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JZL184 is anti-hyperalgesic in a murine model of cisplatin-induced peripheral neuropathy

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Abstract

Cisplatin has been used effectively to treat a variety of cancers but its use is limited by the development of painful peripheral neuropathy. Because the endocannabinoid 2-arachidonoyl-*sn*-glycerol (2-AG) is anti-hyperalgesic in several preclinical models of chronic pain, the anti-hyperalgesic effect of JZL184, an inhibitor of 2-AG hydrolysis, was tested in a murine model of cisplatin-induced hyperalgesia. Systemic injection of cisplatin (1 mg/kg) produced mechanical hyperalgesia when administered daily for 7 days. Daily peripheral administration of a low dose of JZL184 in conjunction with cisplatin blocked the expression of mechanical hyperalgesia. Acute injection of a cannabinoid (CB)-1 but not a CB2 receptor antagonist reversed the anti-hyperalgesic effect of JZL184 indicating that downstream activation of CB1 receptors suppressed the expression of mechanical hyperalgesia. Components of endocannabinoid signaling in plantar hind paw skin and lumbar dorsal root ganglia (DRGs) were altered by treatments with cisplatin and JZL184. Treatment with cisplatin alone reduced levels of 2-AG and AEA in skin and DRGs as well as CB2 receptor protein in skin. Combining treatment of JZL184 with cisplatin increased 2-AG in DRGs compared to cisplatin alone but had no effect on the amount of 2-AG in skin. Evidence that JZL184 decreased the uptake of [³H]AEA into primary cultures of DRGs at a concentration that also inhibited the enzyme fatty acid amide hydrolase, in conjunction with data that 2-AG mimicked the effect of JZL184 on [³H]AEA uptake support the conclusion that AEA most likely mediates the anti-hyperalgesic effect of JZL184 in this model.

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1 INTRODUCTION

The demand for cancer treatments is growing. The American Society for Clinical Oncology [1] projects that by 2030, the number of new cancer cases in the United States will increase by 45%, and cancer will become the nation's leading cause of death. Cisplatin has been used for over 40 years to treat a variety of cancers, and it remains a widely prescribed antitumor drug today [2]. Although cisplatin is effective in reducing tumor growth, its use is limited by severe side effects that include painful peripheral neuropathy. The incidence of sensory neuropathy in patients treated with cisplatin varies between 24% and 92% [3]. The neuropathy can lead to reduction in dose and discontinuation of treatment that, in turn, may affect overall survival. Chemical analyses of tissue from human patients treated with cisplatin show accumulation of platinum in dorsal root ganglia (DRGs) and peripheral nerves - neural tissues that are not protected by a blood-brain-barrier [4].

We developed a model of cisplatin-induced neuropathy in mice to study the interaction of endocannabinoids with the hyperalgesia that occurs as a consequence of chemotherapy. Mechanical hyperalgesia develops in mice treated with cisplatin (1 mg/kg) for 7 days [5], and the hyperalgesia is associated with a cellular marker for injury as well as other biochemical and functional changes in DRG neurons. In addition, cisplatin treatment reduces the amount of the endocannabinoid *N*-arachidonoyl ethanolamine [anandamide (AEA)] in plantar paw skin. AEA is a partial agonist at the cannabinoid type-1 (CB1) receptor [6], and CB1 receptors on nociceptors modulate the threshold for firing of these neurons in response to stimuli that elicit withdrawal reflexes [7] [8]. Thus, reduced activation of CB1 receptors on nociceptors by endogenous ligands promotes hyperalgesia, and CB1 receptor agonists suppress hyperalgesia in many models of pathophysiological pain (reviewed by Kress and Kuner [9]), including preclinical models of chemotherapy-induced peripheral neuropathy [10] as well as chemotherapy-induced pain in humans [11]. Moreover, inhibition of the enzyme that hydrolyzes AEA, fatty acid amide hydrolase (FAAH), increases the level of endogenous AEA and inhibits hyperalgesia in preclinical models of chemotherapy-induced peripheral neuropathy. Co-administration of FAAH inhibitors with cisplatin suppresses the mechanical hyperalgesia caused by cisplatin through a CB1 receptor-dependent mechanism [5,12]; CB2 receptors also contribute to the effect of FAAH inhibitors in cisplatin-treated rats [12].

