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Increasing 2-arachidonoyl glycerol signaling in the periphery attenuates mechanical hyperalgesia in a model of bone cancer pain

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Abstract

Metastatic and primary bone cancers are usually accompanied by severe pain that is difficult to manage. In light of the adverse side effects of opioids, manipulation of the endocannabinoid system may provide an effective alternative for the treatment of cancer pain. The present study determined that a local, peripheral increase in the endocannabinoid 2-arachidonoylglycgerol (2-AG) reduced mechanical hyperalgesia evoked by the growth of a fibrosarcoma tumor in and around the calcaneous bone. Intraplantar (ipl) injection of 2-AG attenuated hyperalgesia (ED_{50} of 8.2 μg) by activation of peripheral CB2 but not CB1 receptors and had an efficacy comparable to that of morphine. JZL184 (10 μg, ipl.), an inhibitor of 2-AG degradation, increased the local level of 2AG and mimicked the antihyperalgesic effect of 2-AG, also through a CB2 receptor-dependent mechanism. These effects were accompanied by an increase in CB2 receptor protein in plantar skin of the tumor-bearing paw as well as an increase in the level of 2AG. In naïve mice, intraplantar administration of the CB2 receptor antagonist AM630 did not alter responses to mechanical stimuli demonstrating that peripheral CB2 receptor tone does not modulate mechanical sensitivity. These data extend our previous findings with anandamide in the same model and suggest that the peripheral endocannabinoid system is a promising target for the management of cancer pain.

Keywords

Endocannabinoid; Monoglycerol lipase; 2-arachidonoyl glycerol; cancer; cannabinoid receptor; pain

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1 INTRODUCTION

Over 60% of individuals with primary or metastatic bone cancer suffer from severe pain [1] making pain a major factor contributing to diminished quality of life in these patients. Typically, bone cancer pain is treated with opioid therapy which produces adverse side-effects including nausea, respiratory dysfunctions, and physical dependence [2]. Moreover, because some patients do not attain sufficient analgesia with opioids, cancer pain management remains a therapeutic challenge. Studies of the endocannabinoid system are unveiling the relevance of this system to the management of pain associated with tissue damage [3,4,5].

The endocannabinoid system includes cannabinoid (CB) receptors (CB1 and CB2), their endogenous ligands and the enzymes responsible for their synthesis and degradation. In addition to anandamide (AEA), 2-arachidonoyl glycerol (2-AG) has been characterized as an endocannabinoid (reviewed by [5]). 2-AG is synthesized by diacylglycerol lipase [6] and hydrolyzed to arachidonic acid and glycerol by monoacylglycerol lipase (MGL) [7,8] as well as serine hydrolase α-β-hydrolase domain 6 (ABHD6) [9,10]. Basal levels of 2-AG are higher than those of AEA in brain and skin [11], and 2-AG acts as a full agonist of CB1 and CB2 receptors in multiple assay systems (reviewed by [12]).

Peripheral anti-hyperalgesic effects of 2-AG have been demonstrated in models of tissue injury. Injection of 2-AG near the site of injury decreases nocifensive behavior in rat models of inflammatory [13,14] and neuropathic pain [15]. Whether the anti-hyperalgesic effect of 2-AG in the periphery is mediated by CB1 or CB2 receptors is dependent on the model and the behavioral assay: The effect of 2-AG in the formalin model of inflammatory pain is selectively blocked by local administration of a CB2 receptor antagonist [13], but both CB1 and CB2 receptor antagonists block the anti-allodynic effect of 2-AG in a model of neuropathic pain [15].

An alternative approach to injection of 2-AG to increase its level in tissue is to inhibit the degradation of what is synthesized endogenously. Whereas MGL accounts for the majority of the degradation of 2-AG in neurons within the brain, the contribution of ABHD6 ranges from 15% [9] to 40% [10]. Early studies used the MGL inhibitor URB602 to increase the level of endogenous 2-AG locally in the brain [16] or in vitro [17]. A local injection of URB602 in the periphery attenuates inflammatory and neuropathic hyperalgesia ([13,15] respectively). However, in tissue homogenates URB602 inhibits fatty acid amide hydrolase, the enzyme that degrades AEA [18]. This observation in conjunction with the low potency of the compound impugns the selectivity of URB602 in vivo in the absence of measures of endocannabinoids under the same experimental conditions. Recently, a more selective inhibitor of MGL has been generated: JZL184 elevates levels of 2AG but not AEA following acute systemic administration [19,20].

We have used a murine model of bone cancer pain that mimics metastatic bone cancer pain in humans [21,22] to address the efficacy of synthetic cannabinoid receptor agonists to reduce tumor-evoked pain [23,24,25]. We also determined that mechanical hyperalgesia in the tumor-bearing paw is associated with a decrease in the level of the endocannabinoid AEA in the associated plantar skin, and treatments that increase the level of AEA locally alleviate the hyperalgesia [26]. In the present study, we extend our investigation of the endocannabinoid system in bone cancer pain to address whether increasing the level of 2-AG locally through intraplantar administration of 2-AG or the MGL inhibitor JZL184 reduces tumor-related mechanical hyperalgesia. Following determination that both pharmacological approaches produced an anti-hyperalgesic effect that was mediated by the CB2 receptor, tissue levels of 2AG and the CB2 receptor were investigated. Although the

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intraplantar injection of a selective CB2 receptor antagonist did not alter sensitivity to a mechanical stimulus in naïve mice, indicating that basal CB2 receptor activity does not modulate the nociceptive mechanical threshold, increasing the level of 2-AG at the site of sensory transduction may be advantageous in the management of tumor-evoked pain in humans.

