## Title

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# A peripherally restricted cannabinoid 1 receptor agonist as a novel analgesic in cancer-induced bone pain

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## Abstract

Many malignant cancers, including breast cancer, have a propensity to invade bones, leading to excruciating bone pain. Opioids are the primary analgesics used to alleviate this cancer-induced bone pain (CIBP) but are associated with numerous severe side effects, including enhanced bone degradation, which significantly impairs patients' quality of life. In contrast, agonists activating only peripheral CB1 receptors (CB1Rs) have been shown to effectively alleviate multiple chronic pain conditions with limited side effects, yet no studies have evaluated their role(s) in CIBP. Here, we demonstrate for the first time that a peripherally selective CB1R agonist can effectively suppress CIBP. Our studies using a syngeneic murine model of CIBP show that both acute and sustained administration of a peripherally restricted CB1R agonist, 4-{2-[−(1E)-1[(4-propylnaphthalen-1-yl)methylidene]-1H-inden-3-yl]ethyl} morpholine (PrNMI), significantly alleviated spontaneous pain behaviors in the animals. This analgesic effect by PrNMI can be reversed by a systemic administration but not spinal injection of SR141716, a selective CB1R antagonist. Additionally, the cancer-induced bone loss in the animals was not exacerbated by a repeated administration of PrNMI. Furthermore, catalepsy and hypothermia, the common side effects induced by cannabinoids, were measured at the supra-therapeutic doses of PrNMI tested. PrNMI induced mild sedation, yet no anxiety nor a decrease in limb movements were detected. Overall, our studies demonstrate that CIBP can be effectively managed by using a peripherally restricted CB1R agonist, PrNMI, without inducing dose-limiting central side effects. Thus, targeting peripheral CB1Rs could be an alternative therapeutic strategy for the treatment of CIBP.

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## Introduction

Cancer-induced bone pain (CIBP) is experienced by over 30% of cancer patients with bone metastases [5,20] and is considered one of the most difficult pain conditions to treat [27]. Current management of CIBP follows the World Health Organization analgesic ladder recommending opioids for moderate to severe pain [66]. However, opioid therapy is not always sufficient in controlling pain [8], and is associated with several dose-limiting side effects contributing to their inadequate pain relief [46]. Additionally, opioids for cancer pain are related to a substance diversion which contributes to the growing opioid epidemic and drug-related deaths [7,33]. Recent studies in both humans [12,19,53] and animals [32] indicate that opioids may exacerbate cancer-induced bone degradation, which counteracts the effects of antiosteolytic therapies and CIBP management [24,43,57,65]. There are also reports showing that sustained opioids promote cancer proliferation and migration [21,34,40,68], which impedes antitumor therapy and largely impair patients' quality of life. Therefore, novel analgesics are urgently needed for patients with CIBP.

Recently, cannabinoids have emerged as attractive alternatives for the treatment of both chronic cancer and non-cancer pain [29,31,42,61,62]. Cannabinoid receptor agonists have also been shown to improve bone integrity by regulating the activities of osteoblasts and osteoclasts [41,60], which is beneficial for the treatment of CIBP. However, the effectiveness of cannabinoids is largely limited by their psychotropic effects via the activation of CB1 receptors (CB1Rs) in the central nervous system (CNS) [16,42]. In addition to their CNS expression, cannabinoid receptors have a wide distribution in peripheral tissues [3,58]. The activation of peripheral cannabinoid receptors exhibits a significant inhibition against different chronic pain conditions [4,22,28,38,48,49], including bone cancer pain [11,29,30]. Importantly, Agarwal and colleagues discovered that cannabinoid-induced analgesia is primarily mediated through the activation of peripheral CB1Rs [2]. Also, some studies identified an increased expression of CB1Rs in the peripheral tissues under pathological conditions [4,56], which results in enhanced potency and efficacy of exogenously applied cannabinoids in the treatment of pain [44]. Based on these studies, a potential strategy to dissociate cannabinoid-mediated analgesia from those psychotropic effects is to target peripheral CB1 receptors. Currently, several peripherally selective CB1 agonists have been developed and produced robust analgesic effects on chronic pain conditions with reduced CNS-mediated adverse effects [1,13,52,67]. Yet, no studies have investigated the efficacy of peripherally selective CB1R agonists in the treatment of established CIBP.

In the present study, we investigated the efficacy and mechanism of a peripherally restricted CB1R agonist, PrNMI [52], in attenuating spontaneous pain by using a preclinical CIBP model. Additionally, we also evaluated the potential CNS-mediated side effects induced by this peripherally restricted CB1R agonist. Our data suggest that PrNMI produces robust analgesic effects with a decreased CNS-mediated adverse effects profile, which may provide a valuable alternative for patients with CIBP.

## Materials and methods

### 1.1 In vitro

**1.1 Cell culture—**Murine mammary tumor line, 66.1, was a kind gift from Dr. Amy M. Fulton [63]. 66.1 cells were cultured in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum, 100 $IU^{-1}$ penicillin and 100 μg/mL streptomycin and then housed at 37°C and 5% $CO_2$. For all assays, cells were counted using a gridded hemocytometer (Hausser Scientific, Horsham, PA).