A second endocannabinoid, 2-arachidonoyl-*sn*-glycerol (2-AG), is a full agonist at CB1 and CB2 receptors, but it is less potent than AEA at CB1 receptors [6]. Because tissue levels of 2-AG are generally more than 100 times greater than those of AEA, 2-AG may contribute to CB1 receptor tone (reviewed by Howlett et al. [13]). In contrast, the potency ratio of 2-AG:AEA is inverted at CB2 receptors such that 2-AG has greater potency [14]. Reports of CB2 receptor-mediated modulation of nociception in naïve subjects are variable and specific to a thermal stimulus. Increased sensitivity to radiant heat in CB2^{-/-} mice has been reported [15] and was consistent with an earlier observation that peripheral CB2 receptor activation reduced thermal sensitivity in rats [16]. However, in another report [17], no changes in sensitivity to cooling, radiant heat, or mechanical stimuli were noted in CB2^{-/-} mice. In contrast, CB2 receptor agonists are anti-hyperalgesic in preclinical models of inflammatory and neuropathic pain (reviewed by Jhaveri et al. [18]; Guindon and Hohmann [19]) where

effects may occur directly on DRG neurons following an induction of CB2 receptors on these cells or indirectly through CB2 receptors on immune cells in the periphery [16] or the central nervous system [17,20]. Several CB2 agonists are currently in clinical trials for treatment of pain [21].

Effects of CB2 receptor agonists are mimicked when degradation of endogenous 2-AG is reduced. Although 2-AG is hydrolyzed by multiple enzymes, monoacylglycerol lipase (MGL) accounts for 85% of the degradation of 2-AG in brain [22]. Thus, local administration of URB602 and JZL184, inhibitors of MGL, is also effective in suppressing thermal and mechanical hyperalgesia in models of inflammation and neuropathic pain through CB1 and CB2 receptor- dependent mechanisms [23,24,25,26,27]. Given the anti-hyperalgesic effect of 2-AG in conjunction with changes in CB2 receptor expression and 2-AG metabolism in pathophysiological conditions, we tested the efficacy of a low dose of the MGL inhibitor JZL184 in a murine model of cisplatin-induced peripheral neuropathy. Specifically, we determined that 1) repeated co-administration of a low dose of JZL184 with cisplatin suppressed mechanical hyperalgesia, 2) the anti-hyperalgesic effect of JZL184 was mediated by CB1 receptors, a bias that may be due to a down-regulation of CB2 receptors in skin and spinal cord, 3) cisplatin treatment reduced the level of 2-AG in plantar skin and DRGs, and 4) AEA most likely mediates the anti-hyperalgesic effect of JZL184 in this model.

2. METHODS

2.1 Animals

Adult, male C3H/HeN mice (25–32 g, National Cancer Institute) were used in these studies. All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.2 Drugs

The chemotherapeutic agent cisplatin [*cis*-diamminedichloroplatinum (II), Sigma-Aldrich, St. Louis, MO] was dissolved in sterile saline. Stock solutions of the CB1 receptor antagonist AM281 [1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide, 18 mM] and the CB2 receptor antagonist AM630 [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; 20 mM] were prepared in DMSO. AM281 has 300-fold greater affinity for the CB1 compared to the CB2 receptor [28]. AM630 has 165-fold greater affinity for CB2 compared to CB1 receptors [29]. For *in vivo* studies, stock solutions of 2-AG (10 µg/µl) and URB597 (cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester, Cayman Chemical, 5 µg/µl) were prepared in ethanol and were diluted to a final concentration in saline. JZL184 (Cayman Chemical, Ann Arbor, MI) was solubilized in DMSO (20 µg/µl) and was diluted to the final dose in a vehicle of DMSO:Tween-80:saline (12:1:87, v:v:v). All other drugs were diluted to the final dose in saline. For *in vitro* experiments, the stock solution of 2-AG (26 mM) was prepared in ethanol and the solution of JZL184 (24 mM) in methyl-2-pyrrolidone:ethanol (1:1, v:v). 2-AG, AM281, and AM630 were purchased from Tocris

(Ellisville, MO). The highest concentrations of organic solvents in a dose were used as vehicle controls.