2 METHODS

2.1 Subjects

Adult male C3H/HeNCr MTV^{-} mice (National Cancer Institute; 25–30 g) were used throughout this study. Mice were housed 4 per cage, allowed free access to food and water, and maintained on a 12-hour light/dark schedule. All behavioral testing was performed during the light cycle. Experiments adhered to the guidelines set forth by the Committee for Research and Ethical Issues of the IASP, and procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

2.2 Maintenance and implantation of fibrosarcoma cells

NCTC clone 2472 fibrosarcoma cells (American Type Culture Collection, Manassas, VA, USA) were maintained as described previously [26]. This clone was derived from a connective tissue tumor in a C3H mouse, rendering the fibrosarcoma cells syngeneic with C3H/He mice [21]. Fibrosarcoma cells (2×10^5 cells in 10 μl of phosphate buffered saline, pH 7.3) were injected into and around the calcaneus bone of the animal's left hind paw while the mouse was anesthetized with isoflurane (2%). Histological studies conducted previously documented that this approach produces a tumor with bone osteolysis [21].

2.3 Measurement of mechanical sensitivity in naïve mice

Mechanical sensitivity was measured using graded von Frey monofilaments with bending forces of 3.9, 5.9, 9.8, 13.7, 19.6 and 39.2 mN. Monofilaments were applied individually to the plantar surface of the hind paw in order of increasing force [16]. Each monofilament was applied 10 times and the withdrawal frequency was calculated.

2.4 Measurement of mechanical hyperalgesia

Response to mechanical stimuli was selected as the dependent measure in the study because this measure is highly reproducible in our hands and effective in resolving sensitivity to cannabinoid receptor ligands. Moreover, touch-evoked pain is prominent in human pain syndromes [27]. Mechanical hyperalgesia in the tumor-bearing paw was defined as an increase in withdrawal frequency in response to a standard mechanical stimulus: a von Frey monofilament that delivers a force of 3.9 mN (0.4 g). Animals were placed on an elevated wire mesh platform, covered individually with glass containers and allowed to acclimate for 30 minutes prior to testing. The monofilament was applied to the plantar surface of each hind paw ten times, and the withdrawal frequency was calculated for each paw as the (number of withdrawal responses/total stimuli) × 100%.

The baseline (pre-tumor) withdrawal frequency for each hind paw was measured on 3 consecutive days preceding implantation of fibrosarcoma cells. The average baseline withdrawal frequency evoked by the 3.9 mN monofilament across several experiments was 13%. Following implantation, the development of mechanical hyperalgesia was monitored daily. Consistent with previous studies [21] an increase in the withdrawal frequency occurred in response to the test stimulus in the tumor-bearing paw. By 10 days after fibrosarcoma cell implantation, the average paw withdrawal frequency increased to 78% in the tumor-bearing paw across several experiments. Approximately 15% of mice did not display mechanical hyperalgesia after implantation of fibrosarcoma cells. On the day of drug

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injections (day 10 or 11 after implantation), only mice that exhibited a withdrawal frequency ≥70% were used in the experiments. Following intraplantar (ipl) drug injections, the withdrawal frequency of each hind paw was measured every 30 minutes for 3.5 hours. The individual scoring behavioral responses following injection of drugs was blinded to the treatment of the animal in all experiments, and at least 2 drug groups were tested in each session.

2.5 Drug solutions and administration

A stock solution of the endocannabinoid 2-AG (Tocris, Ellisville, MO, USA) was prepared in ethanol (10 μg/μl). JZL184 (Cayman Chemical, Ann Arbor, MI, USA) was prepared in DMSO:Tocrisolve™100 (1:12.5, 12.5 μg/μl). The CB1 receptor antagonist AM281 [1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-mo rpholinyl-1H-pyrazole-3-carboxamide; Tocris] and the CB2 receptor antagonist AM630 [6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l](4-methoxyphenyl)methanone; Tocris] were dissolved in DMSO (10 μg/μl). Each receptor antagonist exhibits more than a 100-fold difference in affinity for CB1 and CB2 receptors [28,29].

All drugs were diluted to the final dose in saline for injection in a volume of 10 μl. The highest concentrations of organic solvents in a dose were used as vehicle controls. Drugs or vehicles were injected subcutaneously into the plantar surface of the hind paw.

2.6 Analysis of behavioral data

The effect of each drug on mechanical hyperalgesia in tumor-bearing mice was calculated as a percent of the maximum possible effect on hyperalgesia.

$$\%\text{inhibition of hyperalgesia}=\frac{(\text{predrug response}-\text{postdrug response})}{(\text{predrug response}-\text{pre}-\text{tumor response})}\times 100\%$$

Additionally, the percent maximum drug effect was calculated for use in the dose response analysis.

$$\%\text{maximum drug effect}=\frac{(\text{predrug response}-\text{postdrug response})}{(\text{predrug response}-\max-\text{postdrug response})}\times 100\%$$

Calculating maximum drug effect produced a limited number of values greater than 100 or less than 0, which occurred at high and low doses, respectively. These values were adjusted to 100 and 0, respectively, to determine the anti-hyperalgesic effect of the drug.

