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A cannabinoid 2 receptor agonist attenuates bone cancer-induced pain and bone loss

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Abstract

Aims—Cannabinoid CB_2 agonists have been shown to alleviate behavioral signs of inflammatory and neuropathic pain in animal models. AM1241, a CB_2 agonist, does not demonstrate central nervous system side-effects seen with CB_1 agonists such as hypothermia and catalepsy. Metastatic bone cancer causes severe pain in patients and is treated with analgesics such as opiates. Recent reports suggest that sustained opiates can produce paradoxical hyperalgesic actions and enhance bone destruction in a murine model of bone cancer. In contrast, CB_2 selective agonists have been shown to reduce bone loss associated with a model of osteoporosis. Here we tested whether a CB_2 agonist administered over a 7 day period inhibits bone cancer-induced pain as well as attenuates cancer-induced bone degradation.

Main Methods—A murine bone cancer model was used in which osteolytic sarcoma cells were injected into the intramedullary space of the distal end of the femur. Behavioral and radiographic image analysis was performed at days 7, 10 and 14 after injection of tumor cells into the femur.

Key Findings—Osteolytic sarcoma within the femur produced spontaneous and touch evoked behavioral signs of pain within the tumor-bearing limb. The systemic administration of AM1241 acutely or for 7 days significantly attenuated spontaneous and evoked pain in the inoculated limb. Sustained AM1241 significantly reduced bone loss and decreased the incidence of cancer-induced bone fractures.

Significance—These findings suggest a novel therapy for cancer-induced bone pain, bone loss and bone fracture while lacking many unwanted side effects seen with current treatments for bone cancer pain.

Keywords

CB₂ agonists; AM1241; osteolytic sarcoma; bone cancer pain

Introduction

Many prevalent forms of cancer including lung, breast, prostate and sarcoma metastasize to bone (Coleman 2006; Luger et al. 2001). Bone metastasis is commonly characterized in cancer

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patients by bone pain (Luger et al. 2005; Mercadante 1997). Destruction of the bone causes chronic pain, which often leads to pathological fractures and/or hypercalcemia. The bone destruction induces an "ongoing" pain arising from the tumor bearing bone that significantly compromises the quality of life and functional status of the patient (Jimenez-Andrade and Mantyh 2009). With the progression of tumor-induced bone destruction, breakthrough pain which is an intermittent occurrence of severe pain, manifests itself either spontaneously or following weight bearing or strenuous movement of the affected bone (Luger et al. 2005).

Treatment for bone cancer involves multidisciplinary therapies that include a combination of radiotherapy, hormone or chemotherapy, bisphosphonates (carbon-substituted analogs that inhibit osteoclast function), and analgesic therapy (Mercadante and Falfaro 2007). Analgesic therapy can include treatment with opiates and non-steroidal anti-inflammatory drugs. The use of NSAIDS is limited to the alleviation of mild to moderate pain and have been recently reported to delay bone healing following fracture (for review see O'Connor and Lysz 2008). Chronic use of opiates results in several unwanted side effects including analgesic tolerance, somnolence, constipation, respiratory depression and paradoxical states of hyperalgesia (Vanderah et al. 2000). Recently, we demonstrated that murine bone cancer models treated with sustained morphine not only intensifies pain after a week of treatment but also accelerates bone destruction when compared to vehicle treated animals (King et al. 2007).

Cannabinoid Receptor-2 (CB₂) agonists have been shown to act as an analgesic in acute, chronic, and neuropathic pain (Malan et al. 2003; Ibrahim et al. 2005; Ibrahim et al. 2006; Whiteside et al. 2007). CB₁ receptors are highly concentrated throughout the central nervous system (CNS) and can induce psychotropic side effects. In contrast, CB₂ receptors in the spleen, tonsils, monocytes, B-cells, and T-cells and therefore associated with the immune responses and the peripheral nervous system (Cravitt and Lichtman 2004; Klein et al. 2003; Romero-Sandovall et al. 2008). Although CB₂ receptors are considered peripheral receptors, they have been found in distinct areas of the CNS such as the spinal cord, dorsal root ganglia, and microglia (Pertwee 2001). The presence of CB₂ receptors on neuronal tissue has remained a controversy, most likely due to the lack of specific CB2 receptor antibodies. Previously, studies were unable to show the presence of CB₂ receptors in neuronal tissue (Carlisle et al. 2002; Derocq et al. 1995, Galiegue et al. 1995; Griffin et al. 1999; Sugiura amd Waku 2000). However, recently CB₂ receptors have been identified in areas of the brain such as the cerebellum, cerebral cortex and brainstem of mammalian species such as the rat and mouse (Ashton et al. 2006; Beltramo et al. 2006; Brusco et al. 2008; Gong et al. 2006; Onaivi et al. 2006; Van Sickle et al. 2005). Yet, the functional role of CB₂ receptors in the CNS requires further investigation. Since animal behavior studies have not reported effects on locomotor or psychotropic activity with CB₂ ligands that has been observed with CB₁ or nonselective CB ligands, suggests distinctive roles of these receptors in the CNS (reviewed in Whiteside et al. 2007; Guindon and Hohmann 2008).