2.3 Measure of mechanical sensitivity

Before inclusion in the study, daily baseline measurements of withdrawal frequency to a monofilament that delivered a force of 3.9 mN were obtained for all mice on 3 consecutive days. Mice were isolated under separate glass containers on a mesh platform and allowed to acclimate for at least 30 minutes prior to testing. The monofilament was applied to the plantar surface of each hind paw 10 times (1-2 sec each). The number of withdrawal responses was determined and expressed as a percentage of the number of stimuli applied (withdrawal frequency). These data were used to screen mice for hypersensitivity, and animals that exhibited baseline withdrawal frequencies $\geq 50\%$ were excluded from the study ($<0.5\%$).

The effect of repeated drug treatments on mechanical sensitivity over time was determined approximately 20 h after a drug injection. Similarly, the acute effect of a drug in cisplatin-treated mice was determined 20 h after the 7th dose of cisplatin. Mechanical hyperalgesia was defined as an increase in the frequency of paw withdrawal responses to a monofilament that delivered a force of 3.9 mN to the plantar surface of the hind paw. Withdrawal responses evoked by the monofilament were measured in each hind paw before and every 30 min after drug injection for 2 h. In all behavioral experiments the individual judging behavioral responses was blinded to the treatment of each subject.

The effects of JZL184 and URB597 on the mechanical sensitivity of the hind paws of naive mice were assessed with a repetitive stimulation paradigm [30]. Five von Frey monofilaments with ascending bending forces of 3.9, 5.9, 9.8, 13.7 and 19.6 mN were used. The frequency of withdrawal to each stimulus was determined in the hind paw ipsilateral to the treatment before and 1 h after intraplantar (i.pl.) injection of JZL184, URB597, or vehicle (10 μ l volume). The doses tested were those that reduced mechanical hyperalgesia acutely in cisplatin-treated mice (JZL184: 10 μ g, present study; URB597: 9 μ g, [5]).

2.4 Measurement of 2-AG, AEA and PEA

In order to determine the effect of repeated cisplatin treatment on endocannabinoid levels, mice were euthanized by decapitation under isoflurane anesthesia approximately 24 h after the 7th dose of cisplatin alone, cisplatin+JZL184, or vehicle. Lumbar (L) DRGs L3-L5 as well as samples of plantar paw skin, lumbar spinal cord and midbrain were collected. Upon removal, samples were frozen in liquid nitrogen and remained frozen at -80°C until the time of processing as previously described [31]. During extraction of lipids, 5 pmol of deuterated (*d*)₈-AEA and 100 pmol of *d*₈-2-AG and *d*₈-palmitoylethanolamine (PEA, Cayman Chemical, Ann Arbor, Michigan) were added as internal standards. PEA is included in this report because it is another fatty acid ethanolamide that modulates transduction of noxious stimuli [32]. The total lipid weight was determined by weighing the vials containing the dried organic phases of each sample. These dried samples 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 column (0.5 $\times$ 150 mm)

was used. The amounts of 2-AG, AEA and PEA in unknown samples were estimated from the ratio of the area of the signals of deuterated and non-labeled 2-AG (0.2–2000 pmol), AEA (0.02–200 pmol), or PEA (0.2–2000 pmol) standards. Data are expressed as mol of 2-AG, AEA or PEA per g total lipid extracted from samples with the exception of DRGs (mol/sample). Insufficient recovery of a deuterated standard in one sample and an unusual amount of extracted lipid for one tissue sample resulted in amounts of endocannabinoids that were more than two standard deviations beyond the mean for the treatment group. These values were deleted from the data set for statistical analysis.