2.7 Measurement of AEA and 2-AG

In order to determine whether the tumor condition altered tissue levels of 2-AG, tumor-bearing mice were euthanized by decapitation under isoflurane anesthesia and lumber DRG L3-L5 and samples of plantar paw skin ipsilateral to tumors were collected. Parallel samples were collected from naïve mice. To determine the selectivity and efficacy of JZL184 on endocannabinoid levels in the periphery, samples of paw skin were collected upon euthanasia 2 hr after injection of JZL184 (10 μg, ipl.). Upon removal, samples were weighed, frozen in liquid nitrogen, and kept frozen at −80°C until the time of processing. Endogenous AEA and 2AG were measured as previously described [26]. On the first day of processing, tissues were minced and extracted with 5 volumes of chloroform at 4°C

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overnight. On the second day of processing, samples were then homogenized with an equal volume of methanol:Tris-HCl 50 mM (1:1) containing 5 pmol of deuterated d_8-AEA and 100 pmol of deuterated d_8-2-AG as internal standards. Homogenates were centrifuged at 2500 x*g* for 15 min (4°C); the aqueous phase plus debris were collected and extracted again with 1 volume of chloroform. The organic phases were pooled and evaporated with a gentle stream of nitrogen gas. Vials containing the dried samples were weighed for determination of total lipid weight and were stored at −80°C until analyzed. Targeted isotope-dilution HPLC/atmospheric pressure chemical ionization/mass spectrometry was conducted on each sample. A ZORBAX SB-C18 (0.5 ×150 mm) column was used. The column was maintained at 40°C. The mobile phase A was 0.1% formic acid in 2 mM of ammonium acetate, and phase B was 0.1% formic acid in acetonitrile. The flow rate was 10 μl/min with a gradient that began with 50% A:50% B. The AEA and 2-AG levels in unknown samples were estimated from the ratio of the area of the signals of deuterated and non-labeled AEA (0.2–200 pmol), or 2-AG (2–2000 pmol) standards. Data are expressed as pmol AEA or 2-AG per g tissue weight or total lipid extracted from samples. On three occasions, insufficient recovery of deuterated compounds or an unusual amount of extracted lipids resulted in amounts of endocannabinoids that were more than two standard deviations beyond the mean for the group. These values were deleted from the data set for statistical analysis.

2.8 Western blot analysis of CB2 receptor protein

Samples of plantar paw skin, tibial nerve (~1 cm,) and L3–L5 dorsal root ganglia (DRG) from naive and tumor-bearing mice were dissected, frozen on dry ice, and stored at −80°C until time of processing. Samples of nerve and DRGs were pooled from 3 mice. On the day of processing, samples were sonicated in single-detergent lysis buffer (50 mM Tris-HCl, pH 8.0 with 1% Triton X-100, 150 mM NaCl, 0.02% Na azide, 100 μg/ml PMSF, and 1 μg/ml protease inhibitor mixture (Sigma), and the supernatant was obtained after centrifugation at 800 x*g* for 10 min. The supernatant was concentrated using an Amicon Ultra-0.5 centrifugal filter (Millipore Corporation, Billerica, MA, USA). Western blot analysis was performed on 30 μg of protein/sample. Samples were loaded onto a 10% SDS-PAGE gel, subjected to electrophoresis and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Philadelphia, PA, USA). Samples of one tissue type were processed on the same gel. Nonspecific binding to membranes was blocked by incubation in phosphate-buffered saline with 3% defatted dry milk for 1 h at room temperature. The membranes were probed with a rabbit anti-CB2 receptor antibody (1:500, Cayman) overnight at 4°C. Detection of the primary antibody was performed using a peroxidase conjugate of goat anti-rabbit IgG (1:10,000; Amersham Biosciences, Pittsburgh, PA, USA). Immunoreactivity was visualized using the enhanced chemifluorescence detection reagent (Pierce, Rockford, IL) and X-ray film (Eastman Kodak Company, Rochester, NY, USA). The gel was treated with 0.01% phenylhydrazine for 10 min after detection of CB2 receptor immunoreactivity in order to neutralize the peroxidase activity associated with this antigen. Actin immunoreactivity (rabbit anti-actin antibody,1:500, Sigma) within each sample was then quantified as a loading control. Multiple exposures were done of each film after each antibody detection to insure that measures of density of silver grains with respect to immunoreactivity were within the linear range of the response of the X-ray film. The density of silver grains was quantified using Metamorph (v5.07, Molecular Devices, Sunnyvale, CA, USA). Specificity of the CB2R antibody was confirmed using plantar skin from CB2R$^{-/-}$ mice (B6.129P2-*Cnr2*^{tm1Dgen}/J Jackson Laboratory, Bar Harbor, ME, USA).

2.9 Statistical Analyses

All data are presented as the group mean ± S.E.M. Results were compared between groups and across time using Student's t-test, one-way and two-way analyses of variance (with repeated measures when applicable) followed by the Bonferroni t-test for comparisons

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between groups. For all statistical analyses, a probability value of <0.05 was considered significant. The dose-response data were analyzed using Prism (GraphPad v. 5.01).

3 RESULTS

3.1 2-AG attenuated tumor-evoked mechanical hyperalgesia

Peripheral administration of 2-AG (18 μg, ipl., ipsilateral to the tumor) decreased mechanical hyperalgesia in tumor-bearing mice in a time-dependent manner (Fig. 1A). A reduction in mechanical hyperalgesia occurred at the earliest time point measured (30 min) and persisted through 3 h. The vehicle (20% ethanol in saline) did not alter the withdrawal frequency of tumor-bearing mice. To determine which cannabinoid receptor subtype mediated the antihyperalgesic effect of 2-AG, 2-AG (18 μg, ipl.) was co-injected with either the CB2 receptor antagonist AM630 (4 μg) or the CB1 receptor antagonist AM281 (10 μg). The cannabinoid receptor selectivity of each antagonist at the dose used was validated in previous studies using the same route of administration in the same model [26,30]. AM630 blocked the antihyperalgesic effect of 2-AG (Fig. 1A), however, the CB1 receptor antagonist AM281 had no effect on 2-AG (p=0.454, 2-way ANOVA). Neither dose of AM630 or AM281 administered alone (ipl.) ipsilateral to the tumor reduced mechanical hyperalgesia in tumor-bearing mice (p=0.313 and p=0.662, respectively, n=5 mice/group, two-way ANOVA). Together, these data indicate that CB2 receptors play a principal role in 2-AG-mediated peripheral anti-hyperalgesia.

