CANNABINOID RECEPTOR 2: A NOVEL MULTI-TARGETED APPROACH IN THE TREATMENT OF BREAST CANCER AND RELATED SKELETAL METASTASIS

by

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SIGNED: Katherine Emily Hanlon
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I dedicate this manuscript to my parents Lora Jane and James Casey Hanlon; my brothers Rondel Casey, James Eric, and Edmond Cary Hanlon, and the rest of my family, without all of whom I wouldn't be here today. I make this dedication in particular to two of the most influential women of my life:

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"Keep your dreams alive. Understand to achieve anything requires faith and belief in yourself, vision, hard work, determination, and dedication. Remember all things are possible for those who believe."

-Gail Devers
# TABLE OF CONTENTS

## LIST OF FIGURES

- Figure 1.1: Breast Cancer Image...9

## ABSTRACT

- Title and abstract text...

## CHAPTER 1: INTRODUCTION

1.1 Breast Cancer .............................................................................. 13
1.2 Cancer Pain ................................................................................ 25
1.3 Immunology of the tumor microenvironment......................... 37
1.4 Cannabinoids .............................................................................. 43

## CHAPTER 2: MATERIALS AND METHODS

2.1 In Vitro ....................................................................................... 49
  2.1.1 Cell culture ........................................................................ 49
  2.1.2 Drugs ................................................................................. 49
  2.1.3 Immunoblotting ................................................................ 50
  2.1.4 Sulforhodamine B (SRB) assay ........................................ 50
  2.1.5 Ca\(^{2+}\) imaging ................................................................. 51
  2.1.6 Caspase 3/7 activity ............................................................ 51
  2.1.7 shRNA knockdown of CB\(_2\) receptor ................................ 52
  2.1.8 Immunohistochemistry ....................................................... 52
  2.1.9 Intracellular cAMP .............................................................. 52
  2.1.10 BrdU cell proliferation assay ........................................... 53

2.2 In Vivo ....................................................................................... 53
  2.2.1 Animals .............................................................................. 53
  2.2.2 Surgical Procedures ............................................................ 53
  2.2.3 Drug Treatment ................................................................. 54
  2.2.4 Behavioral Testing Protocols .............................................. 54
  2.2.5 In Vivo Imaging ................................................................. 55

2.3 Ex Vivo ....................................................................................... 56
  2.3.1 Primary tumor burden and metastasis detection ............... 56
  2.3.2 Flow Cytometry ................................................................. 57
  2.3.3 Magnetic assisted cell sorting (MACS) .............................. 57
  2.3.4 Suppression assay .............................................................. 58
TABLE OF CONTENTS- CONTINUED

2.3.5 Cytotoxicity assay ........................................................................................................................................ 58
2.3.6 Bone histology ........................................................................................................................................... 58
2.3.7 Micro-Computed Tomographic Analysis of Tumor-Induced Bone Destruction ................................................. 59
2.3.8 TNFα ELISA ............................................................................................................................................... 59
2.3.9 TRAP/5b and CTX measurement ................................................................................................................ 60

2.4 Statistical Analysis ......................................................................................................................................... 61

CHAPTER 3: DISEASE MODIFICATION OF BREAST CANCER INDUCED BONE REMODELING BY CANNABINOID RECEPTOR 2 AGONISTS ............................................... 62

3.1 Introduction ..................................................................................................................................................... 62
3.2 Acute or sustained CB2 agonist attenuates breast cancer-induced bone pain ......................................................... 65
3.3 CB2 agonist treatment reduces breast cancer-induced bone loss and fracture .............................................................. 73
3.4 CB2 agonists inhibit cancer cell growth in vivo and in vitro .................................................................................. 80
3.5 Antinociceptive effect of CB2 agonists in breast-induced bone cancer is mediated by the cytokine, TNFα .......................................................... 83
3.6 CB2 agonists maintain body weight and increase survival .................................................................................. 86
3.7 Conclusions ..................................................................................................................................................... 89

CHAPTER 4: CB2 INDUCED ATTENUATION OF BREAST CANCER PROLIFERATION: MECHANISMS OF ACTION ................................................................. 95

4.1 Introduction ..................................................................................................................................................... 95
4.2 JWH-015 attenuates proliferation of breast cancer cells in vitro ...................................................................... 99
4.3 JWH-015 attenuation of proliferation is not blocked by CB2 inverse agonist SR144528 ............................................. 102
4.4 Antiproliferative effects of JWH-015 are not blocked by a CB1 inverse agonist, TRPV1 or TRPA1 channel blockers, or the Gαi inhibitor pertussis toxin ............................................................ 105
4.5 4T1 cell survival in vitro is dependent on expression of CB2 ............................................................................ 108
4.6 JWH-015 induces apoptosis without inducing cell cycle arrest in breast cancer cells in vitro ..................................... 111
4.7 JWH-015 induction of apoptosis is dependent on calcium flux in vitro .............................................................. 114
4.8 JWH-015 induces intracellular calcium flux ...................................................................................................... 118
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.9</td>
<td>JWH-015 is not acting through a Gαi or Gαs mechanism in 4T1 cells at relevant concentrations in vitro</td>
<td>121</td>
</tr>
<tr>
<td>4.10</td>
<td>JWH-015 and SR144528 reduced pERK 1/2 phosphorylation and increased pAKT phosphorylation in vitro</td>
<td>124</td>
</tr>
<tr>
<td>4.11</td>
<td>JWH-015 reduces primary tumor burden and decreases metastasis in vivo</td>
<td>127</td>
</tr>
<tr>
<td>4.12</td>
<td>Conclusions</td>
<td>130</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>136</td>
</tr>
<tr>
<td>5.2</td>
<td>4T1 cells induce immunosuppressor cells when inoculated into the mammary fat pad</td>
<td>141</td>
</tr>
<tr>
<td>5.3</td>
<td>JWH-015 decreases the ratio of CD4+CD25+Foxp3+ regulatory T cells to CD4+CD25+Foxp3- effector T cells in tumor bearing animals</td>
<td>144</td>
</tr>
<tr>
<td>5.4</td>
<td>JWH-015 inhibits the suppressive activity of regulatory T cells ex vivo</td>
<td>147</td>
</tr>
<tr>
<td>5.5</td>
<td>JWH-015 does not inhibit the growth or activation of naïve T cells ex vivo</td>
<td>150</td>
</tr>
<tr>
<td>5.6</td>
<td>JWH-015 does not inhibit tumor killing cytotoxic activity of CD8+ cytotoxic T cells ex vivo</td>
<td>153</td>
</tr>
<tr>
<td>5.7</td>
<td>JWH-015 reduces spleen but not bone marrow population of myeloid derived suppressor cells in tumor bearing animals</td>
<td>156</td>
</tr>
<tr>
<td>5.8</td>
<td>Conclusions</td>
<td>159</td>
</tr>
<tr>
<td>6.1</td>
<td>Discussion and Future Directions</td>
<td>161</td>
</tr>
<tr>
<td>A1</td>
<td>List of Publications</td>
<td>167</td>
</tr>
<tr>
<td>A2</td>
<td>Permissions</td>
<td>170</td>
</tr>
<tr>
<td>A3</td>
<td>Human/Animal Subjects Approval</td>
<td>171</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>173</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

1. Acute systemic administration of CB2 receptor agonist JWH015 attenuates breast (66.1) cancer-induced spontaneous and evoked pain on day 14 after femoral inoculation .........................................................67
2. Chronic systemic administration of CB2 receptor agonist JWH015 and AM1241 attenuates spontaneous pain; blocked by a CB2 antagonist but not by a CB1 antagonist .................................................................69
3. Chronic systemic administration of CB2 receptor agonist JWH015 and AM1241 attenuates tactile evoked pain .............................................................71
4. Radiographs of the femora in the presence of either media (control) or breast cancer cells (66.1) on day 14 after inoculation ........................................75
5. Sustained CB2 agonist attenuates breast cancer-induced bone remodeling .............................................................................................77
6. CB2 Agonist decreases 66.1 breast cancer proliferation in vitro and in vivo .................................................................................................81
7. Increased levels of TNFα from exudates from intramedullary space of femur of animals inoculated with 66.1 breast cancer cells ...................84
8. JWH-015 maintains body weight and increases survival time of tumor bearing animals ..............................................................................87
9. JWH-015 attenuates proliferation of breast cancer cells in vitro ..........100
10. JWH-015 attenuation of proliferation is not blocked by CB2 inverse agonist SR144528 .................................................................................103
11. Antiproliferative effects of JWH-015 are not blocked by a CB1 inverse agonist, TRPV1 or TRPA1 channel blockers, or the Gαi inhibitor pertussis toxin ...............................................................................................106
12. 4T1 cell survival in vitro is dependent on expression of CB2 ..........109
13. JWH-015 induces apoptosis without inducing cell cycle arrest in breast cancer cells in vitro ...........................................................................112
15. JWH-015 induces intracellular calcium flux .
16. JWH-015 is not acting through a Gαi or Gas mechanism in 4T1 cells at relevant concentrations in vitro .
17. JWH-015 and SR144528 reduced pERK 1/2 phosphorylation and increased pAKT phosphorylation in vitro .
18. JWH-015 reduces primary tumor burden and decreases metastasis in vivo .
19. 4T1 cells induce immunosuppressor cells when inoculated into the mammary fat pad .
20. JWH-015 decreases the ratio of CD4+CD25+Foxp3+ regulatory T cells to CD4+CD25+Foxp3- effector T cells in tumor bearing animals .
21. JWH-015 inhibits the suppressive activity of regulatory T cells ex vivo .
22. JWH-015 does not inhibit the growth or activation of naïve T cells ex vivo .
23. JWH-015 does not inhibit tumor killing activity of CD8+ cytotoxic T cells ex vivo .
24. JWH-015 reduces spleen but not bone marrow population of myeloid derived suppressor cells in tumor bearing animals .
ABSTRACT

Breast cancer, which in advanced stages often leads to bone metastasis, is the most frequent malignant tumor among women in the U.S. Skeletal metastasis is associated with imbalanced bone remodeling and eventual bone fracture that contributes to incapacitating pain and loss of mobility. Bone cancer pain remains a significant health problem due to the limited repertoire of analgesics available to treat this pain without negatively influencing the quality of life and “bone health” of the patient. Bone cancer results in a marked influx of pro- and anti-inflammatory hematological cells into the medullary cavity resulting in activation of nociceptors that express cytokine and chemokine receptors. Blockade of these factors may result in a significant attenuation in bone cancer pain. The sustained release of cytokines by both primary tumor cells and invading leukocytes into the tumor microenvironment shapes the immune response to tumor invasion and ultimately mediates the shift in immune balance to the predominantly immunosuppressive state. Activation of cannabinoid receptor 2 (CB2), found on immune cells but not neuronal cells, has been shown to inhibit the release of cytokines from leukocytes; this inhibition plays an important role in CB2 agonist’s ability to inhibit pain without producing the CNS side effects commonly associated with CB1. Cannabinoids have also been demonstrated to modulate the tumor microenvironment via effects specific to the tumor cells. Here, we show that the CB2 specific agonist JWH-015 mediates inflammatory factors in vitro and in vivo in the femoral intramedullary cavity in a
murine model of bone cancer while simultaneously attenuating breast cancer induced bone pain and promoting overall health of the bone microenvironment. We demonstrate JWH-015's ability to positively modify the systemic balance of regulatory to effector lymphocytes as well as modulate the suppressive function of regulatory T lymphocytes. We also show that JWH-015 attenuates breast cancer cell proliferation \textit{in vitro} in a concentration dependent manner. Finally, utilizing a murine \textit{in vivo} bioluminescence model, we demonstrate that JWH-015 treatment not only attenuates primary tumor growth, but also rate of metastasis. Taken together, these data establish CB2 as an innovative therapeutic target across multiple stages of breast cancer.
CHAPTER 1: INTRODUCTION

1.1 Breast Cancer

Worldwide, breast cancer is the leading cause of cancer-related deaths among women aged 34-50 [1] and the most commonly diagnosed metastasizing tumor in women of all ages [2]. One in seven women in the United States alone will be diagnosed with breast cancer at some point in her lifetime, contributing to an over $225 billion annual overall cost of cancer in the U.S. [3]. This staggering prevalence has made breast cancer a central focus of investigative effort over the last 70 years. The results of such expansive research efforts are encouraging: not only have there been tremendous advances in understanding the etiology of the disease, significant improvements have been made in detection and diagnosis of breast cancer. These advances have aided in increasing the 10-year survival rates of metastatic breast cancer from 3.3% in 1944 to 22.2% in 2004, with even greater life expectancy in patients with localized disease (76.5%) [4]. Despite advances in understanding cancer as a disease, the need for novel therapeutics is as critical now as it has ever been. The near infinite variety of possible mutations that occur in breast tumors vary widely from patient to patient [5], making protein targeted therapies applicable to only specific subsets of patients and increasing the odds that a particular tumor will develop therapeutic resistance to a given therapy [6]. In addition, there is an inverse relationship between disease progression and efficacy of analgesics,
contributing to the 75-95% of breast cancer patients experiencing chronic debilitating pain that is predominantly associated with metastasis [7].

Breast cancer is not a single disease entity but rather a collection of tumors that differ at the molecular/cellular level as well as by morphology and can be classified based on either parameter [5]. There is no clinical correlation between morphological classification and molecular classification, though disease outcome can be correlated to either classification system separately [8-12].

Several morphological classes of breast tumors exist, including those originating from either ducts (approximately 80% of breast tumors), lobules (10% of reported cases), or connective tissues. Tumors of these three distinct tissue types are further classified as in situ (local) or invasive (metastatic). Other rarer breast cancers include tubular, medullary, mucinous, papillary, and cribriform carcinomas, as well as inflammatory breast cancer, Paget’s disease, and Phyllodes tumors [12-14].

In addition to the morphological differences between breast tumors significant subsets have distinct molecular characteristics. These genetic aberrations of cancerous mammary cells have received the greatest amount of scrutiny as researchers have worked to identify a number of mutations common in significant populations of breast cancer patients; this has led to the sub-classification of breast tumors based on phenotype. Six clinical cellular classifications of breast tumor are currently defined including Luminal A, Luminal
B, Her2/neu positive, basal-like, claudin-low, and normal-like tumors [8]. Luminal A/B tumors represent approximately two thirds of diagnosed human breast tumors [15] and typically respond well to hormone suppressing therapies due to a dependence on estrogen and/or progesterone receptor expression [16, 17]. Her2 (neu in mice), also known as ErbB2, is an EGF tyrosine kinase receptor that is over-expressed by as many as 25-30% of breast tumors and is associated with an aggressive clinical profile, higher risk of recurrence, and reduced survival compared to Luminal A/B tumors [18]. Basal-like breast tumors are commonly referred to as triple negative breast cancer (TNBC) due to the lack of estrogen/progesterone receptor expression and lack of Her2 over-expression [19]. Claudin-low tumors are also triple negative but have an unusually low expression of the critical cell-cell junction protein claudin [20]. Neither basal-like nor claudin-low tumors can be targeted via hormonal depletion or with the humanized Her2 monoclonal antibody trastuzumab and typically have the worst patient prognosis [20]. Normal-like breast tumors bear the greatest resemblance to normal mammary structure; although it has been proposed that the vast majority of tumors falling into this group are primarily categorized as such due to improper histological slide preparation where samples may contain more healthy breast tissue than tumor [20]. There is evidence for differential preference in sites of metastasis among the molecularly distinct classes of breast tumor: Her2 dependent tumors have a higher incidence of brain metastasis; ER+ tumors
show preference for bone while ER- tumors appear to prefer soft tissues such as brain and visceral tissues including liver and lung [21].

The molecular classes of breast cancer are diagnosed based on the presence or absence of a few key molecular differences:

**ER/PR dependence:** Approximately two thirds of breast tumors contain cells that express estrogen receptors (ER) and/or progesterone receptors (PR) [15]. These ER+/PR+ tumors are classified as either luminal A or luminal B depending on the level of hormone receptor expression and proliferation markers such as EGFR or Ki67 [8]. Luminal A tumors have a higher incidence of hormone receptor signaling and lower levels of proliferation markers while luminal B tumors display decreased hormone dependence and increased proliferation marker expression along with worsened clinical prognosis over luminal A [8]. While luminal B tumors have greater intrinsic resistance to direct inhibitors of hormone receptors like tamoxifen, luminal A tumors have a greater propensity to acquire resistance over time [22]. However, once resistance has been established both tumor classes tend to respond to aromatase inhibitors, indicating that the molecular mechanisms behind resistance to direct hormone inhibitors and hormone synthesis inhibitors are distinct [16].

**Her2/neu:** A third major molecular indicator of breast cancer outcome is over-expression of human epidermal growth factor receptor 2 (Her2; neu in mice). Also known as ERBB2, Her2 forms a dimer predominantly with
Her3 to participate in a number of intracellular signaling cascades including MAPK, PI3K, STAT, and others [23]. Her2 is over-expressed (i.e. >30% of cells in a tumor express increased levels) in 25-30% of breast tumors [24, 25]. Interestingly, despite a major subset of breast tumors' dependence on Her2 signaling, Her2 aberrations are not limited to breast cancer or even predominantly female cancers. Her2 over-expression has been detected in a significant number of ovarian cancers, prostate cancers, stomach cancers, and uterine cancers, among others [23, 26-28]. Some efficacy has been seen with trastuzumab (Herceptin©). When used in combination with chemotherapy in patients with Her2+ breast tumors, the overall response rate increases to 50% from 32% and mean survival increases to 25 months from 20 [18]. Despite this, nearly 75% of Her2+ tumors are intrinsically resistant to trastuzumab treatment and a significant portion of those that initially respond develop resistance within one year [29].

**Claudin:** The most recent breast cancer subset to emerge, claudin-low tumors are generally a triple negative (TN) basal-like tumor in which cellular replication is not dependent on either hormone receptors or Her2. Claudin-low tumors are primarily invasive ductal carcinomas with a resemblance to mammary stem cells that lack expression of several cell-cell adhesion proteins [20]. These tumors show increased expression of immune response genes like CD4 and CD79a, which may contribute to
the increased immune infiltration of this tumor subtype [20]. Claudin-low tumors treated with standard chemotherapy can be expected to have outcomes slightly better than basal-like tumors, but still worse than luminal tumors [10].

**BRCA1/2**: Breast cancer susceptibility protein type 1 and type 2 are caretaker genes that aid in error free repair of DNA double strand breaks (DSBs) [30]. Women with heterozygous loss of function mutations in either BRCA1 or BRCA2 have a 60-80% or 40-80% chance of developing breast cancer, respectively, in addition to a significant increase in chance of developing ovarian cancer [30]. To date, the BRCA1/2 genes are one of only few known heritable mutations leading to the occurrence of breast cancer; germline mutations in one of these genes accounts for approximately 10% of all diagnosed breast cancer cases [31]. BRCA complex mediated DNA repair occurs through homologous recombination (HR) and is classified as error free since the sister chromatid is used as a template for repair [30]. Loss in BRCA mediated HR leaves cells dependent on error prone mechanisms for double strand DNA repair, including non homologous end joining (NHEJ), that result in genomic instability [30]. BRCA1/2 mutation induced HR deficiency frequently leads to dependence on poly ADP ribose polymerase (PARP) dependence for DNA repair. PARP inhibitors, however, have failed to show survival benefit in clinical trials against breast cancers with BRCA1/2 mutation [32].
The mutations leading to these distinct phenotypes represent only a few of the aberrations that occur in not just breast cancer, but the entirety of cancer as a disease. Broad classes of mutations are consistently observed in most, if not all, cancers and have been summarized as 'the hallmarks of cancer'. The hallmarks of cancer were first posited by Douglas Hanahan and Robert Weinberg in 2000 in a review article in the journal *Cell* [33]. Hanahan and Weinberg summarized the similarities between the latest ideas in the field of cancer biology; what they found (summarized below) has become the basis for cancer genetic courses all over the world. It is important to note that each of the hallmarks described below are not specific mutations, but rather a class of possible mutations that confer a specific selective advantage to a cancerous cell. While the exact same mutation may occur across a significant proportion of patients, as is the case in Burkitt’s lymphoma, it is not required.

