension of 66.1 mammary adenocarcinoma cells in n 5µl growth medium was injected into the intramedullary space of the femur and the injection site was sealed with dental amalgam. The incision was closed with 9mm stainless steel surgical autoclips. Animals were allowed a 7 day period for surgical recovery and tumor manifestation. At 7 days post-tumor inoculation (dpi), animals were again anesthetized with ketamine/xylazine to remove surgical autoclips and verify sham recovery or tumor progression via radiographic imaging.

Mini osmotic pump implantation. At 7 dpi, mice were anesthetized with ketamine/xylazine (80/20 mg/kg, 10 ml/kg) and an area between the shoulder blades was shaved and disinfected. A 1cm incision was made at or above the shoulder blades and primed Alzet 1007D minipumps containing drug or saline vehicle were implanted subcutaneously. The incision was closed with VetBond surgical adhesive. The use of dual subcutaneous minipumps for analgesic administration has been described previously[24].

2.2.3 Drug Treatments

SR144528, SR141716, morphine sulfate pentahydrate and (+)naloxone HCl were generously supplied by the National Institute on Drug Abuse (NIDA). Morphine and (+)naloxone were dissolved in saline vehicle and administered via subcutaneous (s.c.) osmotic minipump (0.5 µl/hr, 7 days). JWH015 was purchased from Tocris Biosciences (Ellisville, MO). Cannabinoid compounds JWH015, SR144628 and SR141716 were administered intraperitonally (i.p.) in a 10% DMSO/10% Tween-80/80% saline vehicle. MRS5698 and FTY720 were a kind gift from Dr. Salvemini at Saint Louis University. MRS5698 and FTY720 were administered i.p. in a 2% DMSO/98% saline vehicle. Potential vehicle effects were controlled-for through the generation of vehicle treatment groups alongside drug treatment groups.

2.2.4 Behavioral Testing

All observations were conducted between the hours of 7-9am (ie. in the first 2 hours of the room light cycle) by a blinded observer.

Spontaneous pain. Mice were acclimated for 30 minutes in individual plexiglass chambers with a wire mesh floor. Following the acclimation period, number of flinches and time spent guarding the affected paw were observed over 2 minute periods. Flinching was characterized as the rapid lifting and flexion of the ipsilateral hindpaw when not associated with locomotion. Guarding was characterized as the lifting and retraction of the right hind limb under the torso.

Tactile hypersensitivity. Paw withdrawal thresholds were obtained using calibrated von Frey filaments as per the Chaplan up-down method. Mice were acclimated for 30 minutes in individual plexiglass chambers with a wire mesh floor. Following the

acclimation period, Von Frey filaments were applied to the ipsilateral hindpaw for 3-6s. A positive response was indicated by a sharp withdrawal of the paw from the stimulus. The 50% paw withdrawal threshold was determined by the non-parametric up-down method[85].

2.2.5 Radiography

Digital radiographs of the lower extremities were taken following behavioral testing on 0, 7 and 14 dpi (MX20 DC12, Faxitron XRay, Lincolnshire, IL, USA). Bone loss was rated by an observer blinded to treatment group according to a modified 5 point scale[74]: 0 = normal, 1 = small radiolucent lesions indicative of bone destruction (1-3 lesions), 2 = increased number of lesions (3-6 lesions), 3 = full-thickness unicortical fracture, 4 = full-thickness bicortical fracture.

2.3 Ex Vivo

2.3.1 Bone histology and tumor burden analysis

Immediately following behavioral testing on day 14, mice were anesthetized (ketamine 80 mg/kg/xylazine 12 mg/kg, i.p.) and perfused transcardially with 0.1M PBS followed by 10% neutral buffered formalin (Sigma, St. Louis, MO). Femurs were collected and postfixed and decalcified in Decalcifier I agent (Leica Biosystems, IL) for 24 hours at 4°C, then stored in 70% ethanol until processing. Femurs were cut on the frontal plane into 5 μm sections and stained with hematoxylin and eosin (H&E) to visualize normal marrow elements and tumor cells or TRACP5b antibody in order to quantify osteoclasts under bright field microscopy on a Nikon E800 at 4X magnification. Tumor or marrow areas were measured in square millimeters (mm^2) between the epiphyseal plates using Image J software by a blinded observer and with the aid of a pathologist.

2.3.2 Bone marrow extrudate collection

Animals were sacrificed and whole femurs were harvested and cleaned. The proximal and distal ends of the femur were removed and intramedullary contents were flushed with PBS containing Halt protease inhibitor cocktail and EDTA (Thermo Scientific, Rockford, IL). Femur marrow extrudates from 4 mice were pooled into 500µl of solution.

2.3.3 Bone marrow extrudate immunoassays

Ipsilateral and contralateral extrudates were analyzed for protein content using the BCA assay method and quantified via enzyme-linked immunosorbent assay or immunoblotting: relative cytokine and chemokine expression (IL-1β, IL-4, IL-6, IL-12, IL-17a, IFN-γ, TNF-α, MCP-1, MIP-1a, MIP-1b) using a semi-quantitative enzyme-linked immunosorbent array (SABiosciences, Valencia, CA) or quantitative expression of MCP-1 (detection limit: 25.5 pg/ml), MIP-1a (detection limit: 26.3 pg/mL), IL-6 (detection limit: 6.5 pg/mL), TNF-α (detection limit: 8.0 pg/mL), and RANK ligand (detection limit: 4 pg/mL) using enzyme-linked immunosorbent assay (SABiociences; Affymetrix, San Diego, CA; Abcam, MA, USA) according to manufacturer specifications. Alternatively, total protein (20µg) was separated electrophoretically and transferred to a PVDF membrane as previously described [81]. Membranes were probed with polyclonal primary antibodies anti-TLR4 (Abcam), or anti-GAPDH (Cell Signaling Technology).

2.3.4 Serum biochemical assays

Animals were anesthetized with ketamine/xylazine and whole blood was collected by transcardial puncture. Blood was coagulated at room temperature for 1 hour or overnight at 4ºC, and was centrifuged to isolate serum. Serum was stored at -80ºC until utilized for assays. Enzyme immunoassays were used to measure the serum

concentrations of tartrate-resistant acid phosphatase form 5b (TRAP5b) for osteoclast number (Immunodiagnostic Systems, Fountain Hills, AZ, USA) and C-terminal telopeptide α1 chain of type I collagen (CTX) (Immunodiagnostic Systems, Fountain Hills, AZ, USA) for bone loss. Assays were conducted according to the manufacturers' instructions.

2.4 Statistical Analyses

Statistical comparisons between treatment groups were done using ANOVA and pairwise comparisons were made using Tukey's multiple comparison test. Non-parametric data sets were analyzed with the Kruskal-Wallis test and multiple comparisons were made using Dunn's multiple comparisons test. Pairwise comparisons were made with Student's t test. ED_{50} values were calculated using non-linear regression analysis of the log(dose)-response curve. Significance was set at $p<0.05$. All data are presented as mean ± SEM and GraphPad Prism 6.0 (Graph Pad Inc., San Diego, CA, USA) was used to plot data.

CHAPTER 3: PRONOCICEPTIVE OSTEOLYTIC MECHANISMS OF MORPHINE

3.1 Introduction

Opioids including morphine remain the clinical standard for management of CIBP; in accordance with the WHO 3-step analgesic ladder for cancer pain, morphine is often combined with osteoclast inhibitors that are thought to retard painful tumor-associated bone remodeling and skeletal related events (SREs). However, the 3-step strategy is insufficient for 30% of patients experiencing moderate-to-severe cancer pain[20] for reasons of inadequate pain relief or intolerability of side effects such as somnolence, analgesic tolerance, physical dependence or paradoxical hyperalgesia[86]. Furthermore, CIBP patients who do find relief with the use of morphine and other opioids will often experience analgesic failure[87] in part due to the progressive nature of bone metastases and continued bone loss. It has been suggested that chronic opioids may elicit an add-on mechanism of bone loss in patients: chronic diacetylmorphine and methadone maintenance therapies have been associated with osteoporosis risk factors including lowered bone mass and bone mineral density in men[88, 89], and methadone use exacerbates bone loss in female HIV patients[90]. In the elderly, chronic methylmorphine and other opioid use is associated with an increased risk of hip fracture[91]. A nation-wide study in Denmark found that use of seven common opioids (including morphine) was associated with an increased risk of skeletal fracture[25] in both genders at various ages, suggesting that many populations may be vulnerable to the negative impact of chronic opioid use on bone health. Alarmingly, preclinical studies suggest that chronic morphine administration also exacerbates bone destruction, spontaneous fracture and associated pain behaviors in

CIBP[24, 92]; in the present work, we corroborate and explore the mechanism of opioid-induced bone loss (OIBL) in our model of breast cancer-induced bone metastasis (BCBM), suggesting that there may be a causal relationship between morphine administration and worsened bone pathologies in CIBP. The possibility of OIBL in patients with bone metastasis and CIBP is of enormous concern due to the tight coupling of bone integrity to quality of life and functional status of the patient. It is possible that the use of chronic opioid therapies in CIBP patients contributes to analgesic failure and represents a counterproductive effort to anti-osteolytic adjunct strategies.

Hypothesized mechanisms of OIBL. In order to provide a more informed therapeutic strategy for CIBP, it is important to determine the exact mechanism of OIBL. However, to date, no conclusive mechanism has been demonstrated for the effects of opioid on bone: opioid receptors are not known to be expressed on mature osteoblasts or osteoclasts *in vivo*, in spite of studies that have demonstrated *in vitro* μOR expression on immortalized osteoblast-like cells[93]. It has been posited that osteopenia or osteoporosis is a secondary effect to opioid-induced endocrinopathy[94, 95]: μOR activation in the hypothalamus inhibits gonadotropin-releasing hormone (GnRH) and activation of peripheral μOR decreases circulating levels of testosterone in a condition known as opioid-induced androgen deficiency (OPIAD)[94]. However, no study has concluded that loss of BMD occurs exclusively in patients with OPIAD, and most importantly, an association between OPIAD and BMD does not address a direct mechanism for the preclinical observation of opioid-induced osteoclastogenesis and osteolysis. It is vital to investigate mechanism(s) of opioid-induced bone loss in order to

find safer, more effective strategies for utilizing mu opioid receptor agonists in CIBP and to inform future efforts in analgesic development.

Toll-like receptor 4. The ability of morphine and other opioids to mimic the biological actions of lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, has only been uncovered in the past decade[96]. LPS is the classical activator of toll-like receptor 4 (TLR-4), a pattern recognition receptor complex that serves a pivotal function in the innate immune response[97]. The TLR4 receptor complex is found on cells of myeloid-monocytic lineage including macrophages, myeloid progenitor cells, osteoclasts[98] and osteoblasts[99] in the bone. In healthy bone, myeloid (osteoclast) precursors are retained in a non-committed differentiation state that is resistant to TLR4-mediated osteoclastogenesis; it is when precursors are committed to osteoclast lineage through exposure to receptor activator of nuclear factor κ-B ligand (RANKL), tumor necrosis factor-α (TNF-α), or other pro-inflammatory cytokines that LPS readily stimulates osteoclastogenesis[100, 101]. In diseased bone (eg. osteomyelitis, a bacterial infection of the bone), the net effect of TLR4 signaling appears to promote osteolysis and resorption processes: both *in vivo* and *in vitro*, LPS stimulates osteoclastogenesis and bone resorption indirectly through immunomodulation[102, 103] and directly via TLR4[98, 104] to produce osteoclastogenesis and osteoblast expression of RANKL. Indeed, inflammatory bone loss can be modeled with systemic or targeted administration of LPS[103, 105, 106]. In tumor-burdened bone, it is likely that a host of pro-inflammatory, acidic and pro-osteolytic influences collaborate to initiate bone loss and prime tumor-burdened bone for the putative effect of opioids at TLR4. In epithelial origin (eg. breast origin) tumor-burdened bone, infiltrating macrophages—termed tumor-

activated macrophages (TAMs)—can comprise up to 50% of the tumor mass and, in tandem with TLR4-positive osteoclasts, progenitors and osteoclasts, represent an expanded site of TLR4 expression[60]; heightened expression of TLR4 in tumor burdened bone could predispose CIBP patients to TLR4-dependent drug effect. Accordingly, we hypothesized that in circumstances of cancer in the bone (eg. metastasis), individuals may be particularly vulnerable to TLR4-mediated osteoclastogenesis induced by morphine.

Opioid activation of TLR4. In recent years, it has been suggested that several clinically relevant opioids including morphine[107], morphine-3-glucoronide[108], methadone, buprenorphine, fentanyl and oxycodone[109] produce off-target activation of TLR4 that is furthermore non-stereospecific; that is, both natural (opioid-active) and unnatural (inactive at endogenous opioid receptors) enantiomers bind and activate the TLR4 complex with apparently identical affinity[107]. Several studies have provided evidence for the non-stereospecific activation of TLR4 signaling by opioids *in vivo* and *in vitro* [110-112] with an emphasis on the actions of morphine; however, determinations of the affinity and site of binding for morphine at TLR4 are complicated by the unique biochemistry and pharmacology of pattern recognition receptor complexes. Studies in the last few years indicate that morphine does not bind TLR4 directly, but rather produces activation by binding the MD-2 co-receptor in a manner similar to the classical TLR4 agonist, lipopolysaccharide (LPS): LPS bound to MD-2 induces the oligomerization of TLR4 and MyD88-dependent activation of nuclear factor kappa b (NF-κB)[113]. Similarly, morphine was found to bind and oligomerize the MD-2/TLR4 receptor complex resulting in TLR4-mediated NF-κB signaling[107] and likely does so in

the fashion of a partial agonist[114]. Studies have offered contradictory evidence regarding whether morphine binds MD2 competitively[107, 109] or non-competitively[114] with LPS.

Critics of the ability of opioids to bind TLR4 suggest that rather than direct binding of the MD2-TLR4 complex, morphine produces μOR $G_{i/o}$-dependent (indirect) increases in the transcription and translation of TLR4[115]. The indirect hypothesis is however inconsistent with reports of enantiomer independence (ie. TLR4 activation produced by μOR-inactive opioid enantiomers) and studies directly indicating that opioid TLR4-mediated events are insensitive to $G_{i/o}$ inhibition[114]. It is likely that both direct and indirect mechanisms have the potential to contribute to the observed effects of opioids on TLR4 signaling.

The enantiomer independence of opioid binding to TLR4 provides a unique pharmacological tool in preclinical studies: like μOR agonists, μOR antagonists such as naloxone and naltrexone also inhibit TLR4 signaling[116] in a non-enantioselective manner. Because (+)naloxone and (+)naltrexone are devoid of opioid activity and, unlike other TLR4 antagonists such as LPS-RS, are selective for TLR4 over TLR2[116], (+)naloxone is utilized as a specific TLR4 antagonist in pharmacology and microbiology studies alike. A small body of work has used (+)naloxone to isolate TLR4-mediated side effects from the mixed activity of μOR agonists: studies suggest that many detrimental components of chronic opioid administration—including the onset of tolerance[117], abuse potential[118], paradoxical states of hyperalgesia[110], but not respiratory depression[119]—are in part mediated through the pro-inflammatory, pro-oxidative consequences of TLR4-mediated gliosis[107, 110].

No study to date has investigated the consequence of opioid off-target activation of peripherally expressed TLR4, specifically in the contexts of bone metabolism and cancer-induced bone pain. Therefore, we utilized (+)naloxone HCl as a specific TLR4 antagonist in order to evaluate the TLR4-dependence of the pro-osteolytic and pro-nociceptive effects of chronic morphine administration in preclinical CIBP.

3.2 Chronic morphine treatment produces (+)naloxone-sensitive hyperalgesia in CIBP

Our laboratory employs a syngenic model of breast cancer bone metastasis wherein 66.1 mammary adenocarcinoma cells are implanted into immunocompetent BALB/cfC3H female mice via a femoral arthrotomy surgery. At 7 days post-inoculation (dpi) with 66.1 mammary adenocarcinoma cells or control growth medium, animals are evaluated for spontaneous pain behaviors (flinching, guarding) and mechanical hypersensitivity (von Frey threshold) and distributed into equivalent groups. At 7 dpi, 66.1-inoculated mice demonstrated flinching, guarding and tactile allodynia that was absent in sham mice (Fig 3.1A-C). Mice were treated with saline, morphine sulfate or (+)naloxone for 7 days via subcutaneous osmotic minipumps. At 10 dpi, both morphine/saline and morphine/(+)naloxone-treated animals showed a similar decrease in flinching and guarding (Fig 3.1A, 3.1B) and tactile hypersensitivity (Fig 3.1C) as compared to saline/saline-treated controls. However, at 14 dpi analgesia was lost in morphine/saline-treated mice and in fact, morphine/saline-treated animals exhibited a statistical increase in flinching and guarding behaviors and tactile hypersensitivity as compared to tumor-bearing controls. Whereas (+)naloxone/saline-treated animals showed no alteration in pain progression over the disease course,

morphine/(+)naloxone co-treated animals continued to demonstrate morphine analgesia at 14 dpi.

It has been suggested that (+)naloxone can produce an independent antinociceptive effect in models of neuropathic pain[120]; to further validate the use of (+)naloxone as an antagonist in our studies, we performed a supplementary study of acute (+)naloxone administration in CIBP. A single administration of (+)naloxone at 10 dpi had no effect on spontaneous pain behaviors (Fig 3.2A-B); furthermore, (+)naloxone co-administered with morphine in a single administration did not modify morphine analgesia, suggesting that the benefits of (+)naloxone co-administration with morphine are related to the effects of chronic opioid use. These data establish the ability of chronic morphine treatment to produce an exacerbation of cancer-induced bone pain in the 66.1-BALB/cfC3H model, and the sensitivity of this effect to a (+)naloxone, a specific TLR4 antagonist.

3.3 Bone-tumor TLR4 expression in the 66.1-BALB/cfC3H model

We characterized the expression of TLR4 in the bone-tumor environment of the 66.1-BALB/cfC3H model in order to determine whether an increase in total TLR4 expression might account for the susceptibility of CIBP to opioid-induced bone loss. Bone marrow extrudates from 66.1-inoculated mice showed a nearly 3-fold increase in TLR4 expression from sham controls (Fig 3.3A-B). TLR4 was found to be expressed in the RAW264.7 cell line and on osteoclasts differentiated *in vitro* (Fig 3.7A), but not in the 66.1 cell line (data not shown), suggesting that myelo-monocytic cells account for increased TLR4 expression in marrow extrudates.