 CB_2 agonists not only produce antinociceptive and anti-inflammatory effects, but also have been shown to increase bone density (Ofek et al. 2006; Karsak et al. 2005). CB_2 agonists increase the number of osteoblasts (bone forming cells) and inhibit the production of osteoclasts (bone destruction cells) resulting in an overall increase in bone integrity (Ofek et al. 2006). CB_2 knockout mice experience accelerated trabecular bone loss and cortical expansion further demonstrating the importance of the endogenous CB_2 system in the mediation of skeletal maintenance (Ofek et al. 2006). Mice that undergo an ovariectomy result in accelerated bone loss. These ovariectomized mice when treated with sustained CB_2 agonist result in the suppression of osteoclastogenesis and increased osteoblast activity with an overall increase in bone integrity (Ofek et al. 2006)

In this study we will investigate the CB_2 selective agonist AM1241. In animal pain models, AM1241 is consistently reported as a CB_2 agonist, as effects are blocked by CB_2 but not CB_1 selective antagonists and not seen in CB_2 —/— mice. (Malan et al. 2001; Ibrahim et al. 2003; 2005, LaBuda et al. 2005). In contrast to results seen in vivo studies, functional assays attempting to characterize the pharmacological properties of AM1241 have yielded inconsistent results, with activity ranging from agonist, antagonist, or inverse agonist depending on the assay and enantiomer utilized (Yao et al. 2006; Mancini et al. 2009; Bingham et al. 2007). Differences of pharmacological properties observed in vivo and in vitro could be the result of differences in native versus recombinant receptors. Thus, in vitro assays do not necessarily predict in vivo efficacies. Furthermore, AM1241 was used due to its consistency and effectiveness as a CB_2 selective agonist across multiple animal pain models published in the literature.

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

Materials and Methods

Cells

Murine CCL-11 (NCTC clone 2472) sarcoma cells were maintained in NCTC media containing 10% fetal bovine serum and 1% penicillin, passaged every 4 days, and harvested between 2 and 12 passages.

Animals

All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication Number 80-23, 1966) and to the guidelines of the International Association for the Study of Pain (Zimmerman 1983). Male C3H/HeJ mice were 20–25 grams at the time of testing. Mice were purchased from Jackson Laboratories, Bar Harbor, ME. This mouse stain was chosen for histocompatability with the NCTC 2472 tumor line [American Type Culture Collection (ATCC), Rockville, MD], that has been shown to form lytic lesions in bone after intramedullary injection (Clohisy et al. 1995; Clohisy 1996; Schwei et al. 1999; Luger et al. 2001). Mice were maintained in a climate-controlled room on a 12-hour light/dark cycle. Animals were allowed food and water ad libitum.

Surgery

Mice were anesthetized with ketamine (80mg/kg)/xylazine (12mg/kg) i.p. An arthrotomy was performed as previous described (Luger et al. 2005; King et al. 2007). The condyles of the right distal femur were exposed and a hole was drilled to create a space for a needle injection of 25,000 CCL-11 murine sarcoma cells in 5 uL of alpha minimal essential medium containing 1% bovine serum albumin or 5 uL of alpha minimal essential medium alone (control) within the intramedullary space of the mouse femur. Proper placement of needle was confirmed through use of Faxitron x-ray images. The drilled hole was sealed with bone cement. (Schwei et al. 1999)

Drug Treatment

Starting on day 7 after inoculation of the femur with sarcoma cells, mice were injected intraperitoneally with the CB₂ receptor agonist, AM1241 (CB₂ K_i = 3.4 nM, 82 fold selectivity versus CB₁)(Sigma cat. #A6478), (3 mg/kg, dose base on Malan et al., 2003 and preliminary

studies) twice daily (7:00am and 17:00pm) dissolved in a vehicle solution of 10% dimethyl sulphoxide, 10% Tween 80, and 80% saline. Control groups were administered vehicle solution alone.