2.5 Quantification of mRNA by real time RT-PCR

Dorsal root ganglia L3–L5 and plantar skin samples were collected from mice following euthanasia, placed in RNeasy Lysis Buffer (Qiagen) and stored at 4°C. Total RNA was isolated from DRG samples using RNeasy Lipid Tissue Mini Kits (Qiagen) and from plantar paw skin using RNeasy Fibrous Tissue Mini Kits (Qiagen). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen) as per the manufacturer's instructions. Real-time PCR studies were performed with Dy-NAmo HS SYBR Green Master Mix (Finnzymes) using the DNA engine Opticon 2 (MJ Research) through 45 PCR cycles (94°C for 10 s, 57°C for 20 s, 72°C for 30 s). Each cDNA sample was run in triplicate for the murine MGL, FAAH, CB1 receptor, and the reference gene (S15). Primer pair sequences for CB1 receptor, FAAH and S15 were described previously [8]. The primer pair sequence for MGL (GenBank Accession number NM_011844.4) was forward primer 5'- CCT TGC CAA TCC GGA ATC TG -3' and reverse primer 5'- ACA GGT CAA CCT CCG ACT TG -3'. All primers were synthesized by Operon Biotechnologies. Specificity of each amplicon was confirmed by melting curve analysis, evidence of a single band after gel electrophoresis, authenticity of the DNA sequence of the band isolated from the gel, and resolution by BLAST analysis that the sequence of the amplicon was unique to each target. The ratio of fold change in expression of the mRNA of interest for each sample was calculated by normalization of cycle threshold (Ct) values to S15 using the equation derived by Pfaffl [33] to correct for potential differences in PCR primer efficiencies between the target and reference genes. This approach was validated by the lack of an effect of treatments on the expression of S15 and confirmation that data for tissue samples were within the linear range used to determine the efficiency of the primers. The efficiency of the primer set was derived from the slope of its linear graph using the following equation: $\text{efficiency} = 10^{(-1/\text{slope})}$. To control for variability in preparations and assays, data for mRNA (corrected to the reference gene) were normalized to the mean of the control group for each date of sample collection.

2.6 Western blot analysis of CB2 receptor protein

Samples of plantar paw skin, sciatic nerve and lumbar spinal cord were dissected from euthanized mice, frozen on dry ice, and stored at –80°C until time of processing. Samples of skin and nerve were pooled from both sides of one mouse. On the day of processing, samples were homogenized in a bullet blender 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 ×g for 10 min. The supernatant was concentrated using an Amicon Ultra-0.5 centrifugal filter (Millipore Corporation, Billerica, MA). Western blot analysis for

CB2 receptor protein was performed on 25 µg of protein/sample as previously described [34] using rabbit anti-CB2 receptor antibody (dilution 1:500, Cayman). Actin immunoreactivity (rabbit anti-actin antibody, 1:2000, Sigma) within each sample was then quantified as a loading control. The density of immunoreactive bands was quantified using ImageJ (version 1.48r 18, NIH). Data for CB2 immunoreactivity were normalized to the amount of actin immunoreactivity within each sample (i.e., density of CB2 /density of actin). The quality of the material used in the analysis is provided in a supplemental figure.

2.7 Primary cultures of DRG cells

Cultures were prepared from DRGs collected from all levels of the spinal cord as previously described [35]. Cells in the final suspension were plated at a density equivalent to ~3 DRGs/laminin-coated glass coverslip for biochemical studies. Biochemical studies were conducted 20-28 h later.

2.8 [³H]AEA uptake

The assay used on primary cultures of DRGs was reported previously by our laboratory [32]. At the start of the experiment, cultures were washed with HEPES buffer and 0.1% fatty acid free BSA for 10 min at 37 °C. The buffer was then replaced with [³H]AEA (10 µM; American Radiolabeled Chemicals) in HEPES buffer (500 µL) and incubated for 15 minutes at 37 °C. Transport was terminated by removal of extracellular [³H]AEA followed by 3 washes with ice-cold HEPES buffer, 1% BSA. Cells were lysed by incubation in 0.2 M NaOH (500 µL) for 10 min at 75°C. Aliquots (400 µL) were added to scintillation vials and the amount of tritium in each sample was measured by Liquid Scintillation spectroscopy.