3.4 Chronic morphine treatment does not modify bone tumor burden

In order to determine whether changes in tumor burden might contribute to morphine hyperalgesia in our model of CIBP, we evaluated the effect of drug treatment on tumor burden *in vivo* and *in vitro*. There was no significant effect of morphine or (+)naloxone treatment (1nM-10μM) on 66.1 cell viability *in vitro* at 24 or 48 hours (Fig 3.4A-B). To evaluate *in vivo* tumor burden, mice were sacrificed and perfused at 14 dpi and femurs were postfixed and stained with hematoxylin and eosin (H&E). Analysis of the intramedullary space showed that chronic morphine treatment did not modify the 50% baseline intramedullary tumor burden: tumor occupation in morphine/saline and morphine/(+)naloxone-treated tumor-bearing mice was not significantly different from saline/saline-treated controls (Fig 3.4C).

3.5 Chronic morphine treatment accelerates cancer-related bone loss and fracture in a (+)naloxone-sensitive manner.

To evaluate the effect of morphine on CIBP-associated bone loss and spontaneous fracture, radiographs of the ipsilateral femur were scored for bone loss on 0, 7 and 14 dpi in order to monitor tumor progression and bone integrity (Fig 3.5A). At 7 dpi, 66.1-inoculated mice scored significantly higher bone loss ratings than mice that underwent sham surgery. Following 7-day treatment at 14 dpi, morphine/saline-treated mice demonstrated a greater degree of bone loss than 66.1-inoculated vehicle controls; however, bone loss in morphine/(+)naloxone-treated mice was indistinguishable from 66.1-inoculated controls (Fig 3.5B). (+)Naloxone alone had no effect on bone loss. These data suggest that morphine-induced bone loss occurs in the 66.1-BALB/cfC3H model and is mediated in part through a TLR4-dependent mechanism.

3.6 Chronic morphine treatment exacerbates biomarkers of osteolysis in a (+)naloxone-sensitive manner.

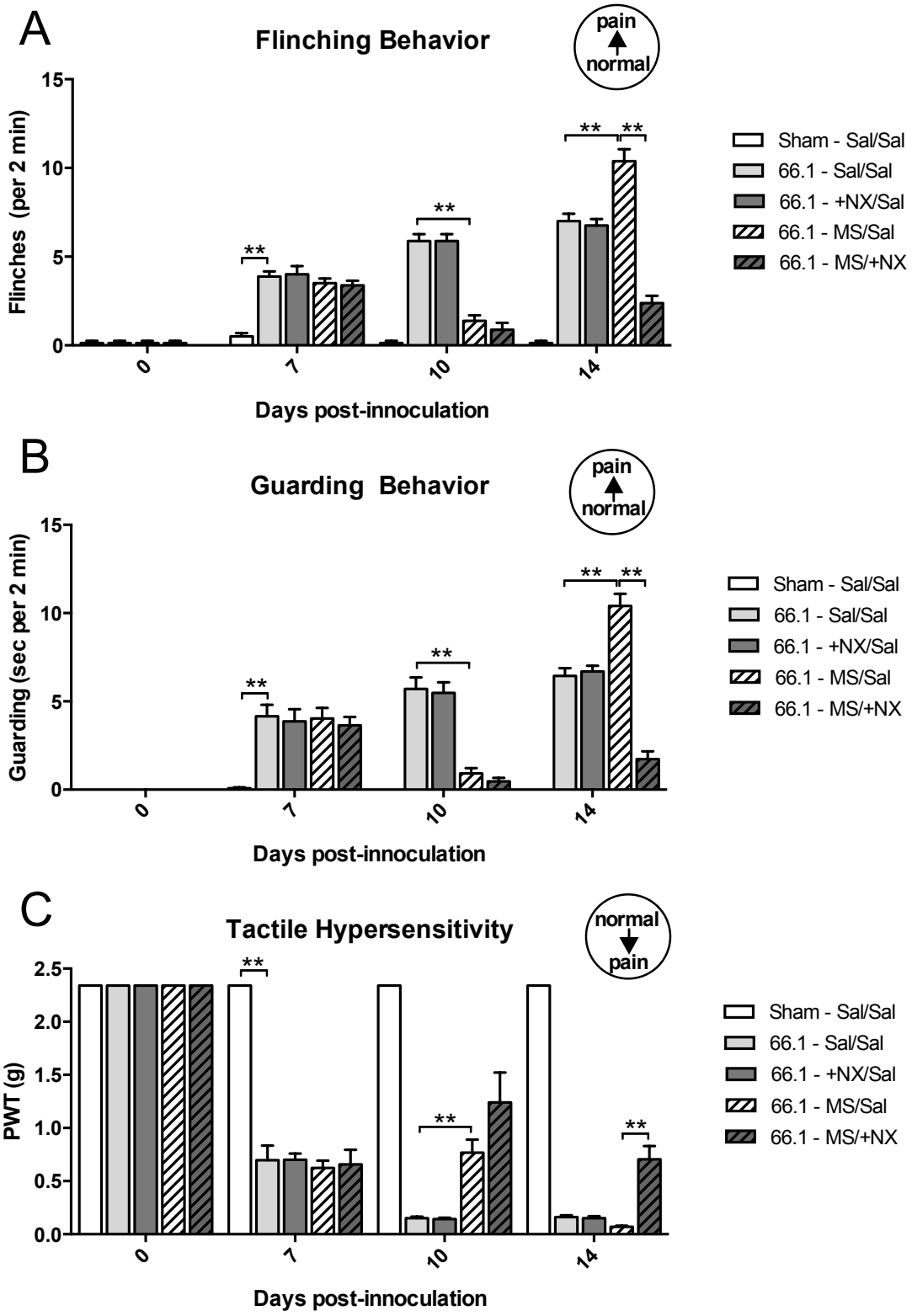
To examine whether morphine-induced bone loss is mediated by an enhancement in osteolytic activity, we evaluated biomarkers of osteoclast function: collagen type-I (CTX) in serum, and RANKL in bone marrow extrudate. Animals were sacrificed at 14 dpi following 7-day drug infusions to collect serum and marrow extrudate from the ipsilateral and contralateral femurs (4 animals pooled per sample). Importantly, At 7 dpi, 66.1-inoculated animals exhibited an increase in serum CTX (Fig 3.6A) and in the ratio of RANLK expression in ipsilateral:contralateral extrudates (Fig 3.6B). Following 7-day drug treatment at 14 dpi, morphine/saline-treated mice displayed an increase in markers of osteolysis as treated animals. Furthermore, serum osteocalcin was quantified to determine whether drug treatment also modified osteoblast activity (Fig 3.6C). Morphine/saline treatment decreased serum osteocalcin as compared to vehicle controls, suggesting that osteoblast suppression is also a mechanism of OIBL in the 66.1-BALB/cfC3H model.

3.7 Morphine enhances osteoclastogenesis in vitro.

Finally, we assessed the ability of morphine to produce osteoclastogenesis *in vitro* through a TLR4-dependent mechanism. Multinucleated osteoclasts were differentiated from RAW264.7 cells and confirmed to express TLR4 (Fig 3.7A). In order to determine whether morphine promotes osteoclastogenesis, we first evaluated the effect of morphine on precursor cell viability. Morphine treatment for 24h had a very mild but significant concentration-dependent effect on the viability of RAW264.7 cells that peaked at 1μM and decayed by 48h (Fig 3.67). In the same dose range, morphine

alone had no effect on osteoclastogenesis, however when morphine was combined with permissive (<50 ng/mL) concentrations of RANKL, morphine enhanced osteoclastogenesis in a manner that exceeded the modest effect on precursor viability (Fig 3.7D). Finally, the ability of morphine to enhance RANKL-mediated osteoclastogenesis was (+)naloxone-sensitive (Fig 3.7C), suggesting that morphine effects on osteoclastogenesis are mediated through TLR4.

Figure 3.1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normal
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10
5
0
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7
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Days post-innoculation
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - +NX/Sal
66.1 - MS/Sal
66.1 - MS/+NX
B
Guarding Behavior
pain
normal
Guarding (sec per 2 min)
15
10
5
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Days post-innoculation
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - +NX/Sal
66.1 - MS/Sal
66.1 - MS/+NX
C
Tactile Hypersensitivity
normal
pain
PWT (g)
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2.0
1.5
1.0
0.5
0.0
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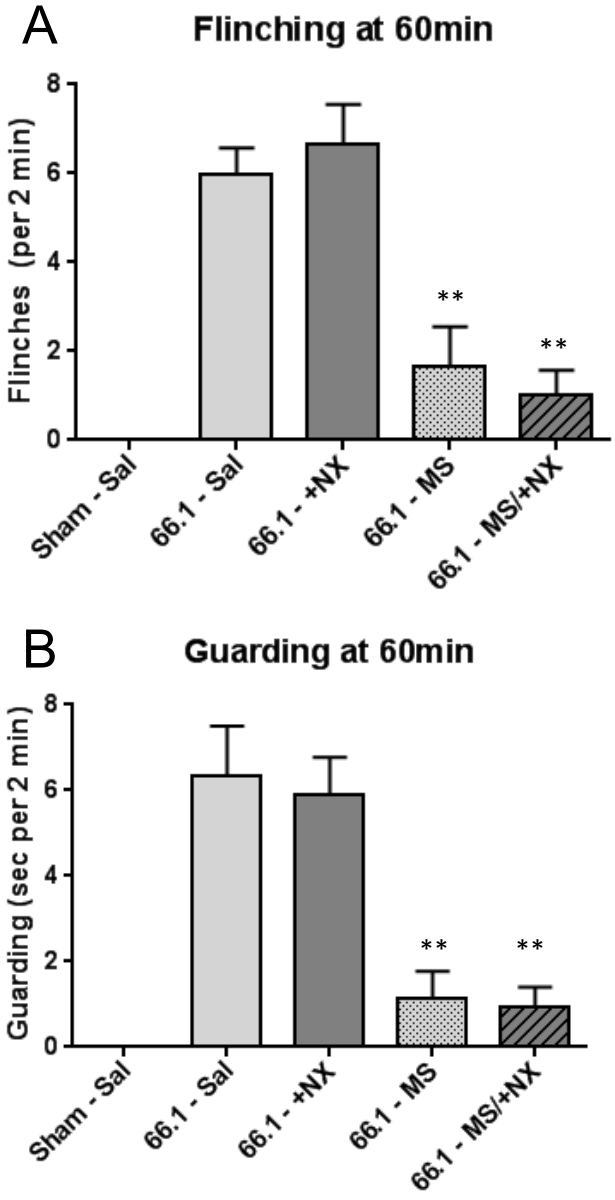
**
0
7
10
14
Days post-innoculation
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - +NX/Sal
66.1 - MS/Sal
66.1 - MS/+NX

Figure 3.1: Morphine analgesic failure in CIBP is (+)naloxone-sensitive. A suspension of 66.1 murine mammary adenocarcinoma cells or media control was injected into the right femur of naïve mice on day 0. Mice were tested prior to surgery and again on days 7, 10 and 14 post-inoculation (dpi). Two osmotic minipumps containing drug or saline vehicle were implanted after behavioral testing on 7 dpi and provided infusions lasting 7 days (until 14 dpi). (a) At 7 dpi, 66.1-innoculated mice displayed significantly more flinching behavior and (b) guarding behavior than sham controls. Chronic morphine/saline treatment (20 mg/kg/day) produced relief of bone cancer-related flinching and guarding on 10 dpi (3 days of treatment), but analgesia was lost by 14 dpi (7 days of treatment): at 14 dpi, both flinching and guarding behaviors in 66.1-morphine/saline mice were significantly increased relative to 66.1-saline/saline controls. Loss of morphine analgesia by day 14 was prevented with (+)naloxone co-treatment (10 mg/kg/day). (+)Naloxone/saline treatment had no effect on cancer pain-like behaviors. (c) At 7 dpi, 66.1-innoculation produced a decrease in Von Frey threshold as compared to sham control indicative of tactile hypersensitivity. Chronic morphine/saline treatment provided partial attenuation of bone cancer-related hypersensitivity at 10 dpi (3 days of treatment) that was lost by 14 dpi. Loss of morphine analgesia was prevented with (+)naloxone co-treatment. (+)Naloxone/saline treatment had no effect on bone cancer-induced tactile hypersensitivity. (n=8)

** indicates significance at $p<0.001$

Figure 3.2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Flinching at 60min
Flinches (per 2 min)
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6
4
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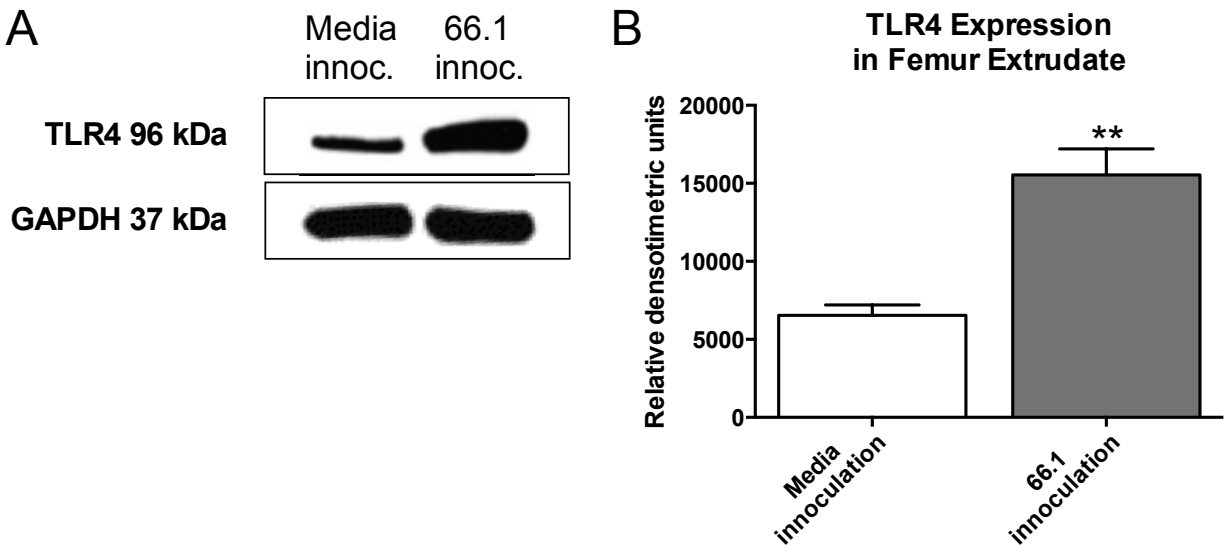
Sham - Sal
66.1 - Sal
66.1 - +NX
66.1 - MS
66.1 - MS/+NX
B
Guarding at 60min
Guarding (sec per 2 min)
8
6
4
2
0
**
**
Sham - Sal
66.1 - Sal
66.1 - +NX
66.1 - MS
66.1 - MS/+NX

Figure 3.2: Acute administration of (+)naloxone alone has no effect on CIBP behaviors or morphine analgesia. A suspension of 66.1 murine mammary adenocarcinoma cells or media control was injected into the right femur of naïve mice on day 0. Mice were tested prior to surgery and again at 10 dpi. Following 10 dpi baseline, mice were distributed into equal groups according to flinching and guarding behavior. Mice were administered a dose of morphine (3 mg/kg), (+)naloxone (3 mg/kg) or saline vehicle (10 mL/kg) and observed again at time of peak effect (t=60min, n=5). (+)Naloxone did not have an independent antinociceptive effect or significantly modify single-dose morphine analgesia. (n=6) ** indicates significance at $p<0.01$.

Figure 3.3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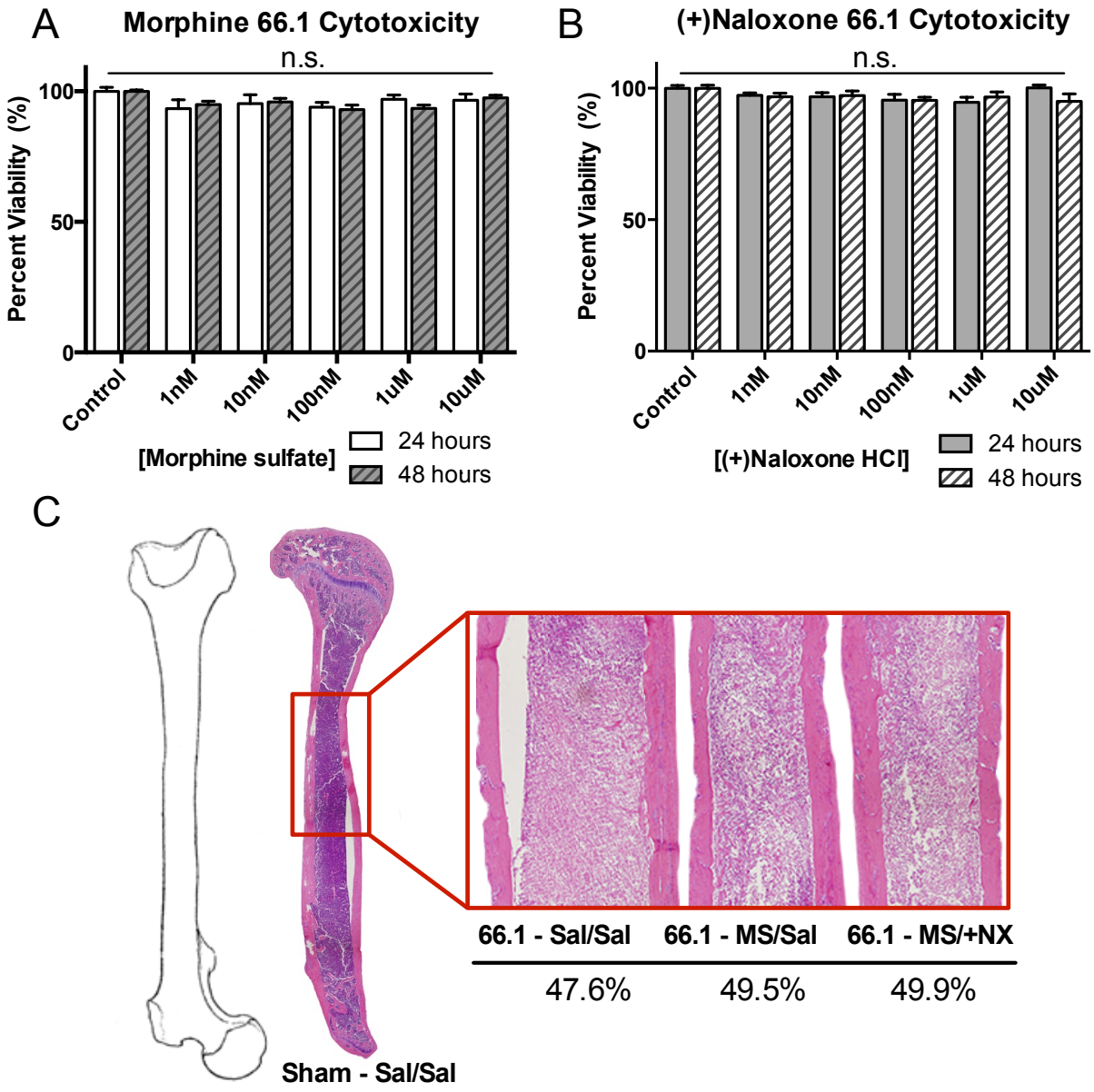
Media innoc.
66.1 innoc.
TLR4 96 kDa
GAPDH 37 kDa
B
TLR4 Expression in Femur Extrudate
Relative densotimetric units
20000
15000
10000
5000
0
**
Media innoculation
66.1 innoculation

Figure 3.3: TLR4 expression is enhanced in tumor-bearing bone. (a) Representative western blot of TLR4 in sham versus 66.1-inoculated ipsilateral femur extrudate at 14 dpi. (b) Quantification of TLR4 expression in sham versus 66.1-inoculated ipsilateral femurs at 14 dpi (n=3 where each n represents 4 pooled femur extrudates, p=0.007).