The effect of 2-AG was also dose-dependent (Fig. 1B). A dose of 18 μg was the maximally effective dose and inhibited mechanical hyperalgesia by 68±4.8%; the ED_{50} was 8.5 μg (5.8–11,2, 95% CI).

In order to establish whether 2-AG reduced mechanical hyperalgesia by a systemic or local mechanism, 2-AG (18 μg, ipl.) was injected into the paw contralateral to the tumor. There was no change in the withdrawal frequency of the tumor-bearing paw compared to the pre-drug response (p=0.761, One-way ANOVA with repeated measures, n=4 mice), indicating that 2-AG exerted its anti-hyperalgesic effect locally.

3.2 Intraplantar injection of an MGL inhibitor mimicked the effect of 2-AG

Injection of JZL184 (10 μg, ipl.), an inhibitor of MGL, into the tumor-bearing paw also attenuated the mechanical hyperalgesia (Fig. 2A). The reduction in mechanical hyperalgesia was first noted 60 min after drug injection, and the effect was no longer evident by 3 hr post injection. The maximum inhibition of mechanical hyperalgesia by JZL184 was 34±7%, which was less than the maximal effect of 2-AG (p<0.005, Student's t-test). A lower dose of JZL184 (4 μg) had no effect on mechanical hyperalgesia and the anti-hyperalgesic effect of a higher dose (40 μg) occurred only at 120 min. Because solubility of the drug in the vehicle (DMSO/Tocrisolve ™100/saline) restricted the range of doses we could administer, an ED_{50} could not be determined.

The involvement of cannabinoid receptor subtypes in JZL184-induced anti-hyperalgesia was investigated by co-injection of JZL184 (10 μg, ipl.) and cannabinoid receptor antagonists ipsilateral to the tumor. The CB2 receptor antagonist, AM630 (4 μg), blocked the anti-hyperalgesic effect of JZL184 (120 min post-drug administration reported in Fig. 2B). Co-administration of the CB1 receptor antagonist AM281 (10 μg) with JZL184 did not diminish the anti-hyperalgesic effect of JZL184 alone. Therefore, in parallel with 2-AG, the anti-hyperalgesia produced by JZl184 was also mediated by CB2 receptors.

To determine whether the anti-hyperalgesic effect of intraplantar injection of JZL184 was mediated by a local mechanism, mechanical hyperalgesia in the tumor-bearing paw was

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determined following injection of JZL184 (10 μg, ipl.) into the paw contralateral to the tumor. At 120 min post drug injection the mean withdrawal frequency in the tumor-bearing paw was 87±3% compared to the pre-drug value of 93±3% (p=0.919, n=3; One-way ANOVA for repeated measures). These data indicate that the anti-hyperalgesic effect observed following intraplantar injection if JZL184 was likely mediated by a local mechanism.

3.3 Levels of 2-AG and AEA in paw skin following treatments

In comparison to AEA, the level of 2-AG was more than 60-fold higher in plantar skin of naïve mice (Table 1; values for skin reported per g of tissue). This difference is consistent with previous reports of the relative amounts of AEA and 2-AG in skin [31,32]. The tumor condition elicited different changes in the levels of 2-AG and AEA in the plantar skin of the tumor-bearing paw and related DRGs. Consistent with our earlier report [26], the level of AEA was lower in the plantar skin and DRGs of tumor bearing mice compared to samples from naïve mice. In contrast, the level of 2-AG was almost 3-fold higher in paw skin ipsilateral to the tumor, but no change occurred in the related DRGs.

In order to address the selectivity of JZL184 in disrupting the degradation of 2-AG over AEA, the levels of 2-AG and AEA were measured in plantar paw skin ipsilateral to the injection of drug. Samples were collected between 100–120 min following drug administration in order to measure 2-AG during the time of the CB2-dependent anti-hyperalgesic effect. Vehicle did not alter the relative amounts of 2-AG and AEA or the effect of the tumor condition (Table 2, values reported per g total lipid). The level of 2-AG increased more than 4-fold following injection of JZL184 in naive mice; a 2-fold increase occurred in skin from the tumor-bearing hind paw. Although the proportional change in 2AG in the skin from tumor-bearing mice was smaller than that in skin from naive mice due to the higher basal level of 2AG, the absolute amount of 2AG that accumulated following drug administration was larger in the skin from tumor-bearing mice. No change occurred in the level of AEA in response to JZL184 in naive or tumor-bearing mice. These data confirm the efficacy and selectivity of JZL184 for inhibition of MGL over fatty acid amide hydrolase in murine skin under the condition in which anti-hyperalgesia was observed.