2.9 [³H]AEA hydrolysis

The effect of JZL184 on hydrolysis of [³H]AEA was determined on primary cultures of DRGs and homogenates of mouse brain (10 µg protein/sample) as described previously for our laboratory [8] [32]. URB597 was included in the assay as a positive control. DRG samples were incubated for 30 min at 37°C with [³H]AEA (0.5 nM), brain homogenates were incubated for 10 min. These incubation times are within the linear response range for these samples. AEA hydrolysis was expressed as a percentage of conversion of total [³H]AEA to ethanolamine.

2.10 Statistical Analyses

Data are reported as the mean±SEM when normally distributed. For these data, differences between treatments were determined using Student's *t* test or one way analysis of variance with Bonferroni *t* test to determine differences between groups. Differences in behavior among multiple treatment groups over time were determined using two way analysis of variance for repeated measures with Bonferroni *t* test to determine differences between groups at specific time points. Data for concentration response were analyzed using Prism software (GraphPad Software, Inc., La Jolla, CA) to determine an ED₅₀. Data that did not meet the criterion of normal distribution are reported as the median [25th -75th percentile], and effects of treatments were analyzed using the Kruskal-Wallis one way analysis of

variance by ranks followed by Dunn's test to determine differences between groups. A significance level of 0.05 was the threshold for stating a treatment effect in all analyses.

3. RESULTS

3.1. Daily treatment with JZL184 blocked development of mechanical hyperalgesia in cisplatin-treated mice

Consistent with previous results [5], the frequency of withdrawal to a force of 3.9 mN applied to the plantar surface of one hind paw increased to approximately 70% in mice treated with cisplatin (1 mg/kg, i.p.) for 7 days (**Fig. 1**; $F_{(3; 72)} = 3.72$, $p=0.005$, 2-way ANOVA). Data are reported as the average of the hind paws because there was no difference in their sensitivity to the mechanical stimulus ($p=0.86$, Paired t test, $n=10$). Daily administration of JZL184 (10 $\mu\text{g}/10\text{ }\mu\text{l}$, s.c., left dorsal hind paw) with cisplatin blocked the occurrence of hyperalgesia in both hind paws (**Fig. 1A**). This dose of JZL184 was chosen because it was the optimal dose for acute suppression of mechanical hyperalgesia in a murine model of tumor-evoked hyperalgesia [34]. Because there was no difference in the mechanical sensitivity of the hind paws following daily unilateral injection of JZL184 ($p=0.98$, $n=14$, Paired t test), data related to repeated JZL184 injections are reported as the average response of the hind paws for each mouse. After the 5th injection of JZL184, the anti-hyperalgesic effect of the drug persisted through 20 h post drug administration. An effect of JZL184 contralateral to the injection site indicates that the drug had a systemic action. Therefore, when CB1 and CB2 receptor antagonists were administered to determine which cannabinoid receptor mediated the effect of JZL184, the drugs were administered by intraperitoneal injection 20 h after the 7th injections of cisplatin and JZL184.

Administration of the CB1 receptor antagonist AM281 (0.33 mg/kg, i.p.) transiently reversed the anti-hyperalgesic effect of JZL184, but the CB2 receptor antagonist AM630 (1 mg/kg, i.p.) had no apparent effect (**Fig. 1B**). Together these data suggest that daily subcutaneous injections of JZL184 had a therapeutic anti-hyperalgesic effect that was mediated by CB1 receptors. Moreover, evidence that the CB1 receptor antagonist acutely reversed the anti-hyperalgesic effect of JZL184 indicates that repeated administration of JZL184 did not block expression of underlying factors responsible for mechanical hyperalgesia.