Figure 3.4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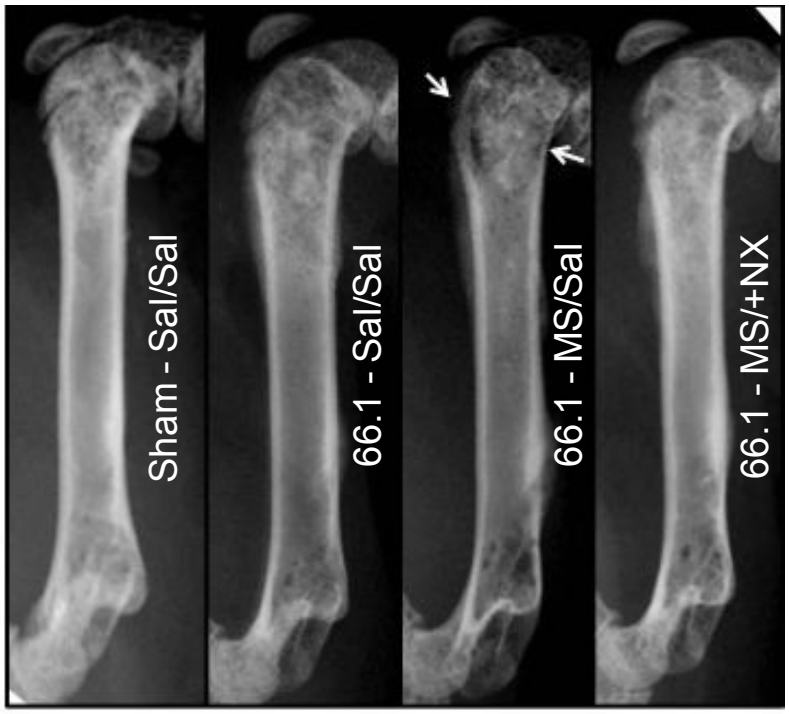
Morphine 66.1 Cytotoxicity
n.s.
Percent Viability (%)
100
50
0
Control
1nM
10nM
100nM
1uM
10uM
[Morphine sulfate]
24 hours
48 hours
B
(+)Naloxone 66.1 Cytotoxicity
n.s.
Percent Viability (%)
100
50
0
Control
1nM
10nM
100nM
1uM
10uM
[(+)Naloxone HCl]
24 hours
48 hours
C
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - MS/Sal
66.1 - MS/+NX
47.6%
49.5%
49.9%

Figure 3.4: Morphine does not modify tumor burden in the 66.1-BALB/cfC3H model. (a) Concentration response of 66.1 mammary adenocarcinoma cell viability to morphine at 24 and 48h; no effect (n=10). (b) Concentration response of 66.1 mammary adenocarcinoma cell viability to (+)naloxone at 24 and 48h; no effect (n=10). (c) Hematoxylin and eosin staining of mouse femurs harvested at 14 dpi. Tumor burden of approximately 50% was not statistically different between treatment groups (n=6).

** indicates significance at $p<0.01$.

Figure 3.5

A


Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - MS/Sal
66.1 - MS/+NX

B

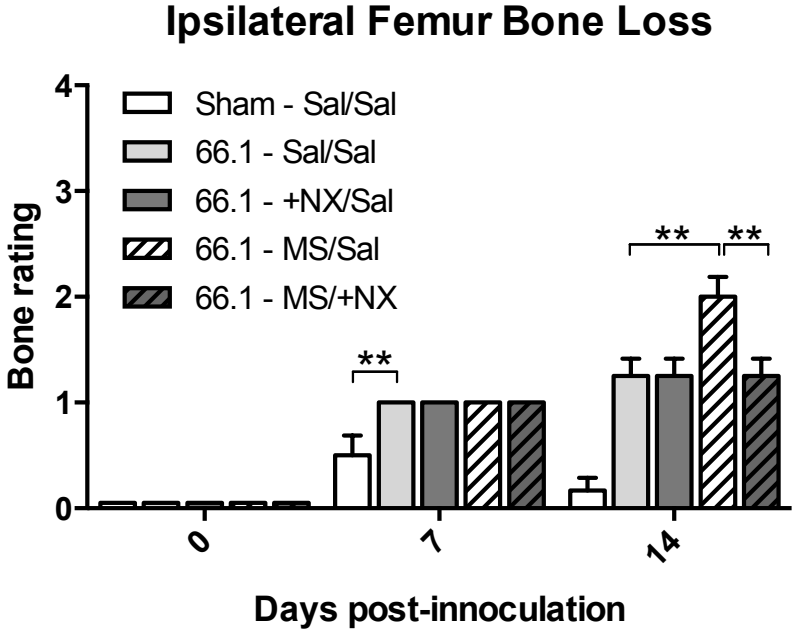

Ipsilateral Femur Bone Loss
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - +NX/Sal
66.1 - MS/Sal
66.1 - MS/+NX
Bone rating
0
1
2
3
4
**
**
**
0
7
14
Days post-innoculation

Figure 3.5: Morphine exacerbates tumor-associated bone loss in a (+)naloxone-sensitive manner. (a) Sample radiograph images of the right femur at 14 dpi. 66.1 inoculation produced radiolucent lesions evident at the distal head of the femur that extended towards the proximal end. Chronic morphine/saline treatment (20 mg/kg/day) in 66.1-inoculated mice increased the number and size of radiolucent lesions (indicated with arrows), and this effect was prevented with (+)naloxone co-treatment (10 mg/kg/day). (b) Bone loss ratings of radiographs taken at 0, 7 and 14 dpi. Chronic morphine/saline treatment accelerated cancer-induced bone loss relative to 66.1-saline/saline controls, and morphine-accelerated bone loss was prevented with (+)naloxone co-treatment. (+)Naloxone/saline treatment had no effect on cancer-induced bone loss. (n=8)

** indicates significance at $p<0.01$

Figure 3.6

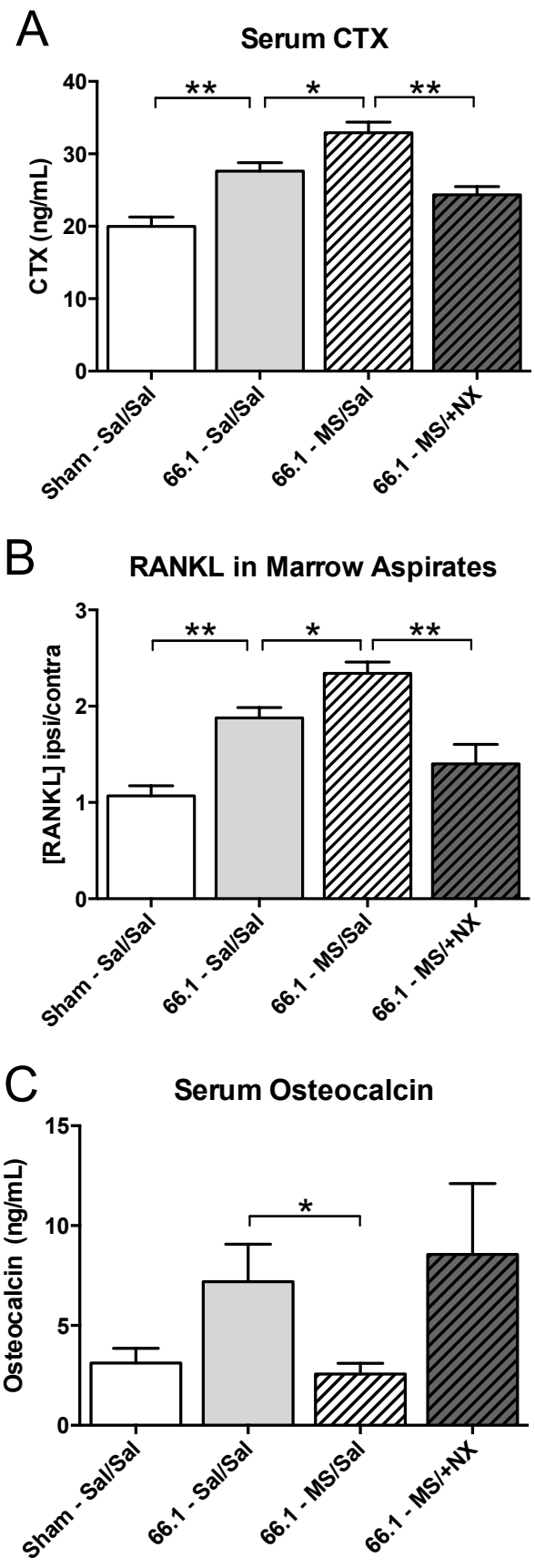
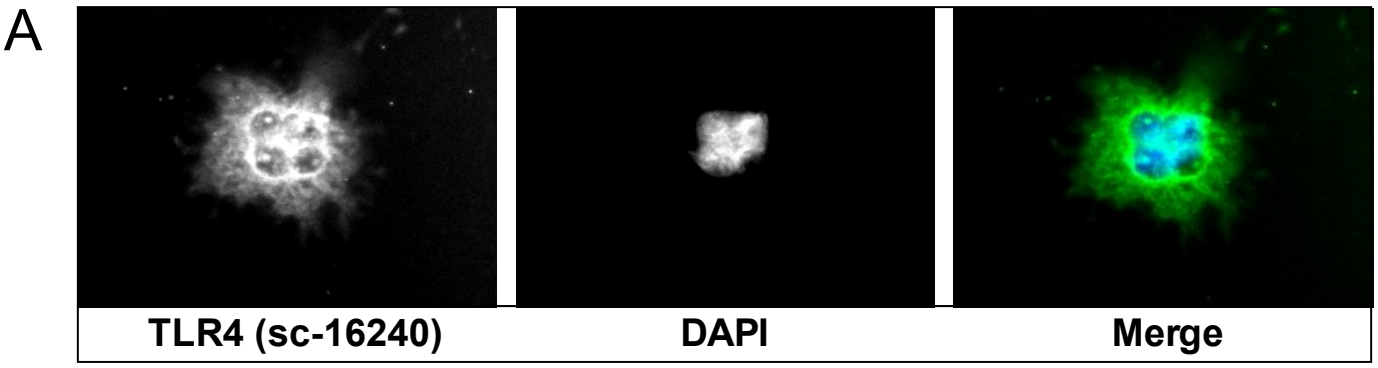
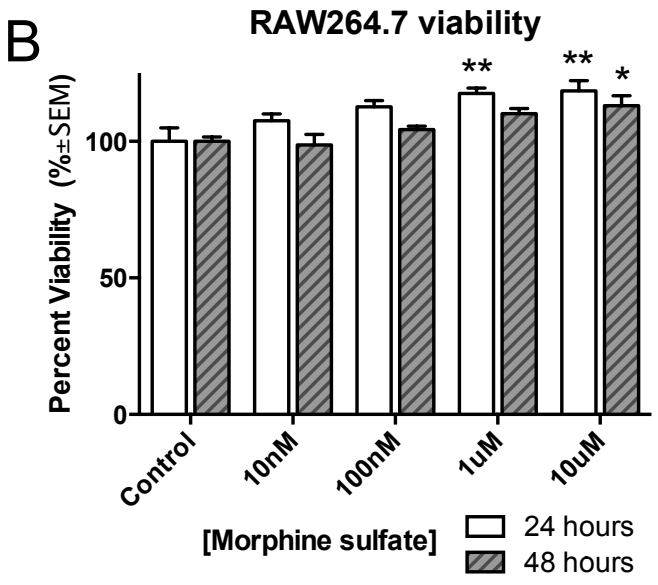

A
Serum CTX
CTX (ng/mL)
40
30
20
10
0
**
*
**
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - MS/Sal
66.1 - MS/+NX
B
RANKL in Marrow Aspirates
[RANKL] ipsi/contra
3
2
1
0
**
*
**
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - MS/Sal
66.1 - MS/+NX
C
Serum Osteocalcin
Osteocalcin (ng/mL)
15
10
5
0
*
Sham - Sal/Sal
66.1 - Sal/Sal
66.1 - MS/Sal
66.1 - MS/+NX

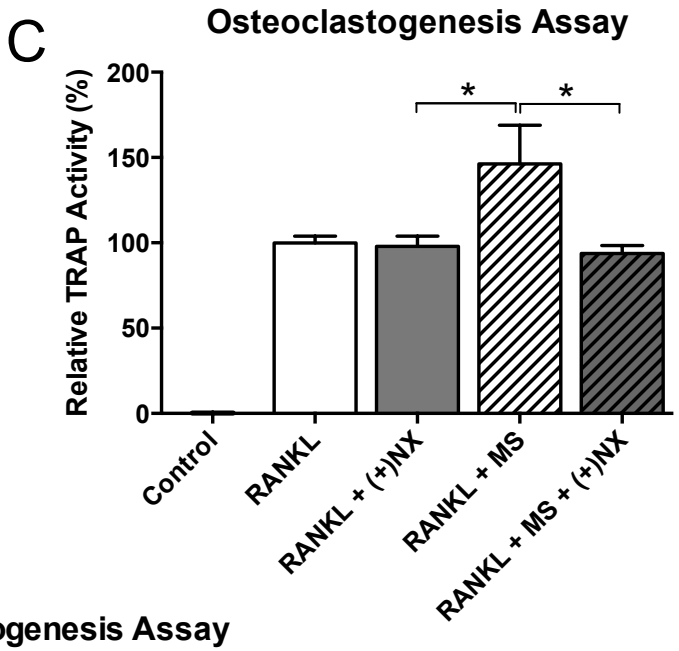
Figure 3.6: Morphine increases local and systemic markers of tumor-associed osteolysis in a (+)naloxone-sensitive manner. (a) Serum concentrations of collagen type-I fragment CTX at 14 dpi (n=8-12). 66.1-inoculated mice had significantly higher serum CTX than sham control mice. Compared to 66.1-saline/saline mice, 66.1-morphine/saline mice showed an elevation of serum CTX (p=0.038) that was prevented by (+)naloxone co-treatment 66.1-morphine/(+)naloxone mice were not significantly different from 66.1-saline/saline controls. (+)Naloxone co-treatment prevented morphine-induced increases in serum CTX and values were not statistically different from 66.1-saline/saline animals. (b) Ratio of RANKL concentrations in the ipsilateral:contralateral femurs at 14 dpi (n=3-6, where each n=4 pooled femur extrudates). Whereas sham control animals had equivalent RANKL concentration in the ipsilateral and contralateral femurs (ratio of 1), 66.1-saline/saline animals displayed a 2-fold increase in RANKL in the ipsilateral femur. 66.1-morphine/saline animals had enhanced RANKL concentrations in the ipsilateral femur relative to 66.1-saline/saline controls (p=0.04), but RANKL expression in 66.1-morphine/(+)naloxone mouse femurs was not significantly different from 66.1-saline/saline controls. (c) Serum osteocalcin concentrations at 14 dpi (n=8-12). 66.1-morphine/saline mice show suppression of serum osteocalcin as compared to 66.1-saline/saline controls (p=0.04).

*indicates significance at $p<0.01$; ** indicates significance at $p<0.01$

Figure 3.7


A
TLR4 (sc-16240)
DAPI
Merge


B
RAW264.7 viability
Percent Viability (%±SEM)
100
50
0
**
**
*
Control
10nM
100nM
1uM
10uM
[Morphine sulfate]
24 hours
48 hours


C
Osteoclastogenesis Assay
Relative TRAP Activity (%)
200
150
100
50
0
*
*
Control
RANKL
RANKL + (+)NX
RANKL + MS
RANKL + MS + (+)NX

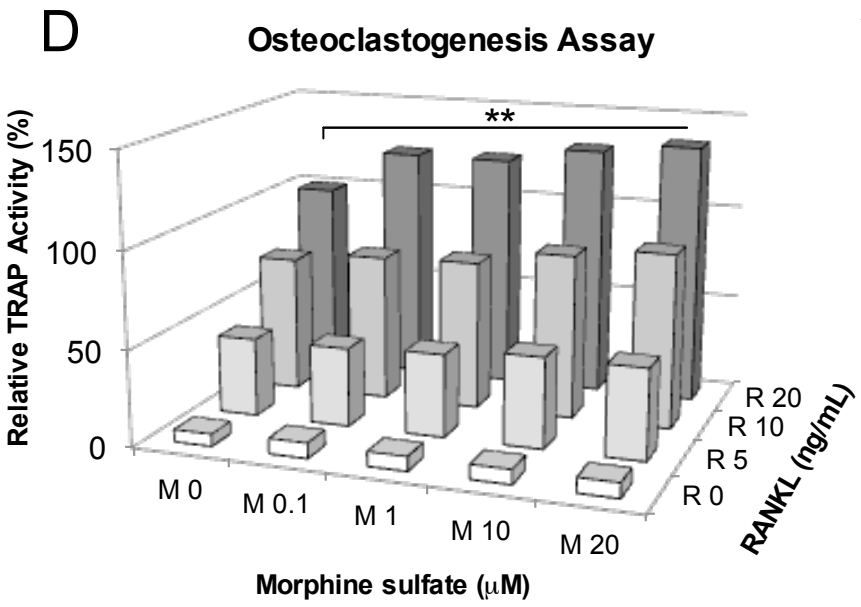

D
Osteoclastogenesis Assay
Relative TRAP Activity (%)
150
100
50
0
**
M 0
M 0.1
M 1
M 10
M 20
Morphine sulfate (μM)
R 20
R 10
R 5
R 0
RANKL (ng/mL)

Figure 3.7: Morphine enhances RANKL-mediated osteoclastogenesis in a (+)naloxone-sensitive manner. (a) ICC on RAW264.7 cells differentiated with RANKL (50 ng/mL). TLR4 is expressed on multinucleated osteoclasts differentiated from the RAW264.7 cell line. (b) RAW264.7 viability concentration-response to morphine. At 24h, viability was mildly enhanced by morphine stimulation for 24h; the pro-viability effect peaked and plateaued at +18% viability with 1μM morphine (significant from control at p=0.0015, n=12). At 48h, the pro-viability effect decayed such that only 10μM morphine produced a significant increase. (c) RAW264.7 cells were differentiated in the presence of RANKL with or without drug treatment for 5 days. TRAP activity was measured in supernatant as an indicator of osteoclastogenesis. Morphine (30μM) co-treatment with low-dose RANKL stimulation (20ng/mL) produced an increase in osteoclastogenesis that was prevented with (+)naloxone co-treatment (10μM) (n=6). (d) RAW264.7 cells were cultured in the presence of varied concentrations of morphine (0.1-20μM) and/or permissive levels of RANK ligand (5-20ng/mL) for 5 days; TRAP activity was measured in culture supernatants. Morphine alone produced no supernatant TRAP activity; however, permissive concentrations of RANKL (20ng/mL) enabled morphine-enhanced osteoclastogenesis (n=3).