**1.2 XTT assay—**XTT cell viability assay (ATCC, Manassas, VA) was performed according to the manufacturer's instructions. Briefly, 66.1 breast cancer cells were plated into a 96-well plate at a density of $1\times10^4$ per well and allowed to grow for 24 hours. Cells were then treated with different concentrations of PrNMI (1 nM – 1 μM) or vehicle for another 24 hours. After treatment, cells were incubated with activated-XTT solution for 2 hours and read at 475 nm and 660 nm using a plate reader.

**1.3 Western blot analysis—**66.1 cells were lysed in the Pierce RIPA buffer (Thermo Scientific, Rockford, IL) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) via ultrasonication. Whole Cell lysates were resolved on 10% SDS-polyacrylamide gels (Criterion TGX; Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA). PVDF Membrane was blocked with 5% BSA in Tris-buffered saline containing 0.5% (v/v) Tween-20 (TBST) for 1 hour at room temperature, and then incubated with rabbit polyclonal anti-CB1R (ab23703, Abcam; 1:3,000 dilution) or mouse monoclonal anti-β-actin (ab8226, Abcam; 1:10,000) diluted in TBST containing 3% BSA overnight at 4°C. After washing with TBST, the membrane was incubated with appropriate secondary antibodies - HRP-linked anti-rabbit IgG (7074, Cell Signaling; 1:10,000 dilution) or HRP-linked anti-mouse IgG (7076, Cell Signaling; 1:30,000 dilution) for 1 hour at room temperature. Membrane was again washed and developed using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA). Bands were detected using GeneMate Blue Lite Autorad films (BioExpress, Kaysville, UT) and quantitated using ImageJ software (Wayne Rasband, Research Services Branch, NIMH, Bethesda, MD). All data were normalized to β-actin in each lane.

### 2. In vivo

**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. Adult female BALB/cAnNHsd mice (18–20 g; Harlan, Indianapolis, IN) were maintained in a climate-controlled room on a 12-hour light-dark cycle and allowed food and water ad libitum. Animals were monitored on days 0, 7, 10 and 14 of the study for clinical signs of rapid weight loss and signs of distress.

**2.2 Intramedullary implantation of 66.1 cells—**To establish CIBP in mice using a syngenic model [54], an arthrotomy was performed as previously described [15]. Briefly, mice were anesthetized with 80 mg/kg ketamine + 12 mg/kg xylazine (in 10 mL/kg, intraperitoneally (i.p.) injection volume) and the condyles of the right distal femur were

exposed. A hole was drilled at the intercondylar notch and into the medullary canal to create an initial core pathway. A placement needle was then inserted to make the final pathway into the bone. Proper placement of the needle was confirmed by radiograph (UltraFocus, Faxitron Bioptics, Tucson, AZ). Next, $8 \times 10^4$ 66.1 cells in 5 μL complete MEM or 5 μL complete MEM alone (as a control) was injected into the intramedullary space of the femur and the injection sites were sealed with bone cement. Muscle and skin were closed in separate layers with 5-0 Vicryl suture and wound autoclips, respectively. All mice were monitored for anesthesia during the surgery to ensure that no whisker movement or toe pinch response was presented. Gentamicin (8mg/kg, 10 mL/kg volume, subcutaneously (s.c.)) was given to all mice after surgery in order to prevent infection. Staples were removed 7 days after surgery.

**2.3 Drug treatment—**All drugs injected into the animals are dissolved in a vehicle solution of 10% dimethyl sulfoxide, 10% Tween-80 and 80% saline for injection (10 mL/kg, i.p.). Acute studies applied one injection of PrNMI (0.1, 0.3, 0.6 or 1 mg/kg, i.p.) or vehicle. Chronic studies consisted of once-daily injection of PrNMI (0.6 mg/kg, i.p., every day [q.d.], days 7–14) or vehicle after femoral inoculation. Separate sets of animals were treated with selective CB1R antagonist SR141716 (RTI International, Research Triangle Park, NC) or CB2R antagonist SR144528 (Tocris, Bristol, UK) (1 mg/kg, i.p., q.d., 10 minutes prior to PrNMI or 5 μg per 5 μl (lumbar puncture, l.p.), day 14, 30 minutes prior to the behavioral testing).

**2.4 Acute and chronic behavioral testing of spontaneous pain—**Spontaneous pain-related behaviors, flinching and guarding, were recorded as previously described [15]. Flinching was characterized by the lifting and rapid flexing of the ipsilateral hindpaw not associated with walking or other movement. Guarding was characterized by fully retracting the ipsilateral hindpaw under the torso. These two behaviors were observed for 2 minutes during a resting state after a 30-minute acclimation period. The number of flinches and the time the hindpaw was retracted during the 2-minute period were recorded. Both flinching and guarding of the cancer-bearing limb are best described as measurements of ongoing pain that is reflective in patients with bone cancer who protect affected limbs [37,51]. Guarding and spontaneous flinching are behaviors observed in which there is, no to very little, contact with a ground surface and becomes more progressive with time (days after femur inoculation). *Acute behavioral testing:* Baseline behaviors of spontaneous pain were recorded seven days after surgery. Mice were then separated into treatment groups and received a single dose of drug. After drug administration, mice were tested over a 24-hour time course until their pain behaviors returned to baseline. C*hronic behavioral testing:* Spontaneous pain behaviors were assessed before surgery (baseline). Mice then received treatment at the same time of each day during day 7–14. Spontaneous pain behaviors were measured 3 hours after treatment on days 7, 10 and 14, based on the time of peak effect determined by the acute studies.