3.2. Acute treatment with 2-AG mimicked the effect of JZL184

Because JZL184 is an inhibitor of MGL, we predicted that local injection of 2-AG would mimic the effect of JZL184. Considering that 2-AG is rapidly metabolized, we compared the acute effect of JZL184 and 2-AG in cisplatin-treated mice. Consistent with the previous results, the average frequency of withdrawal to the 3.9 mN force was approximately 70% 20 h after the 7th dose of cisplatin (baseline, BL, **Fig. 2A, B**). Intraplantar injection of JZL184 (10 $\mu\text{g}/10\mu\text{l}$) transiently reduced the level of mechanical hyperalgesia by approximately 50% in the hind paw ipsilateral to the injection site (**Fig. 2A**), but there was no effect in the contralateral hind paw ($F_{(4,24)} = 1.15$; $P = 0.141$, $n=6$, One-way ANOVA RM). Injection of 2-AG (18 $\mu\text{g}/10\mu\text{l}$, i.pl.) mimicked the effect of JZL184 (**Fig., 2B**), including the absence of an effect in the contralateral hind paw ($F_{(4,30)} = 1.64$; $P = 0.0.19$, $n=7$, One-way ANOVA

RM). When either AM281 (10 µg/10µl, i.pl.) or AM630 (4 µg/10µl, i.pl.) was co-injected with JZL184 and 2-AG, only AM281 blocked the anti-hyperalgesic effect of each drug. Although smaller in magnitude and duration of effect, the data for acute treatment with JZL184 parallel those of chronic treatment with the drug. The parallels in data for 2-AG and JZL184 support the conclusion that 2-AG contributes to the anti-hyperalgesic effect of JZL184, however, anti-hyperalgesic activity of 2-AG at peripheral CB1 receptors is unusual in our experience (e.g., [34]). Indeed, although JZL184 was anti-hyperalgesic in cisplatin-treated mice, it had no effect in naive mice (**Fig. 3A**). In contrast, local inhibition of fatty acid amide hydrolase with URB597 (9 µg/10µl, i.pl.) is analgesic in naive mice (**Fig. 3B**). This dose of URB597 was chosen because it is anti-hyperalgesic in cisplatin-treated mice through a CB1 receptor-dependent mechanism [5].

3.3. The effect of cisplatin on endocannabinoid signaling varied by tissue

The levels of 2-AG and anandamide (AEA) in skin, DRGs, spinal cord and midbrain were quantified in order to develop insights into the biochemistry underlying the interaction of JZL184 with cisplatin-induced hyperalgesia. These tissues were selected because they are sites relevant to initiation of somatosensation (peripheral terminal and soma of somatosensory neurons) as well as ascending transmission and descending modulation of nociceptive processing (spinal cord and midbrain). Repeated injections of cisplatin resulted in persistent decreases in the level of 2-AG in skin and DRGs and an increase in the level of 2-AG in the spinal cord (**Table 1**). Parallel changes occurred in the levels of AEA. In contrast, the level of neither endocannabinoid in the midbrain was affected by repeated cisplatin treatment. When mice were given repeated JZL184 injections (s.c., dorsal hind paw) with cisplatin, the only difference from cisplatin alone was an increase in 2-AG in DRGs. This result confirms the efficacy of the treatment with JZL184 and supports the conclusion that activity at the level of the sensory neurons underlies the anti-hyperalgesic effect of the drug *in vivo*. The changes in skin and DRGs are most likely due to the treatments and not differences in the handling of samples because the level of PEA did not vary with treatment in these tissues. The midbrain also served as a negative control because the drug treatments had no effect on the levels of the endocannabinoids and PEA in this tissue.

To gain a broader perspective for the effect of repeated cisplatin treatment on endocannabinoid signaling, we extended our biochemical analyses to measures of mRNA for MGL and FAAH. The expression of CB1 receptors was inferred from the level of mRNA because we have not been able to validate an antibody that recognizes CB1 receptor protein in murine somatosensory neurons. Conversely, CB2 receptor expression was measured by western blot using an antibody validated in earlier studies [34]; we have not been able to validate CB2 receptor mRNA primers in murine DRGs. Daily treatment with cisplatin for 7 days increased the amount of mRNA for MGL in the skin almost 2-fold, while a smaller increase occurred in mRNA for FAAH (**Table 2**). Increased expression of MGL and FAAH in the skin is consistent with decreased levels of 2-AG and AEA in the same region (Table 1). However, the levels of mRNA for these enzymes decreased in DRGs where cisplatin also decreased levels of 2-AG and AEA. Repeated cisplatin treatment did not affect the amount of CB1 receptor mRNA in either plantar paw skin or DRGs. However,