1. **Self Sufficiency in Growth Signaling (GS autonomy):** Normal, healthy cells require mitogenic growth signals (recall that mitosis is the division stage in cell replication; mitogenic = mitosis inducing). In normal tissues, these mitogenic signals are paracrine or endocrine and usually consist of the growth factor ligand (for example epithelial growth factor, or EGF) binding the growth factor receptor on the cell surface (EGFR). Usually a tyrosine kinase receptor, the growth factor receptor induces one of a number of possible second messenger systems in the cell to stimulate proliferation and modify transcription/translation of many cellular proteins.
In cancer cells, the signal becomes autocrine where the cancerous cell is either capable of manufacturing its own growth signals and responding to them, expresses a constitutively active growth factor receptor, or develops a mutation in which a critical component of the downstream second messenger system becomes constitutively active. A common mutation that confers GS autonomy is activation of the Ras oncogene- a member of the ERK cascade involved in proliferation and survival- approximately 30% of human cancers have a Ras mutation. It is also possible for mutations to occur in cancerous cells wherein growth factor receptors are highly over expressed, so that proliferation is stimulated even when conditions are not optimal. For example, the Her2/neu (Her-2 in humans, neu in mice) mutation occurs in up to one third of breast cancer cases.

2. Insensitivity to Anti-growth Signals: Normal cells respond to antiproliferative signals to maintain tissue homeostasis- only allowing cell cycle progression when it is appropriate to do so, as in wound repair. This ‘brake’ on the cell cycle is bypassed in cancerous cells, most commonly through elimination of the tumor suppressor pRb (protein retinoblastoma, named for the disease in which it was discovered). pRb is a critical protein in cell cycle progression, controlling the check-point between the G₁ and S phase (remember that pRb function is dependent on phosphorylation state: hypophosphorylated pRb binds and sequesters the E2F transcription factor while hyperphosphorylated pRb releases E2F and
allows transcription of genes necessary for G₁-S progression). The mutations responsible for elimination of the pRb tumor suppressor are generally mutations in the TGF-β receptor- TGF-β is a cytokine that controls transcription of a number of proteins, including p15 and p21-themselves tumor suppressors that block the phosphorylation of tumor suppressor pRb. Display of non-functional TGF-β receptors stymies proper TGF-β signaling and allows for early and improper pRb phosphorylation and further results in unchecked continuous cell cycle progression.

3. **Evasion of Apoptosis:** Cell death can happen in a variety of ways, including autophagy, apoptosis, necrosis, cornification and other atypical cell death modalities. Two of the most well studied forms of cellular death are apoptosis and necrosis. While necrosis is often associated with traumatic cell death after injury and is generally highly inflammatory, apoptosis is usually described as the natural end of a cellular life cycle. All cells contain the necessary cellular machinery for apoptosis and induction of one of the apoptotic pathways (extrinsic or intrinsic) is minimally disturbing to surrounding cells- macrophages are programmed to swiftly respond to apoptotic factors, sweeping away cell remnants without inducing inflammation. Cancerous cells possess mutations that allow the bypass of both the extrinsic and intrinsic apoptotic pathways, an
advantage that when paired with other hallmarks of cancer including limitless replicative potential, GS autonomy, and insensitivity to antigrowth signals render these cells virtually immortal. Evasion of apoptosis is often associated with the loss of tumor supressor p53. Functional inactivation of the p53 protein is observed in greater than 50% of human cancers, resulting in the inability to sense DNA damage (cells have multiple checkpoints allowing them to sense damage from external factors such as UV radiation and either repair the injured DNA or induce apoptosis if needed). Loss of functional p53 is also detrimental to normal stress response: hypoxia, which should induce transcription of p53, is a common occurrence in tumors as they increase in size (and correspondingly grow away from oxygenated blood). Conditions remain hypoxic even after the tumor acquires the ability to induce angiogenesis, another hallmark of cancer, as new vessel formation is highly unregulated and results in a hodge-podge intratumoral vascular network.

4. Limitless Replicative Potential: A finite number of possible divisions is an important phenomenon occurring in normal replicating cells discovered by Leonard Hayflick. The Hayflick limit states that a normal replicating cell will replicate 40-60 times during its lifetime before entering senescence and consequently apoptosis- regardless of its sensitivity to growth factors, antigrowth factors, or the development of mechanisms to evade death. This limit is determined by chromosomal telomere length- telomeres are
located at the ends of chromosomes and consist of thousands of repeats of a 6 base pair sequence. With each successive division, beginning with the very first division of a fertilized oocyte, a little bit of the telomere is lost. Once the telomeres are completely gone, the chromosomes, and consequently genes, are open to damage and mutation (for review google the break-fusion-break cycle). In this state, normal cells will enter crisis mode and induce apoptosis- even if they have acquired the other hallmarks of cancer mentioned above. Not so in cancerous cells- these are cells that have found ways around the Hayflick limit. This is commonly done by expressing the enzyme telomerase, a privilege normally reserved for embryonic stem cells. Telomerase rebuilds or lengthens telomeres by repetitively adding the 6 bp sequence 'TTAGGG' to chromosomal ends. This results in evasion of the Hayflick limit, allowing cells to replicate indefinitely.

5. Sustained Angiogenesis: As a general rule, cells must be located within 100 µm of a capillary or other blood supply in order to acquire the nutrients required for survival. In normal development and growth, this necessary proximity is tightly controlled by coordinating angiogenesis with tissue development in an organized manner. Cancerous tissues develop massive venous networks that are highly unorganized. As a result, tumors will generally develop with a necrotic center (unvasculated) with regions of hypoxia and highly vascularized regions in the outer mass. Although a
very complex process, acquiring sustained angiogenesis hinges on a cancerous cell’s ability to produce or induce production of vascular endothelial growth factor (VEGF) by production of HIF-1α. HIF-1α (hypoxic inducible factor 1α), as the name suggests, is produced by cells in a hypoxic environment and stimulates the release of VEGF by macrophages, which are particularly abundant in mammary carcinomas.

6. Tissue Invasion and Metastasis: A tumor is not truly cancerous without the ability to invade surrounding tissue and metastasize, or move to distant sites in the body. In normal cells there are a number of factors in place to ensure that this migration doesn't happen. Among these factors is expression of E-cadherin, which is expressed on all epithelial cells and aids in cell-cell adhesion. Cancerous cells commonly decrease expression of E-cadherin, a move that allows them to survive as singular cells rather than existing only in a structured tissue. To be sure, singular cells have an easier time slipping through the basement membrane and entering the lymphatic system or blood stream than large masses of cells.

While breast cancer therapeutics are moving towards greater specificity by targeting mutated proteins or the pathways they function in as is the case with tamoxifen, primary standards of care still rely heavily on the traditional cancer regimen of surgical resection of eligible tumors followed by adjuvant chemotherapy [34]. There are several classes of standard chemotherapeutics including alkylating agents such as cyclophosphamide, platinating agents
including cisplatin, antimetabolites like methotrexate, antibiotics such as doxorubicin, topoisomerase inhibitors like etoposide, and antimicrotubular agents including the vinca alkaloids and taxanes [35]. All of these chemotherapeutic classes target replicating cells by interfering with either DNA replication or the structural components of mitotic division. This non-specificity makes healthy replicating cells including cells of the gastrointestinal tract, hair follicles, and the immune system additional targets of standard chemotherapeutics and results in a host of detrimental side effects including hair loss, nausea, and immunosuppression [36]. In addition, taxanes, platinating agents, and vinca alkaloids can cause a significant amount of neuropathy and pain, requiring patient monitoring to prevent or stop development of chemotherapy induced peripheral neuropathy (CIPN) by discontinuing treatment or lowering dosing of the offending therapeutic [37]. There is a clear need for development of therapeutic agents with greater specificity towards tumor cells with fewer side effects. While some level of improvement has been made with agents such as the estrogen receptor inhibitor tamoxifen; the Her2/neu antibody trastuzumab; and the VEGF inhibitor Avastin®, less than an ideal percentage of tumors respond to these treatments and those that do quickly develop resistance.

1.2 Cancer Pain

Once breast cancer metastasis occurs, pain becomes the number one complaint [38]. The skeletal metastasis endured by 73% of advanced stage
breast cancer patients [39] is associated with bone remodeling and eventual bone fracture that contribute to incapacitating pain and limited or total loss of mobility [40]. Breast cancer metastasis to bone results in excruciating pain by activating pain fibers within bone [41].

Processing of pain in the body begins with this nociception- the transduction of a noxious stimulus from peripheral nociceptors along an excitatory pathway to the brain for interpretation as pain [42]. From the initial point of nociception, the signal travels along the primary afferent nerve fiber: thinly myelinated Aδ or unmyelinated C fibers forming a synapse with a second order neuron in the spinal cord [42]. Aδ and C fibers, unlike other sensory neurons that have the ability to respond to only one type of sensory input, display a remarkable array of nociceptors capable of detecting both chemical and mechanical input including acid sensing ion channels (TRPV1, ASIC), purinergic receptors activated by ATP, mechanically gated ion channels, and prostanoid receptors activated by inflammatory factors including prostaglandin E\(_2\) (PGE\(_2\)) [43]. From this entrance in the first and second laminae of the dorsal horn, the signal crosses to the contra-lateral side of the spinal column and travels along the spinothalamic tract to a third order neuron in the thalamus, which in turn transmits the information to the cortical and limbic structures, where the stimulus is ultimately interpreted both cognitively and emotionally [42, 43].

Understanding pain transmission occurring as a result of bone metastasis also requires a basic understanding of bone biology: there are three main
osteogenic cells found on the surface of the bone and in the lacunae of the bone matrix: osteoblasts, osteoclasts and osteocytes [44]. Each of these cell types plays an important role in bone remodeling/maintenance. Osteoblasts, which are derived from local stem cells within the mesenchyme termed mesenchymal stem cells (MSCs), are often referred to as osteoprogenitor cells [44]. They are found along the bone surface where they synthesize the organic matrix and regulate mineralization of bone by depositing calcium phosphate. As osteoblasts build-bone they become embedded in the mineralized bone and become osteocytes in which they serve to regulate calcium levels and to further form a collagenous matrix [44]. They stay active by communicating with other osteocytes and can become active osteoblasts if needed [45]. Osteoclasts on the other hand, are bone resorbing cells [46]. Osteoclasts are multinucleated cells that generally lie in apposition to bones undergoing resorption and are rich in enzymes used to degrade calcium phosphate matrix, in particular tartrate resistant acid phosphatase (TRAP) [46].

There are several mechanisms that contribute to cancer induced bone pain. Acid sensing receptors on primary afferents respond to the lower pH surrounding a tumor: apoptosis and necrosis occurring within the tumor contribute to the excess of charged ions in the tumor microenvironment, as does increased osteoclastic bone degradation commonly seen in bone metastasis [47]. Increased osteoclast activity and number results in a significant increase in acid and enzymes causing severe bone degradation, pain and eventual bone
fracture [48]. Bone tumors, including those originating from breast cancer metastasis, result in a marked influx of hematological and inflammatory cells into the medullary portion of the bone that activate nociceptors expressing receptors for cytokines, chemokines and prostaglandins; substances frequently released upon tissue injury to stimulate immune response [49-52]. These factors directly excite as well as sensitize nociceptors in the bone, possibly inducing a central sensitization characterized by neurochemical and cellular changes in the dorsal horn of the spinal cord and higher brain regions that facilitate the transmission and perception of pain in the CNS [53, 54]. Activation of the peripheral nervous system leads to activated immune cells of the CNS including microglia that may play a further role in promoting central sensitization [55, 56].

Inflammatory factors within the bone may promote the proliferation of the metastases [57] resulting in further invasion of the bone, advanced bone degradation and pursuing pain. Metastases to bone results in the increased production of cytokines including TNFα, IL-6, IL-1β and chemokines such as MCP1, MIP1α and MIP1β [58]. Such mediators can act at their corresponding receptors on nociceptive fibers within bone to promote pain, as well as act at receptors on breast cancer cells promoting further proliferation [57]. Several of these cytokine receptors have been found on osteoclast progenitor cells as well as on mature osteoclasts resulting in the increase in number of osteoclasts as well as an increase in their activity resulting in an increase in bone degradation [59]. Over time, as the tumor grows, pain can be associated with nerve damage
resulting from spinal and/or nerve root compression as well as a central sensitization due to persistent peripheral nociceptor activation by increased levels of cytokines and chemokines [53, 60-62].

When metastatic breast cancer invades bone the balanced bone remodeling is disrupted in favor of net bone loss [63]. Tumor-induced bone remodeling is associated with an acidic environment created by the tumor itself, local acidosis associated with tissue injury, and bone resorption by osteoclasts that directly sensitize and excite primary afferent fibers that innervate bone [47]. Both osteolytic activity and osteoblastic activity result in weakening of the normal healthy bone and predispose the patients to skeletal complications including bone pain from loading stress as well as from damage to sensory nerve endings in the intramedullary space, impaired mobility, pathological fracture, spinal cord compression, and symptomatic hypercalcemia [7]. Bone degradation and remodeling results in the release of growth factors including NGF and TGFβ from the bone itself that act back on the cancer cells to propagate their own growth as well as directly activate pain fibers and stimulate osteoclastogenesis to further degrade bone [64]. As the disease progresses and bone remodeling becomes prominent, metastatic bone pain becomes more severe and is correlated with ongoing pain in the area of the tumor characterized as dull in character and constant in presentation [65]. As bone destruction progresses, breakthrough pain, an intermittent episode of extreme pain while on analgesics, may occur spontaneously or more commonly by weight bearing or movement of the affected
bone. Breakthrough pain is very difficult to control, representing the most serious and highly debilitating cancer related events [40, 66, 67].

Cancer pain limits daily activity in 41% of patients reporting mild to moderate pain and 94% of patients reporting moderate to severe pain, leading to severely diminished quality of life in these patients [68]. Advances in cancer therapies have improved survival times of patients with breast cancer, including those with bone metastases, yet treatment for bone cancer pain has not changed. Current pain therapeutics used in the treatment of moderate to severe cancer pain and breakthrough pain, while diverse, do not adequately control pain at tolerable doses. First line therapy includes palliative radiotherapy, non-steroidal anti-inflammatory drugs (NSAIDs) for mild to moderate pain, and opiates for severe pain. These therapies can be supplemented additional therapies, some of which may be disease modifying and may have greater efficacy as prophylactics.

**Palliative Radiotherapy:** Localized beam radiotherapy, wide field radiotherapy, and systemic administration of radioisotopes are non-curative radiation treatments collectively known as palliative radiotherapy and are used most commonly to alleviate pain derived from bone metastasis in patients with advanced disease [69]. Local radiation is used in the treatment of uncomplicated bony metastasis while wide field radiotherapy and systemic radioisotopes are used to alleviate widespread metastasis pain and may also be used as prophylactics to prevent
formation of new symptomatic sites [69]. Palliative radiotherapy differs from curative radiotherapy in that treatment times are generally shorter and fraction size of the radiation dose is larger, resulting in a lower total dose and fewer acute side effects [69]. Palliative radiotherapy is not recommended in earlier stage patients due to severity of long term side effects: patients outliving the duration of benefit can expect an increased risk of several side effects at the site(s) of administration including fracture, radiation induced tumor formation, cognitive decline, heart disease, radiation proctitis (bleeding and urgency of the rectum), lymphedema, fibrosis, epilation (hair loss), and salivary gland dryness [70, 71]. Number and severity of these effects are directly correlated to duration and intensity of radiotherapy. In addition, radiotherapy must be carefully coordinated with administration of chemotherapeutics being used in treatment of the underlying disease to avoid severe hematologic toxicity [69]. In qualifying patients, pain relief is seen by 80% of those responding to treatment within one week. Average time to failure of palliative radiotherapy ranges from 2.5-4 months and re-irradiation is effective in 2/3 of patients [72, 73]. The mechanism behind radiotherapy induced bone cancer pain relief is as yet not understood, although it has been postulated that radiation induced reduction of tumor burden as well as chemical pain modulators contribute to decreased pain sensitivity [74].
**Opiates:** Opioids function by inhibiting pain pathways in the CNS. Three main opioid receptors have been cloned and are classified as µ, δ, and κ [75]. The majority of opioids used in the treatment of moderate to severe pain, including morphine, have the highest affinity for and display action at the µ opioid receptor (MOR). These G-protein coupled receptors (GPCRs) are activated by endogenous opioid peptides including β-endorphins, met- and leu-enkephalins, dynorphins, as well as exogenous opiates; the most notable of which are modified from opium isolated from the poppy plant: morphine, codeine, and thebaine [76]. During a resting state, the GPCR is associated with guanosine diphosphate (GDP). When the MOR binds an opioid, a conformational change is induced in the protein, causing the GDP to be exchanged for a GTP (guanosine triphosphate) [77]. The Gαi and βγ subunits of the activated GPCR then dissociate: the Gαi subunit inhibits the intracellular enzyme adenylate cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [76]. The Gαi subunit also inhibits neuronal calcium (Ca^{2+}) channels and causes them to remain closed. The βγ subunit further dissipates the charge gradient by causing voltage gated potassium (K^+) channels to remain open [76]. The result is inhibition of neuronal excitability [42].
Although well accepted clinically and highly effective in the relief of hyperalgesia, opioids are not as effective at relieving tactile allodynia within the tolerable dose range [78]. Recently we demonstrated that sustained morphine not only intensifies tumor-induced pain but also accelerates tumor-induced bone destruction in a murine model of bone cancer [79] suggesting that chronic morphine for advanced cancer patients may result in increased pain and decreased quality of life over time. Chronic opiate use results in a multitude of unbearable side effects including analgesic tolerance, somnolence, constipation, respiratory depression and paradoxical states of hyperalgesia [80].

**NSAIDs:** Non-steroidal anti-inflammatory drugs like aspirin and ibuprofen function by inhibiting one or both forms of the enzyme cyclooxygenase (COX-1 and COX-2), which catalyze the committing step in the conversion of free arachidonic acid to prostaglandins [81]. While a wide body of research exists indicating that inhibition of cyclooxygenase enzymes may be useful in treating malignancies and mild to moderate cancer pain [82, 83], there are several drawbacks to the long term use of NSAIDs, including deleterious effects on bone healing [54]; cardiotoxicity [84]; and significant gastrointestinal adverse effects such as dyspepsia, ulceration, gastritis, pain and vomiting caused by inhibition of COX-1 [85]. Additionally, NSAIDs are ineffective in treatment of severe and
breakthrough pain, further discouraging use among advanced metastatic patients [86].