**2.5 Radiography—**A digital Faxitron machine (UltraFocus, Faxitron Bioptics, Tucson, AZ) was used to acquire radiographs of mice anesthetized with ketamine/xylazine before surgery and day 14 after surgery. Bone loss was rated by 3 blinded observers trained in

scoring animal radiographs according to the following scale: 0 = normal bone, 1 = 1 to 3 radiographic lesions indicating bone loss, 2 = 4 to 6 radiographic lesions indicating bone loss, 3 = full-thickness unicortical bone loss indicating unicortical bone fracture and 4 = full-thickness bicortical bone loss indicating bicortical bone fracture. Observer scores for each bone on day 14 were averaged.

**2.6 Open field testing—**An open field test was used to investigate the potential sedative effect of PrNMI. The open-field arena (33 cm × 28 cm × 33 cm) is a white box with an open top and a black floor. A rectangle (16.5 cm ×14 cm) was marked in the center of the field. Sessions began by placing the mouse in the center rectangle and ended after 5 minutes. A consistent white noise (~55 dB) and a dim lighting (~24 lux) were applied during the test. The entire session was recorded by a video camera mounted 1.5 m above the floor. The tracking of mouse movement was realized by analyzing the testing videos with an open-source tracking software, EthoWatcher [10].

**2.7 Rotarod—**A rotarod test was used to determine the motor effect of PrNMI. Four days before testing, naïve mice were trained to acclimate to the rotating rod (LE8505 Rota-Rod, Panlab Harvard Apparatus, Spain) at a speed of 10 rpm. A maximal cut-off time of 180 seconds was used to prevent exhaustion. On the day of testing, mice were baselined and reevaluated 3 hours after treatment.

**2.8 Rectal temperature testing—**Animal rectal temperature was measured by using a thermistor probe (Thermoworks, American Fork, UT) to investigate the hypothermia induced by PrNMI. On the testing day, the rectal temperature was measured before treatment and 3 hours after treatment.

**2.9 Ring immobility testing—**As CB1R agonists cause catalepsy in animals [16], a ring immobility test was used to determine the cataleptic effect of PrNMI, as described previously [45]. Briefly, mice were placed on a horizontal metal ring (5.5 cm diameter) attached to a ring stand at a height of 16 cm. Each mouse was observed for 5 minutes and the sum of time it remained motionless was counted. The criterion for immobility was the absence of all voluntary movements including snout and whisker movements, but the movements associated with breathing were excluded. Immobility is described as the percentage of the 5-minute period in which the mouse was motionless.

## 3. Statistical analysis

Two-way ANOVA with the Tukey's multiple comparisons test was used to compare acute and chronic behavioral studies of spontaneous pain; Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare the radiograph results; One-way ANOVA with Dunn's multiple comparisons test was used to analyze XTT assay, rotarod, open field testing, rectal temperature testing and ring immobility testing. All data are presented as mean ± standard error of the mean (SEM) or median ± interquartile range and a value of $P < 0.05$ was accepted as statistically significant. Statistical analyses were run and plots were generated in GraphPad Prism 7.0 (Graph Pad Inc, San Diego, CA). Power analyses were performed on cumulated data by using G*Power 3.1 software to estimate the optimal

numbers required. The adequate statistical separation requires a group size of 8–12 per individual behavioral assay to detect differences (80%) between the drugs and control groups at $\alpha < 0.05$.

## Results

### 1. Acute or sustained administration of PrNMI attenuates cancer-induced bone pain

We assessed the acute antinociceptive effect of PrNMI on spontaneous pain in the syngeneic murine model of cancer-induced bone pain. In this model, 66.1 murine breast cancer cells were inoculated into the right femur of BALB/c mice. Before surgery, no mouse presented with any spontaneous pain behaviors (Fig. 1A, B); but seven days after femoral inoculation, mice injected with 66.1 cancer cells displayed a significant amount of flinching and guarding, which are behavioral signs of spontaneous pain (Fig. 1A, B). A single injection of PrNMI (0.1, 0.3, and 0.6 mg/kg, i.p.) resulted in a significant, time-related reduction of flinching but not guarding in a dose-dependent manner (Fig. 1A, B). This suppression of flinching started 1-hour post-injection and persisted for at least 5 hours (Fig. 1A).

Cancer-bearing mice treated with vehicle presented with spontaneous flinching and guarding starting at day 7 and increasing through day 14 (Fig. 2A, B). Repeated administration of PrNMI (0.6 mg/kg, i.p., q.d., from day 7 to day 14) significantly attenuated both flinching and guarding on day 10 and 14 post-surgery when compared to vehicle-treated cancer-bearing mice (Fig. 2A, B). Pre-treatment with selective CB1R antagonist, SR141716 (1 mg/kg, i.p., q.d., from day 7 to day 14), suppressed this antinociceptive effect produced by PrNMI, while the administration of a selective CB2R antagonist, SR144528 (1 mg/kg, i.p., q.d., from day 7 to day 14), did not (Fig. 2C, D). To confirm that the antinociceptive effect of PrNMI occurs by targeting peripheral cannabinoid receptors, we injected the antagonist (SR141716 or SR144528, 5 μg/5 μl) or the vehicle spinally into the animals receiving chronic PrNMI. The results indicate no significant difference in pain behaviors was displayed between the antagonist and vehicle groups (Fig. 2E, F). Together, these data indicate that the administration of PrNMI attenuates CIBP by targeting peripheral CB1Rs.