*indicates significance at $p<0.01$; ** indicates significance at $p<0.01$.

3.7 Conclusions

Strong opioids such as morphine remain the standard of care in the treatment of moderate-to-severe cancer-induced bone pain; however, one-third of patients do not find adequate relief from pain with the available opioid therapies[20], and it has been posited that opioid-induced bone loss (OIBL) could produce add-on mechanisms of pain in CIBP patients and mechanistically explain a component of opioid analgesic tolerance. For the first time, these data offer evidence that morphine produces TLR4-dependent contributions to pain and bone loss in a clinically relevant model of CIBP, the syngenic 66.1-BALB/cfC3H model. Tumor burden in bone enriched TLR4 expression and may represent an expanded target for OIBL. Chronic morphine treatment exacerbated behavioral indicators of CIBP, and this worsened state was associated with an increase in bone loss and osteolytic activation. Importantly, opioid-related pain and bone loss were attenuated with the co-administration of the non-opioid TLR4 antagonist, (+)naloxone. We then demonstrated that opioids may elicit a direct enhancement of osteoclastogenesis from lineage-committed myeloid precursor cells in a mechanism that appears to be TLR4-mediatd. Our data call into question the use of TLR4-active opioids in metastatic bone pain and underscore the need to develop strategic adjunct therapies or TLR4-inactive opioids for the improved treatment of cancer-induced bone pain. Therapies adjunct to opioid administration should carefully target opioid-related bone loss and hyperalgesia mechanisms in order to improve the efficacy of palliative care in suffering CIBP patients.

CHAPTER 4: A CANNABINOID RECEPTOR 2 AGONIST ATTENUATES CANCER-INDUCED PATHOLOGIES AND PAIN

4.1 Introduction

The endocannabinoid (ECB) system describes a system of lipid messengers and cognate receptors that play a vital role in neuron-glia communication, neuronal signaling, synaptic plasticity and neuroprotection. In humans, endocannabinoids contribute to emotional perception[121], motivation and reward[122], learning and memory[123], appetite[124] and nociception[125]. Endocannabinoids include the lipophilic structures anandamide (AEA) and 2-arachidonyl glycerol (2-AG) that, unlike common neurotransmitters, are not stored in vesicles but are typically generated from membrane lipids *de novo*. To date there are two recognized cannabinoid g-coupled protein receptors (GPCRs), cannabinoid receptors 1 and 2 (CB_1 and CB_2), which function to inhibit adenylyl cyclase by coupling to the $G_{i/o}$ pathway. In the central nervous system, the presynaptic expression CB_1 on neurons accounts for the role of ECBs as retrograde transmitters: presynaptic activation of CB_1 in the CNS negatively regulates glutamatergic[126, 127], GABAergic[128] and cholinergic[129] neurotransmission and plays a vital role in the induction of long-term depression[130]. Alternatively, CB_2 expressed in the CNS is primarily located on glial cells (eg. astrocytes, microglia) and acts as a site of neuron-glia communication: neuronal or autocrine ECB activation of CB_2 regulates glial proliferation[131] and inhibits glial activation and associated neuroinflammatory production of cytokines and reactive oxygen species[132, 133]. Controversially, CB_2 may also be expressed in areas of neurogenesis such as the subventricular zone (SVZ), where an endogenous CB_2-

dependent pathway is thought to play a role in neural progenitor cell proliferation and neurogenesis[134]. Peripherally, CB_2 is predominantly expressed on cells of myeloid, monocytic and lymphoid lineage, including macrophages, B-cells and T-cells in areas including the spleen, tonsils and bone[135]. Accordingly, CB_2 activation plays a regulatory role in inflammation and in the innate and adaptive immune responses[136, 137].

Cannabinoid utility for pain. The FDA has approved the clinical use of few cannabinoid drugs in the United States, including the mixed CB_1/CB_2 agonists Marinol (dronabinol) in 1985 and Cesamet (nabilone) in 2006. In early 2014, the FDA granted Fast Track designation to Sativex oromucosal spray (combination cannabidiol and Δ-tetrahydrocannabinol) for the treatment of cancer pain in patients who do not achieve sufficient relief with chronic opioid therapy. Although nonselective cannabinoids have produced effective relief from pain (ie. greater-than-placebo) in patient populations[138, 139], the CNS depressant and psychotropic (sedation, mental clouding) effects of CB_1 activation can be an unfavorable side effect and is a common reason for discontinuation of therapy in clinical trials[140]. As such, CB_2 has become an attractive target for analgesic development due to its limited expression in the CNS[141] and anti-inflammatory properties[142].

The analgesic efficacy of selective CB_2 agonists is well established in preclinical literature: selective CB_2 agonists including AM1241, JWH015 and JWH133 have provided behavioral attenuation of pain in models of chronic inflammatory pain[143, 144], rheumatoid arthritis[145, 146], post-operative pain[147], neuropathic pain[148-150], chemotherapy-induced neuropathy[151] and diabetic neuropathy[152].

Importantly, CB_2-mediated analgesia has not been reported to produce psychotropic side effects, tolerance or physical dependence[153, 154]. In 2010, our laboratory published pilot work regarding the antinociceptive efficacy of AM1241 in a model of osteosarcoma pain[155]. In this chapter, we have employed the CB_2 agonist JWH015 and specific CB_1 and CB_2 inverse agonists (functional antagonists) for mechanistic studies in a clinically relevant model of breast cancer bone metastasis.

Cannabinoids as a complementary adjunct to opioids in cancer pain.

As discussed in chapter 3, the failure of opioid therapy in cancer pain patients is often a result of analgesic tolerance, disease progression or intolerability of side effects[86, 87]. A substantial degree of CB_2 agonist clinical utility lies in its potential to counteract detrimental aspects of opioid therapy and improve overall analgesia. Furthermore, the development of novel analgesics as adjunct therapeutics to current therapy represents a “path of least resistance” for the clinical emergence of cannabinoid drugs.

Tolerance. Opioid tolerance in cancer pain patients often leads to dose escalation and opioid switching, which can exacerbate unwanted side effects of treatment. Glial activation has a known role in the induction and maintenance of preclinical opioid tolerance: morphine tolerance involves the activation of midbrain astrocytes[156] and spinal microglia[110, 157, 158] and associated neuroinflammatory mediators including monocyte chemoattractant protein-1 (MCP-1)[159], tumor necrosis factor-α (TNF-α)[160], and interleukin-6 (IL-6)[161]. The ability of CB_2 agonists to attenuate or suppress neuroinflammation is widely recognized[162] and furthermore, specific work has pointed to the ability of CB_2 agonists to prevent or attenuate gliosis associated with opioid tolerance[163]: CB_2 activation suppresses morphine-induced release of IL-1β,

TNF-α, IL-6 and nitric oxide by interfering with Akt-ERK1/2 signaling. CB_2 activation also prevents changes in TLR4 signaling associated with glial activation[164] and may provide a relevant strategy for combating tolerance given the involvement of TLR4 in some preclinical models of opioid tolerance[117]. Further investigations should evaluate the ability of cannabinoids to potentiate morphine analgesia.

Opioid-induced bone loss. In the previous chapter, we discussed the concern of OIBL in cancer pain, specifically in the common instance of pain elicited by bone metastases. CB_2 is functionally expressed on myelo-monocytic cells in the bone including osteoclasts and osteoblasts[165]. Activation of CB_2 shifts bone homeostasis to favor bone formation (mineralization) and suppress osteoclast activation[166] and has been hypothesized as an anti-osteoperotic therapy[166, 167]. It follows that the utilization of CB_2 agonists in CIBP may be osteoprotective and improve palliative care by retarding painful bone remodeling and SREs. This strategy is explored in the work herein.

Disease modification. Disease progression (eg. increased tumor burden or metastasis) leads to the disruption of successful pain management in CIBP. Several studies illustrate the anti-cancer properties of cannabinoids[168] and CB_2-specific compounds[169]: in tumoral drug-resistance conferred by multidrug resistance transporter expression, CB_2 activation can overcome resistance to produce cancer cell cytotoxicity[170]. Work from our laboratory has demonstrated the ability of AM1241 and JWH015, selective CB_2 agonists, to reduce bone tumor burden in a model of cancer-induced bone pain[84, 155]. Furthermore, studies performed in a model of murine breast cancer metastasis have demonstrated the ability of a CB_2 agonist to attenuate primary tumor burden and prohibit metastasis[171]. These studies offer that CB_2-

specific strategies may be a prudent choice for the treatment of cancer pain and CIBP.

Analgesic synergy. The ultimate goal of adjunct therapeutic development is to increase analgesic efficacy and improve patient quality of life. In some circumstances, the combination of an adjunct therapy with an opioid can produce a greater-than-additive effect known as synergy. The synergistic interactions of endogenous opioid and cannabinoid systems[172] has lead to the hypothesis that exogenous activation of μOR and CB_2 may produce analgesic synergy; indeed, CB_2 activation produces transcriptional activation of the μOR *in vitro*[173] and co-administration studies of μOR and CB receptor agonists have demonstrated a potential for antinociceptive/analgesic synergy in some states of chronic pain[154]. Early studies evaluating JWH015 in our laboratory have indicated the specific potential for analgesic synergy between μOR and CB_2 in pain elicited through inflammatory mechanisms (data not shown). Furthermore, cannabinoid enhancement of opioid analgesia in primates does not appear to produce an increase in reward or abuse potential[174]. These data lend credibility to the examination of CB_2-specific strategies in CIBP and pharmacological analysis of its interaction with μOR agonists.

Given the potential utilities of cannabinoids in cancer pain, we hypothesized that administration of a CB_2 agonist in the 66.1-BALB/cfC3H model of CIBP would not only demonstrate the antinociceptive properties of CB_2 activation in cancer pain, but also exhibit useful features as an adjunct opioid therapy.

4.2 JWH015 produces antinociception in CIBP

In order to investigate the utility of a selective CB_2 agonist in cancer-induced bone pain, we employed JWH015 (human CB_2 K_i=13.8nM, human CB_1 K_i=383nM) in

the immunocompetent 66.1-BALB/cfC3H model of breast cancer bone metastasis. Spontaneous flinching and guarding behaviors and paw withdrawal thresholds (tactile hypersensitivity) were monitored as indicators of on-going pain. Following 7-day treatment, JWH015 significantly reduced CIBP-associated flinching (Fig 4.1A) and guarding (Fig 4.1B) behaviors as compared to vehicle-treated controls. JWH015 also attenuated tactile hypersensitivity elicited by femoral tumor burden (Fig 4.1C). Importantly, there was no effect of JWH015 in sham surgery animals. Specificity of effect to CB_2 was demonstrated through the administration of CB_1 or CB_2 functional antagonists alone or in combination with JWH015: following 7 day treatment, co-administration of SR144528, a specific CB_2 functional antagonist, prevented the antinociceptive effect of JWH015 on spontaneous flinching (Fig 4.2A) and guarding (Fig4.2C), whereas co-administration of SR141716 had no effect on JWH015 analgesia. Neither SR144528 nor SR141716 alone had any effects on CIBP-associated pain behaviors. These data suggest that JWH015 provides CB_2-mediated relief of ongoing CIBP in the 66.1-BALB/cfC3H model of breast cancer bone metastasis.

4.3 JWH015 decreases bone tumor burden

Given the anti-tumoral effects of CB_2 activation in other preclinical models, we investigated the ability of JWH015 to modify disease as a mechanism of pain relief. Following 7-day administration of JWH015, animals were sacrificed and tumor burden was evaluated *ex vivo*. Indeed, animals treated with JWH015 demonstrated a 43% reduction in intramedullary tumor burden when compared to vehicle-treated controls (Fig 4.3B). These findings were corroborated by the *in vitro* dose-dependent

cytotoxicity of JWH015 in 66.1 mammary adenocarcinoma cells following a 48h treatment period (Fig 4.3A).

4.4 JWH015 treatment reduces tumor-associated spontaneous fracture and osteoclastogenesis.

Bone metastases associated with CIBP produce dysregulation of bone homeostasis, bone loss and a heightened likelihood of spontaneous fracture. Accordingly, we investigated whether JWH015 is osteoprotective in the 66.1-BALB/cfC3H model. Treatment with JWH015 for 7 days reduced the incidence of spontaneous fracture as compared to vehicle-treated controls (Fig 4.4B) quantified via radiographic analyses on days 0, 7 and 14 (Fig 4.4A). Co-administration of SR144528 reversed the osteoprotective effects of JWH015 but had no independent effect, suggesting that JWH015 prevents bone loss through a CB_2-dependent mechanism. To determine whether the effects of JWH015 were directly on osteoclasts, we quantified osteoclast number *ex vivo* at 14 dpi. Tumor-bearing femurs showed a 2-fold increase in osteoclast number that was significantly attenuated with JWH015 treatment (Fig 4.4C). JWH015 was deemed to provide protection from tumor-associated osteolysis and fracture via the CB_2 receptor.

4.5 JWH015 exerts anti-inflammatory actions in the bone-tumor-microenvironment.

Inflammation plays a vital role in tumor self-propagation and additionally serves as a pro-nociceptive influence in CIBP. Because CB_2 agonists are acclaimed for their immunosuppressive function, we investigated the ability of JWH015 to suppress cytokine and chemokine production in the bone-tumor microenvironment as a mode of

antinociception and/or antitumor action. Bone marrow extrudate was collected from ipsilateral (tumor-bearing) and contralateral femurs following 7-day treatment. There were no significant differences between the contralateral femurs of sham or 66.1-inoculated mice, suggesting that early inflammatory changes may be restricted to the bone-tumor microenvironment (data not shown). Initial studies performed semi-quantitative evaluation of inflammatory mediators in order to screen for an effect of JWH015 treatment (Fig 4.5A). Based on the interaction of screened cytokines with JWH015 treatment and based on a literature review of cytokines relevant to pain, two cytokines (IL-6, TNF-α) and two chemokines (MCP-1, MIP-1a) were selected for quantitative assay. Indeed, IL-6, TNF-α, MCP-1 and MIP-1a were subject to a greater-than-2-fold expression increase in tumor-bearing bone as compared to sham-surgerized control femurs (Fig 4.5B-E). JWH015 significantly attenuated the expression of the assayed inflammatory mediators in tumor-bearing bone, with marked effects on cytokine IL-6 (Fig 4.5B) and chemokine MCP-1 (Fig 4.5D).

4.6 JWH015 reduces tumor cell release of pro-inflammatory mediators

The production of pro-inflammatory mediators in the bone-tumor microenvironment is thought to promote tumor growth and viability. To investigate whether the effects of JWH015 *in vivo* could be mediated through the engagement of CB_2, we measured secretion of pro-inflammatory mediators IL-6, TNF-α and MCP-1 from 66.1 mammary adenocarcinoma cells. 66.1 cells produced basal levels of all cytokines measured and 3-hour stimulation with JWH015 significantly decreased the release of IL-6 (Fig 4.6A), TNF-α (Fig 4.6B) and MCP-1 (Fig 4.7A). Co-treatment of JWH015 with SR14458

restored the release of inflammatory mediators from 66.1 cells, implicating CB_2 in JWH015-mediated inflammatory suppression.

We also asked whether the inflammatory mediators affected by JWH015 administration *in vivo* are involved in tumor viability. Treatment with IL-6 (Fig 4.6C) or TNF-α (Fig 4.6D) for 24h produced significant, dose-dependent enhancement of 66.1 cell viability. Neither chemokines MCP-1 (Fig 4.7B) nor MIP-1a (Fig 4.7C) had an effect on 66.1 viability; the absence of a pro-viability effect was expected due to the nature of chemokines as chemoattractants.