**Bisphosphonates:** The bisphosphonates are pyrophosphate analogs with preference for bone matrix: dual phosphate groups on the bisphosphonate molecule bind calcium ions, the largest store of which in the human body is in bone [47]. There are two principal classes of bisphosphonates-nitrogenous and non-nitrogenous. Both nitrogenous and non-nitrogenous bisphosphonates are internalized by osteoclasts upon localization to bone matrix [87]. These two classes differ structurally by the presence or absence of a nitrogen moiety and mechanistically. Nitrogenous bisphosphonates including zoledronate and ibandronate function by inhibiting prenylation, or membrane trafficking of lipid-anchored proteins to the cell membrane, effectively disrupting components of cell structure and proliferation related pathways including ras, rho, and rac proteins that require membrane anchoring for proper function [88]. Non-nitrogenous bisphosphonates like etidronate are metabolized by osteoclasts and replace the terminal phosphate group of adenosine tri-phosphate (ATP), yielding a non-functional ATP molecule that interferes with cellular metabolism [88]. Both of these mechanisms result in apoptosis of the ingesting osteoclast, ultimately reducing osteoclast number and activity and effectively maintaining bone structure, although no survival benefit has been seen with bisphosphonate therapy [87]. Pain alleviating effects
of bisphosphonates are secondary to the primary mechanism of structural maintenance, including pH normalization of the bone microenvironment as a result of reduced osteoclastic bone degradation and the reduction of mechanically induced pain due to greater structural integrity of bone with long term bisphosphonate therapy indicating that bisphosphonates may have greater efficacy as prophylactics [89].

**Denosumab:** Recently approved for the treatment of skeletal related events (SREs) in bone cancer after fast track review by the FDA due to success in osteoporosis models, denosumab is a soluble RANK (receptor activator of nuclear factor κ) receptor that inhibits RANK ligand (RANK-L) activity by binding and sequestering RANK-L [90]. RANK is expressed by osteoclasts and dendritic cells (professional antigen presenting cells of the immune system) and facilitates osteoclast activation upon binding RANK-L expressed by osteoblasts, T-cells and stromal cells [47]. Like the bisphophonates, denosumab does not confer a survival benefit and may have greater efficacy as a prophylactic due to functioning as an osteoclast activation inhibitor [91].

**Local Anesthetics:** Patients suffering from chronic regional pain syndrome (CRPS) may benefit short term from single injection of local anesthetics in the sympathetic ganglia (nerve block) [92]. Local anesthetics may also be used in conjunction with corticosteroids intraspinally. Local anesthetics are typically restricted to emergency and diagnostic use due to disruption of
motor, sensory, and sympathetic function [93]. Oral preparations of local anesthetic such as mexiletine may be used in patients with severe pain that does not improve with opiates in conjunction with other adjuvants; mexiletine is associated with a high rate of adverse effects including nausea, vomiting, tremor, dizziness, unsteadiness and paresthesias. Approximately half of the patients prescribed mexiletine discontinue use due to toxicity [94].

Investigational prospects: Mammary tumor cells, the surrounding stromal cells, and the tumor infiltrating milieu of leukocytes all significantly contribute to the abundance of growth factors, cytokines and other inflammatory mediators in the tumor microenvironment. As metastases are established in the skeletal system these mediators directly activate fibers innervating the bone, making growth factors and inflammatory activators attractive targets for the relief of bone cancer pain and the growth of the metastasizing cells [95]. Nerve growth factor (NGF) is one such target: NGF inhibitors have shown promise in osteoarthritis clinical trials and preclinical models of cancer pain [54, 60, 96-102]. NGF inhibitors may be particularly effective in bone cancer pain due to their potential to prevent neuroma formation- a phenomenon correlated with primary bone tumor formation and metastasis to bone that has been shown to be dependent on NGF [100, 102]. In late 2010 clinical trials of NGF inhibitors, including those being tested by Johnson & Johnson,
Sanofi Aventis, and Pfizer, among others, were halted by the FDA due to potential avascular joint destruction though no speculation could be made on direct cause of the joint degradation because of the blinded study design [103]. Recently the Arthritis Advisory Committee recommended that the FDA reopen trials and while this is not absolute, the FDA has historically followed the AAC’s advice [103]. Another group of potential targets are the endothelins. Endothelin 1, 2, and 3 are over-expressed by multiple tumor types and human studies have shown a correlation between pain severity and endothelin level [104]. Several endothelin antagonists have undergone clinical scrutiny but have thus far failed to gain FDA approval due to lack of improved survival [105]. Further study is needed to determine efficacy as pain alleviating drugs.

1.3 Immunology of the tumor microenvironment

In order to understand the role of inflammation and the immune system in tumor development, a basic understanding of the immune system is necessary. It is helpful to think of immune response in terms of phases. The first phase or immediate response is often termed ‘innate’ immunity. The innate immune response consists largely of natural killer cell and macrophage activity, and is defined as immunity present at birth. Innate immune responses are limited to microbe recognition and are highly non-specific. Within a matter of hours after the innate response is initiated, the second phase or adaptive immune system
kicks in- involving T-cells and/or B-cells. Adaptive immune responses are memory based; the specificity and magnitude of response increases with each successive exposure to a particular antigen. With a near infinite recognition capability, these responses target bacteria, parasites, viral infections, foreign bodies, and tumors with a great deal of specificity.

The adaptive immune system is further classified into humoral (commonly referred to as Th2 response) activities, and cell-mediated responses (commonly referred to as Th1 response). Which system is activated depends on the type of invasion and the resulting cytokine profile released at the site(s) of infection/invasion. In a normal, healthy individual Th1 responses deal with intracellular threats including viruses, yeast, cancerous cells, and intracellular bacteria while Th2 targets extracellular invaders including normal bacteria, parasites, toxins, and allergens. A fully functioning immune system is able to quickly switch back and forth to deal with multiple threats. It should be noted that in general activation of Th2 supresses Th1 response and vice-versa so that both systems are not able to act simultaneously. The opposing nature of these two systems is cytokine mediated: interleukin 4 (IL-4) and IL-10 (important in the Th2 response) inhibit production/release of gamma interferon (IFN-$\gamma$), IL-2 and IL-12 (vital to Th1 activation)- the reverse is also true. In cancer patients, the juxtaposition between Th2 and Th1 is exxagerated: there is a sustained overactivation of Th2 and a near complete supression of Th1 activity. For
complete review of basic immune interactions see reviews completed by Medzhitov and Romagnani [106, 107].

It should also be noted that while ideally both Th1 and Th2 responses ultimately result in the death of their respective targets- it is the killing mechanism and the subsequent level of immunogenicity (an immunogenic stimulus induces or furthers immune response) that remains variable; this is particularly important in cancer. Originally it was thought that apoptotic cellular death is not immunogenic but rather tolerogenic (induces immune tolerance with no further inflammation) and that necrotic death is immunogenic (proinflammatory)[108]. That idea has since been disproven- apoptosis can be either immunogenic or tolerogenic and the same can be said of necrosis [109]. In tumor development, both apoptosis and necrosis occur as the tumor progresses, however the more advanced the disease the more likely that tumor cell death occurs in a tolerogenic manner [110]. This correlates directly to Th2 polarization, suggesting that a successful tumor induces changes in immune reactivity and eventually renders the immune system completely useless in tumor cell elimination.

The process by which the immune system is manipulated in the tumor’s favor is known as cancer immunoediting and consists of three phases [111-113]:

**Elimination**: The elimination phase occurs with complete elimination of cancer cells prior to clinical appearance. Most adults are in or have been in this phase and successfully eradicate cancerous cells by mounting a Th1 response against cancerous cells multiple times throughout their
lifespan. At this point no tolerogenicity exists and inflammatory cytokines properly activate NK cells; dendritic cells properly engulf cancer cells and display tumor antigen to Th1 cells, which in turn activate cytotoxic T-cells, etc.

**Equilibrium:** In the event that a population of tolerogenic tumor cells evade the immune system during the elimination phase, these cells move into equilibrium. At this point most immunogenic cells have been eradicated and the tolerogenic cells have been inadvertently selected for. This stage may persist for several years and the tumor cells may not have acquired all of the hallmarks of cancer yet. There is still attrition of tumor cells (mainly via apoptosis), outgrowth is controlled by cytotoxic T lymphocytes, and the tumor is also kept in check largely by size limitation: if the tumor grows too large it cuts itself off from blood supply. Equilibrium is a critical stage in tumor development as during this phase genetically unstable cells arise and begin to get selected for. The unstable cells begin to display immunosubversion tactics:

- Decreased MHC I expression- prevents recognition and killing by CTLs
- Release of soluble MIC-A/B- serves as an antagonist of NKG2D receptors on the NK cells (evading non-self recognition)
- Release of soluble MHC I- effectively antagonizing CD4+ T-cells
Expression of immunosuppressive cytokines including TGF-β and IL-10

- Upregulation of BCL-2 (evasion of apoptosis)
- Downregulation of tumor specific antigens
- Counter attack: release of TNFα or other death ligands by cancer cells
- Induction of immunosuppressive cells: tolerogenic DCs, TAMs, T-reg, MDSCs

**Escape**: Complete immunosubversion- by this stage all tumor cells are able to undermine immunosurveillance mechanisms and ‘edit’ the immune system to create a more tumor friendly environment. The tumor escape variants replicate quickly, developing into a clinically apparent tumor.

In recent years it has also been recognized that inflammation can potentiate tumor metastases and promote tumor growth [57]. Novel anti-cancer therapeutic strategies include mechanisms to inhibit inflammatory mediators including cytokines. In breast cancer, macrophages are one of the most abundant innate immune cell types termed tumor-associated macrophages (TAMs) that have been found to enhance angiogenic programming by production of pro-angiogenic factors such as cytokines, chemokines, VEGF and proteases [114-121]. Breast cancer metastases to bone results in a significant inflammatory/immune response including a significant increase in macrophages, monocytes, dendritic cells, leukocytes and neutrophils [57, 122-124]. In addition
to promoting angiogenesis, inflammatory cells promote metastatic dissemination by enhancing migratory/invasive potential of neoplastic cells through the production of cytokines [115, 125, 126]. Breast cancer cell release of TNFα demonstrates an autocrine and/or a paracrine secretion that result in breast cancer self proliferation [127]. Although there is a delicate balance between antitumor immunity and tumor-originated proinflammatory activity, the net outcome of persistent inflammatory microenvironment is enhanced tumor promotion, accelerated tumor progression, invasion of surrounding tissues and angiogenesis [125, 128, 129]. Several pro-inflammatory cytokines are known to promote tumor growth, such as TNFα, IL-1β and IL-6 [123, 130]. These studies demonstrate inflammatory cell production of cytokines may be general promoters and proliferators of metastatic breast carcinomas.

Studies have identified IL-6 and its major intracellular effector STAT3 as protumorigenic in breast cancer [131]. IL-6 levels are significantly elevated in breast cancer patients [131]. IL-6 is released from breast cancer cells and act in both an autocrine and paracrine fashion through IL-6R/gp130 receptors expressed by the breast cancer cells contributing to cellular transformation and growth [132]. Malignant transformation results in a tumor-infiltrating inflammatory/immune response resulting in the accumulation of macrophages, leukocytes and dendritic cells. Macrophages and dendritic cells are potent IL-6 producers and can be activated by molecular “danger” signals produced by dying breast cancer cells [133]. It is thought that immune/inflammatory cells in close
proximity with breast cancer cells are capable of producing prodigious amounts of “start-up” IL-6, required for early tumor promotion [133]. These studies suggest that cytokines released from breast cancer cells as well as from cancer recruited immune cells result in self proliferation of the breast cancer cells and that inhibition of the different cytokines may indeed decrease proliferation.

1.4 Cannabinoids

The endocannabinoid system (ECS) is a complex and far reaching network comprised of the nonspecific endogenous cannabinoids N-arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoyl glycerol (2-AG), the cannabinoid hydrolyzing enzymes fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MAGL), and the two receptors that have been cloned to date: cannabinoid receptor type 1 (CB1) and type 2 (CB2) [134, 135]. Endogenous cannabinoid ligands and many synthetic cannabimimetic compounds also display activity at a number of other receptor classes, including transient receptor potential cation channel, subfamily V, member 1 (TRPV1) channels and the peroxisome proliferator-activated receptors (PPARs) of the nucleus [136-140]. The promiscuity of cannabinoids is due in part to their lipophilic properties, and contributes greatly to the complexity of the ECS.

In addition to limited expression in the immune system, lung, kidneys, and liver [141, 142], CB1 is one of the most prevalently expressed GPCRs on neurons in the CNS and plays a role in a wide variety of neuronal processes
including locomotor activity, pain transmission, satiety, mood, and memory [141, 143-148]. The CB1 receptor couples primarily to $G_{i/o}$ and is found in the highest density in GABAergic neurons, though CB1 has also been shown to act via $G_{i/o}$ in excitatory neurons [145]. This results in inhibition of neurotransmitter release via a blockage of voltage dependent $Ca^{2+}$ channels and activation of inwardly rectifying $K^+$ channels. In addition, CB1 affects the intracellular focal adhesion kinase cascade, the mitogen activated protein kinase (MAPK) cascade, phosphatidylinositol 3-kinase (PI3K) pathway, and modulates production of nitric oxide through nNOS [145, 149-151]. Pharmacological manipulation of the CB1 receptor is significantly impacted by the constitutive activity of the receptor. The CB1 receptor is not only constitutively active, but it has the ability to sequester $G_{i/o}$ proteins - either preventing other receptors from signaling at all, or increasing the signaling of other receptors through $G_s$ or $G_q$, ultimately forcing a non-native response [152-154]. Review of the literature reveals some degree of discrepancy with the claim of constitutive activity since it can be difficult to distinguish between endogenous ligand activity and constitutive activity of a receptor in an intact biological system; however it is generally accepted that the CB1 GPCR does behave constitutively in most systems. This constitutive activity makes discovery of neutral antagonists of the receptor exceedingly difficult, if possible at all. Long after the introduction of so-called selective antagonists of CB1 including AM251 and SR141716A (Rimonabant©), it was revealed that many if not all are actually inverse agonists [155, 156]. The search for a neutral antagonist of CB1
has been extensive, and only recently have compounds begun to surface claiming to be neutral antagonists- most notably NESS 0327 [157]. Further characterization is still needed to determine the true activity of these compounds.

CB2 receptors, on the other hand, are found in spleen, osteoclasts, on T and B lymphocytes and other immune cells, including microglia and dendritic cells and have long been known to be immunosuppressive [158], though the specific mechanisms and circumstances surrounding this observation lack definition and are in direct opposition to the known functions of endogenous cannabinoids acting at CB2 during development. While CB2 receptors are not found on mature neurons, neural progenitor cells do express CB2 and respond with increased proliferation when agonized by CB2 selective compounds [159]. CB2 is also known to be critical in development of the innate and adaptive immune systems: CB2 knockout mice are immunodeficient as evidenced by the lack of CD4+ memory T-cells, and downregulation of multiple subsets of B cells, NK and NKT cells [160]. Post complete thymic development (around age 14 in humans) the cannabinoid receptors tend to have a contrary response in immune cells, having been demonstrated to deplete T cell populations and are a prevalent topic in autoimmune research [161, 162]. Little to no evidence exists however in perhaps the most useful potential role of cannabinoids: cancer immunotherapy. A small handful of studies over the last decade have demonstrated that nonspecific cannabinoid agonists have little if any effect on NK and NKT cells, and suggest that these compounds may selectively target T
regulatory cells over effector T cells [163, 164]. Available data focuses on nonspecific compounds with a slightly higher selectivity for the cannabinoid receptor 1 (CB1) rather than the more obvious target- cannabinoid receptor 2 (CB2).

Like CB1, CB2 is traditionally characterized as coupling to $G_{\alpha_i/o}$, however unlike CB1, agonism of CB2 receptors does not result in psychotropic episodes [145]. Recent studies have demonstrated that a selective CB2 agonist JWH-133 inhibited cocaine self-administration and conditioned place preference suggesting that CB2 activation in the CNS may inhibit the rewarding effects of other drugs [165]. When considered along with the marked anti-inflammatory effects of CB2 compounds [158], this makes CB2 an ideal target for cancer pain alone or in combination with current analgesics such as an opioid. Compounds selective for cannabinoid receptor 2 have been shown to be effective in multiple models of inflammatory and chronic pain in both acute and chronically administered settings [166-168]. Recent studies have identified an increase in mRNA for CB2 receptors in the CNS after nerve injury with upregulation in the CNS associated with microglia after inflammation: DeLeo and colleagues have demonstrated that CB2 receptor activation within the spinal cord after L5 nerve injury resulted in an increase in CB2 receptor expression on microglia and perivascular cells with a reduction in hypersensitivity using the CB2 selective agonists JWH015 [169]. They concluded that CB2 agonists may offer pain relief by modulating the immune response and microglia function under chronic pain.
conditions without inducing tolerance or CNS side effects. Newly developed preclinical models of bone cancer pain also indicate that CB2 agonists may be useful in cancer pain and are based on novel synthetic cannabinoid agonist’s antinociceptive/antihyperalgesic effects and enhancement in bone growth/strength in models of osteoporosis [170].

Astoundingly, CB2 receptors are also upregulated in a number of different solid tumor lines [24]; the most surprising of which are epithelial carcinomas, where CB2 expression is not expected. Preliminary data from our group and others demonstrates that CB2 specific agonists attenuate tumor cell proliferation both in vivo and in vitro: ∆⁹-tetrahydrocannabinol (THC) and CB2 selective agonist JWH-133 have been demonstrated to exert considerable antitumoral effects in the MMTV-neu mouse model of breast cancer which over-expresses the EGF receptor HER2 [24]; similarly, CB2 selective JWH-015 has been shown to retard growth of the highly aggressive spontaneous breast tumors occurring in PyMT transgenic mice [171]; cannabidiol, a CB1 and CB2 agonist inhibited human breast cancer cell line MDA-MB-231 growth in vitro via apoptosis and autophagy- notable due to the low expression of HER2/3 in MDA-MB-231 cells; anandamide, a CB1 and CB2 agonist, has been shown in multiple models to inhibit breast cancer cell growth and migration.

Cannabinoid effects on bone remodeling through receptors on osteoclasts are also of critical importance, particularly in the context of bone metastasis. CB2 agonists inhibit osteoclasts- preventing the adsorption of calcium from bone
matrix, and thus facilitating bone healing and growth [172, 173]. For example, CB2 receptor activation on osteoclasts and osteocytes by the selective CB2 agonist HU-308 significantly suppressed osteoclast activity and osteoclastogenesis (production of osteoclasts) considerably reducing the activity of osteoclasts in trabecular and cortical bone [173]. Bone density in CB2 knockout mice was significantly lower when compared to wild type littermates [172]. In addition, CB2 knockout mice displayed a markedly accelerated age-related trabecular and cortical bone remodeling [173]. On the other hand, cytokines generated during inflammation and metastases including TNFα, IL-6 and IL-1β potentiate osteoclastogenesis (production of new osteoclasts) stimulating bone resorption [174]. Hence, normal bone mass, in part, is maintained by levels of endogenous endocannabinoids acting at CB2 receptors that modulate endogenous cytokines and osteoclastogenesis. CB2 receptors, therefore comprise a multifaceted approach in the treatment of advanced stages of cancer: a direct effect on growth of tumor cells, a direct effect in selective depletion of immunosuppressor cells, a modulation of the tumor micro-environment including the many cytokines and chemokines resulting in a decrease in pain by reducing bone resorption.
CHAPTER 2: MATERIALS AND METHODS

2.1 In Vitro

2.1.1 Cell culture:

Murine mammary tumor line 66.1 cells were cultured in Minimum Essential Medium eagle (MEM) with 10% fetal bovine serum (FBS), 100 IU\(^{-1}\) penicillin and 100 µg/ml streptomycin (P/S). BT20 cells were cultured in MEM supplemented with P/S/G (100 IU\(^{-1}\) penicillin, 100 µg/ml streptomycin, and 29.2 mg/mL L-glutamine). 4T1, 410.4, MCF7 and MDA-MB-231 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and P/S. MDA-MB-468 cells were cultured in RPMI supplemented with P/S/G. Except as specified for individual assays, the cell lines were plated in 10 cm tissue culture dishes, allowed to grow exponentially, and housed in an incubator at 37°C and 5% CO2.