### 2. Sustained PrNMI does not alter bone integrity in mice with cancer-induced bone pain

To determine if the effect of sustained PrNMI on bone remodeling contributes to PrNMI-induced analgesia, radiographic images of all mice were taken on days 0 and 14 post-surgery (Fig. 3A). Bone loss was rated by three blinded observers according to an established scale (See **Materials and methods**). Mice injected with media only displayed a mild bone loss in the femurs (16 out of 20 mice have a score of 1 or less) (Fig. 3A, B). In contrast, almost all cancer-bearing mice experienced severe bone loss (21 out of 24 mice have a score of 2 or more) (Fig. 3A, B). Sustained treatment with PrNMI (0.6 mg/kg, i.p., q.d., from day 7 to day 14 after surgery) did not mitigate or worsen this cancer-induced bone degradation compared to vehicle group (Fig. 3A, B).

### 3. PrNMI does not alter cancer cell viability in vitro

The 66.1 murine breast cancer cells used in this study were identified to express CB1Rs (Fig. 4A). To investigate if PrNMI alters tumor viability that may indirectly influence

spontaneous pain, 66.1 breast cancer cells were treated in vitro with varying concentrations of PrNMI (1 nM – 1 μM) or vehicle for 24 hours, and an XTT assay was performed. As compared to vehicle-treated cells, none of the PrNMI treatments significantly changed cell viability (Fig. 4B). Overall, these data indicate that PrNMI at the concentrations tested does not promote nor reduce cancer cell viability in vitro.

### 4. Central side effects are induced by PrNMI in naïve mice

Previous studies suggested that brain-permeant CB1R agonist administration lead to central adverse consequences including sedation, motor incoordination, hypothermia and catalepsy [16,42], which largely limits their value as therapeutic reagents. Here we performed open field, rotarod, core temperature and ring immobility tests to determine whether PrNMI produces these adverse effects. In the open field test, a single administration of PrNMI (0.6 mg/kg, i.p.) significantly decreased the distance that mice traveled within a 5-minute period (Fig. 5A). However, the tracking patterns, center time and moving time performed by these naïve mice were not altered (Fig. 5B, C, D). In addition, PrNMI (0.6 mg/kg, i.p.) did not reduce the time that naïve mice spent on the rotating rod when compared to vehicle-treated mice (Fig. 6A). Rectal temperature in naïve mice was significantly decreased when treated with 1 mg/kg PrNMI compared to vehicle group, a dose 10-fold higher than the approximate $ED_{50}$ analgesic dose (0.1 mg/kg) (Fig. 6B), yet mice treated with 0.6 mg/kg or lower doses of PrNMI did not show a significant decrease in their body temperature (Fig. 6B). PrNMI also produced a significant increase in the time that mice spent motionless in the ring test when mice were treated with 0.6 mg/kg or a higher dose of PrNMI (Fig. 6C).

## Discussion

Cancer-induced bone pain is one of the most common types of chronic pain in cancer patients, which presents in more than 30% of the cancer patients who have bone metastasis [5,20]. Currently, opioids are the primary medications for CIBP [66]. However, opioids are not always sufficient in pain management and are associated with severe adverse effects and contribute to the growing opioid epidemic and drug-related deaths [7,8,12,19,32,33,46,53]. Cannabinoids are considered a promising alternative analgesic to opioids, having demonstrated potent anti-allodynic effects in multiple chronic pain conditions, including CIBP [17,23,29,31,42,61,62]. Nevertheless, cannabinoids have had limited success in the clinic due to their central side effects induced by the activation of CB1Rs in the CNS [16,42]. Recent studies demonstrate activation of only peripheral cannabinoid receptors can produce significant antinociceptive effects on different chronic pain models [2,11,22,28–30,38,48,49]. Consistent with these studies, the use of peripherally restricted cannabinoids also exerts profound anti-allodynic effects on several neuropathic and inflammatory pain states [1,13,52,67]. Furthermore, limited central side effects were induced by these peripherally restricted agonists compared to typical cannabinoids [1,13,52,67].

In the present study, we evaluated the analgesic effect of a restricted selective cannabinoid PrNMI in a murine model of CIBP. Our results show that both a single injection and repeated administration of PrNMI significantly alleviates CIBP-induced spontaneous pain behaviors, including flinching and guarding. Additionally, our antagonist studies suggest

that CB1Rs but not CB2Rs mediate the analgesic effect of PrNMI in mice. These results are consistent with our previous finding that PrNMI suppresses mechanical allodynia in a rat model of neuropathic pain by activating CB1Rs rather than CB2Rs [52]. Spinal application of SR141716A, a selective CB1R antagonist, failed to block PrNMI analgesia confirming peripheral selectivity of the compound. Thus, a peripherally-restricted Cannabinoid is sufficient to attenuate CIBP in mice.