Figure 4.1

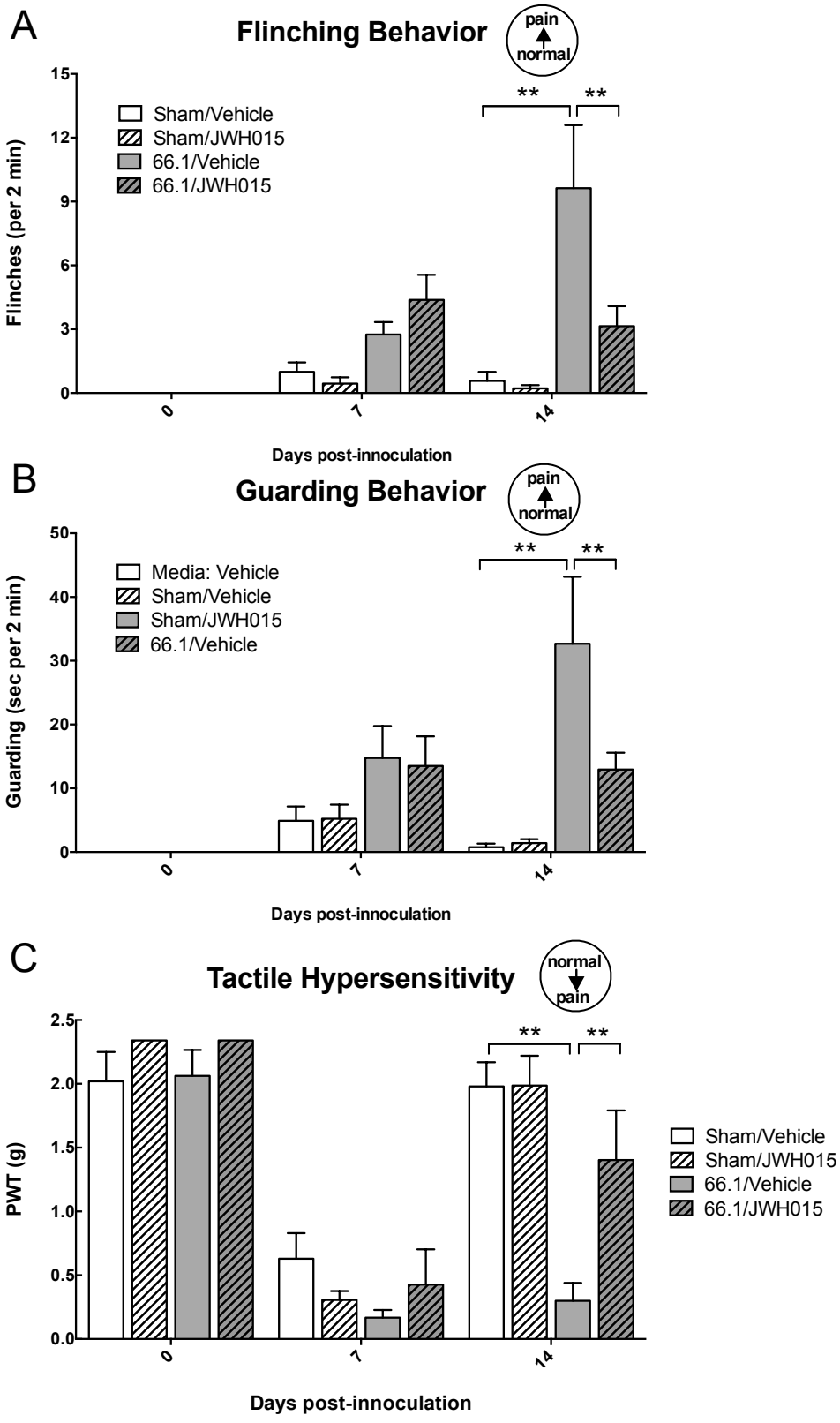

A
Flinching Behavior
pain
normal
Flinches (per 2 min)
Sham/Vehicle
Sham/JWH015
66.1/Vehicle
66.1/JWH015
**
**
Days post-innoculation
B
Guarding Behavior
pain
normal
Guarding (sec per 2 min)
Media: Vehicle
Sham/Vehicle
Sham/JWH015
66.1/Vehicle
**
**
Days post-innoculation
C
Tactile Hypersensitivity
normal
pain
PWT (g)
Sham/Vehicle
Sham/JWH015
66.1/Vehicle
66.1/JWH015
**
**
Days post-innoculation

Figure 4.1: JWH015 attenuates behavioral indicators of CIBP. A suspension of 66.1 murine mammary adenocarcinoma cells or media control was injected into the right femur of naïve mice on day 0. Mice were tested prior to surgery and again on days 7 and 14 post-inoculation (dpi). Beginning on day 7, animals received once daily vehicle or drug treatment i.p. (10mL/kg, n=8 per group). (a) At 14 dpi, 66.1/vehicle mice displayed significantly more flinching behavior and (b) guarding behavior than sham controls. Once daily treatment with JWH015 (6 mg/kg/day, i.p.) produced relief of bone cancer-related flinching and guarding at 14 dpi. Sham/vehicle-treated mice were not significantly different from sham/JWH015-treated mice. (c) At 14 dpi following 7 days of treatment, 66.1/vehicle-treated mice demonstrated a significant decrease in Von Frey threshold as compared to sham controls, indicative of tactile hypersensitivity. Once daily treatment with JWH015 (6 mg/kg/day) produced attenuated tactile hypersensitivity as compared to 66.1/vehicle-treated controls at 14 dpi. Sham/vehicle-treated mice were not significantly different from sham/JWH015-treated mice. ** indicates significance at $p<0.001$

Figure 4.2

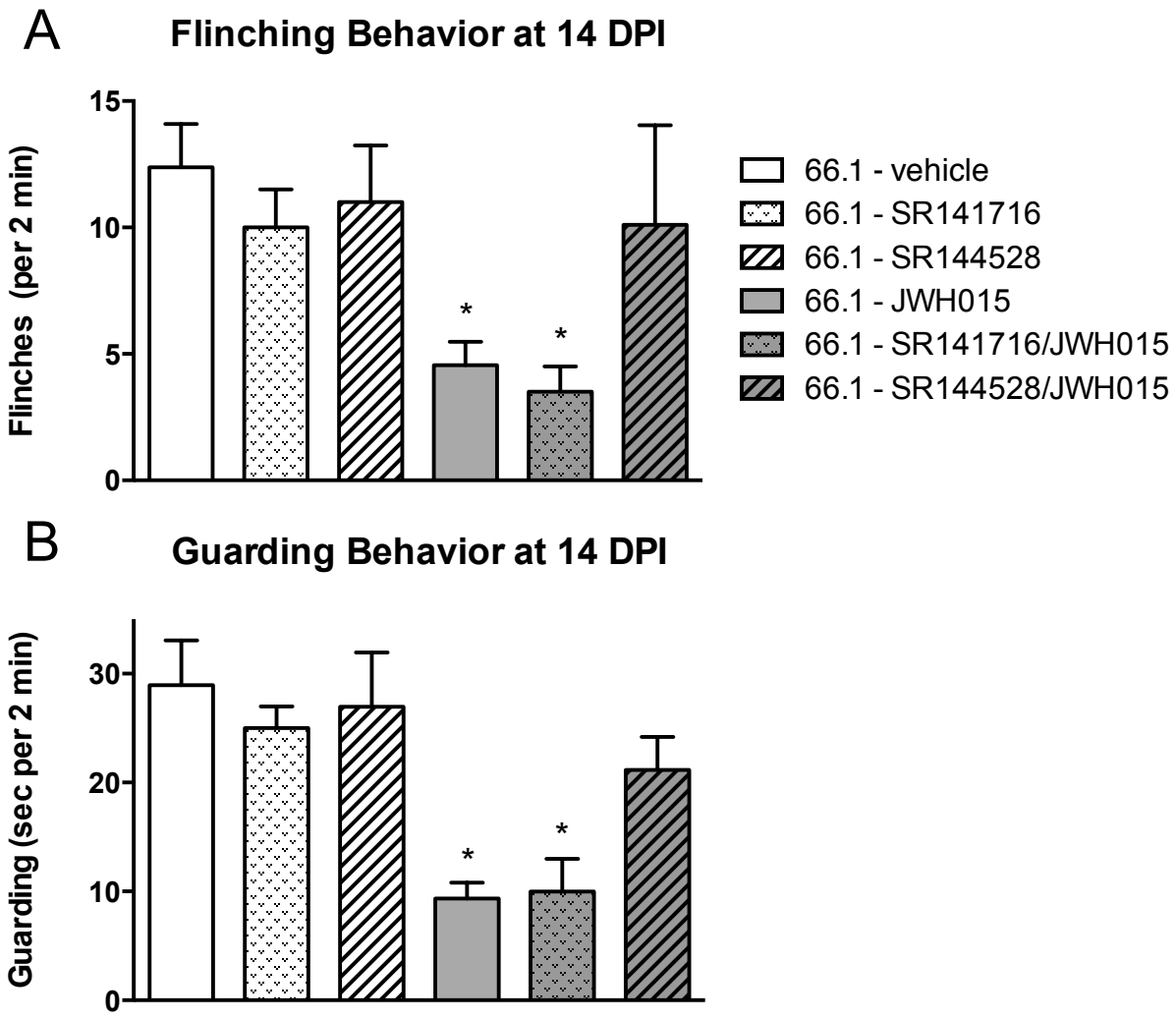

A
Flinching Behavior at 14 DPI
Flinches (per 2 min)
15
10
5
0
*
*
66.1 - vehicle
66.1 - SR141716
66.1 - SR144528
66.1 - JWH015
66.1 - SR141716/JWH015
66.1 - SR144528/JWH015
B
Guarding Behavior at 14 DPI
Guarding (sec per 2 min)
30
20
10
0
*
*

Figure 4.2: JWH015 antinociception in CIBP requires CB_2 but not CB_1. A suspension of 66.1 murine mammary adenocarcinoma cells was injected into the right femur of naïve mice on day 0. Mice were tested prior to surgery and again on days 7 and 14 post-inoculation (dpi). Beginning on day 7, animals received once daily vehicle or drug treatment i.p. (10mL/kg). Following 7-day treatment, (a)flinching and (b)guarding behaviors in tumor-bearing animals treated with CB_1 antagonist SR141716 (3 mg/kg/day, i.p.) or CB_2 antagonist SR144528 (3 mg/kg/day, i.p.) were not significantly different from vehicle control. Mice treated with JWH015 (6 mg/kg/day, i.p.) showed a significant attenuation of pain that was reversed with co-administration of SR144528 (3 mg/kg/day), but not with SR141716 (3 mg/kg/day). (n=10-16)

* indicates significance at $p<0.05$.

Figure 4.3

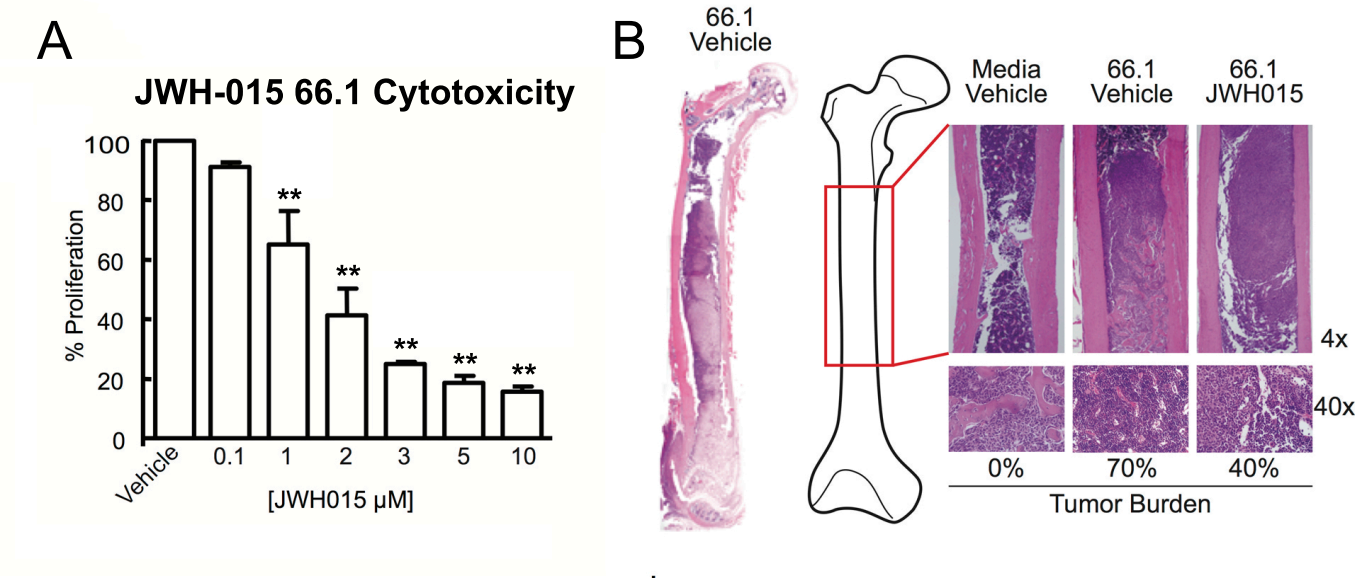

A
JWH-015 66.1 Cytotoxicity
% Proliferation
100
80
60
40
20
0
**
**
**
**
**
Vehicle
0.1
1
2
3
5
10
[JWH015 µM]
B
66.1
Vehicle
Media
Vehicle
66.1
Vehicle
66.1
JWH015
4x
40x
0%
70%
40%
Tumor Burden

Figure 4.3: JWH015 reduces tumor burden and demonstrates antitumoral effects *in vitro*. (a) 66.1 mammary adenocarcinoma cells were treated with JWH015 (0.1-10μM) or media control (n=18). JWH015 produced a dose-dependent decrease in cell viability. **indicates $p<0.001$ (b) Hematoxylin and eosin staining of mouse femurs harvested at 14 dpi. JWH015 treatment produced a 43% decrease in tumor burden (70% intramedullary occupation in media-treated animals versus 40% intramedullary occupation in JWH015-treated animals, n=4).

** indicates significance at $p<0.01$.

Figure 4.4

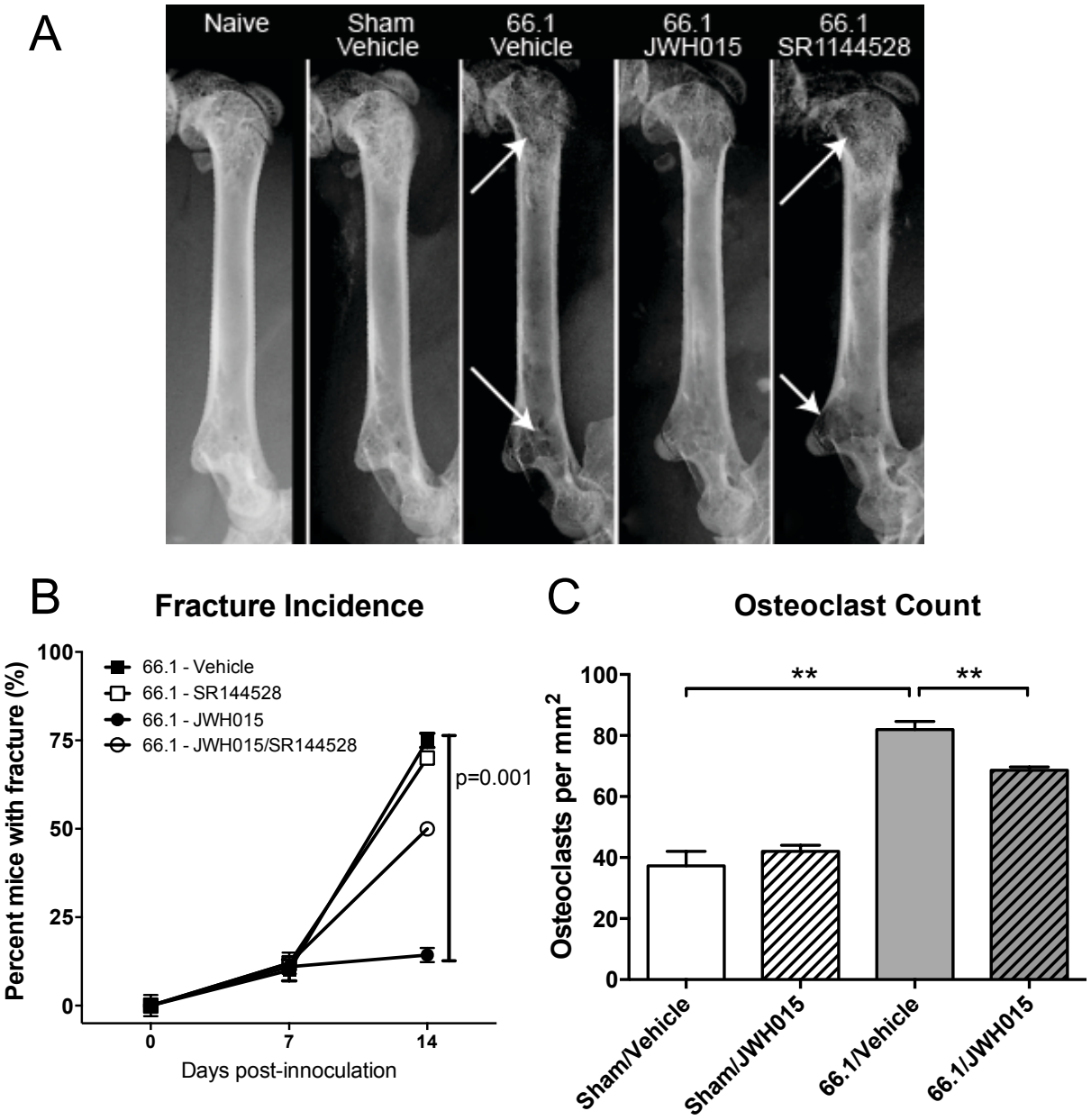

A
Naive
Sham Vehicle
66.1 Vehicle
66.1 JWH015
66.1 SR1144528
B
Fracture Incidence
66.1 - Vehicle
66.1 - SR144528
66.1 - JWH015
66.1 - JWH015/SR144528
p=0.001
Percent mice with fracture (%)
Days post-innoculation
C
Osteoclast Count
Osteoclasts per mm2
**
**
Sham/Vehicle
Sham/JWH015
66.1/Vehicle
66.1/JWH015

Figure 4.4: JWH015 is osteoprotective against tumor-associated fracture and osteoclastogenesis. (a) Sample radiograph images of the right femur at 14 dpi. 66.1 inoculation produced radiolucent lesions evident at the distal head of the femur that extended towards the proximal end (indicated with arrows). (b) Treatment of tumor-bearing mice with JWH015 (6 mg/kg/day) for 7 days reduced the incidence of spontaneous fracture as compared to vehicle-treated controls. Furthermore, cotreatment of JWH015 with SR144528 (3 mg/kg/day, i.p.) partially abolished JWH015-induced osteoprotection. Fracture incidence in mice treated with SR144528 alone was not significantly different from vehicle control. (n=8) (c) Tumor-bearing bone displayed a 2-fold increase in the number of TRACP5b-positive osteoclast cells. Osteoclast number was significantly attenuated in the tumor-bearing bone of animals treated with JWH015 treatment (6 mg/kg/day) for 7 days. (n=4) ** indicates significance at $p<0.01$