2.1.2 Drugs:

The National Institute on Drug Abuse (NIDA) provided SR144528 and SR141716. Pertussis toxin and capsazepine were purchased from Sigma-Aldrich (St Louis, MO). JWH-015, nifedipine, ω-conotoxin MVIIC, and HC-030031 were purchased from Tocris (Ellisville, MO). All compounds were dissolved in DMSO and added to fresh culture media such that the effective DMSO concentration was 0.1% per well.
2.1.3 Immunoblotting:

Cells were lysed, protein extracts separated electrophoretically and transferred to a PVDF membrane as previously described. The membrane was incubated with either polyclonal primary anti-CB2 antibody (Cell Signaling Technology), monoclonal primary anti-phospho-ERK antibody, or monoclonal primary anti-phospho-AKT antibody (Cell Signaling Technology) followed by horseradish peroxidase-conjugated secondary IgG antibody (Cell Signaling Technology) and developed using a chemiluminescent system (Amersham Biosciences). α-tubulin (Cell Signaling Technology) was used as loading control. Quantification of western blot was performed using gel analysis in ImageJ.

2.1.4 Sulforhodamine B (SRB) assay:

Cell proliferation in response to different treatments was measured as previously described [175]. Briefly ~ 1.6 x 10^4 cells per well were plated on six well plates and allowed to grow overnight. On day 2, complete medium was replaced with Optimem (Invitrogen) serum free medium and incubated for 16 hours. Cells were then stimulated with experimental compound on the morning of day three in varying concentrations. 48 hours later cells were fixed with ice cold 50% trichloroacetic acid (TCA) (500 uL/well) for one hour at 4 °C. Cells were then washed with deionized water and stained with SRB dye (2 mL/well) for 10 min at room temperature. Cells were washed with 1% acetic acid and the bound SRB
dye was solubilized with 1M unbuffered tris for ten minutes on a plate shaker. Optical density of each well was read at 540 nm using a plate reader (Biomek).

2.1.5 Ca\(^{2+}\) imaging:

Cells were cultured to 50% confluency in dual chamber slides in complete media. On the day of imaging: cells were switched to serum low, antibiotic free Optimem media (Invitrogen) and equilibrated for 1 hour in a 5% CO2 incubator at 37 C. Lyophilized fura-2 AM dissolved at 1mg/mL in DMSO constituted stock solution which was then diluted to 5mg/mL in Optimem. Cells were incubated for 1 hour in 5ug/mL Fura-2 AM for loading, followed by a 30 minute incubation in Optimem without Fura-2 AM to allow de-esterification of the fluorescent dye. Cells were imaged on a Nikon fluorescent microscope equipped with a CCD camera. 340nm and 380nm were simultaneously recorded and the absorbance ratio at 340nm (Ca\(^{2+}\) bound Fura-2) over 380nm (free Fura-2) was plotted for a period of at least 60 minutes.

2.1.6 Caspase 3/7 activity:

Caspase-Glo 3/7 assay (Promega) was used to measure caspase 3 and 7 activities in 4T1 and MCF7 cells according to manufacturer instructions. Results were normalized to total protein and expressed as a percentage of control.
2.1.7 shRNA knockdown of CB₂ receptor:

CB₂ shRNA lentiviral particles (Santa Cruz Biotechnology) were used to transiently knockdown CB₂ receptor expression in 4T1 cells according to manufacturer instructions.

2.1.8 Immunohistochemistry:

4T1 or MCF7 cells were allowed to grow to 50% confluency in dual chamber slides (Fisher Scientific). Cells were treated with either JWH-015 or SR144528 for 0-24 hours and fixed with 10% formalin for 20 minutes at room temperature. Cells were permeabilized with 0.25% Triton X-100 for 30 minutes and blocked with 5% BSA for 30 minutes at room temperature. Cells were incubated in polyclonal Ki67 antibody (Abcam) diluted 1/200 in 5% BSA overnight at 4°C. Cells were incubated for 2 hours at room temperature with Alexa Fluor 488 (Invitrogen) anti-rabbit IgG secondary antibody, mounted with Flurosheild mounting medium with DAPI (Abcam) and visualized on a fluorescent microscope (Zeiss).

2.1.9 Intracellular cAMP:

A cAMP complete EIA kit (Enzo Life Sciences) was used to measure forskolin induced cAMP formation in response to JWH-015. Assay was conducted per manufacturer instruction and normalized to total protein.
2.1.10 BrdU cell proliferation assay:

BrdU cell proliferation assay (Invitrogen) was performed in triplicate according to manufacturer instruction after seeding ~10,000 cells per well in a 96 well plate and treated with increasing concentrations of JWH-015 for 48 hours where indicated. Briefly, after treatment cells were incubated for 16 hours with BrdU for incorporation and fixed to the plate. Plates were washed to remove excess BrdU. Plates were incubated with a peroxidase-conjugated antibody against BrdU and read at 450nm.

2.2 In Vivo

2.2.1 Animals:

All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the Guidelines by the National Institutes of Health and the International Association for the Study of Pain. Female BALB/cfC3H mice (Harlan, IN) were 15-20 grams prior to initiation of study. Mice were maintained in a climate-controlled room on a 12-hour light/dark cycle and allowed food and water ad libitum.

2.2.2 Surgical Procedures

Intramedullary implantation of 66.1 cells: Mice were anesthetized with ketamine/xylazine. An arthrotomy was performed. The condyles of the right distal femorises were exposed and a hole was drilled to create a space for injection of $1 \times 10^5$ 66.1 cells in 5 uL complete MEM or 5 uL complete MEM without cells.
in control animals within the intramedullary space of the mouse femoris. Injections were made with an injection cannula affixed via plastic tubing to a 10 μL Hamilton syringe. Proper placement of the injector was confirmed through use of Faxitron x-ray imaging. Holes were sealed with bone cement [176].

**Mammary fat pad injections:** Mice were orthotopically injected with 4T1-Luc cells (5x10^5) into the c-9 mammary fat pad as previously described [177].

### 2.2.3 Drug Treatment:

The CB2 receptor agonist JWH015, with a Kᵢ at CB2 = 13.8nM and 30 fold selectivity over CB₁, and the CB2 inverse agonist SR144528 were dissolved in a vehicle solution of 10%-dimethyl sulfoxide, 10%-Tween-80, and 80%-saline. All injections were made at a volume of 10ml/kg and consisted of once daily intraperitoneal (i.p.) injections for 21 days (8-28). Randomly assigned animals were treated with JWH-015 (6 mg/kg i.p), JWH-015 (20 mg/kg i.p), SR144528 (1 mg/kg i.p), or vehicle.

### 2.2.4 Behavioral Testing Protocols:

**Analysis of Acute Pain:** Spontaneous pain (flinching and guarding), and tactile allodynia were measured 0, 30, 60, 90, and 120 mins after a single dose of drug was administrated in a blinded fashion. Breast cancer-induced hypersensitivity was shown to return to baseline levels 2 hours after drug administration.

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

**Spontaneous Pain:** Flinching and guarding were observed for duration of two
minutes during a resting state. Flinching was characterized by the lifting and
rapid flexing of the right hind paw when not associated with walking or
movement. Flinches were recorded on a five-channel counter. Guarding was
characterized by the lifting the right hind limb into a fully retracted position under
the torso. Time spent guarding over the duration of two minutes was recorded
and measurements were performed in blinded fashion [79].

**Tactile Allodynia:** The assessment of tactile allodynia consisted of measuring the
withdrawal threshold of the paw ipsilateral to the site of tumor inoculation in
response to probing with a series of calibrated von Frey filaments using the
Chaplan up-down method [178] with the experimenter blinded to treatment
groups. The 50% paw withdrawal threshold was determined by the non-
parametric method of Dixon [179].

**2.2.5 In Vivo Imaging:**

**Radiography:** A digital Faxitron machine was used to acquire live radiographs on
days 0, 7, and 14 of the intramedullary inoculation model. Bone loss was rated
by a blinded third party expert in animal radiographs according to the following
scale: 0 = normal, 1 = bone loss observed with no fracture, 2 = full thickness
unicortical bone loss indicating unicortical bone fracture, 3 = full thickness
bicortical bone loss indicating bicortical bone fracture. From this rating, the incidence of fractures was reported and used to calculate the percent of animals with fractures. Before capturing images, mice were anesthetized with ketamine/xylazine [180].

Bioluminescence imaging: Animals inoculated into the mammary fat pad with 4T1-Luc cells were live-imaged on a weekly basis as previously described (in vivo luc model). Briefly, 4T1-luc tumor bearing animals were anesthetized with inhaled isoflurane and injected i.p. with K+ D-luciferin (Molecular Imaging Products) 5 minutes prior to imaging (5 minute exposure in a dark chamber). Dark images were assigned intensity map coloring in ImageJ and overlayed on 100 mSec light exposures of the same animals.

2.3 Ex Vivo

2.3.1 Primary tumor burden and metastasis detection:

28 days post tumor inoculation animals were sacrificed, primary tumors excised, and mass recorded. Common sites of mammary tumor metastasis were harvested (brain, lung, femur, kidney and spleen) and homogenized in the presence of protease inhibitor cocktail (Sigma) for ex vivo detection of metastasis using a dual luciferase reporter assay (Promega) performed according to manufacturer instruction. Data is represented as % of treatment group testing positive for luciferase activity.
2.3.2 Flow Cytometry:

Spleen, inguinal lymph nodes, and whole blood were harvested 28 days post mammary fat pad inoculation of 4T1 cells. Blood was taken by cardiac puncture into heparinized tubes and the spleen and lymph nodes were excised immediately following. Solid tissues were homogenized and strained through a 100μM nylon filter (BD Biosciences) to create single cell suspensions in RPMI. RBC lysis buffer was used to perform red blood cell lysis according to manufacturer instructions (EBiosciences) and the remaining leukocytes were counted and stained with colorimetric antibodies for analysis via flow cytometry (LSRII, BD Biosciences) according to manufacturer instruction (EBiosciences).

2.3.3 Magnetic assisted cell sorting (MACS):

Single cell suspensions of leukocytes were prepared as described above however cells were incubated with antibody coated magnetic beads (Miltenyi Biotec) rather than fluorescent conjugated antibodies. Columns (Miltenyi Biotec) placed on strong magnetic fields were used to separate cells according to manufacturer instruction. Immediately after sorting the primary lymphocyte cultures were incubated with IL-2 in the presence or absence of CD3+CD28+ T cell activating Dynabeads (Invitrogen) in preparation for activity or suppression assays (described below).
2.3.4 Suppression assay:

Splenic CD4+CD25+ regulatory T cells were isolated via MACS from mammary tumor bearing mice that had either been treated with daily i.p. injections of either vehicle or JWH-015 (6mg/kg or 20mg/kg) for 7 days. Isolated T-regs were co-cultured with naïve T lymphocytes (CD62+) from naïve mice of the same age as tumor bearing mice and suppression of naïve T cell activation and expansion by the regulatory T cells (1x10^5 of each cell type per well in a round bottom 96 well cell culture plate for 48 hours in the presence of Dynabeads) was measured with a BrdU cell proliferation assay. Results are expressed as mean percent suppression.

2.3.5 Cytotoxicity assay:

CD8+ T lymphocytes were isolated via MACS from mammary tumor bearing mice treated either with vehicle or JWH-015 (7 days, 6 mg/kg, i.p.). Ability of these CD8+ cytotoxic T lymphocytes to kill 4T1 tumor cells ex vivo was measured using a cytotoxicity assay (Promega) according to manufacturer instruction after lymphocytes were co-cultured with 4T1 cells for 18 hours. Results are expressed as mean percent dead cell activity per treatment group.

2.3.6 Bone histology:

Immediately following behavioral testing on day 14, mice were anesthetized (ketamine/xylazine, 100mg/kg i.p.) and perfused transcardially with 0.1 M PBS followed by 10% neutral buffered formalin (Sigma, St Louis, MO,
USA). Femurs were collected and post-fixed in picric acid with 4% formalin at 8°C overnight and decalcified in 10% EDTA (RDO-Apex, Aurora, IL) for 14 days. Femora were cut in the frontal plane into 5 um sections and stained with hematoxylin and eosin (H&E) to visualize normal marrow elements and cancer cells under bright field microscopy on a Nikon E800 at 4X magnification. Tumor or marrow areas within the femur (6 bones per treatment) were measured in mm² between the epiphyseal plates using Metamorph imaging software by a blinded observer with the aid of a pathologist.

2.3.7 Micro-Computed Tomographic Analysis of Tumor-Induced Bone Destruction:

On day 14 after intramedullary tumor cell implantation, On day 14, mice were anesthetized (ketamine/xylazine, 100mg/kg i.p.) and perfused transcardially with 0.1 M PBS followed by 10% neutral buffered formalin (Sigma, St Louis, MO, USA). Femurs were collected and post-fixed in picric acid with 4% formalin at 8°C overnight and stored in PBS until scanned. A desktop eXplore Locus SP MicroCT imaging system (GE Healthcare, London, Ontario, Canada) was used to visualize densitometric and architectural parameters in tumor-bearing animals treated with a cannabinoid 2 agonist (JWH015) or vehicle (10% dimethyl sulfoxide, 10% Tween 80, and 80% saline) and compared to control (naive) animal bones. The conebeam CT scanner used a 2300 x 2300 CCD detector with current and voltage set at 80 µA and 80 KVp, respectively. A 360° scan was
performed with a 2100-ms integration time with images reconstructed at 16-μm³ resolution in Reconstruction Utility software (GE Healthcare). From the two-dimensional slices obtained, a three-dimensional reconstruction was created using MicroView analysis software (GE Healthcare). µCT parameters used to assess disease progression included bone mineral density [54].

2.3.8 TNFα ELISA:

Immediately after euthanasia, femurs were extracted from mice treated with media or 66.1 cells in the presence of absence of JWH015. PBS was used to remove bone exudate from intramedullary space. TNFα levels were measured using an ELISA kit (Invitrogen) and performed according to manufacturers’ instructions.

2.3.9 TRAP/5b and CTX measurement:

Animals were deeply anesthetized and whole blood was collected by transcardial puncture. Blood coagulated at room temperature for 1 hour, and was centrifuged to isolate serum. Serum was stored at -80°C until utilized for assays. Enzyme immunoassays were used to measure the serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAP5b) for osteoclast number (Immunodiagnostic Systems, Fountain Hills, AZ, USA) and C-terminal telopeptide 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 Analysis:

SRB assays, quantified western blots, caspase 3/7 assays, cAMP assays, and primary tumor mass were analyzed using one-way analysis of variance followed by students Neuman-Kuels testing for multiple comparisons in FlashCalc [181]. Differences were considered to be significant if p≤0.05. When possible, potencies (or A\textsubscript{50}) were determined by regression analysis of dose–response curve (log dose [x] vs response [y]) using a 95% confidence interval according to the method of analysis of the Graded Dose–Response [182]. For the calculations of A\textsubscript{50}’s, the minimal possible response was set to 0%.

For behavioral data, statistical comparisons between treatment groups were done using ANOVA. Pairwise comparisons were made with Student’s t-test, multiple comparisons between groups were done using Bonferroni’s Multiple Comparison Test. Dose–response effects were done with linear regression analysis of the linear portion of the log dose–response curve. Survival studies utilized the Kaplan Meier estimator with a Log-rank Mantel-Cox test and Gehan-Breslow Wilcoxon test. Significance was set at p<0.05. All data are presented as mean ± SEM and GraphPad Prism 5.0 (Graph Pad Inc., San Diego, CA, USA) used to plot data.
CHAPTER 3: DISEASE MODIFICATION OF BREAST CANCER INDUCED
BONE REMODELING BY CANNABINOID RECEPTOR 2 AGONISTS

3.1 Introduction

The World Health Organization (WHO) estimates that the number of women living with breast cancer globally will rise to 15 million by the year 2020 [183] with a significant portion of these patients in advanced stages of the disease. Breast cancer is the second leading cause of cancer death in women [183] with nearly 200,000 diagnoses each year in the U.S. alone. As the cancer most commonly metastasizes to bone [184], breast cancer cells in the bone microenvironment cause bone loss, fractures, anemia and severe pain [57]. Despite marked advances in chemotherapeutics for early stage breast cancers, few new therapies are effective in slowing disease progression and increasing survival in advanced disease. Recent findings with a B-RAF inhibitor in advanced stages of melanoma emphasize the need for treatments that effectively reduce pain and increase survival time in terminally ill patients [185]. Reports also highlight the importance of patient quality of life in late stages of cancer as well as how family and physicians surrounding the patient may be affected [185].

The most commonly prescribed treatments for skeletal-related events (SREs) in advanced-stage breast cancer patients with bone metastasis and pain are bisphosphonates, radiation, Nonsteroidal Anti-inflammatory Drugs (NSAIDs) and opioids [91]. Although these therapies reduce pain and SREs, they bring
about unwanted side effects and an overall decrease in quality of life without increasing patient survival or slowing disease progression [91]. The FDA recently allowed a fast track review of denosumab (Xgeva™), a human monoclonal antibody targeting receptor activator of nuclear factor-κ ligand (RANKL), due to the inadequacy of current therapies for treating advanced stage breast cancer patients. Denosumab demonstrated a greater efficacy in preventing SREs in breast cancer patients with bone metastasis than the bisphosphonate zoledronic acid, however neither bisphosphonates nor denosumab have shown a survival benefit [91]. Additionally, both treatments induce a small but significant subset of patients to develop serious adverse events including osteonecrosis of the jaw and disruption of normal serum calcium levels [91]. Cancer pain caused by bone metastases is inadequately managed by a combination of opiates and NSAIDS, as dictated by the guidelines set by the WHO Ladder Approach for Relief of Cancer Pain. NSAIDs, while effective at reducing inflammatory and musculoskeletal pains [186], have been shown to aid in bone destruction and prevention of proper bone remodeling, thus decreasing bone strength in both animal models [187] and in human studies [188]. Recently we demonstrated that sustained morphine not only intensifies tumor-induced pain but also accelerates tumor-induced bone destruction in a murine model of bone cancer [79]. Chronic opiate use results in a multitude of often unbearable side effects including analgesic tolerance, somnolence, constipation, respiratory depression and paradoxical states of hyperalgesia [189]. Neither palliative care
nor therapeutics approved for use in advanced stages of breast cancer have improved in the last 30 years despite the persistence of the disease; for these reasons, alternative therapies must be developed.

One possible alternative includes the activation of CB$_2$ receptors, which have been shown to have analgesic activity in acute, chronic, inflammatory and neuropathic pain without producing psychoactive or rewarding behavior [166, 168]. Additionally, CB$_2$ receptors are integral components of normal bone metabolism [172, 173, 190]. Activation of CB$_2$ receptors improves bone integrity by stimulating the proliferation of osteoblasts and inhibiting the proliferation and activation of osteoclasts [170, 173, 190]. Recently, CB$_2$ agonists have also been shown to have anti-tumor potential in the MMTV-neu mouse, ErbB2–driven breast cancer model [24].