The exact mechanisms by which peripherally restricted CB1R agonists produce their anti-allodynic effects on CIBP are poorly understood. A probable mechanism is the suppression of nociceptor activity within the tumor-bone microenvironment. Cancer metastasis to the bone results in the damage and sprouting of primary afferent fibers, as well as inflammatory responses in the tumor-bone microenvironment, which activates nociceptive neurons and subsequently produces pain [14,36]. CB1Rs are known to be expressed on the peripheral terminals of primary afferents [3], and are upregulated under multiple pathological conditions [4,56]. Previous studies showed that peripheral application of CB1R agonists or increase in endocannabinoids greatly attenuated both chronic cancer and non-cancer pain locally, as well as decreased spontaneous activity and sensitization of nociceptors [22,28–31,48,49], indicating the peripheral terminals of nociceptors are a critical site of cannabinoid-induced analgesia. By using a conditional peripheral CB1R knockout mouse strain, Agarwal, et al. nearly completely blocked the antinociceptive effect of systemically administered cannabinoids, thus demonstrating that cannabinoid-induced analgesia primarily occurs through the CB1R receptors distributed on peripheral terminals of nociceptors [2]. Our present study demonstrates that the activation of peripheral CB1Rs can effectively suppress CIBP in a mouse model of CIBP since the selective CB1R antagonist given spinally did not block the PrNMI analgesic effect while systemic administration significantly attenuated the effects. Based on these studies, it is likely that peripherally selective agonists activate CB1Rs on peripheral nociceptors, thus reducing CIBP. Further study would investigate if the knockout of CB1Rs on the nociceptors within the tumor-bone microenvironment may prevent the antinociceptive effect of peripherally restricted CB1R agonists.

We also investigated the effect of PrNMI on bone remodeling in our murine CIBP model because cancer-induced bone fracture is one of the major components contributing to bone cancer pain [39]. In a normal healthy bone, bone mass is maintained by the balance between the activities of osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells [6]. When cancer cells invade the bone, the balance is disrupted, eventually leading to net bone loss [9]. Cannabinoid receptors are involved in this modulation of bone remodeling. CB1Rs are mainly expressed on the nerve fibers innervating bone [59,60]. Osteoblasts and osteoclasts also express CB1Rs, but at low levels [26,50]. In contrast, CB2Rs are mainly expressed by osteoclasts, osteoblasts and osteocytes [25,41]. CB2R activation was identified to produce anti-osteolytic effects in different animal models of bone loss. Studies by Lozano-Ondoua et al., demonstrated that CB2R agonism can result in a significant decrease in cancer-induced bone loss by directly inhibiting osteoclast activity, playing a role in the CB2 mechanism of antinociception [35]. Ofek et al. and Sophocleous et al. found that the activation of CB2Rs can inhibit age-related and ovariectomy-induced osteoporosis by promoting osteoblast differentiation and suppressing osteoclast function [41,55]. However,

the functional role of CB1Rs in bone remodeling is still controversial. Previous studies by Tam et al. suggest the CB1Rs present on skeletal sympathetic nerve terminals promote bone formation by suppressing norepinephrine release [60]. Idris et al. reported that genetic deletion of CB1Rs prevents ovariectomy-induced bone loss by inhibiting the activity of osteoclasts [25]. Our chronic studies show that the peripherally restricted CB1R agonist PrNMI does not significantly reduce bone loss. Differences may be explained by the chronicity of receptor inactivation in CB1R-KO versus a 7-day agonist activity in our studies. Furthermore, differences may be due to mouse strains or the construct used for genetic mutation [6].

The psychotropic actions mediated by central CB1Rs represent the most troubling side effects that limit the clinical use of CB1R agonists [16,42]. Catalepsy, hypothermia, motor incoordination, and sedation are the classical indicators of central CB1Rs activation [16]. Here, we used open field test, rotarod test, rectal temperature test and ring immobility test to determine the CNS actions of PrNMI. Our data indicate that PrNMI does not induce motor incoordination in naïve mice. In addition, hypothermia is not seen at any of the antinociceptive doses, but is detected at a 10-fold higher dose than the approximate analgesic $ED_{50}$ dose (0.1 mg/kg). Catalepsy was not present at a dose of 0.3 mg/kg but was moderately-induced by PrNMI at 0.6 and 1.0 mg/kg as compared to our previous publication using a centrally acting CB1 agonist, WIN55,212-2 [38]. Interestingly, the open field test shows a decrease in total travel distance but no differences was observed in tracking patterns, center time or moving time. Although there are no published studies that have previously reported such findings, the idea that animals may have a reduction in overall travel distance but no significant change in activity on the rotarod, center time and most importantly moving time might suggest that the peripherally restricted cannabinoids may cause animals to move more in a stationary place. A review by Walsh and Cummins [64] described decreases in ambulation in an open field test typically indicates locomotor function and emotionality (anxiety/sedation) yet, our lack of an effect using rotarod and the no change in center time suggest no locomotor/sedation or anxiety activity, respectively. Further studies will need to confirm whether a peripherally restricted cannabinoid may result in an overall decrease in distance traveled and/or other changes in motor performance such as increased grooming. Our previous pharmacokinetic findings in rats demonstrated minimal CNS access of PrNMI after systemic administration, particularly compared to other reported peripherally restricted CB1R agonists, as well as typical cannabinoids [1,13,52,67]. Overall, PrNMI produces limited central actions yet full antinociceptive efficacy in inhibiting bone cancer pain.