3.4 CB2 receptor tone in naïve mice

Several lines of evidence indicate that CB1 receptors contribute to the threshold for nociception: First, genetic deletion of CB1 receptors in nociceptors results in thermal and mechanical hyperalgesia compared to wild type mice [33]. Secondly, mechanical hyperalgesia occurs in naïve mice following intraplantar injection of a CB1 receptor antagonist [26]. Therefore, intraplantar injection of the CB2 receptor antagonist AM630 was used to determine whether basal CB2 receptor tone in skin regulates sensitivity to mechanical stimuli. We used the dose of AM630 (4 μg) that blocked the effect of 2-AG on mechanical hyperalgesia in tumor-bearing mice. Responses to monofilaments of 3.9 to 39.2 mN (0.4–4 g) were measured at 2 h following intraplantar injection of AM630, a time point at which intraplantar injection of AM630 blocked the anti-hyperalgesic effect of 2-AG (Fig. 1A). Compared to the vehicle control (20% DMSO), AM630 did not alter the response to any mechanical stimulus within the range tested at 2 hr post drug administration (Fig. 3). Although the basal level of 2-AG in skin of naïve mice is more than 60-fold greater than that of AEA (see above), these data suggest that CB2 receptors do not modulate mechanical sensitivity in naïve mice.

3.5 Expression of CB2 receptor protein in tumor-bearing mice

Given that the anti-hyperalgesic effects of 2-AG was mediated by local CB2 receptors, the expression of CB2 receptor protein was determined by analyzing Western blots of peripheral

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tissues from naïve and tumor-bearing mice. The absence of an immunoreactive band at 45 kD in plantar skin from $CB2^{-/-}$ mice confirmed the selectivity of the antibody used to detect CB2 receptor protein (Fig. 4A). The amount of CB2 receptor in DRGs from both naïve and tumor-bearing mice was at the limit of detection, so reliable conclusions could not be drawn for these samples. There was no difference in the level of CB2 receptor protein in tibial nerve ipsilateral to tumors compared to nerve from naïve mice. However, samples of the tumor included significant amounts of CB2 receptor protein, and CB2 receptor protein was higher in plantar paw skin ipsilateral to tumors compared to skin from naïve mice (Fig. 4B). On the basis of these data it is likely that effects of CB2 receptor agonists on mechanical hyperalgesia are mediated by non-neuronal cells which may include keratinocytes as well as fibrosarcoma and immune cells.

4 DISCUSSION

Pain related to tumor growth is often difficult to manage, and approximately two-thirds of patients experience pain with advanced disease [34], particularly with metastases to bone [1]. These data compel novel approaches to the management of tumor-related pain. The present results demonstrate that 2-AG inhibited mechanical hyperalgesia in a murine model of bone cancer pain by a local CB2 receptor-dependent mechanism. The effect was mimicked by JZL184, an inhibitor of MGL, which increased the endogenous level of 2-AG in tumor-bearing mice. The maximum effect of 2AG was comparable to intraplantar administration of morphine which reduced mechanical hyperalgesia by 53% in this model [30]. It is likely that the anti-hyperalgesic effect is mediated by the activation of CB2 receptors on non-neuronal cells in the skin, but the cellular mechanism underlying the effect remains to be resolved.

4.1 Pharmacology of the anti-hyperalgesic effect of 2-AG

Evidence that the anti-hyperalgesic effect of 2-AG was mediated by activation of CB2 and not CB1 receptors is noteworthy in light of reports that 2-AG is a full agonist at both CB1 and CB2 receptors [35], and 2-AG activates CB1 receptors in the brain [16,36]. Conversely, AEA administered by the same route is anti-hyperalgesic by activation of CB1 and not CB2 receptors in this model [26]. The apparent selective effect of 2-AG for peripheral murine CB2 receptors is consistent with the anti-nociceptive effect of this endocannabinoid in rats in the formalin model of nocifensive behavior [13]. However, the data contrast with a report in a more relevant model of persistent hyperalgesia: Both CB1 and CB2 receptors contribute to the anti-hyperalgesic effect of 2-AG in assays of mechanical and thermal sensitivity in a rat model of neuropathic pain [15]. The variability in observations most likely reflects underlying differences in the pathophysiology of the models of peripheral injury and accompanying neurochemical changes in somatosensory neurons [37]. We [26,38,39] and others [40] have shown that chemicals released from cancer cells modify the neurochemistry and excitability of DRG neurons *in vitro*. Importantly, the anti-hyperalgesia following 2-AG in the present study was not mediated systemically because 2-AG injected into the contralateral paw did not alter mechanical hyperalgesia exhibited by the tumor-bearing paw.

In addition to the receptor selectivity of 2-AG in reducing mechanical hyperalgesia, it was curious that the anti-hyperalgesic effect of 2-AG (18 μg, equivalent to 48 nmol) occurred within the context of a level of 2-AG in plantar skin of the tumor-bearing hind paw that was approximately 3-fold higher than that in skin from naïve mice. The higher level of 2AG in the skin of tumor bearing mice can be attributed in part to the synthesis of 2AG by fibrosarcoma cells (Khasabova, unpublished observation). Evidence that exogenous 2-AG as well as inhibition of MGL with a dose of JZL184 that increased levels of 2-AG more than 2-fold had the same anti-hyperalgesic effect supports the biological relevance of the observation. The tissue chemistry underlying this apparent conundrum, however, is not

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known. There may be compartmentalization of 2-AG within cells, such as the fibrosarcoma cells, and not in the interstitial fluid where CB2 receptors mediating the anti-hyperalgesic effect are localized. Using *in vivo* microdialysis in nucleus accumbens, JZL184 was shown to increase recovery of 2-AG in the dialysate following neuronal depolarization [19]. Alternatively, the increased expression of CB2 receptors in the tumor-bearing hind paw may reflect the induction of a receptor with a lower affinity for 2-AG thereby requiring a higher level of 2-AG for its activation.