In this study we evaluate the efficacy of CB$_2$ agonists in attenuating bone reduction, bone pain, and cancer proliferation in a murine model of breast-induced bone cancer. Our data suggest that CB$_2$ agonists may provide cancer patients with bone metastases a superior alternative to current available therapeutics.
3.2 Acute or sustained CB$_2$ agonist attenuates breast cancer-induced bone pain

Flinching and guarding behaviors were observed to determine the acute effects of JWH015 on bone cancer-induced spontaneous pain. Von Frey filaments were used to determine the withdrawal thresholds of the ipsilateral hind paw. Fourteen days after intra-femoral cancer (66.1) inoculation, mice displayed significant bone cancer-induced flinching, guarding and decreases in hind paw withdrawal thresholds (Figure 1 A, B, C). JWH015 resulted in a significant, time-related reduction in flinching and guarding with an increase in paw withdrawal thresholds when compared to vehicle treated mice (Figure 1A, B, C). Pain behaviors returned to a hypersensitive state in cancer treated animals 240-320 minutes after JWH015 administration. Control animals inoculated with media showed no flinching, guarding or mechanical hypersensitivity when administered JWH015 or vehicle (data not shown).

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

The pre-administration of the CB$_2$ antagonist (SR144528), but not the CB$_1$ antagonist (SR141716), abolished the antinociceptive effect produced by the
CB2 agonist (Figure 2C, D). The treatment of either antagonist alone had no effect on flinching or guarding. By day 7 post surgery, cancer-inoculated animals began to display behavioral signs of tactile sensitivity (Figure 3), as well as the media treated animals due to the invasive surgery. On day 14 however, media control animals’ mechanical thresholds were back to baseline whereas cancer treated animal’s demonstrated significant mechanical hypersensitivity. On day 14, animals treated with vehicle demonstrated significant breast cancer-induced mechanical hypersensitivity. Animals treated with sustained JWH015 or AM1241 demonstrated a significant attenuation of cancer-induced mechanical hypersensitivity (Figure 3).
Figure 1. Acute systemic administration of CB$_2$ receptor agonist JWH015 attenuates breast (66.1) cancer-induced spontaneous and evoked pain on day 14 after femoral inoculation (time 0). Bone cancer-induced spontaneous (A) flinches (B) guarding and (C) evoked paw withdrawal were all significantly attenuated in a time related fashion in animals administered JWH015 (i.p.) compared to vehicle treated animals ($p \leq 0.05$; $n = 15$ mice per group) (*Data collected together with Lozano-Ondoua, AN).
Figure 2

(a) Number of Flinches in 2 min (± SEM)

(b) Guarding in 2 min (sec ± SEM)

(c) Number of Flinches in 2 min (± SEM)

(d) Guarding in 2 min (sec ± SEM)
Figure 2. Chronic systemic administration of CB$_2$ receptor agonist JWH015 and AM1241 attenuates spontaneous pain; blocked by a CB$_2$ antagonist but not by a CB$_1$ antagonist. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral inoculation animals demonstrated bone cancer-induced (A) flinching and (B) guarding. CB$_2$ agonists, JWH015 (6 mg/kg, i.p., q.d.) or AM1241 (6 mg/kg, i.p., q.d.) was administered after behavioral measurements and continued for 7 days. Spontaneous (A) flinching and (B) guarding in cancer bearing animals was significantly reduced by JWH015 (p=0.0005; n = 16) and AM1241 (p=0.0001; n = 12) compared to animals that received vehicle on day 14 (n = 20). No significant difference was observed in media only control animals between vehicle treated (n = 15), JWH015 treated (n = 12), or AM1241 treated (n = 12) animals. The attenuation of bone cancer-induced (C) flinching and (D) guarding by JWH015 (n = 15) on day 14 was blocked by the pre-treatment with the CB$_2$ antagonist SR144528 (3 mg/kg, i.p., q.d. 30 min prior to agonist; n = 10; ) but not by the CB$_1$ antagonist SR141716 (3 mg/kg, i.p., q.d. 30 min prior to agonist; p=0.003; n = 15). Either CB$_2$ antagonist (n = 10) or CB$_1$ antagonist (n = 15) alone had no significant effect on the bone cancer-induced pain as compared to vehicle (n = 12) (*Data collected together with Lozano-Ondoua, AN).
Figure 3

![Graph showing Paw Withdrawal Threshold (g ± SEM) across Preinjury, Day 7, and Day 14 for different treatments: Media, Vehicle, Media, AM1241, 66.1, AM1241, Media, JWH015, 66.1, JWH015. Statistical significance indicated for Day 7 and Day 14 compared to Preinjury with p-values: p=0.003, p=0.006, and p=0.04.]
Figure 3. (A) Chronic systemic administration of CB$_2$ receptor agonist JWH015 and AM1241 attenuates tactile evoked pain. Animal femora were injected with either breast cancer cells (66.1) or media only as a control after baseline (pre-injury) behavioral measurements. On day seven after femoral inoculation of 66.1 cells animals demonstrated bone cancer-induced tactile allodynia. CB$_2$ agonists, JWH015 (6 mg/kg, i.p., q.d.) or AM1241 (6 mg/kg, i.p., q.d.) was administered after behavioral measurements and continued for 7 days. Tactile allodynia in cancer bearing animals was significantly reduced by JWH015 ($p<0.0001; n = 16$) and AM1241 ($p=0.006; n = 16$) compared to animals that received vehicle on day 14 ($n = 16$). No significant difference was observed in media only control animals between vehicle treated ($n = 17$), JWH015 treated ($n = 15$), or AM1241 treated ($n = 15$) animals (*Data collected together with Lozano-Ondoua, AN).
3.3 CB2 agonist treatment reduces breast cancer-induced bone loss and fracture

Radiographic images were taken following behavioral testing to determine the effect of sustained JWH015 on cancer-induced bone degradation. No bone loss or fractures were observed in animals injected with media and treated with vehicle (Figure 4A). Cancer-induced bone loss (evidenced by the presence of radiolucent areas in the proximal and distal femoral heads) increased in tumor-bearing mice treated with vehicle. At day 14 post-cell injection, 68% of tumor-bearing mice treated with vehicle displayed femoral fractures, while no mice injected with media displayed femoral fractures (Figure 4A, 5A). Sustained JWH015 treatment significantly reduced the amount of cancer-induced bone loss with a 40% reduction in the incidence of fractures (Figures 4A, B, 5A). Animals inoculated with media and treated with JWH015 had no observable changes in bone structure. The pre-administration of the CB2 antagonist significantly attenuated the ability of JWH015 to decrease cancer-induced fracture (Figure 4A, B, 5A). Similar findings were seen with AM1241 (Figure 5A). Antagonist alone in either cancer or media inoculated animals produced no significant change from controls (Figure 4A, 5A).

At day 14, femora were analyzed using micro-CT (Figure 4B, C). In this cancer model, we observe osteolytic and osteoblastic characteristics, resulting in ectopic periosteal bone remodeling. This is consistent with human breast cancers where 70% of breast lesions in bone are osteolytic, 15% are osteoblastic, and 15% take on a mixed growth pattern [191]. A clear loss of
cortical and trabecular bone in the cancer-inoculated (66.1) animals administered vehicle (Figure 4B) was apparent compared to controls (Figure 4B). BMD was significantly lower in cancer-inoculated animals compared to control animals (Figure 5B). Sustained treatment with JWH015 significantly attenuated BMD compared to vehicle treated cancer-inoculated animals (Figure 5B).

To confirm a CB$_2$ agonist attenuates breast cancer-induced bone loss, the bone resorption markers, TRAP5b and CTX, were measured. CTX concentration in cancer-inoculated mice treated with vehicle significantly increased compared to control animals injected with media (Figure 5D). Cancer-inoculated mice treated with JWH015 attenuated increased levels of CTX (Figure 5D). Additionally, the levels of TRAP5b were significantly increased in cancer-inoculated animals treated with vehicle when compared to media-inoculated control animals (Figure 5C). Sustained JWH015 treatment attenuated breast-induced bone cancer up regulation of TRAP5b (Figure 5C).
Figure 4. (A) Radiographs of the femora in the presence of either media (control) or breast cancer cells (66.1) on day 14 after inoculation. Mice received either vehicle or CB$_2$ agonists (JWH015, 6 mg/kg, i.p., q.d.) in the absence or presence of the CB$_2$ antagonist (SR144528, 3 mg/kg, i.p., q.d. 30 min prior to agonist) from days 7 to day 14 after femoral inoculation. Bone loss (hypodense at proximal and distal ends) identified in cancer (66.1) treated animals as compared to media only (control) animals. JWH015 (6 mg/kg, i.p., q.d. days 7-14) attenuates breast cancer-induced bone loss compared to (B) cancer inoculated, vehicle administration (10-10-80%DMSO,Tween 80, saline). The attenuation of bone cancer-induced bone loss by JWH015 on day 14 was inhibited by the pre-treatment with the CB$_2$ antagonist SR144528 (3 mg/kg, i.p., q.d. 30 min prior to agonist). SR144528 (3 mg/kg, i.p., q.d. 30 min prior to agonist) alone did not result in any differences. (B, C) Micro-computed tomographic analysis of tumor-induced bone destruction in the absence or presence of JWH015. Cortical bone loss is demonstrated in breast cancer (66.1) inoculated animals as compared to control, naïve or media treated animals. Radiographs in all panels are representative of images obtained of femurs obtained from each animal in figure 2 (*Data collected together with Lozano-Ondoua, AN).
Figure 5

(a) % Mice with fractures (+/- SEM)

(b) BMD (mg per cm² ± SEM)

(c) TRACP-5b (U/L ± SEM)

(d) CTX (ng/mL ± SEM)
Figure 5. Sustained CB₂ agonist attenuates breast cancer-induced bone remodeling. (A) Based on radiographic images, the number of animals with a clear cortical bone fracture was counted from the different groups of mice at day 0, 7 and 14. Either CB₂ agonists, AM1241 (n = 15) or JWH015 (n = 30) significantly reduced the number of animals with cancer-induced cortical fracture of the femoris by day 14 (p=0.001) as compared to vehicle (n = 30) and this was blocked by the pre-administration of the CB₂ antagonist, SR144528 (p=0.001; n = 10). Antagonist alone (n = 10) had no significant effect. (B) Using micro-CT, bone mineral density (BMD) was calculated indicating a significant decrease in intra-femoral cancer (66.1) treated animals administered vehicle from day 7 to day 14 (p=0.047; n = 9,). Intra-femoral cancer (66.1) treated animals that received CB₂ agonist JWH015 (n = 9, pooled from 3 experiments) from day 7 to day 14 demonstrated a significant reversal from the intra-femoral cancer vehicle treated animals (p=0.039; n = 9, pooled from 3 experiments). No difference was observed between JWH015 (n = 6, pooled from 2 experiments) and vehicle treated (n = 6, pooled from 2 experiments) of non-tumor bearing mice. (C) TRAP5b was measured in animals on day 14 as a marker of osteoclast activity. TRAP5b levels were significantly higher in animals that received intra-femoral breast cancer cells (66.1) (p=0.045) compared to intra-femoral media treated animals. This increase in cancer-induced TRAP5b levels was significantly reduced in JWH015 (6mg/kg, i.p., q.d. from day 7 to 14) treated animals (p=0.039; n=8). (D) CTX was measured in animals on day 14 as a marker of bone
resorption. The amount of CTX was significantly higher in animals that received intra-femoral breast cancer cells (66.1) (p=0.006; n=5) compared to intra-femoral media treated animals. This increase in cancer-induced CTX levels was significantly reduced in JWH015 (6mg/kg, i.p., q.d. from day7 to 14) treated animals (p=0.01; n=5) (*Data collected together with Lozano-Ondoua, AN).
3.4 CB2 agonists inhibit cancer cell growth in vivo and in vitro

The murine breast cancer cells used in this study, 66.1, were found to express the CB2 receptor (Figure 7A). Breast cancer cells were treated in vitro with multiple concentrations of JWH015 or AM1241. In a concentration-dependent manner, both JWH015 and AM1241 (Figure 6A,C) significantly decreased the percent proliferation compared to vehicle treated cells. Additionally, in a concentration-dependent manner, JWH015 significantly decreased the percent activity of BrdU, another measure of proliferation (Figure 6D). Morphological analysis demonstrated that breast cancer cells occupied 70% of the medullary cavity on day 14 in animals treated with vehicle, whereas breast cancer cells occupied 40% of the medullary cavity on day 14 after JWH015 treatment (Figure 6B).
Figure 6

(a) Graph showing % Proliferation (± SEM) against [JWH015 µM] with Vehicle, 0.1, 1, 2, 3, 5, and 10 µM. The bars indicate significant differences with p-values: 0.1 µM (p=0.0019), 1 µM (p=0.0001).

(b) Images of tissue sections with different tumor burdens (0%, 70%, 40%) treated with Vehicle or JWH015 at various concentrations (Media, Vehicle, 66.1 µM JWH015).

(c) Graph showing % Proliferation (± SEM) against [AM1241 µM] with Vehicle, 1, 2, 3, 5, and 10 µM. The bars indicate a significant difference at 1 µM (p=0.001).

(d) Bar graph showing BrdU % Activity (± SEM) with Vehicle, 10nM, 100nM, 1µM, 3µM, and 10µM JWH015. Significance levels: 10nM (p=0.045), 1µM (p<0.0001), 3µM (p=0.002), 10µM (p=0.0002).
Figure 6. CB\textsubscript{2} Agonist decreases 66.1 breast cancer proliferation in vitro and in vivo. (A,C) SRB assay indicates breast cancer cells (66.1) treated with JWH015 or AM1241 demonstrate a concentration related decrease in their ability to proliferate over a 48 hour period in a 6-well plate compared to vehicle treated cells (p≤0.001; n = 18 per group). (B) Tumor burden using H&E staining of a femoris from intra-femoral cancer animals administered either vehicle or JWH015. JWH015 reduces the number of 66.1 cells within the intramedullary space compared to vehicle treated animals (p=0.01; n =4 per group). (D) BrdU assay demonstrates breast cancer cells (66.1) treated with JWH015 decrease their ability to proliferate in a 96-well plate compared to vehicle treated cells (p≤0.0001; n = 3 per group) (*Data collected together with Lozano-Ondoua, AN).
3.5 Antinociceptive effect of CB2 agonists in breast-induced bone cancer is mediated by the cytokine, TNFα

Intramedullary bone exudates were collected from mice treated with breast cancer cells (66.1) or media (control) and tested for TNFα levels. TNFα levels were significantly higher in cancer-inoculated animals as compared to media inoculated animals on day 14 (Figure 7B). Animals that were inoculated with 66.1 cells and received chronic CB2 agonists (6mg/kg, i.p. q.d., from day 7-14) resulted in a significant reduction in TNFα levels upon bone aspiration on day 14 as compared to vehicle treated, 66.1 inoculated animals (Figure 7B).
Figure 7

a. CB₂ receptor (39 kDa) → 
α tubulin (50 kDa) →

b. 

<table>
<thead>
<tr>
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<th>TNFα (pg/mg total protein ± SEM)</th>
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<tr>
<td>Naive</td>
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<tr>
<td>66.1, AM1241</td>
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p = 0.002, p = 0.005, p = 0.019
Figure 7. (A) Western blot confirming that 66.1 breast cancer cells express the CB₂ receptor with a molecular weight of 39kDa. The membrane was stripped and re-probed with mouse anti α-tubulin as a loading control. Blot displayed is representative of four western blots run from separate cell pellets on each run. (B) Increased levels of TNFα from exudates from intramedullary space of femur of animals inoculated with 66.1 breast cancer cells and treated with vehicle (day 14; p<0.05; n=4) as compared to animals inoculated with media. Cancer inoculated animals treated with AM1241 or JWH015 significantly decreased 66.1-induced levels of TNFα (p<0.05; n=4) (*Data collected together with Lozano-Ondoua, AN).
3.6 CB2 agonists maintain body weight and increase survival

Animal weights were significantly decreased from baseline (17.21 g ± 0.25) by day 21 in cancer-inoculated, vehicle treated animals (14.30 g ± 1.17) compared to cancer treated animals that received sustained JWH015 or AM1241 (16.87 g ± 0.43; 17.03 g ± 0.15, respectively) (Figure 8A). Animals with cancer-inoculated femora and sustained vehicle resulted in a significant decrease in survival by day 21 compared to a non-significant change in survival from control in the JWH015 or AM1241 treated group (Figure 8B).
Figure 8

a. 

![Graph showing body weight changes over days with different groups labeled: Media: Vehicle, Media: AM1241, Media: JWH015, 66.1: Vehicle, 66.1: AM1241, 66.1: JWH015. There is a significant difference indicated by *p=0.001.](image)

b. 

![Graph showing percent change over days with two groups labeled: Vehicle, JWH015.](image)
Figure 8. (A) On day 7 animals are equally divided by weight and post-surgery behavioral baselines. Cancer-inoculated animals treated with vehicle weigh significantly less on day 21 compared to control animals and cancer-inoculated animals treated with JWH015 (p=0.001, n=4-8 per group) (B) Survival studies utilized the Kaplan Meier estimator. The percent of mice surviving to day 21 was significantly reduced in intra-femoral cancer treated (66.1), vehicle (i.p., q.d. from day7 to 21) animals (p=0.032) whereas animals administered JWH015 or AM1241 were not statistically different from control (n = 4 per group) (*Data collected together with Lozano-Ondoua, AN).
3.7 Conclusions

Epidemiological studies show that 1 out of 7 women in the U.S. will develop breast cancer in their life-time [183]. The National Institutes of Health estimates overall costs of cancer in the U.S. at $206 billion with breast cancer being the most frequent malignant tumor [183]. Up to 80% of patients with advanced breast cancer develop bone metastases associated with bone loss and fracture which contribute to incapacitating pain and limited or total loss of mobility [40, 89, 192, 193]. Pain, the first symptom in many cancer patients, substantially decreases the quality of life [40, 89, 192, 193]. Skeletal related events (SREs) and pain due to breast cancer metastases are treated using radiation therapy, opiates and bisphosphonates [194-196]. Nephrotoxicity, osteonecrosis of the jaw, hypocalcemia and flu-like symptoms are associated with bisphosphonate therapy [197, 198]. Chronic pain treatments for advanced stage breast cancer have not been developed, while patients are inadequately treated and suffer from the unwanted side effects of opioids and NSAIDs [186-188].

Here we show that either a single injection of a CB$_2$ agonist or sustained administration significantly attenuates spontaneous and evoked pain behaviors in an animal model of breast-induced bone cancer pain. Inhibition of pain behaviors by the CB$_2$ agonist are similar in efficacy to a single injection of morphine [7], suggesting that CB$_2$ agonists have the ability to directly inhibit pain activity. Our antagonist studies support CB$_2$ agonist activity via CB$_2$ receptors and not CB$_1$ receptors. Antinociceptive effects by CB$_2$ receptors in our bone cancer model
are believed to be via an anti-cytokine/anti-inflammatory activity. The anti-inflammatory effects are supported by a significant decrease in the cytokine TNF\(\alpha\) levels after JWH015 administration as compared to vehicle treated animals. Cancer metastasis to the bone initiates an immune response within the bone and the nervous system [57]. This immune response activates nociceptors and subsequently generates pain [61]. A mechanism of pain inhibition by CB\(_2\) agonists is proposed by decreasing pronociceptive cytokines such as IL-6, TNF\(\alpha\), and IL-1\(\beta\) released from infiltrating immune cells [199-201] and from the cancer cells themselves [123, 125]. Studies have demonstrated that IL-1\(\beta\), TNF\(\alpha\) and IL-6 are released from macrophages, monocytes, breast cancer and glial cells to promote nociception [127, 202, 203]. IL-1\(\beta\) and IL-6 have been shown to increase the expression of COX-2 [204, 205], nerve growth factor (NGF) [206] while increasing the production and release of other cytokines to promote nociception. TNF\(\alpha\) acts directly at its receptor, TNFR1, to produce nociception and indirectly via increasing prostanoids and sympathetic amines [61, 207]. Activation of CB\(_2\) receptors on immune cells has been shown to inhibit the release of a number of cytokines from monocytes and macrophages in animal [56, 200, 208, 209] and human studies [199, 210]. Activation of the peripheral nervous system leads to active immune cells of the CNS, including microglia, that contribute to central sensitization [56]. In addition to CB\(_2\) agonist action in bone, recent studies suggest that CB\(_2\) agonists act in the CNS to inhibit microglia-induced nociception [56]. Overall, the ability of a CB\(_2\) agonist to inhibit many
nociceptive substances results in a wide-ranging antinociceptive agent for bone cancer pain.