Figure 4.5

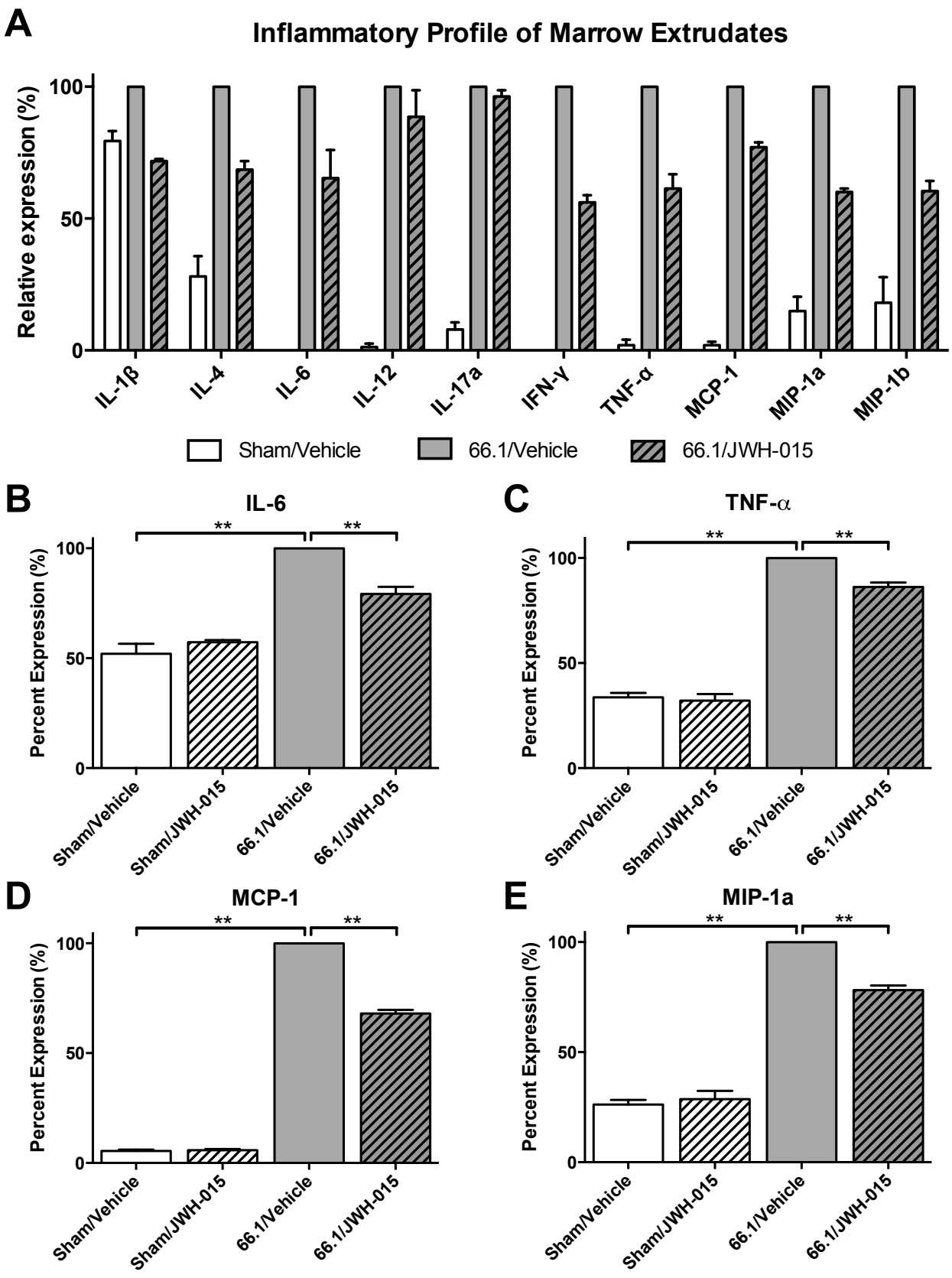

A
Inflammatory Profile of Marrow Extrudates
Relative expression (%)
100
50
0
IL-1β
IL-4
IL-6
IL-12
IL-17a
IFN-γ
TNF-α
MCP-1
MIP-1a
MIP-1b
Sham/Vehicle
66.1/Vehicle
66.1/JWH-015
B
IL-6
C
TNF-α
D
MCP-1
E
MIP-1a
Percent Expression (%)
**
Sham/Vehicle
Sham/JWH-015
66.1/Vehicle
66.1/JWH-015

Figure 4.5: JWH015 suppresses the secretion of inflammatory mediators in the bone-tumor microenvironment. (a) A semi-quantitative ELISA was used to evaluate the effect trend of JWH015 (6 mg/kg/day, i.p. 7 days) on inflammatory mediators in tumor-bearing marrow extrudate at 14 dpi (n=2). (b) IL-6 and (c) TNF-α were highly expressed in 14 dpi tumor-bearing bone extrudate (n=3, where each n=4 pooled extrudates) and expression was partially attenuated by JWH015 treatment (6 mg/kg/day, 7 days). For chemokines MCP-1 (d) and MIP-1a (e), expression was significantly higher in tumor-bearing femurs and JWH015 treatment for 7 days partially attenuated chemokine expression (n=3).

** indicates significance at $p<0.01$.

Figure 4.6

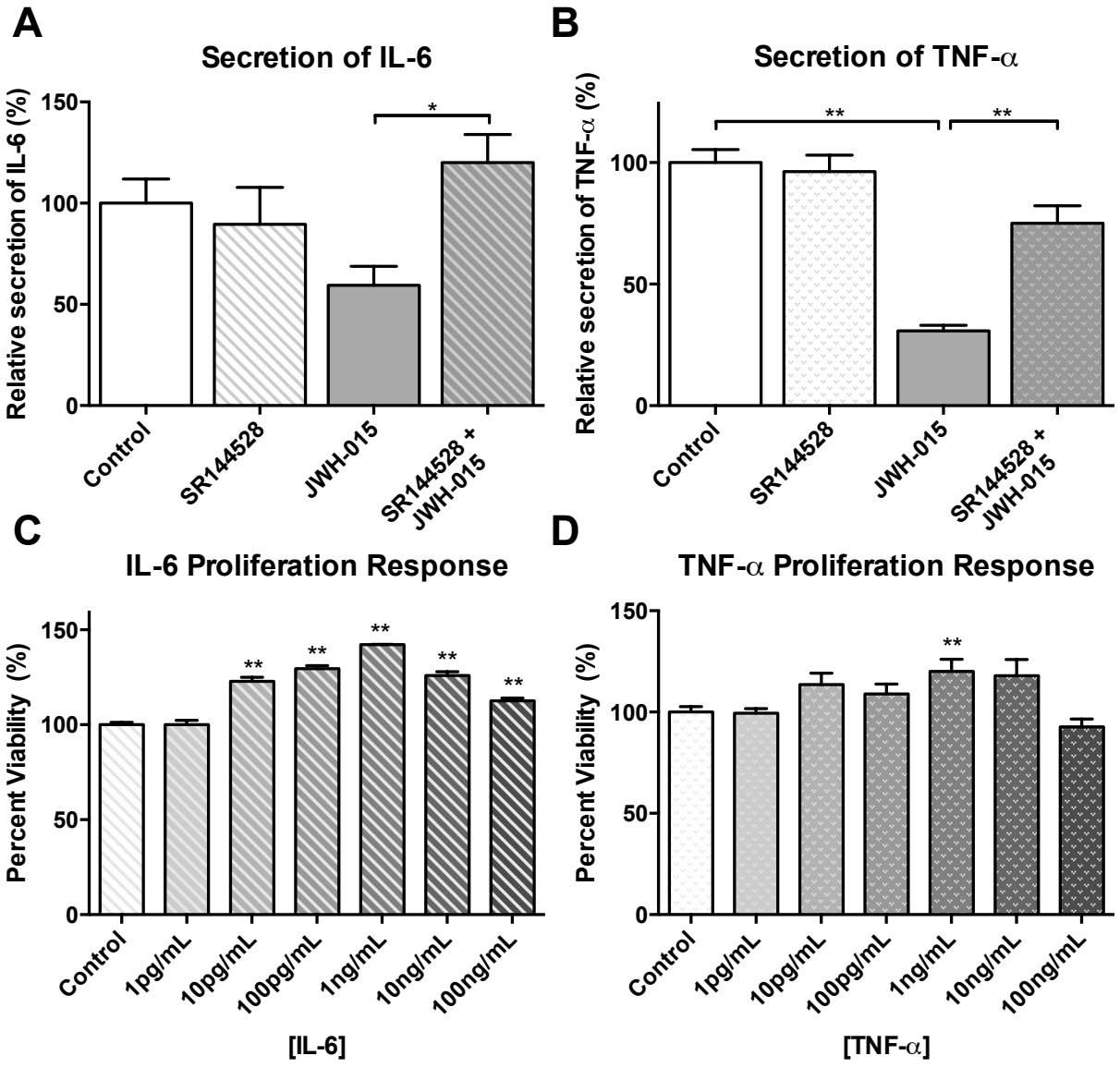

A
Secretion of IL-6
Relative secretion of IL-6 (%)
150
100
50
0
*
Control
SR144528
JWH-015
SR144528 + JWH-015
B
Secretion of TNF-α
Relative secretion of TNF-α (%)
**
**
100
50
0
Control
SR144528
JWH-015
SR144528 + JWH-015
C
IL-6 Proliferation Response
Percent Viability (%)
150
100
50
0
**
**
**
**
**
Control
1pg/mL
10pg/mL
100pg/mL
1ng/mL
10ng/mL
100ng/mL
[IL-6]
D
TNF-α Proliferation Response
Percent Viability (%)
150
100
50
0
**
Control
1pg/mL
10pg/mL
100pg/mL
1ng/mL
10ng/mL
100ng/mL
[TNF-α]

Figure 4.6: JWH015 produces CB_2-dependent suppression of IL-6 and TNF-α secretion from 66.1 cells. (a) 66.1 mammary adenocarcinoma cells were seeded overnight and then treated for a 3-hour period with media control or JWH015 (1μM) in the presence or absence of SR144528 (100nM). 66.1 cells produced basal concentrations of (a) IL-6 and (b) TNF-α. Treatment with JWH015 significantly attenuated basal cytokine production in an SR144528-sensitive manner; SR144528 treatment alone had no effect on basal cytokine secretion (n=6). (c) Concentration-response of 66.1 cells to exogenous IL-6 (1pg/mL-100ng/mL) and (d) exogenous TNF-α (1pg//mL-100ng/mL) stimulation for 24 hours. IL-6 produced concentration-dependent enhancement of viability that peaked at 1 ng/mL (n=6) whereas TNF-α only produced a significant enhancement of viability at 1 ng/mL (n=6).

* indicates significance at $p<0.05$, ** indicates significance at $p<0.01$.

Figure 4.7

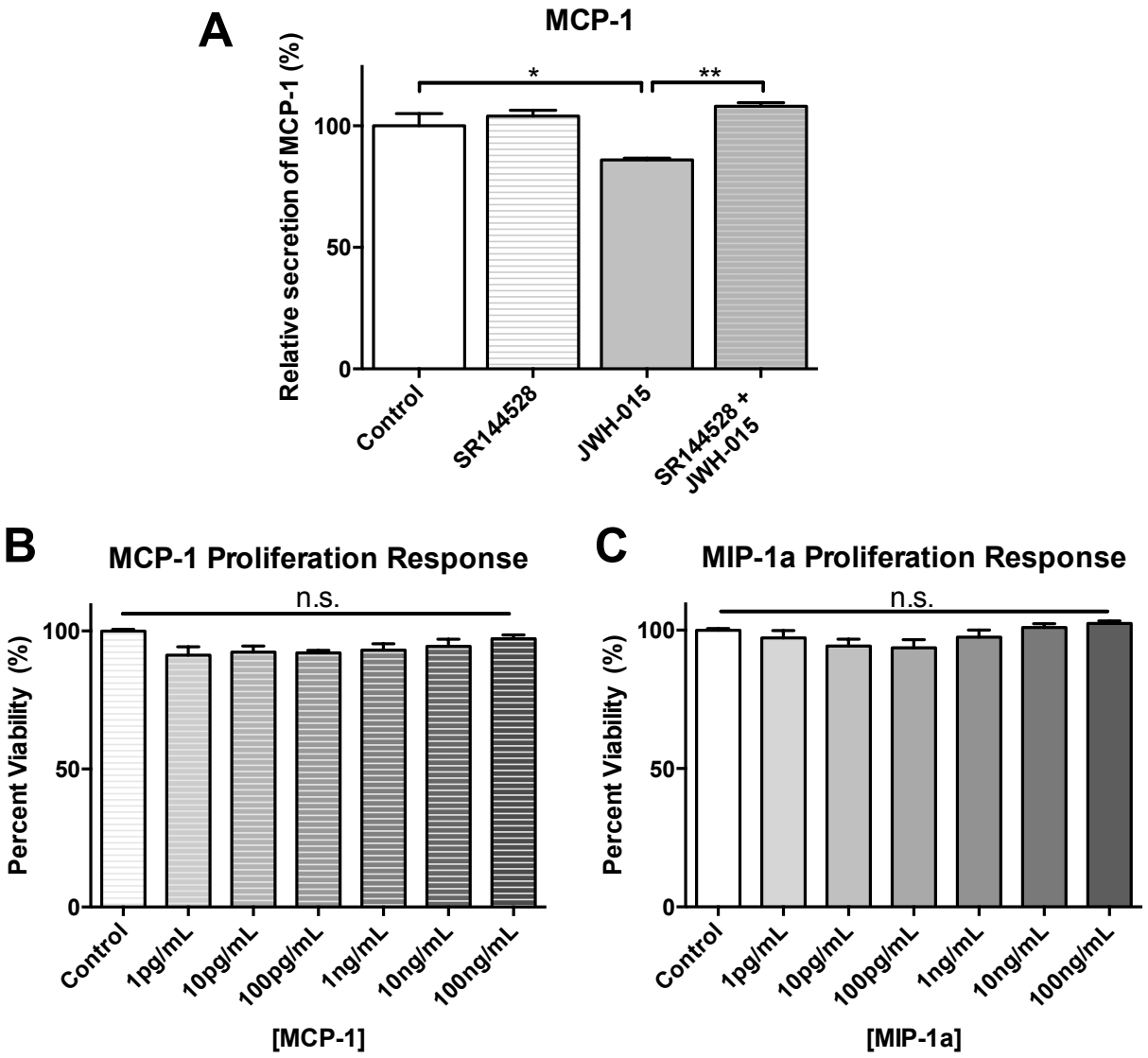

A
MCP-1
Relative secretion of MCP-1 (%)
*
**
100
50
0
Control
SR144528
JWH-015
SR144528 + JWH-015
B
MCP-1 Proliferation Response
n.s.
Percent Viability (%)
100
50
0
Control
1pg/mL
10pg/mL
100pg/mL
1ng/mL
10ng/mL
100ng/mL
[MCP-1]
C
MIP-1a Proliferation Response
n.s.
Percent Viability (%)
100
50
0
Control
1pg/mL
10pg/mL
100pg/mL
1ng/mL
10ng/mL
100ng/mL
[MIP-1a]

Figure 4.7: JWH015 produces CB_2-dependent suppression of MCP-1 secretion from 66.1 cells. (a) 66.1 mammary adenocarcinoma cells were seeded overnight and then treated for a 3-hour period with media control or JWH015 (1μM) in the presence or absence of SR144528 (100nM). 66.1 cells produced basal concentrations of MCP-1. Treatment with JWH015 significantly attenuated basal MCP-1 production in an SR144528-sensitive manner; SR144528 treatment alone had no effect on basal MCP-1 secretion (n=6). (b) Concentration-response of 66.1 cells to exogenous MCP-1 (1pg/mL-100ng/mL) and (d) exogenous MIP-1a (1pg//mL-100ng/mL) stimulation for 24 hours. Neither chemokine elicited a significant change in viability (n=6).

* indicates significance at $p<0.05$, ** indicates significance at $p<0.01$.

4.7 Conclusions

Cannabinoids have gained significant recent attention for the non-FDA approved usage of medicinal marijuana in the palliative care of suffering cancer patients. Few cannabinoid drugs are available for use in cancer pain, and those with approval (Marinol, Cesamet) possess mixed CB_1/CB_2 activity that may offer unwanted psychotropic effects to patients. In this work, we demonstrate the clear utility of a CB_2-specific strategy, JWH015, as an analgesic for the treatment of cancer-induced bone pain: JWH015 as a stand-alone therapy produced CB_2-dependent relief of spontaneous cancer pain behaviors in the 66.1-BALB/cfC3H model of breast cancer bone metastasis. Additionally, JWH015 demonstrated key disease modifying effects that may make it a strategically prudent adjunct to current opioid therapies: JWH015 produced CB_2-mediated osteoprotective effects and decreased primary tumor burden, effectively reducing two add-on mechanisms of pain that contribute to opioid failure. Lastly, the anti-inflammatory and neuroprotective effects of JWH015 may have implications for combating opioid tolerance.

Advanced-stage cancer patients remain in desperate need of alternative strategies for relief from cancer pain. In this chapter, we offer evidence that a nonpsychotropic CB2 agonist may serve as a useful adjunct therapy in the treatment of metastatic breast cancer and cancer-induced bone pain. By improving palliative care, patients will experience a greater quality of life that ultimately benefits treatment success and survival.

CHAPTER 5: ADENOSINE RECEPTOR 3 AGONIST ATTENUATES CANCER-INDUCED PATHOLGIES AND PAIN

5.1 Introduction

Adenosine is a purinergic nucleoside that is comprised of an adenine base attached to a ribose sugar. Physiological adenosine occurs intracellularly as the result of 5'-adenosine monophosphate (5'-AMP) metabolism or extracellularly through the metabolism of released nucleotides by ecto-5'-nucleotidases[175]. Adenosine that is generated in the extracellular space or released through nucleoside transporters can act at four g-coupled protein receptor (GPCR) subtypes including A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R to mediate an array of physiological actions[176]. In the central and peripheral nervous systems, adenosine is a vital extracellular signaling molecule that is most known for its positive regulatory effects on cerebral blood flow[177, 178] and for its action as an inhibitory fine-tuning mechanism for excitatory neurotransmitter systems[179, 180]. There is a complex interplay between the neuroinhibitory effects of $G_{i/o}$-coupled receptors A_1R and A_3R, which reduce neurotransmitter release at presynaptic terminals and depress neuronal firing at postsynaptic sites[181-183], and the neuroexcitatory effects of G_s-coupled A_{2A} and A_{2B} receptors, which can promote the release of glutamate and acetylcholine but also GABA in the CNS[184-186].

The ability of adenosine to act as a neuromodulator and neuroprotectant is of chief interest to pain research; however, adenosine has a notoriously short physiological half-life (<10 seconds) that has complicated its analysis. Strategies to potentiate the half-life of adenosine using inhibitors of AK, an enzyme which regulates intra- and extra-cellular concentrations of adenosine, has uncovered the robust antinociceptive

properties of endogenous adenosine[187-189]. Previous work has demonstrated that A_1AR activation[187] and to a lesser extent A_{2A}AR activation[190] mediate the antinociceptive effects of adenosine in models of neuropathic pain[187, 191]; however, the therapeutic utility of A_1AR and A_2AR agonists is limited by profound cardiovascular side effects[192]. Studies of adenosine in neuropathic pain have not evaluated a role for the G_i-coupled A_3 receptor [191, 193] until recently: our collaborators at Saint Louis University demonstrated that IB-MECA and CI-IB-MECA, two drugs with moderate specificity for A_3R, provide A_3-dependent antinociception in the murine chronic constriction injury (CCI) model of neuropathic pain and in several models of rodent chemotherapy-induced peripheral neuropathy (CIPN)[194] Both IB-MECA and CI-IB-MECA, in clinical trials for other indications[195], have not reported serious cardiovascular side effects[196, 197]. These data suggest that A_3 may be a viable target for CIBP.