Finally, exogenous 2-AG was less effective in reducing mechanical hyperalgesia at doses higher than 18 μg. Decreased efficacy at a high dose also occurs with the synthetic CB2 receptor agonist AM1241 [30] and is most likely related to CB2 receptors expressed by cells at the injection site. CB2 agonists promote the recruitment of eosinophils resulting in the release of inflammatory mediators [12,41] that would cause pronociceptive effects to counter-balance the anti-hyperalgesic effect of 2-AG. Alternatively, we cannot exclude the possibility that elevated tissue levels of 2-AG following exogenous administration of the endocannabinoid results in increased hydrolysis of the 2-AG and the generation of intermediates such as leukotrienes and prostaglandin E2 (PGE_2) which promote hyperalgesia [42,43,44].

4.2 Anti-hyperalgesia mediated by JZL184

This is the first evidence that JZL184 promotes anti-hyperalgesia through a peripheral mechanism in a model of persistent pain. Importantly, we demonstrated that JZL184 (10 μg) elevated the level of 2-AG and not AEA near the site of injection at the dose that was maximally effective in reducing mechanical hyperalgesia in the tumor-bearing paw. The absence of an effect of JZL184 on AEA is consistent with evidence that the anti-hyperalgesic effect of AEA by the same route of administration in this model was mediated solely by CB1 receptors [26], and the anti-hyperalgesic effect of JZL184 was not blocked by the CB1 receptor antagonist at any time-point during its effectiveness. It is noteworthy that the anti-hyperalgesic effect was mediated by CB2 receptors because two recent studies have demonstrated that systemic administration of JZL184 is anti-hyperalgesic in a murine model of neuropathic pain by a CB1 receptor-dependent mechanism [36,45]. This effect following systemic drug administration is most likely mediated centrally because the dose of JZL184 (16 mg/kg, equivalent to approximately 400 μg/mouse [19] was 40 times that used in the present study, and the authors documented a 5–6 fold increase in 2-AG within the central nervous system. Our data also differ from a recent report that intraplantar injection of JZL184 had no effect on the acute mechanical hyperalgesia produced by intraplantar injection of capsaicin in rats [46]. Effectiveness of the peripheral dose of JZL184 in rats was established by its inhibition of capsaicin-induced nocifensive behaviors and thermal hyperalgesia. Whereas the difference in species may contribute to the difference in results, it is also likely that long-term changes underlying sensory transduction in tumor-bearing mice are more relevant (see below).

Although systemic administration of JZL184 increases the level of 2-AG in brain for more than 8 hr [19], the anti-hyperalgesic effect following intraplantar administration was no longer evident 3 h after drug administration. The effect was likely specific to JZL184 as mechanical hyperalgesia was still reduced at 3 h following exogenous administration of 2-AG. The reason for the short duration of action of JZL184 is not known. In addition to the increased generation of arachidonic acid by ABHD6 when MGL is inhibited, JZL184 may promote the accumulation of yet to be defined intermediates that counter-balance its effect on mechanical hyperalgesia.

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4.3 CB2 receptors and anti-hyperalgesia

In the periphery CB2 receptors are predominately expressed by keratinocytes [47,48] and immune cells [49,50]. Compared to skin from naive mice, CB2 receptor protein was higher in plantar skin ipsilateral to tumors and was present in tumors, but the cellular location remains to be determined. The antibody used for detection of protein by Western blot was not specific in immunohistochemistry. In contrast to reports of increased expression of CB2 receptor in DRG following peripheral nerve injury [51,52], this did not occur in tumor-bearing mice. Even though nerve injury has been shown in this model [53], the level of CB2 receptor protein was unchanged in the distal portion of the tibial nerve ipsilateral to the tumor-bearing paw. The relatively low level of CB2 receptor protein detected in DRGs was consistent with levels of CB2 receptor mRNA that were also at the limit of detection using quantitative real-time PCR (Seybold, unpublished observation). These data contrast with an increase in CB1 receptor mRNA in DRGs and receptor protein in tibial nerve ipsilateral to tumors in tumor-bearing mice [26]. Thus, in this murine model of tumor pain, CB2 receptors on keratinocytes as well as tumor and immune cells most likely reduce hyperalgesia indirectly by inhibiting the secretion of algogenic substances that increase the excitability of nociceptors. We speculate that CB2 receptors on keratinocytes [47,48] may inhibit the release of ATP. An increase in the interstitial level of ATP is associated with the development of tumors in skin and nocifensive behavior that is reduced by a P2X receptor antagonist in a murine model of skin cancer pain [54]. Additional support for a role of ATP in tumor-related pain is evidence of increased expression of the P2X3 receptor on epidermal nerve fibers in the murine model used in the present experiments [55].

Peripheral administration of the CB2 receptor antagonist did not alter mechanical sensitivity indicating that this sensory modality is not modulated in naïve mice by a basal level of CB2 receptor activation. These data are interesting in light of the high levels of 2-AG in skin relative to AEA and evidence that basal activation of CB1 receptors affects the threshold for nociception in naïve mice [26,33]. Moreover, peripheral CB2 receptors do not play a tonic role in modulating mechanical hyperalgesia in tumor bearing mice as local administration of the CB2 receptor antagonist did not alter mechanical hyperalgesia (Khasabova and Seybold, unpublished observation). Similarly, genetic deletion of CB2 receptors did not alter development of mechanical or thermal allodynia in a murine model of neuropathic pain [36].