Analysis of Pain

Animals were tested for movement-evoked pain, spontaneous pain, and tactile allodynia before surgery (baseline) and at days 7, 10, and 14 following surgery in a blinded fashion (tester was blinded to treatment groups). All testing was performed one hour after the first daily treatment.

Movement Evoked Pain—This test evaluated the severity of pain the mouse experienced during normal ambulation. The mouse was placed in an empty mouse pan and limping and guarding behavior of the right leg was observed for two minutes. As the mouse walked across the empty pan, the use of the afflicted hind limb was rated with the following scale: 0 = no use of hind limb at all, 1 = partial non-use, 2 = limp and guard, 3 = limp, and 4 = normal use (Luger et al., 2001). Observer of movement evoked pain was blinded to the treatment conditions.

Spontaneous Pain—These tests were intended to quantify the painful behaviors during a resting state. Flinching and guarding were observed for 2 minute durations. Flinching was characterized by the mouse's lifting of his right foot (ipsilateral to the inoculation of sarcoma cells) off the floor when not associated with walking or movement. However, if a mouse shook his foot while walking, the behavior was counted as a flinch. The number of flinches was recorded on a five-channel counter. Guarding was characterized by holding of the mouse's right hind limb (ipsilateral to inoculation with sarcoma) up off the floor. Guarding behavior was recorded over a 2 minute period (King et al. 2007). Observer of spontaneous pain was blinded to the treatment conditions.

Tactile Allodynia—The von Frey test assessed tactile hypersensitivity of the right hind limb in which cancer had been induced with the slight touch of calibrated filaments that would not evoke pain in healthy, uninjured animals. Animals were placed in raised plexiglass chambers with wire grid floors. Animals acclimated to the environment for 30 minutes before testing was implemented. The targeted hind limb was probed with von Frey filaments with logarithmically incremental stiffness that were equivalent to weights ranging from 0.03 to 2.34 grams. Starting with the 3.61, the filament was applied perpendicularly to plantar surface of the targeted hind limb while the animal was sitting still with his foot on the floor for duration of 3 seconds. If the mouse began to walk around while being probed, the same filament was reapplied for another 3 seconds. If the mouse did not respond to the touch of the filament, testing proceeded with the next higher filament until the movement of hind limb occurred or cutoff (highest filament used was 4.56) was reached. The 4.56 filament is chosen as the cutoff due to larger filaments ability to push the paw up with out a response. Upon paw withdrawal, the next lighter filament was applied until the mouse did not withdraw his paw or cutoff was reached (lowest filament used was 2.44). Once the mouse responded, he was tested with four more filaments (Chaplan et al. 1994). The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon (1980). Tester of von Frey evoked pain was blinded to the treatment conditions.

Acute Testing

Flinching, guarding and tactile allodynia were performed as described above. Animals baseline behavioral response were tested on Day 10 after sarcoma innoculation. Animals then received either a single injection of CB₂ agonist AM1241 (6 mg/kg, i.p., equivalent to single days dose in the chronic studies) in the presence or absence of the CB₂ antagonist, SR144528 (1 mg/kg, i.p.)(a kind gift from National Institute on Drug Abuse). Either antagonist or vehicle (control) were administered 8–10 minutes prior to agonist or vehicle (control). Flinching, guarding and

tactile allodynia were performed 30 and 60 mins after AM1241 administration in a blinded fashion. Sarcoma-induced hypersensitivity was tested 2 hours after drug administration for return to baseline levels.

Determination of Bone Destruction

Faxitron Specimen Radiography System MX-20 (Faxitron X-ray Corporation, Wheeling, Illinois) was used to acquire radiographic images. Before capturing images, mice were anesthetized with ketamine/xylazine (Luger et al. 2001) and the individual that rated the bones was blinded to the treatment groups. Bone loss was rated by 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 (King et al. 2007).

Statistical Analysis

Statistical comparisons between treatment groups were done using ANOVA. Pairwise comparisons were made with Student's t-test, multiple comparisons between groups were done using Newman–Keuls Multiple Comparison Test. For the rating assays, limb use and bone loss, statistical comparisons were made with the Kruskal-Wallis. For all analysis, significance was set at p < 0.05.