The leading reason that CIBP remains a significant health problem today is the limited efficacy of analgesics available to treat this pain without impairing the patient's quality of life and the bone health of the patients. Opioid therapy is the primary treatment of moderate to severe bone cancer pain following cancer metastasis to bone [66]. Although opioids are very effective analgesics, they cause numerous unwanted side effects which limit the dose used. Recent studies by our group demonstrate that chronic morphine accelerates bone degradation in a murine model of sarcoma-induced bone loss [32]. Additionally, opioid analgesics cause a variety of psychotropic and life-threatening side effects, including somnolence, agitation, dizziness, cognitive impairment, hyperesthesia and respiratory

depression [47]. As a result, the administration of opioids for CIBP significantly impairs the ability of patients to partake in daily events and effectively engage with their family and friends reducing their overall quality of life. Recently we have shown that the combination of analgesic therapies, CB2 agonist and a mu opioid, for chronic pain can synergistically decrease the pain behaviors while also significantly reduce unwanted effects of both drugs [18]. In the present study, we identified a peripherally restricted CB1R agonist as a promising alternative to the treatment of CIBP. Our results indicate that PrNMI can exert a profound analgesic effect on bone cancer pain and should be further tested in the presence of additional analgesics such as a CB2 agonist or an NSAID for cancer-induced pain. Importantly, PrNMI did not exacerbate cancer-induced bone destruction, did not enhance cancer proliferation and produced no severe side effects at therapeutic doses. Therefore, the use of peripherally restricted CB1R agonists in the treatment of CIBP is a highly favorable and safe alternative to current clinical therapy.

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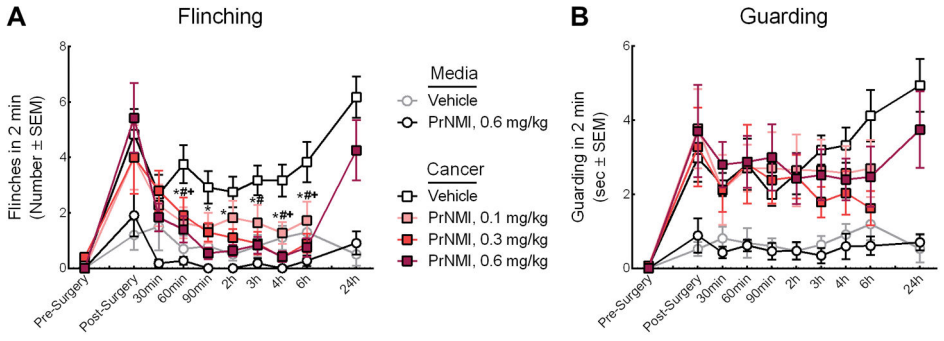


**Figure 1. Acute administration of PrNMI attenuates spontaneous pain in a cancer-induced pain model**

On day 7 after femoral inoculation with 66.1 breast cancer cells or cell-free media, animals were treated with PrNMI (0.1, 0.3, or 0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.), and spontaneous pain behaviors including **(A)** flinching and **(B)** guarding were recorded in a 2-minute period at various time points. **(A)** Spontaneous flinching was significantly reduced by PrNMI compared with animals that received vehicle. **(B)** No significant difference was observed in spontaneous guarding between PrNMI-treated and vehicle-treated cancer-bearing mice. For both flinching and guarding, no significant difference was observed in media-only control animals between PrNMI-treated and vehicle-treated groups. *$p < 0.05$, 0.6 mg/kg PrNMI vs. vehicle; #$p < 0.05$, 0.3 mg/kg PrNMI vs. vehicle; +$p < 0.05$, 0.1 mg/kg PrNMI vs. vehicle. Values represent the mean ± SEM, n = 10–12 per group.