A_3R antagonists as a complementary adjunct to opioids in cancer pain.

A_3R agonists have already shown antinociceptive potential in preclinical CIPN, a condition that is responsible for approximately 10% of cancer pain patients[194]. However, a greater inquiry lies in the ability of A_3R agonists to provide relief of cancer-induced bone pain, a multifaceted and robust pain state, in a manner complementary to opioid therapy. The development of novel anaglesics as opioid adjuncts allows the effective clinical use of opioids to continue while providing a new alternative for patients who do not achieve adequate pain relief with opioids alone.

Opioid-induced bone loss. We have previously explored the calamitous pro-osteolytic effect of OIBL in our model of breast cancer bone metastasis. Bone loss and

associated skeletal related events are a source of severe pain in CIBP. Adenosine has a recognized role in bone metabolism[198, 199] and the action of adenosine in bone typically favors osteoblast differentiation and bone formation[200, 201]. The A_3R agonist IB-MECA prevents inflammatory bone loss in a rodent model of arthritis[202] and stimulates marrow hematopoesis[203]. Therefore, it may be hypothesized that A_3R agonists could provide osteoprotection against tumor-associated bone loss and OIBL.

Disease modification. Given the role of tumor burden in CIBP, disease modification can have a profound role in pain state and analgesic efficacy. Adenosine appears to have an important role in the regulation of tumor growth and viability[204]. Several adenosine agonists have shown utility as anti-cancer agents, with a recent emphasis on A_3R[205]: IB-MECA and Cl-IB-MECA have demonstrated anti-tumor effects in colon and prostate carcinoma and lymphoma[206], and Cl-IB-MECA has been recommended as a chemotherapy adjunct for paclitaxel in the treatment of metastatic melanoma[207]. Moreover, Cl-IB-MECA has shown promising anti-tumor effects in a preclinical model of breast cancer bone metastasis[208], making A_3 a convincing target for CIBP. Importantly, the activation of the G_s-coupled A_{2A} and A_{2B} receptors appears to have a converse role in tumor progression, promoting angiogenesis[209] and adaptive immune suppression[210]; given the typical interplay between G_i-coupled and G_s-coupled adenosine receptors, it follows that A_3-specific strategies should be emphasized in CIBP for their potential anti-tumor effects.

5.2 A_3R is functionally expressed at spinal and supraspinal sites involved in pain processing.

To date, the distribution of the A_3R in the CNS is poorly understood[211-219]. In preliminary studies, we collaborated with the laboratory of Dr. Daniela Salvemini at Saint Louis University to evaluate distribution of the A_3R in pain-related areas of the central nervous system. Both mRNA transcripts (data not shown) and protein for A_3R were detected in the rodent spinal cord and rostral ventromedial medulla (RVM) (Fig 5.1), demonstrating an expression pattern of the A_3R consistent with a role in antinociception (Fig 5.1A, B). In parallel studies, the rodent model of chronic constriction injury (CCI) was utilized to evaluate whether A_3R expression in the RVM and spinal cord are functional. Adenosine half-life was potentiated through the systemic administration of ABT-702, an adenosine kinase inhibitor. Systemic administration of ABT-702 produced anti-allodynia that was attenuated with intrathecal or intra-RVM injection of a specific A_3R antagonist, MRS1523[220]. These data are consistent with endogenous adenosine accumulation and A_3R activation as a mechanism of antinociception in the spinal cord and RVM. Accordingly, it was concluded that A_3R is functionally expressed at spinal and supraspinal sites involved in nociceptive processing.

5.3 MRS5698 attenuates behavioral indicators of cancer-induced bone pain

Given the validity of A_3R as an antinociceptive target and the efficacy of IB-MECA in CIPN cancer pain, we asked whether A_3R activation could produce antinociception in the robust and complex pain state of CIBP. We employed the agent MRS5698, an A_3R agonist that displays high affinity for A_3AR ($\leq$3 nM) and excellent

selectivity ($\geq 10^4$ fold selectivity over human, rat and mouse A_1AR or $A_{2A}AR$)[221]. We evaluated spontaneous flinching and guarding in the 66.1-BALB/cfC3H model of breast cancer bone metastasis between 10-12 dpi until a pain state was established with a baseline of ≥ 5 flinches and ≥ 10 seconds guarding over a 2-minute period. Systemic administration of MRS5698 elicited a dose-dependent decrease in spontaneous flinching behavior (Fig 5.2A) and guarding behavior (Fig 5.2C) with a peak effect at 60 minutes (ED_{50} 0.14 mg/kg for flinching, ED_{50} 0.63 mg/kg for guarding) and resolution of the antinociceptive effect by 240 minutes. MRS5698 antinociception at peak effect was comparable to that of morphine at 60 minutes post-injection (Fig 5.2B, 5.2D). During treatment, animals displayed no obvious signs of sedation or motor impairment. These data demonstrate a robust dose-dependent antinociceptive effect of acutely administered MRS5698, a specific A_3 agonist, in CIBP.

Figure 5.1

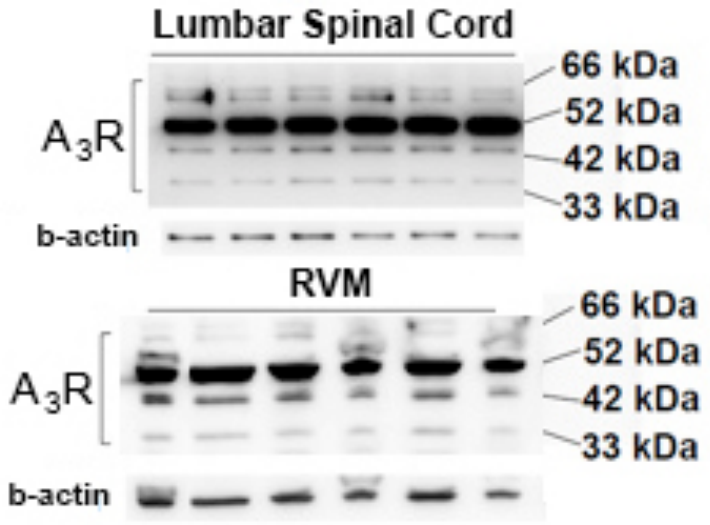

Lumbar Spinal Cord
66 kDa
52 kDa
42 kDa
33 kDa
A3R
b-actin
RVM
66 kDa
52 kDa
42 kDa
33 kDa
A3R
b-actin

Figure 5.1: Expression of A_3R in the rodent spinal cord and RVM. Central expression of glycosylated (42, 42 and 55 kDa) and unglycosylated (33 kDa) A_3R isoforms in rodent spinal cord (L4-L6) and rostral ventromedial medulla (RVM) (n=6 shown). B-actin shown as a loading control.

Figure 5.2

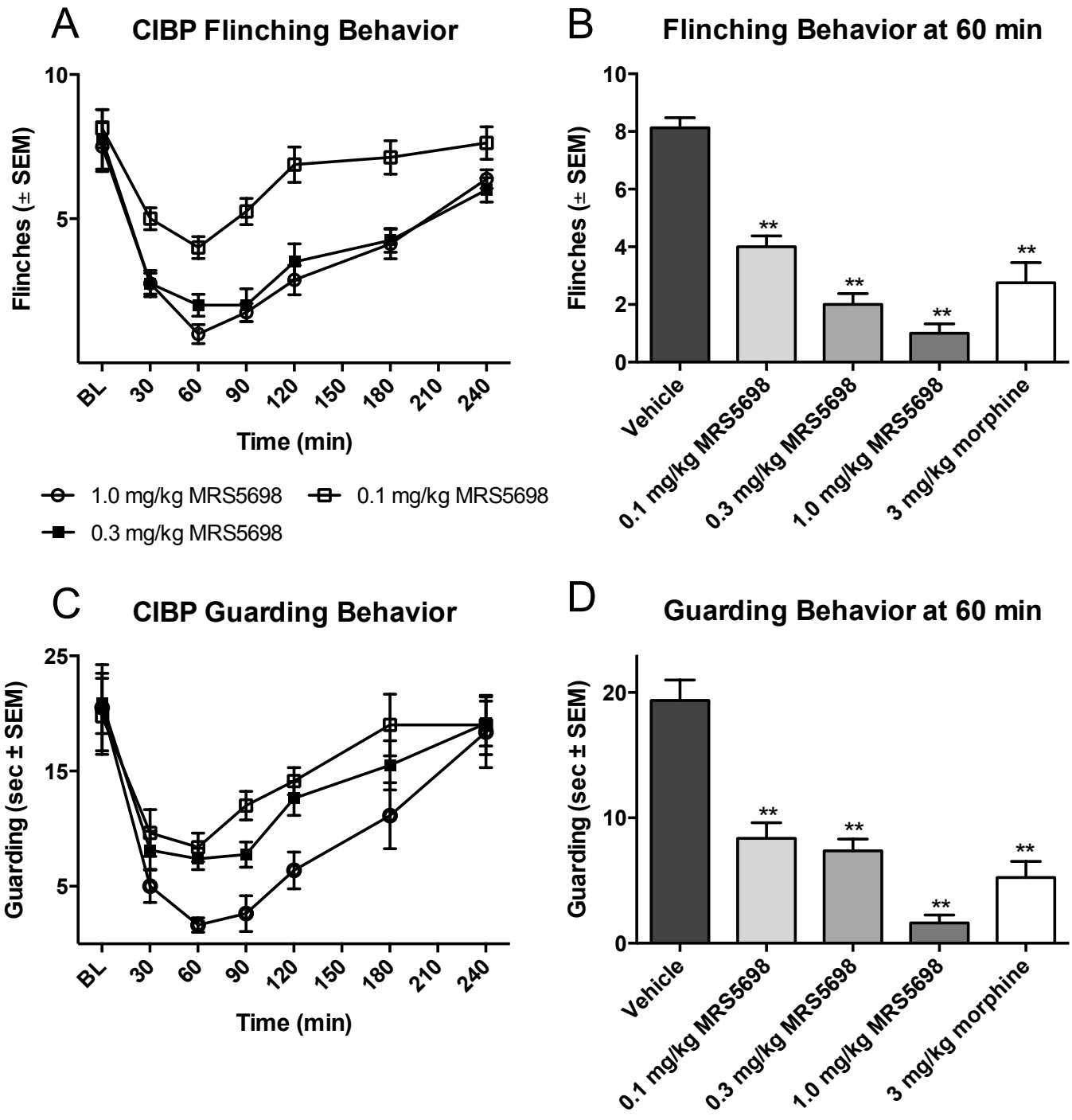

A
CIBP Flinching Behavior
Flinches (± SEM)
10
5
BL
30
60
90
120
150
180
210
240
Time (min)
1.0 mg/kg MRS5698
0.1 mg/kg MRS5698
0.3 mg/kg MRS5698
B
Flinching Behavior at 60 min
Flinches (± SEM)
10
8
6
4
2
0
**
Vehicle
0.1 mg/kg MRS5698
0.3 mg/kg MRS5698
1.0 mg/kg MRS5698
3 mg/kg morphine
C
CIBP Guarding Behavior
Guarding (sec ± SEM)
25
15
5
BL
30
60
90
120
150
180
210
240
Time (min)
D
Guarding Behavior at 60 min
Guarding (sec ± SEM)
20
10
0
**
Vehicle
0.1 mg/kg MRS5698
0.3 mg/kg MRS5698
1.0 mg/kg MRS5698
3 mg/kg morphine

Figure 5.2: MRS5598 alleviates behavioral indicators of CIBP. A suspension of 66.1 murine mammary adenocarcinoma cells or media control was injected into the right femur of naïve mice on day 0. Mice were tested prior to surgery and again between days 10-12 post-inoculation (dpi). When animals baselined ≥5 flinches and ≥10 seconds of guarding, animals were dosed (10 mL/kg, i.p.) accordingly with 2% DMSO vehicle or vehicle containing MRS5698. (a) Dose-response time course for flinching behavior demonstrating a dose-dependent reversal of pain behavior that resolved at 240min. (b) Time of peak effect occurred at t=60min; peak efficacy was achieved with MRS5698 0.3 mg/kg (0.3 and 1.0 mg/kg n.s. at t=60min) and was not significantly different from morphine 3 mg/kg (positive control). (c) Dose-response time course for guarding behavior demonstrating a dose-dependent reversal of pain behavior that resolved at 240min. (d) Consistent with flinching data, time of peak effect occurred at t=60min; peak efficacy was achieved with MRS5698 1.0 mg/kg and was not significantly different from morphine 3mg/kg (positive control). (n=8)

** indicates significance from vehicle treatment at p<0.0 1.

5.4 Conclusions

Adenosine receptor agonists are increasingly available in the clinical context due to on-going trials involving the A_3R agonists IB-MECA and Cl-IB-MECA[196, 197] in arthritis and cancer. We have demonstrated a clear utility for A_3R-specific strategies in the 66.1-BALB/cfC3H model of CIBP using MRS5698, an orally bioavailable A_3R agonist that displays greater selectivity for A_3R than IB-MECA or Cl-IB-MECA[221]. MRS5698 produced dose-dependent relief of CIBP-associated pain behaviors. Recent work utilizing MRS5698 in preclinical models of neuropathic pain has demonstrated that A_3R agonists do not produce analgesic tolerance or conditioned-place preference in rodents[220], making them useful adjuncts to opioids. In the context of CIBP, the hypothesized osteoprotective and anti-tumor effects of A_3 engagement warrant investigation and indeed, this work has laid a foundation for future investigations of MRS5698 in our laboratory's model of CIBP. Future studies should determine if, in addition to providing acute relief from pain, A_3R agonists also protect against pathological nociceptive changes when administered over a chronic timecourse. Furthermore, the interactions of A_3R agonists and μOR agonists are unknown to date; given that adenosine via A_3R acts as an inhibitory neuromodulator, the potential for synergistic interaction of adenosine with the opioidergic system should be investigated. The results of these studies will not only support the potential usage of FDA-approved A_3R agonists as adjunct therapeutics for cancer pain, but also inform future drug development for the treatment of chronic pain as a whole.

CHAPTER 6: A PARTIAL AGONIST OF SPHINGOSINE-1-PHOSPHATE RECEPTOR 1 ATTENUATES CANCER-INDUCED PATHOLOGIES AND PAIN

6.1 Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that plays an important role in cell growth, migration and survival. S1P is generated from ceramide, a lipid component of cell membranes: ceramide is hydrolyzed by ceramidases and phosphorylated by sphingosine kinases (SK1, SK2) to produce S1P[222]. S1P initiates signaling through a family of five cognate G protein-coupled receptors ($S1PR_{1-5}$) that are widely distributed the brain, heart, lung, stomach, intestine, adrenal gland and lymphoid tissue[222, 223]. S1P signaling typically enhances cell survival whereas the signaling of its precursor, ceramide, elicits pro-apoptotic signaling in a mechanism known as the ceramide/S1P rheostat[224].

Modulators of S1P signaling have recently gained attention in the neuropharmacology community due to the innovation of a sphingosine analog S1P agonist/functional antagonist, FTY720 (fingolimid): in 2010, FTY720 became the first orally available agent approved by the FDA for the treatment of relapsing-remitting multiple sclerosis (RR-MS) [225-227], an autoimmune disorder characterized by central neuroinflammation, demyelination, and neurodegeneration. FTY720 is thought to provide neuroprotection in RR-MS by inhibiting the motility of autoaggressive lymphocytes[228, 229] and regulating neuroinflammation via of $S1P_1$, $S1P_3$ and $S1P_5$ on astrocytes[230, 231], oligodendrocytes and microglia[232].

S1P has emerged as key mediator in the development of peripheral and central nociceptive sensitization[233, 234]. Peripherally, S1P via $S1PR_1$ increases the

excitability of small diameter sensory neurons and contributes to nerve growth factor (NGF)-induced sensitization of sensory neurons [235-239]. S1P signaling also enhances the activity of presynaptic NMDARs to result in increased glutamate release from primary afferent terminals[240]. Intraplantar injection of S1P or $S1PR_1$ agonists [241, 242] evokes profound tactile hypersensitivity and the subsequent formation of a peripheral inflammatory response [241, 243, 244]. In the CNS, S1P is thought to elicit glial activation and associated neuroinflammatory responses that contribute to nociceptive sensitization; accordingly, S1P is elevated in the dorsal horn in preclinical models of neuropathic pain[245] and in preclinical morphine tolerance[246, 247]. These studies suggest that targeting S1P could be a useful strategy in pain states like CIBP that incorporate neuropathic and inflammatory features.

S1P modulators as a complementary adjunct to opioids in cancer pain.

Disease modification. It is thought that the ceramide/S1P rheostat plays an important role in the survival of malignant cells. To this end, chemotherapeutic strategies have harnessed the ceramide-to-S1P pathway in the development of novel anticancer agents by promoting ceramide (eg. ceramidases inhibition), reducing S1P bioavailability (eg. SphK inhibition) or attenuating S1P/$S1PR_1$ signaling with anti-S1P antibodies or $S1PR_1$ modulators [224, 248]. These strategies have been fruitful in attenuating primary tumor burden and retarding cancer progression. Importantly, S1P signaling may also have implications for chemotherapy-related changes in disease progression: attenuation of S1P signaling chemotherapy-induced neuropathy via paclitaxel reduces associated mechano-hypersensitivity[249]. Taken together, these findings suggest that S1P

modulators can be designed to protect against the progression of CIPN as well as cancer progression.

Tolerance. The $S1P_1$ receptor was recently demonstrated to play a role in morphine-induced tolerance in naïve rodents: co-administration of FTY720 with morphine prevented the development of morphine tolerance in a dose-dependent manner[250], suggesting that S1P signaling interacts significantly with opioid systems. While this study currently stands alone in the representation of FTY720's adjunct utility for opioid tolerance, it is a powerful finding that demands further exploration[234]. One hypothesis is that the inhibitory effects of FTY720 on microglial $S1P_1R$ suppresses neuroinflammation in a manner parallel to other agents which have a role in preventing opioid tolerance[251].