Results

The CB₂ agonist, AM1241, attenuated bone cancer-induced spontaneous pain

In animals injected with media, flinching and guarding behaviors were not observed. By days seven and ten following arthrotomy surgery and femur inoculation with sarcoma, spontaneous pain was elicited. Mice that received sarcoma cells displayed spontaneous flinching and guarding starting at day 7 with continued behavior until day fourteen as compared to control, media only animals (Figure 1A, B). The sustained systemic (i.p.) treatment of AM1241 began on day 7 post surgery, and flinching and guarding behaviors observed on days 10 and 14. At day 10, tumor bearing mice with AM1241 showed a reduction in flinching when compared to tumor bearing treated mice with vehicle (i.p.), however the effect was not significant until day 14 (p<0.001) (Figure 1A). The sustained systemic (i.p.) treatment of AM1241 resulted in a decrease in guarding by day 14 in sarcoma treated mice when compared to vehicle treated animals (p<0.05) (Figure 1B).

Treatment with AM1241 reduces sarcoma-induced evoked pain

Von Frey filaments were used to measure the hindpaw response thresholds of mice to determine the effect of AM1241 treatment on sarcoma-induced tactile hypersensitivity. On day 7 after sarcoma inoculation and prior to either AM1241 or vehicle, animals' mechanical thresholds were not different from baseline values on day 0. However at days 10 and 14 post surgery, animals began to display behavioral signs of tactile sensitivity as compared to animals injected with media (Figure 2A). Beginning on day 10, tumor bearing mice treated with vehicle displayed significantly lower paw withdrawal thresholds compared to sarcoma-induced, AM1241 treated animals (p<0.05) (Figure 2A). On day 14 after surgery animals treated chronically with vehicle demonstrated significant sarcoma-induced mechanical hypersensitivity as compared to the contralateral leg (data not shown). More importantly is that the animals treated with sustained AM1241 demonstrated a significant block of sarcoma-induced mechanical hypersensitivity (p<0.001) (Figure 2A). In addition to mechanical testing using von Frey filaments, limb use was rated in mice (Honore et al. 2000) to evaluate the effect of AM1241 on movement-evoked pain. Sarcoma-induced animals treated with both vehicle and AM1241 displayed limping by day 10, yet by day 14, there was a significant difference in

movement-evoked pain between AM1241 and vehicle treated groups. Sarcoma-induced mice treated with vehicle alone displayed partial non-use or limping and guarding compared to control (media) treated animals. Sustained administration of AM1241 from day 7 until day 14, significantly reversed the sarcoma-induced loss of limb use by day 14 (P<0.001) (Figure 2B). These data suggest that sustained AM1241 significantly reduces sarcoma-induced evoked pain.

AM1241 treatment reduces sarcoma-induced bone loss and fracture

Radiographic images were taken following behavioral testing to determine the effect of AM1241 treatment on sarcoma-induced bone loss. Bones were rated with the following scale: 0 = normal, 1 = bone loss observed with no fracture, 2 = unicortical bone loss indicating unicortical bone fracture, 3 = bicortical bone loss indicating bicortical bone fracture (Luger et al., 2001). Radiographs were taken prior to surgery eliminating the possibility of baseline group differences. Throughout the time course of the experiment, bone loss was not observed in animals injected with media and treated with vehicle or AM1241. Sarcoma-induced bone loss increased in tumor bearing mice as compared to sham mice. Sarcoma treated animals with vehicle from day 7 to day 14 resulted in a significant amount of bone loss (Figure 3C). Sustained AM1241 from days 7 until day 14 significantly reduced the amount of sarcoma-induced bone loss when compared to the vehicle treated animals (P<0.001) (Figure 3D). Bones were scored by a blind observe with expertise in bone radiology. Animals with sarcoma and vehicle had severe bone loss with all animals having unicortical fracture (Figure 3E). Sustained AM1241 from day 7 until day 14 significantly reduced bone loss by blind scoring with only 2 out of 10 animals demonstrating unicortical bone loss (Figure 3E).

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

Flinching and guarding behaviors were observed in order to determine the acute effects of AM1241 on sarcoma-induced spontaneous pain. Animals were observed for behavioral baselines 10 days following surgeries and given a single injection of AM1241 (6mg/kg, i.p.) or vehicle. Behavioral measurements of sarcoma-induced flinching and guarding were taken 30 and 60 minutes after injection in a blinded fashion (observer blinded to treatment groups AM1241 or vehicle). Baselines resulted in significant sarcoma-induced flinching and guarding (Figure 4A and 4B). However, 30 minutes and 60 minutes following injection with AM1241 animals showed a significant reduction in flinching (p<0.001) and guarding (30 min, p<0.05) (60 min, p<0.001) when compared to vehicle treated mice (Figure 4A and 4B). The preadministration (8–10 min prior to AM1241) of the CB2 antagonist, SR144528 (1 mg/kg, i.p.) resulted in a significant attenuation of the AM1241 effects (p<0.001) in both flinching and guarding (Figure 4A and 4B) demonstrating that the reduction of sarcoma-induced spontaneous pain by AM1241 is CB2 receptor mediated. The antagonist alone had no significant effect on sarcoma-induced flinching and guarding. (Figure 4A and 4B). All behavioral studies were carried out in a blinded fashion.