4.4 Conclusion

Taken together, the data demonstrate that peripheral 2-AG signaling may be a significant target to exploit for the management of cancer pain. In contrast to AEA, which inhibits nociception through CB1 receptors on DRG neurons [3,33], CB2 receptors occurred in skin but were not associated with somatosensory neurons of tumor-bearing mice. Thus, peripheral effects of 2-AG on mechanical hyperalgesia are most likely mediated by keratinocytes, fibrosarcoma and (or) immune cells. Dual pharmacological modulation of peripheral AEA and 2-AG signaling that directly and indirectly affects DRG neurons may be a novel approach to reducing cancer pain without the side effects associated with systemic cannabinoid administration.

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Abbreviations

2-AG	2-arachidonoyl glycerol
AEA	anandamide
ACPA	arachidonylcyclopropylamide
AM1241	(2-iodo-5-nitrophenyl)-(1-(1-methylpiperidin-2-ylmethyl)-1H-indol-3-yl)methanone
AM281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l](4-methoxyphenyl)methanone
CB	cannabinoid
DMSO	dimethylsulfoxide
DRG	dorsal root ganglion
ED_{50}	effective dose for 50% effect
MGL	monoacylglycerol lipase

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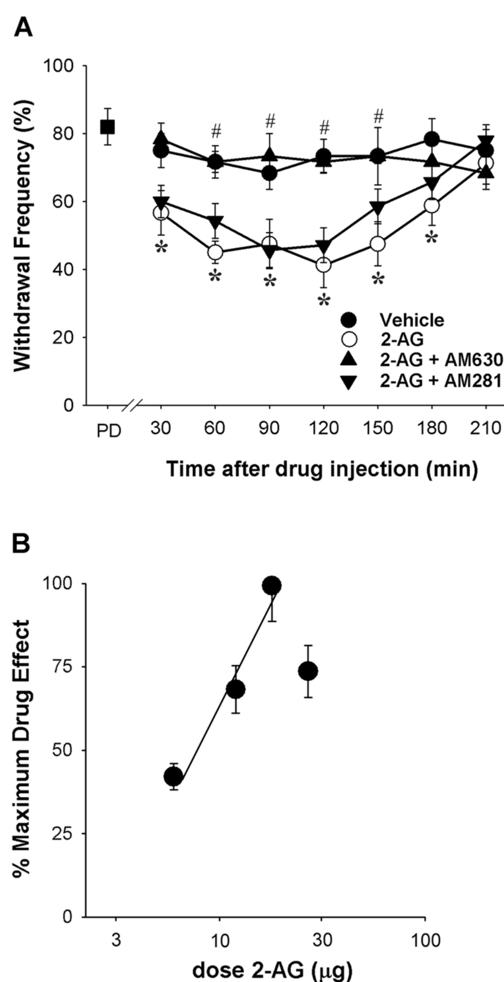

Figure 1.
Effect of intraplantar injection of 2-AG on mechanical hyperalgesia **A.** 2-AG attenuated tumor-evoked mechanical hyperalgesia by a CB2-dependent mechanism ($F_{3,26}$=33.06, p<0.001, two-way ANOVA). Mechanical hyperalgesia was confirmed before drug administration (PD=pre-drug). The dose of 2-AG was 18 μg (i.pl.), the dose of AM630 was 4 μg (i.pl.), and the dose of AM281 was 10 μg (i.pl.). *Different from vehicle at p<0.01; #different from 2-AG at p<0.001 (n=6–8 mice/group; two-way ANOVA with Bonferroni's multiple comparisons test). **B.** The effect of 2-AG was dose-dependent (r^2= 0.57, DF=16); dose is plotted on a log scale.

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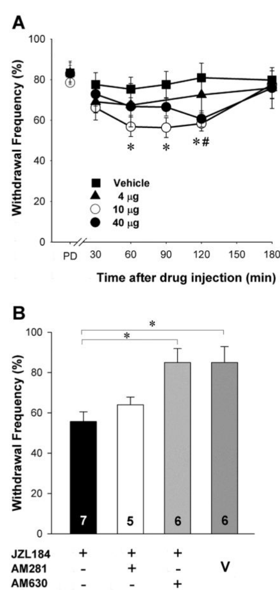

Figure 2.

Effect of intraplantar injection of JZL184 on mechanical hyperalgesia. **A.** JZL184 attenuated tumor-evoked mechanical hyperalgesia ($F_{3,169}$=18.42, p<0.001, two-way ANOVA). Mechanical hyperalgesia was confirmed before drug administration (PD=pre-drug). **B.** Co-administration of the CB2 receptor antagonist AM630 (4 μg) with JZL184 (10 μg) eliminated the anti-hyperalgesia produced by JZL184. Co-administration with the CB1 receptor antagonist AM281 (10 μg) had no effect on the antihyperalgesic effect of JZL184. *Different at p<0.05, one-way ANOVA with Bonferroni's t-test, number inside bar represents sample size).