Another major component of bone cancer pain and decreased quality of life/survival is osteolysis and bone fracture [211]. CB$_2$ receptors are expressed on osteoclasts and have the ability to modify bone structure [173]. CB$_2$ agonists attenuate ovariectomy-induced bone loss in mice [173]. Our radiographic and µCT analysis indicate that breast cancer cells have both osteolytic and osteoblastic effects on bone. Treatment with JWH015 in cancer-bearing mice reduces the incidence of fractures. µCT analysis indicates that breast cancer significantly decreases the BMD, while sustained treatment with JWH015 significantly decreased the cancer-induced loss in BMD. Clinically, this suggests bone cancer metastasis, regardless of time of lesion, predisposes patients to skeletal complications such as impaired mobility, fractures, hypercalcemia, and pain [7, 91]. Our studies suggest that treatment with a CB$_2$ agonist attenuates breast cancer-induced bone loss and helps stabilize normal bone activity. Based on recent studies demonstrating that a selective CB$_2$ agonist significantly suppressed osteoclast activity and osteoclastogenesis [173, 212], combined with our findings of a significant reduction in bone loss markers, we believe that CB$_2$ agonists in breast-induced bone cancer are reducing osteoclast activity and osteoclast numbers, and hence decreasing bone loss.

Bone degradation results in the release of growth factors including NGF and TGFβ from the bone that act on cancer cells to propagate their own growth
These growth factors directly activate pain fibers and stimulate osteoclasts to degrade bone [64]. Tumor cells themselves release several growth factors and cytokines that activate RANKL, stimulating osteoclastogenesis and disrupting normal bone turnover. Several studies have indicated that CB$_2$ agonists inhibit breast cancer cell proliferation. The cannabinoid receptors CB$_1$ and CB$_2$ are over-expressed in human breast tumors compared to normal breast tissue [171] and we demonstrate here that the murine breast cancer cell line expresses CB2 receptors. In vitro and in vivo analysis further revealed that JWH133, a CB$_2$ agonist, inhibited tumor growth and metastasis [171]. Furthermore, MMTV-neu mice with ErbB2-driven metastatic breast cancer express the CB$_2$ receptor and that JWH133 reduced tumor growth, tumor number, and lung metastases [24]. Our data has further supported these observations in the murine mammary 66.1 cell lines and that treatment with the selective CB$_2$ agonists AM1241 and JWH015 significantly inhibit proliferation of the breast cancer cell line 66.1 in vitro and in vivo. Future studies are likely to determine the molecular pathway in which CB2 agonists successfully inhibit breast cancer proliferation. Using H&E staining we found that sustained JWH015 treatment in breast cancer-inoculated animals significantly reduced the percentage of tumor burden within the intramedullary cavity of the femoris supporting antiproliferative effects in vivo. Unlike existing advanced stage treatments, we found that sustained treatment with the CB$_2$ agonists significantly increased survival and helped maintain animal body weight. Patients who
develop metastatic breast cancer have an average survival of 1.5 to 3 years [213]. A predictor of a poor prognosis is linked to the lack of treatment available once tumor cells have migrated to the bone [41]. Although advances have been made in breast cancer detection and early stage treatment, there have been relatively few advances in late stage drug development for cancer proliferation, SREs and pain [41]. Recent preclinical trials with a B-RAF inhibitor in advanced skin cancer patients resulted in an increase average survival rate of 8-12 months with a significant reduction in tumors and pain. Although the cancer returned, the extended months without pain resulted in great satisfaction to both the patient and family members [214]. Here we have shown using an established advanced cancer model that CB₂ agonists, unlike drugs approved for late stage breast cancer, can result in the increase in survival time. Some groups have postulated that cannabinoid-mediated inhibition of tumor growth may induce apoptosis through modulation of the Ras-MAPK/ERK and PI3K-AKT pathways [24, 215], yet further studies are needed to identify the molecular mechanism of CB₂-mediated inhibition of tumor growth.

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

As the second leading cause of cancer death in women [2], breast cancer has been a central focus of investigation over the last fifty years. The results of these expansive research efforts have been tremendous advances in understanding the etiology of breast tumors, which has led to sub-classification of tumor type and phenotypically targeted adjuvant therapies used in conjunction with the classic regimen of surgical resection, radiation therapy, and chemotherapy. Five main classifications of breast tumor are currently defined including Luminal A; Luminal B; Her2/neu positive; basal-like; and claudin-low tumors [20, 216]. Luminal A/B tumors represent approximately 50% of diagnosed human breast tumors, and typically respond well to hormone suppressing therapies [25]. Her2 (neu in mice), also known as ErbB2, is an EGF tyrosine kinase receptor that is over-expressed by as many as 33% of breast tumors and is associated with an aggressive clinical profile, higher risk of recurrence, and reduced survival compared to Luminal A/B tumors [217]. Trastuzumab (Herceptin©), a Her2 monoclonal antibody, was approved by the FDA in 1998 for treatment of metastatic breast cancers over-expressing Her2 [218]. Basal-like breast tumors are commonly referred to as triple negative breast cancer (TNBC) due to the predominant lack of estrogen/progesterone receptor
expression and lack of Her2 over-expression [20]. Claudin-low tumors are also typically triple negative in addition to an unusually low expression of the critical cell-cell junction protein claudin [20]. Neither basal-like nor claudin-low tumors can be targeted via hormonal depletion or with trastuzumab; these tumor types typically include the worst patient prognosis [9].

Despite the recent leaps forward in the study of breast cancer genetics, a significant number of tumors either do not respond or quickly develop resistance to the resulting novel adjuvant therapies [22]. Most notably, trastuzumab is effective in only 25% of Her2 positive patients and of those 15% still go on to develop recurrence and metastasis [29, 219, 220]. Additionally, no new developments have been made in the treatment of basal-like triple negative tumors or claudin-low tumors, leaving at best 50% of breast cancer patients to rely on standards of care that date back to the introduction of Doxorubicin (Adriamycin) as a chemotherapeutic agent in the 1970’s [221]. Together, these data demonstrate a clear need for the development of novel therapies viable in the treatment of basal-like, claudin-low and therapy resistant breast tumors.

In this study, we evaluate cannabinoid receptor 2 (CB2) specific compounds in the treatment of breast cancer in vitro and in vivo. The rationale for this approach is multi-fold: nonspecific cannabinoids [222-225], CB2 selective [226-228], as well as cannabinoid receptor 1 (CB1) specific compounds [229-231] have yielded similar antiproliferative results in several tumor models, yet with unclear antiproliferative mechanisms. The lack of neuronal expression of
CB2 receptors precludes selective CB2 compounds from resulting in the psychotropic effects that typically accompany CB1 activation [173]. Our group and others have shown that CB2 agonists displaying a high degree of selectivity for the CB2 receptor simultaneously decrease tumor cell proliferation and significantly attenuate cancer induced bone pain without displaying psychoactive or addictive properties [167, 232, 233]. Additionally, CB2 receptors are markedly upregulated in many breast tumors [24] - a curious finding given that many of these tumor lines are of epithelial origin while in normal adults CB2 receptor expression is primarily limited to cells of the immune system and bone maintenance cells [145].

There are three distinct systems impacted by the use of CB2 agonists in the treatment of cancer: the tumor microenvironment, the bone microenvironment, and the immune system. Here, we focus on the direct effects on and mechanisms by which the CB2 specific agonist JWH-015 affects the tumor microenvironment. We show that JWH-015 inhibits proliferation and induces apoptosis of the breast cancer cell lines (murine) 4T1 and (human) estrogen receptor positive MCF7 in vitro and attenuate primary tumor formation and metastasis in vivo. We demonstrate that these effects occur through a Ca\(^{2+}\) dependent mechanism and not via a G\(\alpha_i\) dependent mechanism as cannabinoids are traditionally described to act. Using shRNA against the CB2 receptor we demonstrate a direct correlation between the level of CB2 receptor expression and breast cancer cell proliferation. In addition, we show that mediation of the
PI3K/AKT and MAPK/ERK pathways by JWH-015 may be responsible for the decrease in breast cancer proliferation.
4.2 JWH-015 attenuates proliferation of breast cancer cells in vitro

The sulforhodamine B (SRB) assay was used to determine the relative rate of mouse and human (4T1 and MCF7, respectively) breast tumor cell proliferation \textit{in vitro} in the presence of the CB$_2$ specific agonist JWH-015 (figure 9A-D). Single dose administration of JWH-015 over a 48 hour period after serum starvation for 16 hours dose dependently reduced proliferation of 4T1 cells ($A_{50} = 2.8 \, \mu M; \, 95\% \, C.I. = 2.56–3.07$) and MCF7 cells ($A_{50} = 4.16 \, \mu M; \, 95\% \, C.I. = 3.24–5.34$).
Figure 9: JWH-015 attenuates proliferation of breast cancer cells in vitro. In vitro proliferation of 4T1 and MCF7 cells were measured using SRB assay under serum starved conditions in response to increasing concentrations of JWH-015. Percent activity at all concentrations (A, C) and dose response curves generated (B, D) are presented.
4.3 JWH-015 attenuation of proliferation is not blocked by CB2 inverse agonist SR144528

Pretreatment with the CB2 inverse agonist SR144528 1 hour prior to administration of JWH-015 did not block the antiproliferative effects of JWH-015 in 4T1 cells (figure 10A; A50 = 2.475 µM; 95% C.I. = 1.77-3.46) or MCF7 cells (figure 10C; A50 = 2.475 µM; 95% C.I. = 1.77-3.46). When SR144528 was administered alone in either 4T1 cells (figure 10B; A50 = 4.88 µM; 95% C.I. = 4.12-5.77) and MCF7 cells (figure 10D; A50 = 7.01 µM; 95% C.I. = 3.53-13.92) proliferation was also inhibited.
Figure 10

A

4T1, % Proliferation

SR 144528 (uM)

B

4T1, % Proliferation

[JWH015] (uM) + 1 uM SR144528

C

MCF7, % Proliferation

SR 144528 (uM)

D

MCF7, % Proliferation

[JWH-015] (uM) + 1 uM SR144528
Figure 10: JWH-015 attenuation of proliferation is not blocked by CB2 inverse agonist SR144528. 4T1 and MCF7 cells were treated with the CB2 inverse agonist SR144528 alone (A,C) or in the presence of increasing concentrations of JWH-015 (B,D) and the corresponding dose response curves graphed after completion of SRB assay.
4.4 Antiproliferative effects of JWH-015 are not blocked by a CB1 inverse agonist, TRPV1 or TRPA1 channel blockers, or the Gαi inhibitor pertussis toxin

In order to clarify whether the observed reduction in tumor cell proliferation occurs in response to a non-CB2 mediated interaction or an alternate coupling pathway, SRB assays were carried out in the presence of JWH-015 and either the CB1 inverse agonist SR141716a, the transient receptor potential cation channel subfamily V member 1 (TRPV1) antagonist capsazepine, the transient receptor potential cation channel subfamily A member 1 (TRPA1) antagonist HC-030031, or the Gαi inhibitory molecule pertussis toxin (figure 11). SR141716a did not significantly alter the A50 of JWH-015 in 4T1 cells (A50 = 3.0 µM; 95% C.I. = 1.82–4.94) (figure 11A). Blockade of intracellular mechanisms downstream of Gαi with pertussis toxin did not alter the antiproliferative effect of JWH-015 in 4T1 cells (A50 = 3.82 µM; 95% C.I. = 3.22–4.94) (figure 11B). Similarly, TRPV1 and TRPA1 channels were also ruled out as potential sources of primary cellular effect: pretreatment with the TRPV1 antagonist capsazepine did not alter the antiproliferative effects of JWH-015 in 4T1 cells (A50 = 2.65 µM; 95% C.I. = 2.18–3.21), nor did pretreatment with the TRPA1 antagonist HC-030031 alter JWH-015 activity in 4T1 cells (A50=0.32 µM; 95% C.I. 0.14-0.69) (figure 11C-D). Similar results were seen when SR141716a, pertussis toxin, capsazepine and HC-030031 were administered to MCF7 cells prior to treatment with JWH-015 (data not shown).
Figure 11

A

4T1, % Proliferation

\[0.1 \quad 3 \quad 10\]

[JWH015] (uM) + 1 uM SR141716

B

4T1, % Proliferation

\[0.1 \quad 3 \quad 10\]

[JWH015] (uM) + 1 uM Capsazepine

C

4T1, % Proliferation

\[1 \quad 3 \quad 10\]

[JWH015] (uM) + 100ng/mL PTX

D

4T1, % Proliferation

\[0.1 \quad 3 \quad 10\]

[JWH015] (uM) + 1 uM HC030031
Figure 11: Antiproliferative effects of JWH-015 are not blocked by a CB1 inverse agonist, TRPV1 or TRPA1 channel blockers, or the G\textsubscript{\alphai} inhibitor pertussis toxin. 4T1 cells were evaluated for proliferative response with SRB assay by pretreatment with either SR141716a (A), capsazepine (B), pertussis toxin (C), or HC030031 (D) followed by increasing concentrations of JWH-015. Graphs displayed are the corresponding dose response curves.
4.5 4T1 cell survival in vitro is dependent on expression of CB2

To further characterize whether attenuation of proliferation by JWH-015 in breast cancer cells is a CB2 receptor mediated effect after verification that breast cancer cells express the CB2 receptor (figure 12A), short-hairpin RNA (shRNA) targeted against CB2 mRNA was delivered via lentiviral particles into 4T1 cells *in vitro* to knock down CB2 receptor expression. CB2 receptor knockdown was verified via western blot 72 hours post transfection (figure 12B). 24 hours post knockdown, the SRB assay was used to measure relative proliferation. No difference was observed between wells receiving no lentiviral particles (polybrene only) and wells receiving control scrambled shRNA containing 100,000 particles. The remaining wells received from 5000 to 100,000 CB2 shRNA containing particles. Assay needs to be replicated to achieve a high enough n to calculate for statistical significance. It should be noted that although according to manufacturer instruction a stable cell line expressing fewer or no CB2 receptor is obtainable, we were unable to achieve a stable line of CB2 knockdown in 4T1 cells due to loss in surviving cells in experimental but not control wells <72 hours post transfection with CB2 shRNA. This phenomena was not limited to lentiviral particle transfection- similar results were seen using small interfering RNA (siRNA) targeted to the CB2 receptor, as well as CB2 shRNA DNA vectors transfected via Lipofectamine 2000 (data not shown).
Figure 12

A

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CB2 (39 kDa)  
α tubulin (50 kDa)

B

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</tbody>
</table>

C

Optical Density

Polybrene, Control, 0.25 ul, 0.5 ul, 1.0 ul, 2.5 ul, 5.0 ul

[shRNA]
Figure 12: 4T1 cell survival in vitro is dependent on expression of CB2. (A) Western blot of CB2 receptor expression in multiple breast cancer cell lines. (B) Western blot of CB2 receptor expression after knockdown with shRNA particles. (C) Relative proliferation of cells receiving CB2 knockdown. *preliminary data, additional studies need to be completed prior to calculating statistical significance.
4.6 JWH-015 induces apoptosis without inducing cell cycle arrest in breast cancer cells in vitro

To determine whether loss of breast cancer cell proliferation is the direct result of cell killing or is an effect secondary to senescence, Ki67 imaging was done in vitro using a Ki67 primary antibody and a FITC conjugated secondary antibody (figure 13A). No observable difference was seen between the number of cell nuclei taking up Dapi stain and those expressing FITC fluorescence in naïve cells, media treated cells, or JWH-015 (10μM) treated cells over a 24 hour period.

To verify that, consistent with other cancer models [171, 225, 234], JWH-015 attenuates proliferation in 4T1 and MCF7 breast cancer cells by inducing apoptosis, caspase 3/7 activity was assessed. JWH-015 induced activation of the early caspases in a dose dependent manner in vitro 12 hours post treatment (figure 13B-C) in 4T1 cells and MCF7 cells (p<0.05). 4T1 cells exhibited peak caspase 3/7 activity at 1 and 3 hours post administration of 10 μM JWH-015 (not shown).
Figure 13

(A) Dapi (nuclear stain) and Ki67 (FITC) staining in 4T1 cells.

(B) 4T1 JWH015 (μM) effect on Caspase-3/7 activity.

(C) MCF7 JWH015 (μM) effect on Caspase-3/7 activity.

Naive 4T1 in complete media

Vehicle treated 4T1 in serum depleted media (24 hours)

10μM JWH015 treated 4T1 in serum depleted media (24 hours)
Figure 13: JWH-015 induces apoptosis without inducing cell cycle arrest in breast cancer cells in vitro. (A) Ki67 immunohistochemistry of 4T1 cells in naïve, serum starved, and cells treated with 10µM JWH-015. (B) Caspase 3/7 activity in 4T1 and MCF7 cells in response to increasing concentrations of JWH-015. Activity is reported as percent activity normalized to control vehicle treated cells.
4.7 JWH-015 induction of apoptosis is dependent on calcium flux in vitro

To determine whether induction of apoptosis by JWH-015 occurs by altering cellular calcium, SRB assays were carried out in the presence of JWH-015 and either the ryanodine receptor antagonist dantrolene, Ca2+ free media, or a combination thereof using 4T1 cells. Cells cultured in Ca2+ free media for 16 hours prior to treatment with JWH-015 shifted the antiproliferative dose response curve significantly rightward ($A50 = 9.80 \mu M; 95\% \text{ C.I.} = 7.57-12.69$) (figure 14A-B). Similarly, blockade of intracellular calcium flux by pretreatment with Dantrolene shifted the JWH-015 antiproliferative dose response curve in 4T1 cells significantly rightward compared to JWH-015 alone ($A50 = 18.77 \mu M; 95\% \text{ C.I.} = 10.56-33.00$) (figure 14C). A nearly complete inhibition of JWH-015 induced antiproliferative effect was observed when SRB assays were performed in Ca2+ free media with Dantrolene pretreatment (figure 14D): the dose response curve was shifted rightward over 40 fold ($A50 = 112 \mu M; 95\% \text{ C.I.} = 60-211$), signifying the importance of calcium movement in JWH-015 induced attenuation of proliferation. Because depletion of extracellular calcium with Ca2+ free media may also deplete intracellular calcium stores, in order to determine whether JWH-015 induced apoptosis is dependent entirely on intracellular calcium flux or a combination of intracellular and extracellular flux, JWH-015 SRB assays were performed in the presence of either the L-type calcium channel blocker nifedipine (figure 14E) ($A50 = 1.89 \mu M; 95\% \text{ C.I.} = 0.96-3.72$) or the N/P/Q-type calcium
channel inhibitor w-conotoxin MVIIIC (figure 14F) (A50 = 0.41 µM; 95% C.I. = 0.21-0.79). Neither nifedipine (A50 = 3.28 µM; 95% C.I. = 2.57-4.15) or w-conotoxin (A50 = 1.32 µM; 95% C.I. = 0.76-1.98) blocked the apoptosis inducing effects of JWH-015 on MCF7 cells (data not shown).
Figure 14: JWH-015 induction of apoptosis is dependent on calcium flux in vitro. (A,B) 4T1 cells were cultured in media depleted of Ca$^{2+}$ and evaluated for relative proliferation with SRB assay after treatment with increasing concentrations of JWH-015. Assay was repeated in the presence of dantrolene in normal experimental media (serum starved) (C) and calcium depleted media (D). 4T1 cells were pre-incubated with nifedipine (E) or ω-conotoxin MVIIIC (F) prior to administration of JWH-015.
4.8 JWH-015 induces intracellular calcium flux

Fura-2 AM staining was evaluated in vitro in live cultures to determine whether treatment with JWH-015 induced intracellular calcium flux (figure 15A-B). Vehicle treated cells (figure 15A) did not have altered f380/340 values until 50mM potassium chloride was washed over the cells as positive control 65 minutes after recording start. JWH-015 (10µM; figure 15B) treated cells demonstrated a slight reduction in cytoplasmic Ca2+ at approximately 35 minutes, followed by a rise in intracellular calcium from 45-60 minutes post JWH-015 administration. This timeframe corresponds to the 1 hour peak seen in caspase 3/7 activity. As a positive control 50mM potassium chloride was administered at 60 minutes.
Figure 15

A

Ca2+ flux in vehicle treated cells

B

Ca2+ flux in JWH-015 (10\(\mu\)M) treated cells
Figure 15: JWH-015 induces intracellular calcium flux. 4T1 cells were incubated with cell permeant Fura-2 and imaged for 5 minutes prior and immediately after administration of vehicle or JWH-015 for 60 minutes. Graphs presented are ratio of Fura-2 fluorescence at 380 nm / 340 nm.
4.9 JWH-015 is not acting through a $G_{\alpha_i}$ or $G_{\alpha_s}$ mechanism in 4T1 cells at relevant concentrations in vitro

To ascertain whether the CB$_2$ receptor may engage $G_{\alpha_i}$ or $G_{\alpha_s}$ in 4T1 cells to induce apoptosis, cAMP assays were carried out in the presence of either JWH-015 with forskolin stimulation (figure 16). At a concentration of 1 and 10 nM, JWH-015 exhibits cAMP accumulation inhibition consistent with the traditionally defined $G_{\alpha_i}$ coupling ($p<0.5$), however at all other tested concentrations (from 100 nM to 10 $\mu$M), no significant change in cellular cAMP was observed ($p>0.05$) $G_{\alpha_i}$ or $G_{\alpha_s}$ coupling at higher, antiproliferative concentrations.
Figure 16

Intracellular cAMP

Table 1: A table showing the effect of different concentrations of forskolin on intracellular cAMP levels.