Acute treatment with AM1241 reduces sarcoma-induced evoked pain; blocked by the CB₂ antagonist SR144528

VonFrey filaments were used to measure the hindpaw response thresholds of mice to determine the acute effect of AM1241 treatment on sarcoma-induced touch evoked hypersensitivity. Animals were tested 10 days following sarcoma innoculation and given a single injection of AM1241 (6mg/kg, i.p.) or vehicle (control). Behavioral measurements were taken before injection, 30 and 60 minutes after injection. Animals treated with acute AM1241 demonstrated a significant attenuation of sarcoma-induced touch evoked hypersensitivity compared to control (vehicle) (Figure 4C). Although 30 minutes following AM1241 injection did not result in a significant attenuation of evoked responses the 60 minute time point resulted in a significant

attenuation of evoked responses (p<0.05) when compared to vehicle treated animals and/or baseline thresholds (Figure 4C). The pre-administration (8–10 min prior to AM1241) of the CB₂ antagonist, SR144528 (1 mg/kg, i.p.) resulted in a significant attenuation of the AM1241 effects (p<0.001) in evoked responses (Figure 4C) demonstrating that the reduction of sarcomainduced evoked pain by AM1241 is CB₂ receptor mediated. The antagonist alone had no significant effect on sarcoma-induced touch evoked hypersensitivity. (Figure 4C). All behavioral studies were carried out in a blinded fashion.

Discussion

Many epithelial-derived cancers including sarcoma, breast, prostate and lung commonly metastasize to bone (Coleman 2006). Once cancer metastasis occurs, bone pain can significantly impact the quality of life and functional status of the patient (Rubens et al. 1998; Solomayer et al. 2000). In advanced stages, skeletal metastasis is associated with bone remodeling and eventual bone fracture that contributes to severe and difficult to control pain with limited or total loss of mobility. Here we utilized an animal model of bone cancer metastases using sarcoma cells that results in behavioral signs of spontaneous and evoked pain. Similar to what was reported by Schwei et al. (in 1999), we found that the animals developed severe bone loss by day 14 after inoculation with the sarcoma cells.

Here we demonstrated the acute effects of a CB₂ agonist as well as how sustained administration of a CB₂ agonist for seven days attenuates both spontaneous and evoked pain behaviors. Sustained administration was slightly decreased when compared to the acute antinociceptive effect suggesting tolerance. However, in the CB₂ sustained studies the CB₂ agonist was tested after 14 days as compared to after 10 days in the acute study suggesting an escalation in pain behavior from day 10 to day 14 and therefore more likely a decrease in the potency of CB₂ antinociception versus tolerance. Compound administration was by the systemic route suggesting that the effects may have been both locally as well as in the central nervous system. CB₂ receptors are found in the spleen, tonsils, monocytes, osteoclasts, macrophages, B-cells, and T-cells and are therefore associated with the immune responses, as well as the peripheral nervous system but not directly with the central nervous system (Cravatt and Lichtman 2004). Recent studies have identified an increase in mRNA for CB2 receptors in the CNS after nerve injury (Zhang et al. 2003; Wotherspoon et al. 2005) with upregulation in the CNS associated with microglia after inflammation (Romero-Sandoval et al. 2008; Van Sickle et al. 2005), yet their receptor activation in the CNS lack unwanted psychoactive effects (Romero-Sandoval et al. 2008). Cancer metastases to bone results in the activation of the immune response within the bone and within the central nervous system. The activation of CB₂ receptors on immune cells results in the attenuation of inflammatory factors including cytokines (Rajesh et al. 2007; Klein et al. 2003; Massi et al. 2006). Studies from our group along with others have demonstrated that the activation of CB₂ receptors by specific agonists will inhibit inflammatory, acute and chronic pain without the psychoactive effects demonstrated by activation of CB₁ receptors or opiates (Ibrahim et al. 2005; Malan et al. 2003; LaBuda et al. 2005; Sagar et al. 2005; Whiteside et al. 2007; Yamamoto et al. 2008). A recent study by DeLeo and Colleagues have shown that CB2 receptor activation within the spinal cord after L5 nerve injury resulted in an increase in CB₂ receptor expression on microglia and perivascular cells with a reduction in hypersensitivity using the CB₂ selective agonists JWH015; a compound lacking CNS unwanted side effects (Romero-Sandoval et al. 2008). They concluded that CB2 agonists may offer pain relief by modulating the immune response and microglia function under chronic pain conditions without inducing tolerance or CNS side effects.