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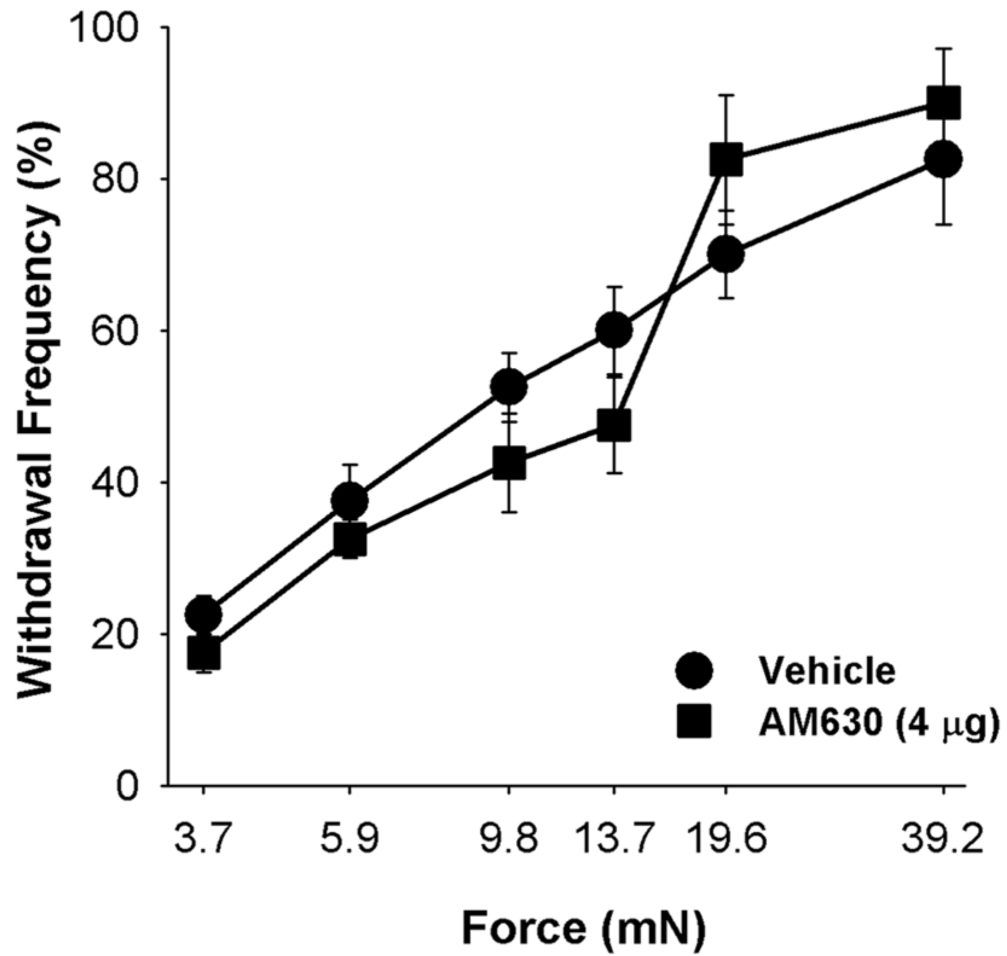

Figure 3.

The CB2 receptor antagonist AM630 did not alter sensitivity to mechanical stimuli in naive mice at 2 h following injection of 4 μg (ipl.) ipsilateral to the testing site ($F_{20,96}$=1.14, *p*=0.325, two-way ANOVA, n=4 mice/group).

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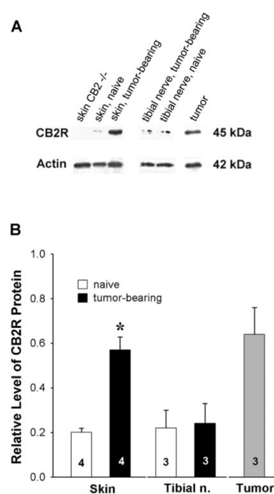

Figure 4.

Expression of CB2 receptor protein increased in plantar skin ipsilateral to tumors in tumor-bearing mice. Western blot analysis was used to determine the expression of CB2 receptor protein; the amount of CB2 receptor protein was normalized to the amount of actin within each sample. **A**. Representative examples of CB2 receptor (CB2R) and actin protein detected by Western blot. Note the absence of CB2 receptor-immunoreactivity in skin from the CB2−/− mouse. **B**. In densitometric analyses of images of blots, the amount of CB2 receptor-immunoreactivity was normalized to the amount of actin-immunoreactivity within each sample. *Different from plantar skin of naive mice at $p<0.05$ (Student's *t*-test). Values inside the bars represent the sample size.

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Table 1

Effect of tumors on the levels of AEA and 2AG in skin and DRGs.

Sample	Treatment	AEA	2-AG
Plantar paw skin[#]	Naive	26.1 ± 2.1 (4)	1572 ± 354 (4)
	Tumor-bearing	10.7 ± 0.2 (6)*	4284 ± 586 (6)**
DRG[†]	Naive	0.9 ± 0.05 (4)	4.6 ± 0.7 (4)
	Tumor-bearing	0.5 ± 0.03 (4)**	7.0 ± 1.7 (4)

[#]Data for skin are expressed as pmol/g tissue;

[†]data for DRGs are expressed as pmol/DRG; L3-L5 DRG ipsilateral to the tumor were pooled from 1 mouse and comparable samples were collected from naive mice.

*Different from naive within the same endocannabinoid at p<0.05,

**different at p<0.001 (Student's t test; AEA data for skin were converted to $\log_{10}$ for statistical analysis). Numbers in parentheses represent the same size.

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Table 2

Effect of JZL184 on levels of AEA and 2-AG in hind paw skin.

Condition	Treatment	AEA pmol	2-AG nmol
Naïve	Vehicle	550 ± 104 (8)	45 ± 6 (9)
	JZL184	455 ± 85 (5)	208 ± 32[#] (5)
Tumor-bearing	Vehicle	135 ± 51[*] (5)	190 ± 113[*] (4)
	JZL184	230 ± 23 (5)	409 ± 62[#] (5)

Endocannabinoid values were normalized to the g of lipid extracted from the sample.

Statistical analyses were conducted on the $\log_{10}$ of the individual values.

[*] Different from naïve/vehicle at $p<0.05$,

[#] different from vehicle in corresponded group at $p<0.05$, one-way ANOVA within endocannabinoid with Bonferroni's *t* test. Numbers in parentheses represent the same size.