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<thead>
<tr>
<th>Concentration</th>
<th>cAMP (pg/mg total protein)</th>
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[JWH015]
Figure 16: JWH-015 is not acting through a Gai or Gas mechanism in 4T1 cells at relevant concentrations in vitro. A cAMP ELISA was conducted on supernatants collected from 4T1 cells stimulated with forskolin and treated with vehicle or increasing concentrations of JWH-015.
4.10 JWH-015 and SR144528 reduced pERK 1/2 phosphorylation and increased pAKT phosphorylation in vitro

4T1 cells were treated with either JWH-015 (10mM) or SR144528 (10mM) over a time-course ranging from 5 min to 24 h in normal or Ca2+ free media and western blots were run to evaluate pERK 1/2 and pAKT phosphorylation (figure 17A). Blots were re-probed with α-tubulin for loading control. Image J was used to evaluate relative density of scanned blot images and data was normalized to relative density of control (data is averaged from 3 separate experiments; figure 17B-C). JWH-015 (10mM) reduced phosphorylation of ERK in serum low cultures at 1, 3, and 6 hours post administration (STATS; figure 17B) This JWH-015 induced reduction in phosphorylation of ERK is reversed in serum low/Ca2+ free cultures. Early and late induction of AKT phosphorylation occurs (5 and 15 minutes; 3, 6, 12, and 24 hours post treatment; figure 17C) in serum starved cells administered JWH-015; this is attenuated by culturing cells in serum starved, calcium free media. *Additional blots need to be evaluated prior to calculating statistical significance.
Figure 17: JWH-015 and SR144528 reduced pERK 1/2 phosphorylation and increased pAKT phosphorylation in vitro. (A) Western blot of pERK and pAKT in 4T1 cells treated with 10µM JWH-015 in normal or calcium depleted media. (B, C) Blot quantification analysis comparing normal media (left bars) to calcium depleted media (right bars) of pERK (B) and pAKT (C).
4.11 JWH-015 reduces primary tumor burden and decreases metastasis in vivo

In order to determine whether the profound effects of JWH-015 in vitro were also observable in an in vivo model, 4T1 cells were transfected with luciferase and the resulting stable 4T1-Luc cell line was injected into the c-9 mammary fat pad of female Balb/c mice. Tumors were allowed to establish for 7 days and the mice were then treated with either JWH-015 (6 or 20 mg/kg, i.p.), SR144528 (1 mg/kg, i.p.), or vehicle (i.p.) for 21 days. The mice were imaged on a weekly basis (figure 18A) beginning on day 7 for 28 days to track primary tumor growth and sites of metastasis. On day 28 all mice were sacrificed and primary tumors resected. Consistent with the in vivo luminescence images, administration of JWH-015 significantly reduced primary tumor mass (6mg/kg p<0.05; 20mg/kg p<0.05), as did 1mg/kg SR144528 (p<0.05) (figure 18B). Common metastasis sites were harvested for ex vivo analysis; total number of detectable metastasis was reduced in the JWH-015 group compared to vehicle (figure 18C).
Figure 18

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</table>

A

Day 7

Day 14

Day 21

Day 28

B

Primary Tumor mass (g)

C

% Positive for Metastasis

* *
Figure 18. JWH-015 reduces primary tumor burden and decreases metastasis in vivo. (A) In vivo bioluminescence imaging of tumor bearing animals treated over a 21 day time course after allowing tumor to establish for 7 days. Animals were treated with either control vehicle, SR144528, or one of two doses of JWH-015. (B) On day 28 animals were sacrificed and primary tumor resected. Graph represents tumor mass by treatment group. (C) Metastasis was evaluated post-mortem using a dual luciferase reporter assay on samples obtained from common metastatic sites in tumor bearing animals.
4.12 Conclusions

Despite marked advances in chemotherapeutics for early stage breast cancer there are few new therapies effective in slowing progression in advanced stages of the disease. Genotypic profiling of human breast cancers over the last several years has led to broad phenotypic sub-classification of breast tumors, shed light on the complexity of the disease, and demonstrated some of the genetic intra-tumoral differences responsible for the success or failure of protein specific therapeutics. There is a clear need for novel therapeutics in the treatment of late stage and chemotherapy resistant breast cancer.

The cannabinoid receptors are classically defined as members of the seven transmembrane spanning G protein coupled receptor family (GPCRs). Two distinct cannabinoid receptors have been cloned: cannabinoid receptor 1 (CB1)[235], which is one of the most abundantly expressed GPCRs on neurons, and cannabinoid receptor 2 (CB2)[236, 237]- found primarily in cells of the immune system and on bone maintenance cells[173]. In physiologically normal systems CB1 and CB2 were first reported to couple predominantly with G\(\alpha_i\)[238, 239], yet several more recent studies have demonstrated CB2 receptor signaling via G\(\alpha_q\) and other alternative coupling pathways [240, 241]. However, the mechanism(s) by which CB2 receptor cannabinoids exert their effects on breast cancer cells are as yet incompletely defined.
Several groups have shown that both nonselective cannabinoid agonists and CB2 specific agonists decrease breast cancer proliferation in vitro and in vivo: Δ⁹-tetrahydrocannabinol (THC) and CB2 selective agonist JWH-133 have been demonstrated to exert considerable antitumoral effects in the MMTV-neu mouse model of breast cancer which overexpresses the EGF receptor HER2[24]; similarly, CB2 selective JWH-015 has been shown to retard growth of the highly aggressive spontaneous breast tumors occurring in PyMT transgenic mice[171]; cannabidiol inhibited human breast cancer cell line MDA-MB-231 growth in vitro via apoptosis and autophagy- notable due to the low expression of HER2/3 in MDA-MB-231 cells[225]; anandamide, an endogenous cannabinoid CB1 agonist, has been shown in multiple models to inhibit breast cancer cell growth and migration[242, 243], and several other studies have demonstrate CB2 agonist efficacy in breast cancer systems[244-247], however the mechanism(s) of how CB2 agonist may alter breast cancer proliferation is absent.

In vitro, we have demonstrated that JWH-015 reduces breast cancer cell proliferation by inducing cell cycle independent apoptosis of breast cancer cells. We were unable to block the in vitro JWH-015 activity in breast tumor cells with co-administration of the CB2 inverse agonist SR144528, suggesting either that JWH-015 is either acting in a non-classical manner at concentrations effective in the tumor microenvironment, or that JWH-015 is acting through a CB2 independent mechanism. As such, agonists and antagonists of GPCRs are defined based on their Gα activity: specifically the effect on intracellular levels of
cyclic adenosine monophosphate (cAMP)[248]. The $\beta\gamma$ subunits however, which dissociate from the $\alpha$ subunit upon binding ATP in response to the conformational change in the receptor induced upon ligand binding, also mediate considerable intracellular effects through channels and pathways including MAPK/ERK and PI3K/AKT[249] that are not typically considered when classifying a compound with affinity to a receptor. We have established that the apoptosis of breast tumor cells induced by JWH-015 is not occurring through a $G\alpha_i$ coupled mechanism in two distinct assays: by blocking the $G\alpha_i$ pathway with pertussis toxin prior to treatment with JWH-015 and by direct measurement of cellular cAMP in response to treatment with JWH-015. Additionally, we have ruled out interaction with the CB1 receptor since the CB1 receptor antagonist SR141716a did not block the antiproliferative effects of JWH-015. Studies have demonstrated that the endogenous cannabinoid anandamide could act as low efficacy agonist at the transient receptor potential TRPV1 calcium channels [246]. Here we have ruled out either TRPA1 or TRPV1 channels as the target for JWH-015 antiproliferative effects by blocking the channels with HC-030031, and capsazepine, respectively. We further ruled out JWH-015’s effects via L-type and N/PQ-type calcium channels using nifedipine and $\omega$–conotoxin MVIIIC, respectively.

Although the effects of JWH-015 in breast cancer proliferation in vitro is not mediated by TRPA1, TRPV1, L-type, or N/PQ-type calcium channels we
have shown that it is a calcium dependent process. We have demonstrated that by blocking either intracellular calcium flux with the ryanodine receptor antagonist dantrolene, or depleting extracellular calcium using media devoid of Ca2+, a significant rightward shift (19 and 10 fold, respectively) of the JWH-015 antiproliferative dose response curves is observed. Notably, when both intracellular and extracellular calcium are restricted simultaneously, the rightward shift (40 fold) of the JWH-015 dose response curve observed is synergistic. Further, we show that in vitro treatment with JWH-015 induces intracellular calcium flux 45-60 minutes post administration of the compound in 4T1 cells. Interestingly, immediate calcium flux is not observed, as would be expected in the case that JWH-015 were primarily acting through integral membrane calcium channels. This data is supported by work done by Felder et al suggesting that CB2 receptors do not modulate ion channels directly[250]. Taken together, these data suggest that intracellular calcium flux is a secondary event necessary for induction of apoptosis that is stimulated by another primary second messenger event activated by administration of JWH-015.

In order to determine whether the CB2 receptor itself mediates the primary event associated with JWH-015 induction of apoptosis in breast cancer cells, we first confirmed expression of CB2 via western blot and then performed shRNA transient knockdown of the CB2 receptor in 4T1 cells, demonstrating that CB2 expression is necessary for tumor cell survival. Additionally, we show that 4T1 cells transiently expressing a significant reduction in CB2 receptors do not
display the same dose dependent attenuation of proliferation seen in wild type 4T1 cells, suggesting that JWH-015 is acting primarily at the CB2 receptor in 4T1 mammary carcinoma cells. Consistent with findings on the intracellular activity of cannabinoids by other groups[223, 251-254], we have demonstrated that CB2 selective JWH-015 reduces phosphorylation of ERK 1/2 and induces phosphorylation of AKT at time points relevant to peak measured caspase 3/7 activity suggesting that the apoptosis induced by JWH-015 in breast cancer cells may be mediated by the MAPK/ERK and PI3K/AKT pathways. Such findings are in line with recent reports demonstrating CB2 activation of the PI3K/AKT and MAPK/ERK pathways in hippocampal neurons.

Our study characterizes the actions of the CB2 selective agonist JWH-015 on breast cancer cells in vitro and in vivo in a syngenic murine model without compromising immune function or inducing oncogenic mutation in the host- two factors that make this model more representative of the clinical presentation of cancer progression and metastasis. Additionally, because we transfected 4T1 cells with a luciferase reporter prior to host inoculation, the model utilized allows non-invasive in vivo tracking of primary tumor growth and metastasis over the entire duration of tumor establishment and treatment. In the studies presented herein, we show a drastically reduced profile of metastasis on treatment with JWH-015 as well as reduced primary tumor burden in vivo.

In conclusion, we have demonstrated here that the CB2 agonist JWH-015 induces apoptosis in a non-\(\text{G}\alpha_i\) mediated, calcium dependent, cell cycle
independent mechanism involving the reduction in the activated anti-apoptotic proteins MAPK/ERK and PI3K/AKT intracellular pathways. These are findings that should be considered with our previous studies indicating that JWH-015 significantly modifies the bone microenvironment in metastatic disease [233] by decreasing bone wasting in advanced stages of cancer and by significantly reducing spontaneous and evoked pain while simultaneously reducing fracture occurrence [233]. Given the current state of available breast cancer therapies, JWH-015 and other CB2 selective agonists demand attention as adjuvant therapies, particularly in patients experiencing bone metastasis where CB2 selective compounds may not only improve disease outcome, but also increase patient quality of life.
CHAPTER 5: RESTORATION OF IMMUNE HOMEOSTASIS BY CANNABINOID RECEPTOR 2 AGONISTS IN ADVANCED STAGE BREAST CANCER

5.1 Introduction

Although the involvement of cytokines and other inflammatory mediators in tumor progression has been clearly established and continues to be a well studied area, it has only been within the last decade that support for theories of immunosubversion and immunoediting have gained support. Fueled by flawed animal models and an incomplete understanding of the immune system as a whole, many innovative experiments have been dismissed even though the earliest predictions of the importance of immune function in tumor development date back to 1909 when Paul Ehrlich proposed that the human immune system contains a mechanism to recognize ‘self’ vs ‘non-self’ and that cancer cells’ digression from ‘self’ allowed recognition and targeting by immune cells [255]. The idea was later expounded upon by Burnet in 1957 [256] and Thomas in 1982 [257] and has ultimately resulted in the current theory of immunosurveillance [258], which states that there are both cell intrinsic and cell extrinsic (immune) barriers to tumor formation. The cell intrinsic barriers are evidenced by the hallmarks of cancer [33] and consist of tumor suppressor genes and intracellular repair mechanisms- the loss of which lead to distinct selective advantages of tumor cells. Cell extrinsic barriers to tumor formation lie in the immune system, primarily in the form of NK and CD8+ CTLs [259]. It has been demonstrated time
and again that mice with compromised immune systems develop more spontaneous tumors more quickly than wild type mice— for review see Dunn [111]. In humans, the development of Kaposi’s sarcoma in immunocompromised (particularly HIV+) patients offers further support that a normally functioning immune system blocks tumor formation [260]. Recently, several groups have demonstrated mechanisms employed by tumor cells to subvert and ultimately escape immune regulation including downregulation of MHCII molecules [261] to prevent recognition by and activation of CD8+ CTLs, release of soluble MICA/B molecules [262] which effectively agonizes NKG2D receptors on NK cells and prevents recognition as ‘non-self’. The result of both of these mechanisms utilized simultaneously is the inability of the immune system to recognize cancer cells as existing at all, let alone as ‘non self’. Many other mutations confer selective immune advantage to tumor cells, not the least of which is the release of a variety of cytokines and chemokines by the tumor cells themselves that not only affect sensory input and local inflammation, but also induce changes in the immune system at large- skewing immune response to a tolerogenic Th2 profile that is much more friendly to tumor development than the cytotoxic Th1 response [263-265]. It is this polarization and induction of a variety of immunosuppressive cells including myeloid derived suppressor cells, regulatory T cells, regulatory B cells, and immature dendritic cells that points to near complete inefficacy of the immune system against cancer cells by the time a primary tumor is clinically apparent [266-269]. Re-manipulation to restore homeostasis in the immune
system has proven difficult: attempts to activate a Th1 response are hindered by the inability to distinguish between regulatory and effector lymphocytes with certainty without lysis of the lymphocyte [270], a fact that makes it increasingly difficult to overcome the rampant immune suppression in late stage disease. Additionally, while great advances have been made in pre-clinical animal models, clinical trial design is a severely limiting factor in that phase I/II trials only allow enrollment of end stage disease patients refractory to established chemotherapy when the basic principles of tumor immunology clearly indicate that the ideal candidates for such therapy are much earlier stage patients that unlike end stage terminal patients have not had remaining viable immune cells ablated by toxic chemotherapeutics and may have a better chance at re-establishment of regulatory/effecter immune homeostasis. It has been hypothesized that a possible workaround is simultaneous knockdown of immune suppressive mechanisms and activation of Th1 response- a method currently under investigation with the combination of Sipuleucel T, a dendritic cell vaccine, and the CTLA-4 inhibitor ipilimumab in the treatment of hormone refractory prostate cancer [271]. This approach is very promising- Sipuleucel T alone confers a survival benefit of approximately 4 months [272] and similarly, ipilimumab has been shown to increase survival [273]. Still, development of personalized dendritic cell vaccines is a costly and arduous task. Investigation into alternative therapies must be continued.
As yet unexplored in this context, CB2 receptors are found in spleen, osteoclasts, on T and B lymphocytes and other immune cells, including microglia and dendritic cells and have long been suspected to be immunosuppressive [158], though the specific mechanisms and circumstances surrounding this observation lack definition and are in direct opposition to the known functions of endogenous cannabinoids acting at CB2 in development. While CB2 receptors are not found on mature neurons, neural progenitor cells do express CB2 and respond with increased proliferation when agonized by CB2 selective compounds [159]. CB2 is also known to be critical in development of the innate and adaptive immune systems: CB2 knockout mice are immunodeficient as evidenced by the lack of CD4+ memory T-cells, and downregulation of multiple subsets of B cells, NK and NKT cells [160]. Post complete thymic development (around age 14 in humans) the cannabinoid receptors tend to have a contrary response in immune cells, having been demonstrated to deplete T cell populations and are a prevalent topic in autoimmune research [161, 162]. Little to no evidence exists however in perhaps the most useful potential role of cannabinoids: cancer immunotherapy. A small handful of studies over the last decade have demonstrated that nonspecific cannabinoid agonists have little if any effect on NK and NKT cells, and suggest that these compounds may selectively target T regulatory cells over effector T cells [163, 164]. Available data focuses on nonspecific compounds with a slightly higher selectivity for the cannabinoid
receptor 1 (CB1) rather than the more obvious target- cannabinoid receptor 2 (CB2).