Due to the fact that the CB_2 receptors are located on immune cells including macrophages (Klein et al. 2003), we believe that the significant reduction in pain behaviors is due to a

reduction in the many inflammatory mediators that are released when cancer invades the bone. Metastases to the bone results in the accumulation of macrophages termed tumor-associated macrophages (TAMs) which have been found to enhance angiogenic programming by producing pro-angiogenic factors such as cytokines, chemokines, VEGF and proteases (Condeelis and Pollard 2006; Theoharides and Conti 2004; Coussens et al. 1999; Lin and Pollard 2007; Ribatti et al. 2001; Ribatti et al. 2004; Benelli et al. 2003; Sinha et al. 2007; DePalma et al. 2005; Bunt et al. 2006; and Lin and Pollard 2004). Cancer metastases to bone results in a significant inflammatory/immune response including a significant increase in macrophages, monocytes, dendritic cells, leukocytes and neutrophils (DeNardo and Coussens 2007; DeNardo et al. 2008; Bertolini et al. 1986; Coussens and Werb 2002; Kim et al. 2006). The number of macrophages present in tumor stroma correlates with increased microvessel density, tumor size, tumor proliferation and decreased survival in cancer patients (O'Sullivan and Lewis 1994; Leek and Harris 2002; Bolat et al. 2006; Tsutsui et al. 2005; and Ohno et al. 2004). It is well known that certain cytokines can enhance and even cause nociception (Verri et al. 2006). Recent studies have demonstrated that the cytokines IL-1β, TNFα and IL-6 are released from macrophages, monocytes and glial cells to promote nociception indirectly via increasing prostanoids and sympathetic amines, as well as by direct activation of receptors on nociceptive fibers (Verri et al. 2006). Recent studies by Li and colleagues have shown that peripheral nerve stimulation, as what would be seen in bone cancer, results in the increase expression of IL-6, TNFα and IL-1β in the dorsal horn of the spinal cord leading to intracellular changes on secondary neurons that may lead to central sensitization (Kawasaki et al. 2008). In the end, these pronociceptive cytokines are released from cancer-induced infiltrating immune cells as well as from the tumor cells promoting pain and continual tumor proliferation, creating a "feed-forward" painful and destructive process that may be inhibited by CB2 receptor activation.

Studies here demonstrate that sustained CB₂ agonist maintain bone integrity when compared to vehicle treated animals. There was a significant reduction in sarcoma-induced bone loss and a reduction in the number of unicortical fractures due to the administration of the AM1241. Bone integrity is maintained by osteogenic cells found on the surface of the bone and in the lacunae of the bone matrix including osteoblasts and osteoclasts (Putnam et al. 2007). Osteoblasts are found along the bone surface where they synthesize the organic matrix and regulate mineralization of bone resulting in bone-building (Putnam et al. 2007). Osteoblast activity is regulated by CB2 agonists. The selective CB2 agonist HU-308 enhanced osteoblast number and bone building activity (Ofek et al. 2006). Bone marrow-derived primary monocytic cultures showed a dramatic (205%) increase in the expression of osteoblast-like cells following application of a selective CB₂ agonist (Scutt and Williamson 2007). Osteoblasts in part, control the cells that breakdown bone called osteoclasts by releasing RANKL, a member of the TNF cytokine superfamily, osteoptegrin and IL-6. Osteoblasts themselves can be suppressed either directly or indirectly by cytokines including IL-1β and TNFα (Li et al. 1992, Bertolini et al. 1986, Stashenko et al. 1987, and Canalis 1987). Osteoblasts are influenced by cancer cells to release cytokines that enhance osteoclast activity (Kinder et al. 2007). Osteoclasts are cells that are derived from the monocyte-macrophage lineage (Boyle et al. 2003) and have high levels of CB₂ receptors (Ofek et al. 2006, Scutt and Williamson 2007, Idris et al. 2005, and Bab and Zimmer 2008). Osteoclasts resorb bone by creating a local acidic microenvironment to dissolve bone and activate proteases to break down bone (Putnam et al. 2007). Osteoclast function is regulated by a number of mediators including endogenous cannabinoids and cytokines (TNFα, IL-6) (Zoux et al. 2008). For example, CB₂ receptor activation on osteoclasts and osteocytes by the selective CB2 agonist HU-308 significantly suppressed osteoclast activity (Ofek et al. 2006) and osteoclastogenesis (production of osteoclasts) (Ofek et al. 2006; Scutt and Williamson 2007; George et al. 2008) considerably reducing the activity of osteoclasts in trabecular and cortical bone (Ofek et al. 2006). Bone density in CB2 knockout mice was significantly lower when compared to wild type littermates (Karsak et al. 2005). In addition,