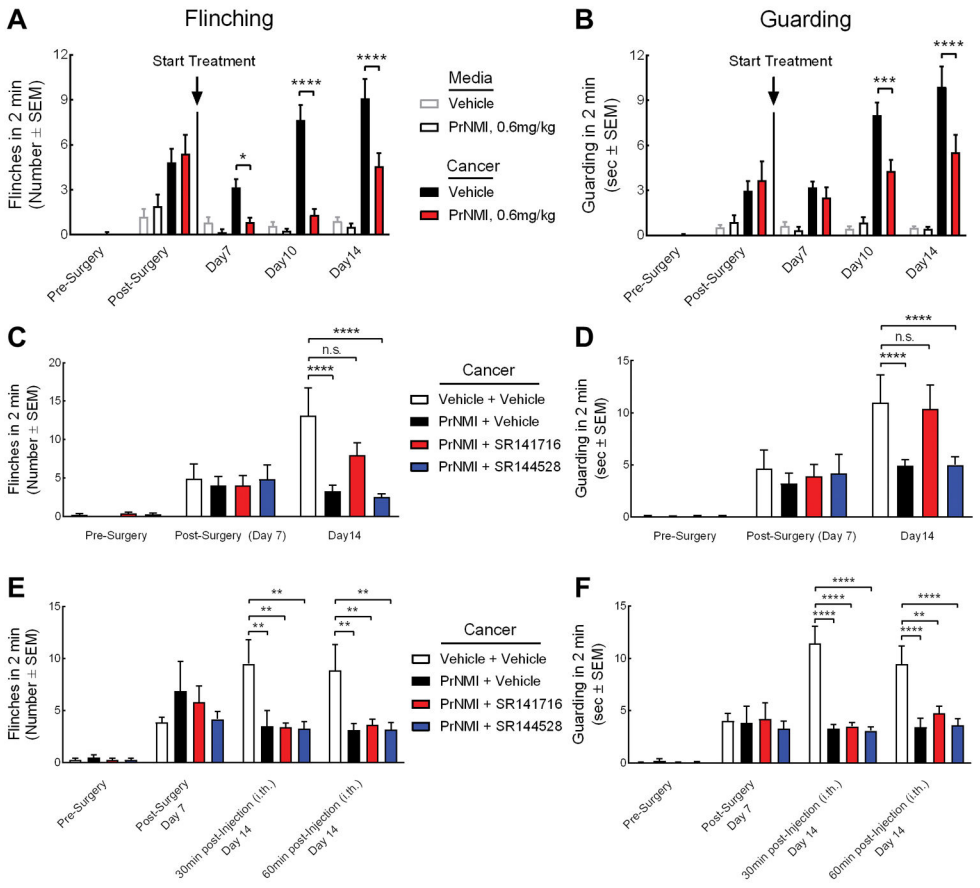


**Figure 2. Sustained administration of PrNMI attenuates spontaneous pain in a cancer-induced pain model through actions at peripheral CB1Rs**

On day 7 after femoral inoculation, animals demonstrated bone cancer-induced **(A)** flinching and **(B)** guarding. PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was administered after behavioral measurements on day 7 and continued to day 14 (q.d.). Spontaneous flinching and guarding were recorded at 3-hour time points after treatment on days 7, 10 and 14. **(A, B)** Spontaneous flinching and guarding were significantly reduced by PrNMI compared to animals that received vehicle on day 10 and 14. For both flinching and guarding, no significant difference was observed in media-only control animals between PrNMI-treated and vehicle-treated groups. **(C, D)** The attenuation of bone cancer-induced flinching and guarding by PrNMI on day 14 was inhibited by pretreatment with the selective CB1R antagonist SR141716 (1 mg/kg, i.p., q.d., 10 minutes prior to PrNMI) but not inhibited by the selective CB2R antagonist SR144528 (1 mg/kg, i.p., q.d., 10 minutes prior to PrNMI). **(E, F)** Spinal administration of either SR141716 or SR144528 (5 μg per 5 μl, 2.5 hours post PrNMI treatment) did not inhibit the antinociceptive effect of PrNMI suggesting actions at peripheral receptors. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s., not significant; values represent the mean ± SEM, n = 8–12 per group.

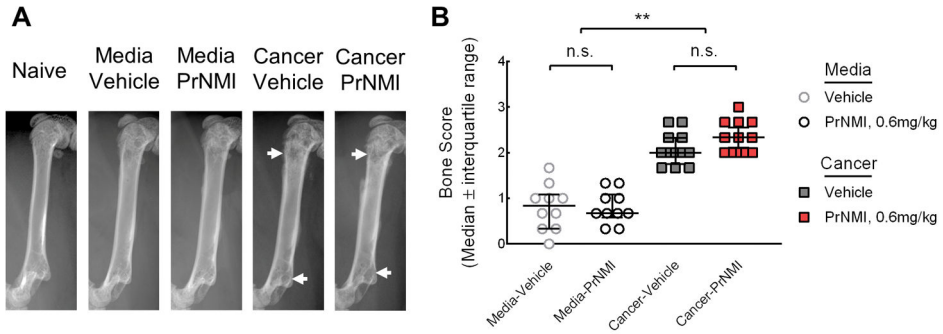


**Figure 3. Sustained administration of PrNMI does not alter bone integrity**

Animal femurs were inoculated with 66.1 breast cancer cells or cell-free media after baseline (presurgery) behavioral measurements. PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was administered on postsurgery days 7 to 14 (q.d.). On day 14, live radiographs were taken. Bone loss was rated according to an established scale (See **Materials and methods**). **(A, B)** Cancer-bearing mice had more severe bone loss than sham mice. PrNMI treatment did not significantly change cancer induced bone loss. **$p < 0.01$, n.s., not significant; values represent the median ± interquartile range, n = 10–12 per group.

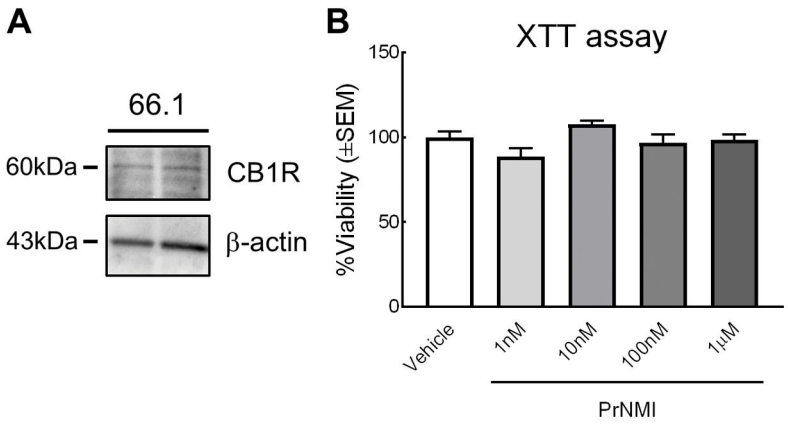


**Figure 4. PrNMI does not alter the viability of 66.1 breast tumor cells in vitro**

**(A)** CB1 receptors are expressed on 66.1 breast cancer cells. **(B)** 66.1 breast cancer cells do not have a change in their viability when treated with PrNMI. 66.1 breast cancer cells were plated into a 96-well plate at a density of $1\times10^4$ per well. 24 hours later, cells were treated with different concentrations of PrNMI. After a 24-hour incubation, the cell viability was tested by using XTT assay. Values represent the mean ± SEM, n = 12 per group.