Here, we investigate the potential of cannabinoid receptor 2 agonist JWH-015 to positively modify the critical effector/regulator immune balance in a murine model of metastatic breast cancer.
5.2 4T1 cells induce immunosuppressor cells when inoculated into the mammary fat pad

In order to evaluate effects of 4T1 mammary tumor on immune profile in Balb/c mice, animals were inoculated into the mammary fat pad with 200uL of media only or media containing $1 \times 10^6$ 4T1 cells. Tumors were allowed to establish for 14 days before the mice were sacrificed and spleen, bone marrow and inguinal lymph nodes harvested. Single cell suspensions of leukocytes were prepared from freshly isolated tissue by straining tissue through 100uM nylon mesh filters (BD Biosciences). Red blood cell lysis was performed according to manufacturer instruction using RBC lysis buffer (Ebiosciences) and the remaining leukocytes were spun down at 500xg, resuspended in advanced RPMI and counted using a hemocytometer. $1 \times 10^6$ leukocytes per animal ($n=4$ per group) were stained for either CD4, CD25, and Foxp3 (figure 19A-B), or CD11b, Gr-1, F4/80, CD11c, and MHCII (figure 19C-D) and evaluated using an LSRII flow cytometer (BD Biosciences). Data was analyzed with FloJo software and graphs herein are median representatives of all animals analyzed.
Figure 19

A  Non Tumor Bearing

CD25

Foop3

B  Tumor Bearing

CD25

Foop3

C  Non Tumor Bearing

CD25

Gr-1

D  Tumor Bearing

CD25

Gr-1
Figure 19: 4T1 cells induce immunosuppressor cells when inoculated into the mammary fat pad. Flow cytometric analysis of spleen from naïve (A,C) or tumor bearing (B,D) animals. Splenocytes were stained with markers for regulatory T cells (A,B) or MDSCs (C,D).
5.3 JWH-015 decreases the ratio of CD4+CD25+Foxp3+ regulatory T cells to CD4+CD25+Foxp3- effector T cells in tumor bearing animals

In order to determine whether JWH-015 modifies the expression of regulatory T cells in tumor bearing animals, mammary fat pad tumors were inoculated into naïve mice as described above. Tumors allowed to establish for 7 days. Beginning on day 8, mice were given either vehicle, 6 mg/kg JWH-015, or 20 mg/kg JWH-015 once daily i.p. for 7 days. Spleens and the corresponding inguinal lymph nodes were harvested on day 14 and stained with CD4, CD25 and Foxp3 fluorescein conjugated antibodies per manufacturer instruction (Ebiosciences) prior to flow cytometric analysis (figure 20A-B). Images presented are representative of typical splenocytes per treatment group (n=6 per treatment group). Mean ratios of regulatory T cell to effector T cell (figure 20C) and effector T cell to total CD4+ (figure 20D) were calculated (p<0.05). JWH-015 reduced the ratio of regulatory to effector T splenocytes in tumor bearing mice but not media control mice. Similarly, JWH-015 induces a trend of increasing CD4+CD25+ effector T cells to total CD4+ populations in splenocytes of tumor bearing mice but not media control mice. Similar results were observed in leukocyte populations isolated from lymph nodes of tumor bearing and non-tumor bearing mice (data not shown).
Figure 20

Vehicle vs. JWH-015

**CD25**

**Foxp3**

**CD4+CD25+Foxp3+/CD4+CD25+Foxp3-**

**T-reg / T-effector**

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<th>JWH-015 20mg/kg</th>
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<td>JWH-015 6mg/kg</td>
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<td>++</td>
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**CD4+CD25+Foxp3+/CD4+CD25+Foxp3-**

**T-effector / Total CD4+**

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Figure 20: JWH-015 decreases the ratio of CD4+CD25+Foxp3+ regulatory T cells to CD4+CD25+Foxp3- effector T cells in tumor bearing animals. Flow cytometric analysis of splenocytes isolated from tumor bearing animals treated with vehicle (A) or JWH-015 (B) and stained with antibody for CD4, CD25, and Foxp3. (B,C) Ratio of total hits calculated for CD4+CD25+Foxp3+/CD4+CD25+Foxp3- (C) and CD4+CD25+Foxp3-/total CD4+ (D).
5.4 JWH-015 inhibits the suppressive activity of regulatory T cells ex vivo

To evaluate the suppressive efficacy of remaining regulatory T cells isolated from tumor bearing animals, 4T1 mammary fat pad tumors were inoculated as previously described and allowed to establish for 7 days. On day 8 treatment with either vehicle or 6 mg/kg JWH-015 (once daily i.p.) commenced for a period of 7 days. On day 15, spleens and inguinal lymph nodes were isolated, single cell suspensions were prepared, and cells were incubated with CD4/CD25 antibody coated magnetic beads according to manufacturer instruction (Miltenyi Biotec). Cells were sorted in a strong magnetic field and the resulting CD4+CD25\textsuperscript{high} cells were cultured in the presence of IL-2 for 18 hours prior to co-culture with naïve T cells isolated from naïve age matched mice stimulated with CD3+CD28+ Dynabeads (Invitrogen). Naïve-naïve wells were used as proliferative control. BrdU assay was used to evaluate proliferation of the ex vivo activated naïve T cells (figure 21). T cells co-cultured with regulatory T cells isolated from vehicle treated tumor bearing animals proliferated significantly less than activated T cells with no suppressive co-culture (naïve-naïve) (p<0.05), indicating that CD4+CD25\textsuperscript{high} regulatory T cells isolated from tumor bearing mice are suppressive lymphocytes. Conversely, CD4+CD25\textsuperscript{high} lymphocytes isolated from JWH-015 treated tumor bearing mice did not significantly alter the proliferation of naïve T cells stimulated with Dynabeads (p>0.05).
Figure 21

% Suppression

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<td>T-regs isolated from tumor bearing mice cocultured with naïve T cells from naïve mice</td>
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Figure 21: JWH-015 inhibits the suppressive activity of regulatory T cells ex vivo. Regulatory T cells isolated from tumor bearing animals treated with either vehicle or JWH-015 were co-cultured with naïve T cells and stimulated with Dynabeads. Suppressive activity is measured using BrdU assay and presented as inhibition of proliferation.
5.5 JWH-015 does not inhibit the growth or activation of naïve T cells ex vivo

To determine whether JWH-015 alters the activation or proliferation of naïve T cells, naïve CD4+ T lymphocytes were isolated from naïve mice and either stimulated with Dynabeads (+) or not (-) in the presence of increasing concentrations of JWH-015 for 48 hours (figure 22). No concentration of JWH-015 tested inhibited the proliferation of either activated or non-activated T cells as compared to vehicle treated cells (p<0.05).
Figure 22

![Bar graph showing the effect of different concentrations of [JWH015] on % activity.](image)

- Naive
- Vehicle
- 100pM
- 100nM
- 1µM
- 10µM

% Activity (±SEM)

[JWH015]
Figure 22: JWH-015 does not inhibit the growth or activation of naïve T cells ex vivo. Naïve T cells were cultured either in the presence or not of Dynabeads and treated with increasing concentrations of JWH-015. BrdU assay was performed to measure relative proliferation.
5.6 JWH-015 does not inhibit cytotoxic tumor killing activity of CD8+ cytotoxic T cells ex vivo

CD8+ cytotoxic T lymphocyte activity was also evaluated ex vivo with CD8+ T cells magnetically isolated from tumor bearing mice either treated with vehicle or 6 mg/kg JWH-015 (once daily i.p.) for 7 days after allowing tumor establishment for 7 days. CD8+ cells were isolated from spleen and whole blood of tumor bearing animals and co-cultured ex vivo with 4T1 tumor cells for 18 hours prior to analysis with a cytotoxicity assay (Promega). No significant difference between the cytotoxic activity was observable in wells containing CTLs isolated from either blood or spleen of JWH-015 treated animals as compared to control vehicle treated animals, suggesting that JWH-015 does not impair the killing ability of cytotoxic T lymphocytes (figure 23; p>0.05).
Figure 23

% Cytotoxicity

% Dead cell/total cell activity

Vehicle  JWH-015  Vehicle  JWH-015

Spleen CD8+ 4T1  Blood CD8+ 4T1
Figure 23: JWH-015 does not inhibit tumor killing activity of CD8+ cytotoxic T cells ex vivo. CD8+ cells were isolated from spleen and whole blood of tumor bearing animals treated either with vehicle or JWH-015 and co-cultured with 4T1 breast cancer cells. Cytotoxicity was evaluated using a cytotoxicity assay and is presented as percent dead cell activity normalized to 4T1 only wells.
5.7 JWH-015 reduces spleen but not bone marrow population of myeloid derived suppressor cells in tumor bearing animals

Due to the severe induction of myeloid derived suppressor cells in spleen and bone marrow of 4T1 mammary fat pad tumor bearing mice, we evaluated whether treatment with JWH-015 6 mg/kg; i.p.) once daily for 14 days after allowing tumors to establish for 7 days could alter the splenic or bone marrow populations of MDSCs. While a trend towards decrease in splenic MDSC populations were observed, evaluation of bone marrow did not reveal a decrease in MDSCs in JWH-015 treated animals as compared to vehicle treated animals (figure 24). Further studies and a larger n are needed to verify statistical significance.
Figure 24

- Isolated from spleen of vehicle treated animals
- Isolated from spleen of JWH-015 treated animals
- Isolated from bone marrow of vehicle treated animals
- Isolated from bone marrow of JWH-015 treated animals
Figure 24: JWH-015 reduces spleen but not bone marrow population of myeloid derived suppressor cells in tumor bearing animals. Spleen and bone marrow were isolated from tumor bearing animals treated either with vehicle or JWH-015 and stained for markers of MDSC.
5.8 Conclusions

Using a syngenic murine model of breast cancer metastasis in non-immunocompromised mice we have demonstrated the skewed immune profile that develops as breast cancer progresses into metastatic disease. We have shown the development of regulatory T cells and myeloid derived suppressor cells that can be at least partially mediated by treatment with JWH-015, though it is apparent that this type of adjuvant therapy may be better directed towards regulatory T cells. Complimentary to previous studies that have shown little to no effect of cannabinoid agonists on NK cells [163], we have demonstrated that chronic treatment with JWH-015 does not inhibit the activation or proliferation of effector CD4+ T-cells and does not alter the killing ability of CD8+ cytotoxic T cells. Importantly, we have also shown that JWH-015 reduces the ratio and activity of CD4+CD25+Foxp3+ regulatory T cells. That JWH-015 appears to be specific towards the down-regulation of immunosuppressive regulatory T cells is of utmost importance to late stage cancer patients who are likely not only experiencing severe immunosuppression due to the disease itself, but who will also likely have remarkably few viable effector immune cells post- rigorous toxic chemo- and radio therapy regimens. Further studies are necessary to pinpoint the mechanisms by which JWH-015 is able to modify the immune landscape in this model as well as best time, level, and duration of treatment. This will be somewhat difficult to accomplish given the complexity and fluidity of interaction
between tumor and immune environments. Still, these preliminary studies highlight JWH-015 and other CB2 specific compounds as potential adjuvant therapies that may change the face of chemo- and immuno- therapy.
CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

Claiming more than 40,000 American and 450,000 worldwide lives per year [183], breast cancer is the most prevalent metastasizing cancer in women. Despite marked advancements in detection, understanding the cell intrinsic and extrinsic factors leading to breast cancer development, and etiology of the disease available chemotherapeutics offer little more to patients experiencing invasive disease than the equivalent of a flimsy patch on a main waterline break. Relapse rates are high [274] and time to therapy resistance is staggeringly short [18] while first line therapeutics are associated with a host of side effects nearly as devastating as the disease itself. Traditional chemotherapeutics induce many side effects including but not limited to severe nausea and vomiting, hair loss, weakness, neuropathy, anorexia and weight loss, constipation, anemia, uncontrolled bleeding and impaired wound healing, memory changes, mood disorders, increased risk of infection, severe fatigue, edema, dry or itchy skin, cotton-mouth, dry throat, secondary tumor formation, and in extreme cases death [36]. The rationale for the use of these toxic chemotherapeutics is a cold and sobering fact: the alternative is death.

Breast cancer patients over the last six decades have seen aggressive treatment regimens consisting of surgical resection where possible followed by intense combinations of radiotherapy and chemotherapy with little change in the mainstays of chemotherapy since the introduction of doxorubicin in the early
1970’s [36, 258, 275]. Some progress has been made with adjuvant therapies with the development of hormone depletion and other protein specific therapies in more recent years. Tamoxifen, an estrogen receptor antagonist, is successful in approximately 58% of ER dependent tumors not over-expressing Her2 and 36% of ER+/Her2+ tumors [276]. Responders generally experience an average time to tamoxifen resistance of 7 months and 4 months with Her2 + and - tumors, respectively, and enjoy greater overall survival (median of 31 months vs 24 months without tamoxifen adjuvant therapy) [276]. Trastuzumab (Herceptin), a Her2 monoclonal antibody, also increases life expectancy in patients with Her2 over-expressing tumors when administered as an adjuvant therapy with median disease free survival time increase of approximately 3 months [277]. Even with these advances in adjuvant therapies primary systemic chemotherapy has not been replaced and 10-year survival of invasive breast cancer hovers around 20% [4]. Mechanisms behind the main line chemotherapeutics are similar: disruption of DNA replication and/or structural integrity of dividing cells. These avenues were pursued for good reason- tumors contain millions of rapidly dividing cells. Shutting down cellular replication can help stop the advancement of the disease. Unfortunately, the major cost to this approach is the loss of normally replicating cells, including those of the immune system, hair follicles, and epithelial cells of the gastrointestinal tract and skin.

In addition to the shortcomings of cancer therapies treating underlying disease, there is a major shortfall of analgesics capable of alleviating cancer-
induced pain. A recent study in cancer patients demonstrated a significant and severe lack in treatments for cancer pain with a higher incident in minority patients [278]. Reports also highlight the importance of patient quality of life in late stages of cancer as well as how family and physicians surrounding the patient may be affected [185]. Primary therapies for cancer pain include radiotherapy, bisphosphonates, NSAIDs, and opiates, all of which are associated with their own drawbacks. Clearly, there is a need for innovative new therapies with far fewer and more tolerable side effects for disease treatment as well as pain therapy.

The studies presented herein demonstrate the great amount of potential that CB2 selective compounds possess as possible breast cancer therapy. CB2 agonists are promising agents for pain relief, particularly breast cancer induced bone pain. We have shown that not only are these compounds capable of acute and chronic alleviation of spontaneous and evoked bone pain but that they are also capable of positively modifying the bone microenvironment. These benefits alone make pursuit of a CB2 compound worthwhile in a subset of patients who are predominantly elderly and already predisposed to fracture. The best analgesics for this type of pain available hinder bone healing [54], cause paradoxical pain [75], and increase chances of secondary tumor formation long term [274]. Future studies are necessary to further elucidate central and peripheral mechanisms of these effects, however at this juncture it appears that in the context of pain control these CB2 molecules are acting peripherally to
inhibit tumor cell and tumor associated leukocyte release of cytokines, inhibiting peripheral nociceptors, alleviating mechanical strain by reducing tumor burden, normalizing pH of the bone microenvironment to prevent ASIC and TRP channel activation by blocking osteoclast activity, and possibly preventing central activation of microglia. Explorations should be made to verify these theories as well as multiple time and dose regimens alone and in combination with other analgesics to determine the most beneficial course of action.

In terms of direct effect on tumor cells, a significant amount of work remains, including determination of the site of action. It is clear that these compounds alter proliferation of breast cancer cells, however unlike in the bone microenvironment, it is unclear whether the proliferative control stems from CB2 receptors or an unidentified receptor. While there are many experiments that could be done, one experiment that should take precedence is the evaluation of proliferation in a non-CB2 expressing cell line, hence identifying the significance of the CB2 receptor in the CB2 agonist mediated effects. In addition, future studies in CB2 and CB1 KO mice will help identify whether antiproliferative effects are mediated via identified cannabinoid receptors in animals.

Of particular interest are the effects of JWH-015 on the immune system. With the recent shift in approach to discovery of novel chemotherapeutics from obliterative therapies to those that attempt to restore immune homeostasis and boost natural immunity against invading tumor cells, CB2 agonists could prove to be very useful. The preliminary data presented here suggests that JWH-015
selectively targets regulatory T cells without disrupting the activation, proliferation, or activity of vital Th1 associated effector cells. This is also an area that needs intense further study to determine whether these effects are secondary to activity on tumor cells themselves or if the primary effect is in the immune system allowing greater intrinsic immunity to the tumor. Determining compound primary site of action can be achieved using an immunocompromised murine model and a murine CB2 knockout animal. In addition, several characterization studies need to be completed to determine complete effect on immune profile, including number and activity of NK cells, macrophages, CD4+, CD8+, and dendritic cells. Several doses and differing time courses need to be tested in all of these models to determine time of best response. Finally, all of the studies presented here use JWH-015 as a single neoadjuvant with no radiotherapy, primary tumor resection, or combination with other standards of care. It is highly likely that CB2 compounds will be far more successful in the adjuvant setting, particularly since the time of appearance of clinical breast cancer, often metastatic, detrimental changes in the immune system have already taken place. It is unlikely that a single miracle compound will have the ability to combat disease and restore immune homeostasis simultaneously and without assistance. One might suggest that the greatest benefit a CB2 compound can offer will be in conjunction with an immunostimulator, such as an intratumoral TLR-9 agonist or a dendritic cell vaccine. Simultaneous pain alleviation, bone normalization, primary and metastatic tumor shrinkage, immunosuppressive
knockdown by a CB2 agonist, and Th1 stimulation by vaccine would be the ultimate combination therapy that could be a true game changer and possibly offer patients a lifetime of tumor free survival rather than only a period of months while also sparing the host of devastating side effects seen with traditional chemotherapy.
APPENDIX A: LIST OF PUBLICATIONS
PUBLICATIONS:


6) **Hanlon KE**, Ondoua-Lozano AN, Largent-Milnes TM, Chandramouli A, Umaretiya PJ, Nelson MA., Mantyh PW and Vanderah TW. *Cannabinoid receptor 2 compounds in the attenuation of breast cancer proliferation: mechanisms of action*. (Breast Cancer Res, manuscript in review)


8) Bagrodia NQ, **Hanlon KE**, Largent-Milnes TM, Petrouv R, Hruby VJ, and Vanderah TW. *Novel dual acting opioids in the inhibition of chronic pain* (manuscript in preparation)

9) **Hanlon KE**, Ondoua-Lozano AN, Symons AM, Largent-Milnes TM, Umaretiya PU, Mantyh PW, Larmonier N, and Vanderah TW. *Cannabinoid receptor 2*
restores immune homeostasis via selective knockdown of regulatory T cells in metastatic breast cancer (manuscript in preparation)

ABSTRACT PRESENTATIONS:

1) **Hanlon KE**, Ondoua-Lozano AN, Largent-Milnes TM, Chandramouli A, Sukhtankar DD, Nelson MA., King T, Mantyh PW and Vanderah TW. Activation of the CB₂ receptor attenuates breast cancer induced bone pain and promotes bone health in a murine model of breast cancer metastases. *Society for Neuroscience*; Chicago, IL (October 2009)


APPENDIX B: PERMISSIONS

NOT APPLICABLE
APPENDIX C: HUMAN/ANIMAL SUBJECTS APPROVAL
Memorandum

Date: May 14, 2012

To: Kathleen Hanlon

From: Andi Mitchell
IACUC Assurance Coordinator

Re: IACUC Certification Confirmation

This memo is to confirm that you have completed the basic certification required by the University of Arizona IACUC for inclusion on approved protocols.

Your UA IACUC certification number is #8157, and you have completed the following training modules:

- Guinea Pig module
- Hamster module
- Introduction to Animal Hazards
- Laws and Regulations
- Micro-isolator training (hands-on)
- Rodents
- Surgery and Anesthesia
- Zoonotic Diseases

Please feel free to contact the IACUC Office if you have any questions.

Thank you,

Andi
REFERENCES