CB₂ knockout mice displayed a markedly accelerated age-related trabecular and cortical bone remodeling (Ofek et al. 2006).

The CB_2 agonists may also act by decreasing the activation of microglia in the central nervous system (Romero-Sandoval et al. 2008). Sustained administration of CB_2 agonists may result in changes in receptor number or intracellular regulation. Future studies will investigate endogenous cytokine levels, immunohistochemistry for activated microglia, and changes in receptor number. Additional reasons for the CB_2 receptor agonists in inhibiting pain include their ability to inhibit bone degradation, a process that entails an acidic environment that activates nociceptive fibers (Mantyh et al. 2002).

Conclusion

Cancer metastasis to bone results in excruciating pain that often reduces the quality of life and results in the prescription of compounds such as NSAIDs and opiates that have been shown to either attenuate bone healing or even enhance bone degradation (King et al. 2007). There is a great need for better analgesics in bone cancer pain that will help maintain the bone structure while reducing pain. Here we have demonstrated that a CB₂ agonist administered acutely or chronically for 7 days significantly attenuates both spontaneous and evoked pain behaviors. Unlike what we have shown with sustained morphine in the sarcoma cancer model (King et al. 2007), the sustained administration of the CB₂ agonist resulted in the inhibition of bone loss. In addition, CB₂ agonist do not result in the many unwanted side effects of current analgesic therapies due to its lack of direct activity on neuronal pathways within the rewarding and respiratory pathways of the CNS suggesting that CB₂ agonists may be an ideal treatment for bone cancer pain.

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Figure 1A

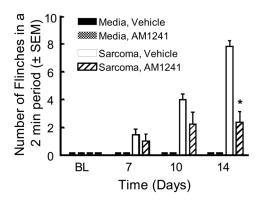


Figure 1B

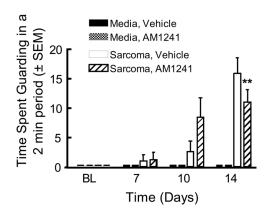


Fig. 1. The CB₂ agonist, AM1241, attenuates spontaneous pain behaviors in a murine bone cancer model. Sarcoma cells or cell medium were injected into the intramedullary space of the femur. Beginning on day 7, vehicle or AM1241 (i.p, 3 mg/kg twice daily) was administered to animals. Flinching and guarding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

Figure 2A

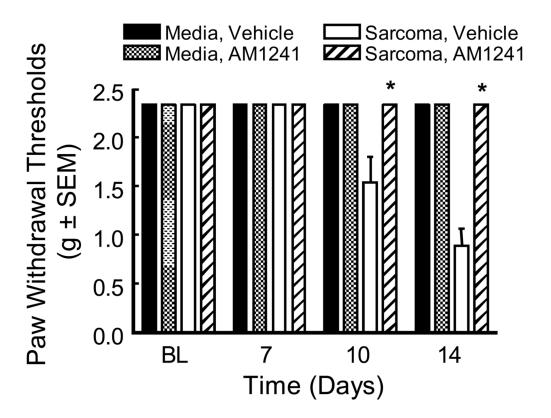


Figure 2B

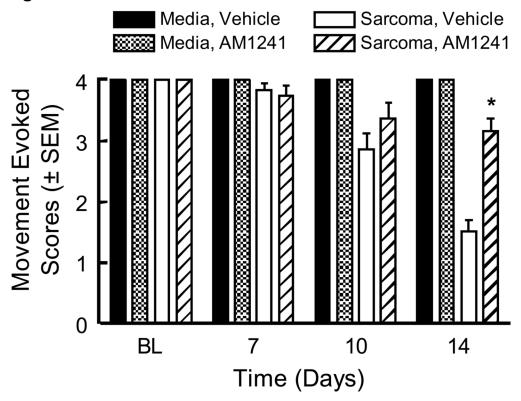
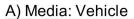