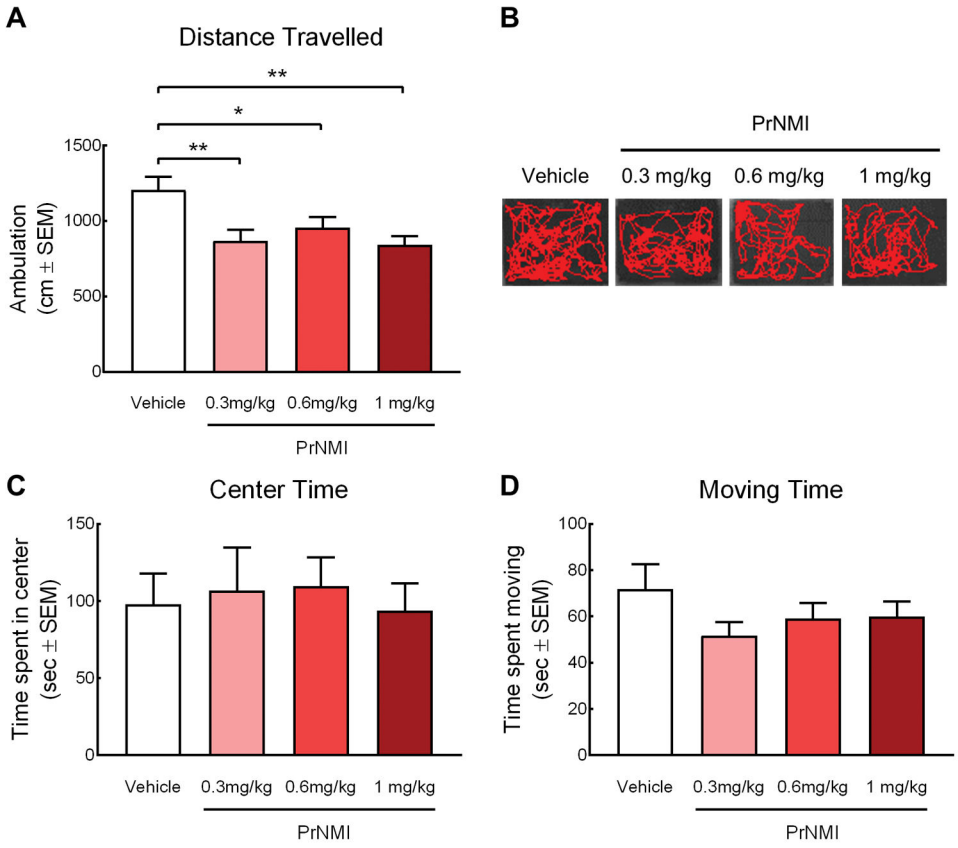


**Figure 5. PrNMI administration does not produce anxiety nor a decrease in ambulation, yet results in a decrease in the distance traveled in the open field test**

3 hours prior to open field test, mice were injected with either PrNMI (0.3, 0.6 or 1 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg). **(A)** Mice given an acute administration of PrNMI did not show a significant difference in the **(A)** tracking patterns, **(C)** center time (the time the mouse stepped both front limbs in the center rectangle), and **(D)** moving time (the time the mouse moved at least one hind limb). However, significant difference was observed in the **(B)** travel distance between PrNMI-treated and vehicle-treated mice. *$p < 0.05$, **$p < 0.01$; values represent the mean ± SEM, n = 12 per group.

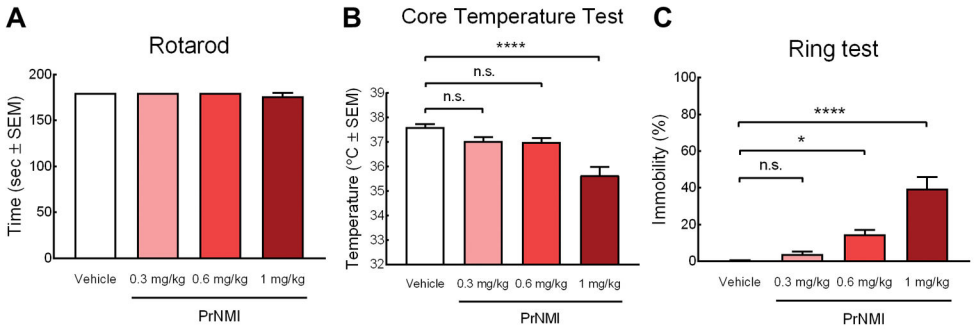


**Figure 6. PrNMI administration at analgesic doses for CIBP does not impair motor function or hypothermia**

**(A)** PrNMI does not impair animal locomotion at all testing doses. **(B)** Hypothermia was induced by PrNMI at the highest dose tested (1mg/kg). **(C)** Catalepsy was seen only at the highest dose tested in the cancer-induced flinching (0.6 mg/kg) as well as at the higher dose of 1 mg/kg. No catalepsy was measured at the dose of 0.3 mg/kg. In all three tests, mice were intraperitoneally injected with either PrNMI (0.3, 0.6 or 1 mg/kg) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg). All tests were performed before treatment and 3 hours post injection. *p<0.05, ****p<0.0001, n.s., not significant; values represent the mean ± SEM, n = 10–12 per group.