6.2 FTY720 attenuates behavioral indicators of cancer-induced bone pain

Studies investigated the ability of FTY720 to alleviate cancer-induced bone pain in the 66.1-BALB/cfC3H model of breast cancer bone metastasis. We evaluated spontaneous flinching and guarding behaviors between 10-12 dpi until a pain state was established with a baseline of $\geq$5 flinches and $\geq$10 seconds guarding over a 2-minute period. Systemic administration of FTY720 produced a dose-dependent decrease in spontaneous flinching behavior (Fig 5.2A) and guarding behavior (Fig 5.2C) with a peak effect at 90 minutes (ED_{50} 0.16 mg/kg for flinching, ED_{50} 0.31 mg/kg for guarding) (Fig 5.2B, 5.2D) and resolution of the antinociceptive effect by 360 minutes. Compared to morphine, FTY720 was late-acting and long-lasting (data not shown). FTY720 antinociception was not compared to morphine at time of peak effect due to a lack of morphine effect at t=90 minutes. During treatment, animals displayed no obvious signs

of sedation or motor impairment. These data demonstrate an efficacious and dose-dependent antinociceptive effect of acutely administered FTY720 in murine CIBP.

Figure 6.1

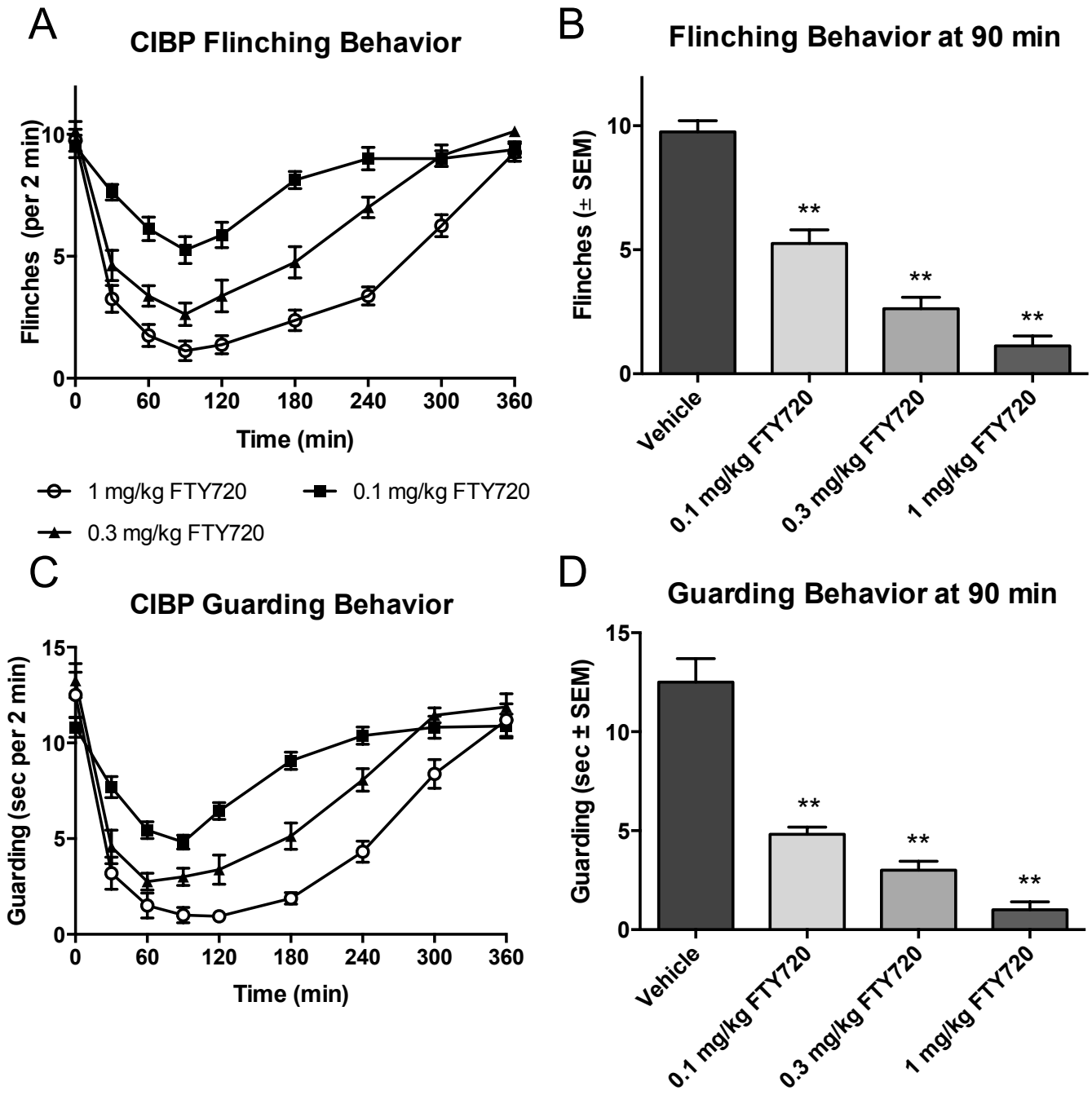

A
CIBP Flinching Behavior
Flinches (per 2 min)
Time (min)
1 mg/kg FTY720
0.1 mg/kg FTY720
0.3 mg/kg FTY720
B
Flinching Behavior at 90 min
Flinches (± SEM)
Vehicle
0.1 mg/kg FTY720
0.3 mg/kg FTY720
1 mg/kg FTY720
C
CIBP Guarding Behavior
Guarding (sec per 2 min)
Time (min)
D
Guarding Behavior at 90 min
Guarding (sec ± SEM)

Figure 6.1: FTY720 alleviates behavioral indicators of CIBP. A suspension of 66.1 murine mammary adenocarcinoma cells or media control was injected into the right femur of naïve mice on day 0. Mice were tested prior to surgery and again between days 10-12 post-inoculation (dpi). When animals baselined ≥5 flinches and ≥10 seconds of guarding, animals were dosed accordingly (10 mL/kg, i.p.) with 2% DMSO vehicle or vehicle containing FTY720. (a) Dose-response time course for flinching behavior demonstrating a dose-dependent reversal of pain behavior that resolved at 240min. (b) Time of peak effect occurred at t=60min; peak efficacy with 0.3 mg/kg was not statistically different from 1.0 mg/kg FTY720. (c) Dose-response time course for guarding behavior demonstrating a dose-dependent reversal of pain behavior that resolved at 360min. (d) Consistent with flinching data, time of peak effect occurred at t=60min; peak efficacy with 0.3 mg/kg was not statistically different from 1.0 mg/kg FTY720. (n=8)

** indicates significance from vehicle treatment at p<0.0 1.

6.3 Conclusions

In the current work, we evaluated the antinociceptive potential of FTY720, a novel S1P modulator that produces functional antagonism of the $S1P_1R$, in the 66.1-BALB/cfC3H model of cancer-induced bone pain. Systemic administration of FTY720 produced an efficacious and dose-dependent relief of spontaneous pain behaviors including flinching and guarding. This preliminary data provides a foundation for a future post-doctoral project investigating the mechanisms of FTY720 pain relief; whereas initial speculation about the efficacy of FTY720 assumed that lymphoid sequestration regulated by the $S1P/S1P_1R$ axis was responsible for neuroprotection[252], we now hypothesize that it the suppression of monocyte-derived inflammation (eg. microglia) is an equivalent mechanism of FTY720 action. Future studies will first investigate the robustness of FTY720 analgesia in models of CIBP and CIPN and seek to determine whether peripheral mechanisms of monocyte suppression in the bone microenvironent or central mechanisms of neuroinflammatory suppression have roles in mediating FTY720 analgesia. Furthermore, we will evaluate the potential disease modification of CIBP by FTY720 (eg. tumor-related bone loss, tumor progression) and interactions with μOR analgesia in order to assess the qualification of FTY720 as a suitable co-therapy for the management of cancer-induced bone pain.

CHAPTER 7: DISCUSSION AND FUTURE DIRECTIONS

7.1 Introduction

According to the World Health Organization, there were 32.6 million individuals living with cancer in 2012 and 14.1 million new cancer diagnoses over the course of that year. Given the prevalence of cancer pain in one-third of the patient population[4], a staggering 11 million individuals were affected by cancer pain in 2012. When considering the breadth of cancer pain, a sense of urgency is appropriate in the research and development of novel analgesics and adjunct therapeutics. In this dissertation, we have identified a critical pitfall of opioid therapy in patients suffering with cancer-induced bone pain—that is, the off-target action of morphine at TLR4 and associated bone pathology—and proposed several adjunct therapies that demonstrate both independent analgesic efficacy in our model of breast cancer bone metastasis and properties that lend these drugs to combination with opioids. Here, we will highlight common themes in the proposed therapeutics and discuss the implications of these themes for cancer pain palliative care.

7.2 The rising importance of neuroprotection in cancer pain.

Cancer-induced bone pain is a complex and multifaceted pain disorder where pain elicited by tumor burden in the bone can be compounded by neuropathic changes and chronic inflammation over time. Neuropathic and inflammatory complications can contribute to the persistence of pain following remission[6], and similar to cancer-pain, non-cancer pain is also subject to the pitfalls and failures of opioid therapies[253]. Since the advent of combined chemotherapeutic strategies, oncology has made leaps and bounds towards increasing the life span of cancer patients who would have

formerly been considered terminal; as such, it is becoming increasingly important to develop combined palliative care for cancer pain that does not only address immediate patient comfort, but also protects patients from future maladaptive changes that could decrease quality of life during end-stage cancer or, more optimistically, recovery. In one respect, the WHO 3-step ladder supports this perspective through the use of anti-osteolytic adjunct therapies in CIBP patients[16]; however, there are no widely used neuroprotective strategies in these patients, where protection from neuropathic changes may be as important to the present and future comfort of the patient as osteoprotective strategies. One reason for the dearth of emphasis on neuroprotective strategy may be due to a misunderstanding of the terminology in the context of cancer-induced bone pain: as was highlighted in the introduction, few studies have focused on the neuropathic characteristics of CIBP. Studies of sensory and sympathetic nerve fibers in proximity to tumor-bearing tissue has demonstrated the induction of pathological nerve sprouting [70-73], specifically in models of cancer metastasis to bone from primary breast[74] and prostate[75] tumors. Preventing the formation of pathological neuroma-like structures in the bone through the use of anti-growth factor therapy (blockade of NGF/TrkA) provides pain relief in preclinical CIBP models[76, 79] and is an example of a neuroprotective approach to cancer pain that has potential to reduce the likelihood of non-cancer pain following remission. To this end, A_3-specific strategies may prove useful: the ATP/adenosine axis plays an important role in neuroplasticity and specifically, therapeutic use of adenosine has been employed in a rodent model of epilepsy to reduce pathological sprouting of hippocampal mossy fibers[254]. The effect of adenosine on the pathological sprouting of peripheral afferent fibers in the context of

CIBP is currently unknown; future studies should evaluate the potential of adenosine agonists to elicit suppression of nerve sprouting in the periphery in order to determine the utility of this effect in preventing neuropathic changes associated with CIBP.

A second consideration in defining “neuroprotection” for CIBP is the role of neuroinflammation. In this dissertation, we identify TLR4-mediated neuroinflammation as one consequence of chronic opioid use, and a potential contributor to tolerance and states of paradoxical hyperalgesia[110, 117]. Ongoing research in our laboratory has corroborated the neuroinflammatory activation of spinal glia in cancer-induced bone pain and the relevance of this mechanism in pain progression. Preclinically, we and others have utilized (+)naloxone to attenuate opioid neuroinflammation associated with pain[57, 120, 255]; however, this strategy may be unwise in cancer patients due to the suppression of innate immunity by (+)naloxone and subsequent predisposal of patients to bacterial infection[256]. Accordingly, it may be prudent to develop strategies to combat neuroinflammation in CIBP while treading lightly to avoid peripheral immunological side effects that do not benefit cancer treatment. CB_2 agonists have provided useful suppression of neuroinflammation in a number of pain states[257] and confer protection against the development of neuropathic pain in preclinical models[258]. While CB_2 agonists are thought to suppress adaptive immunity[259], this translates to an anticancer effect in leukemias and lymphomas[260]. The attention given to CB_2 agonists for their analgesic and anticancer properties has perhaps detracted from emphasis on studies to evaluate the safety of cannabinoids in individuals vulnerable to immunocompromised states. The physiological relevance of putative cannabinoid immunosuppression remains unclear and warrants further research

attention, particularly in the context of cancer, so that CB_2-specific strategies can move forward as agents for the management of cancer pain. Similar considerations should be taken with other immunomodulatory analgesics, including the S1P modulator FTY720; however, it is important to note that the neuroprotective efficacy of FTY720 in Phase II clinical trials peaked at a dose that was suboptimal for the induction of lymphopenia[225], suggesting that immunomodulatory side effects may be averted by determining an appropriate therapeutic index for FTY720-mediated analgesia.

It is important to underscore that the goal of palliative care is to improve the quality of life of patients and their families in part through the prevention of suffering as well as through the relief of suffering. On this premise, it may be prudent for oncologists to work more closely with pain management specialists in order to include both anti-osteolyic and neuroprotective agents in the treatment of cancer pain.

7.3 Reinventing the ladder: should opioids remain the mainstay of palliative care?

The WHO 3-step ladder for the management of cancer pain has become the clinical standard for chronic pain as a whole, incorporating opioid therapies as the backbone of steps 2 and 3 for moderate-to-severe pain. In this dissertation, we highlight the limitations of morphine, an opioid that is commonly utilized in the management of cancer pain, and posit 3 non-opioid therapies that provide stand-alone analgesia in a clinically relevant model of cancer-induced bone pain. An important question arises: should opioids remain the backbone of clinical analgesia?

In short, the opinion of this dissertation is: yes. Despite a narrow therapeutic index, several disconcerting side effects and years of research criticizing opioid analgesia[261], clinicians remain reliant on the success of opioid analgesics in cancer

pain and as a foundation for palliative care. The work put forth herein emphasizes the development of analgesics that can translate into adjunct therapies to enhance opioid pain relief. While a stand-alone novel analgesic is the ideal result of therapeutic development, novel strategies are more easily transitioned to the clinic as opioid adjunct therapies: clinicians are unwilling to discontinue opioid therapy in patients even if there is only partial relief from pain, which is prohibitive for clinical trials of new stand-alone analgesics. Clinicians are, however, more amenable to introducing cotherapies that do not prohibit opioid use.

The opioidergic system is also highly plastic in its therapeutic potential. μOR agonists demonstrate analgesic synergy—that is, a greater than additive analgesic effect—with several co-therapies including acetaminophen[262], norepinephrine reuptake inhibitors[263], α2-adrengergic receptor agonists[264] and even other μOR agonists[265]. Our understanding of analgesic synergy is incomplete and there exists room for significant innovation on this front. Furthermore, analgesic research is only beginning to grasp the function and prospect of other opioid receptors such as the δOR[266] in μOR analgesia. To this end, ligands with mixed opioid receptor activity are being utilized preclinically: μOR ligands with mixed δOR agonist activity has been used to augment μOR analgesia[267], while conversely ligands with δOR antagonist properties delay μOR tolerance and show an improved side effect profile[268]. A greater understanding of the relationship between the δOR and μOR analgesia alone has tremendous potential to improve the tolerability and efficacy of opioid treatment[266].

With good reason, opioids remain the foundation for the clinical approach to pain. The anxiolytic and even sedative-hypnotic qualities of narcotics, while perceived as detrimental, contribute to the whole of opioid analgesia in providing relief from physical and emotional aspects of pain in higher primates. Opioids provide comprehensive inhibition of nociceptive signaling at the level of the periphery, spinal cord, supraspinal sites and cortical sites[269, 270], making opioid analgesia a truly robust strategy for many instances of pain control. Opioids will continue to be used in the treatment of cancer-induced bone pain and for chronic pain as a whole. It is the obligation of pharmacological research to fine-tune the mechanisms surrounding opioid analgesia and provide a broad range of specific co-therapeutic strategies that will allow clinicians to tailor analgesia to a given pain condition and ultimately improve the standard of care for patients suffering with pain.

APPENDIX A: LIST OF PUBLICATIONS

PUBLICATIONS

A. M. Symons-Liguori, L. M. Slosky, N. M. Sayers, T. W. Vanderah. (2014) Morphine produces toll-like receptor 4-dependent osteolysis and hyperalgesia in a model of metastatic bone pain. *PAIN* (submitted).

J. W. Little, A. Ford, **A. M. Symons-Liguori**, et al. (2014) Selective modulation of pathological pain states by endogenous adenosine A3 receptor activation. *Brain.* (submitted, under review).

K. E. Hanlon, A. N. Lozano-Ondoua, P. J. Umaretiya, **A. M. Symons-Liguori**, et al. (2014) Modulation of tumor cell viability by cannabinoid receptor 2 selective agonist is dependent on intracellular calcium flux. *Molecular Pharm.* (submitted, under review).

Lozano-Ondoua, **A. M. Symons-Liguori**, T. W. Vanderah (2013) Cancer-induced bone pain: Mechanisms and models. *Neurosci. Letters* 557A: 52-59.

A. M. Symons-Liguori and T. W. Vanderah (2013) The Delta Opioid Receptor in H. Ko and S. M. Husbands (Eds.), *Research and Development of Opioid-Related Analgesics* p.223-244.

Washington, DC: American Chemical Society Books.

Lozano-Ondoua, K. Hanlon, **A. M. Symons-Liguori**, et al. (2013) Disease modification of breast cancer-induced bone remodeling by cannabinoid 2 receptor agonists. *J. Bone Mineral Res.* 28(1): 92-107.

POSTER PRESENTATIONS

A. M. Symons-Liguori, N. M. Sayers, L. M. Slosky, T. W. Vanderah (2013) Non-opioid actions of morphine in a murine model of cancer-induced bone pain. 2013 Society for Neuroscience meeting abstracts, Abstract GGG29.

A. M. Symons-Liguori, N. M. Sayers, T. W. Vanderah. (2013) Pro-osteolytic effects of chronic morphine administration in a model of breast cancer-induced bone pain. 2013 Experimental Biology meeting abstracts, Abstract # 4388.

N. M. Sayers, **A. M. Symons-Liguori**, A. N. Lozano-Ondoua, T. W. Vanderah. (2013) Inflammatory signaling as a therapeutic target for the treatment of breast cancer-induced bone pain. 2013 Experimental Biology meeting abstracts, Abstract #3972.

A. N. Lozano-Ondoua, N. Williams, L. J. Anderson, **A. M. Symons-Liguori**, A. Bloom, P. W. Mantyh, T. W. Vanderah. (2012) Cannabinoid receptor-2 agonist attenuates cancer-induced bone pain via glial pro-inflammatory mediators.

APPENDIX B: PERMISSIONS

Not applicable.

APPENDIX C: HUMAN/ANIMAL SUBJECTS APPROVAL

THE UNIVERSITY OF ARIZONA

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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