Fig 2. The CB_2 agonist, AM1241, attenuates evoked pain behaviors in a murine bone cancer model. Tactile allodynia and movement evoked pain were tested. A) AM1241 (i.p.) treatment blocked tactile allodynia in cancer-induced mice compared to cancer-induced mice treated with vehicle on days 10 and 14 (*p<0.001). Tactile allodynia was not observed in animals injected with media and treated with AM1241 or vehicle. B) AM1241 (i.p) treatment significantly alleviate movement evoked pain on day 14 (*p<0.001) but not on day 10 in tumor bearing mice treated with AM1241 when compared to cancer-induced mice treated with vehicle. Movement evoked pain was not observed in mice injected with cell medium, treated with AM1241 or vehicle





B) Media: AM1241



C) Sarcoma: Vehicle
Fracture

Bone Loss



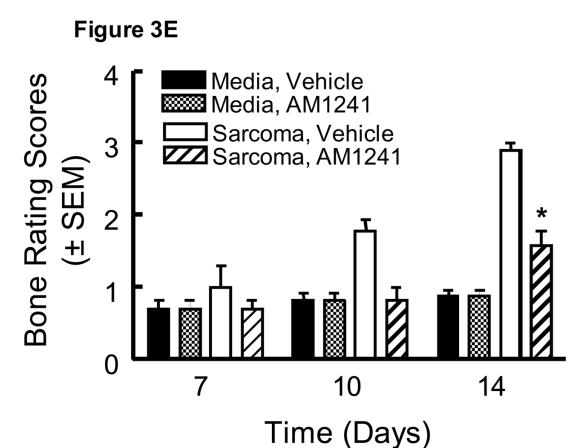


Fig 3.

AM1241 reduces sarcoma-induced bone loss. Representative radiographic images of femur injected with medium of sarcoma cells. The femurs were treated with vehicle or AM1241 Bone is reduced in tumor bearing animals treated with vehicle as compared to animals treated with AM1241. A) Bone injected with media and treated with vehicle. B) Bone injected with media and treated with AM1241. C) Bone injected with sarcoma cells and treated with vehicle. D) Bone injected with sarcoma cells and treated with AM1241. E) Bone rating scores demonstrating AM1241 treatment reduced the occurrence of unicortical bone fractures in sarcoma-induced mice compared to sarcoma-induced mice treated with vehicle. Set to scale 1.5 mm.



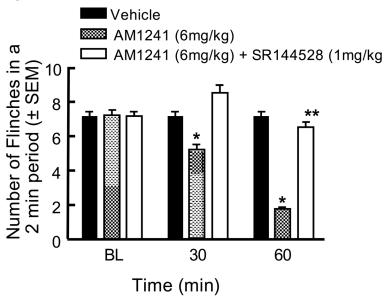


Figure 4B

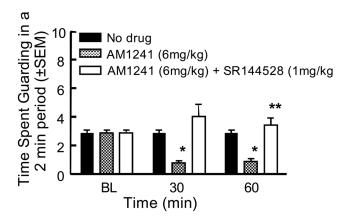


Figure 4C

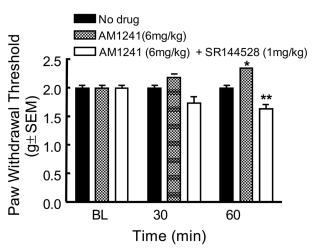


Fig 4. Acute treatment of AM1241 reduced sarcoma-induced spontaneous and evoked pain. These effects were blocked by a single pretreatment of the CB_2 antagonist SR144528. The number of sarcoma-induced flinches (A) and guarding (B) on day 10 was significantly reduced by AM1241 (6 mg/kg, i.p) treatment compared to vehicle treated mice with a significant difference observed at 30 and 60 minutes (*p<0.001). This effect was blocked in animals pretreated (8–10 min) with SR144528 (1 mg/kg, i.p.), at the 60 minute time point (**p<0.001). C) AM1241 (6mg/kg, i.p) treatment attenuated sarcoma-induced evoked responses compared to vehicle treated mice at 60 minutes (*p<0.05). Treatment of SR144528 (i.p.) prior to AM1241 blocked the antiallodynic effects of AM1241 (**p<0.001). Vehicle or antagonist alone had no significant effects.