the level of CB2 receptor protein was reduced more than 75% in plantar paw skin from cisplatin-treated mice compared to control samples. A similar decrease occurred in spinal cord samples from cisplatin-treated mice (vehicle: 1.11 ± 0.43 , cisplatin: 0.20 ± 0.13 , $p < 0.05$, $n = 4$, Student's t test on values in $\log_{10}$). CB2 receptor protein was at the limit of detection in sciatic nerve and, therefore, not quantified. The decrease in amount of CB2 receptor protein may account for the absence of an interaction of AM630 with the anti-hyperalgesic effect of JZL184.

3.4. AEA mediates the effect of 2-AG and JZL184 in cisplatin-treated mice

Given that local injection of neither JZL184 nor a CB2 receptor antagonist (AM630; [34]) modulated mechanical sensitivity in naive mice, we tested whether 2-AG can have an indirect effect on DRGs by competing for uptake of AEA, thereby increasing the availability of AEA for activation of CB1 receptors. 2-AG inhibited the uptake of [3 H]AEA by primary cultures of dissociated DRGs with an EC_{50} of $\sim 30 \mu\text{M}$ (Fig. 4A). Similarly, pretreatment with JZL184 ($1 \mu\text{M}$) decreased [3 H]AEA uptake in a time-dependent manner (Fig. 4B), with a significant effect observed at 30 min. This concentration of JZL184 reduced AEA hydrolysis in primary cultures of DRGs by 75% over 30 min and had a greater effect on activity in mouse brain homogenate (Table 3). Together these data support the conclusion that the anti-hyperalgesic effect of JZL184 in cisplatin-treated mice was most likely mediated by AEA activation of CB1 receptors.

4. Discussion

Repeated treatment of mice with cisplatin changed multiple elements of endocannabinoid signaling in tissues related to transduction and transmission of nociception. Decreased activation of CB1 receptors on somatosensory neurons most likely contributes to the mechanical hyperalgesia precipitated by cisplatin, and co-administration of a low dose of JZL184 with cisplatin suppressed mechanical hyperalgesia. Evidence that a CB1 receptor antagonist was more effective than a CB2 receptor antagonist in reversing the anti-hyperalgesic effect of JZL184 and that CB2 receptor protein decreased in plantar skin of cisplatin-treated mice support the conclusion that CB1 receptors mediate the observed JZL184 suppression of mechanical hyperalgesia in cisplatin-treated mice. Moreover, evidence that JZL184 inhibited FAAH, and that 2-AG and JZL184 reduced AEA uptake by primary cultures of DRGs support the conclusion that AEA mediates the activation of CB1 receptors on nociceptors.

4.1 Cisplatin treatment altered elements of endocannabinoid signaling

This report extends our measures of endocannabinoid signaling in tissues of cisplatin-treated mice [5] and complements an earlier report on cisplatin-treated rats [12]. The cumulative dose in both rodent models was comparable (7 mg/kg over 1 week in mice; 9 mg/kg over 3 weeks in rats). In both species, treatment with cisplatin decreased the level of 2-AG in the paw skin and DRGs and increased the level of 2-AG in the spinal cord. An increase in expression of MGL in skin may contribute to the decrease in 2-AG in that tissue. Given the duration of the cisplatin treatment, the decrease in MGL mRNA in DRGs may be a feedback response to the decrease in 2-AG in DRGs or a direct effect of cisplatin on that tissue. In

addition to 2-AG we noted a decrease in the level of AEA in both plantar paw skin and DRGs of cisplatin-treated mice, replicating our initial report for the effect of cisplatin on AEA in skin [5]. Consistent with the report in rats, we observed a 2-fold increase of AEA in the spinal cord. The changes in FAAH mRNA in skin and DRGs paralleled those of MGL. Because the disposition of cisplatin is limited to peripheral tissues [4], the increases in 2-AG and AEA levels in the spinal cord are most likely compensatory responses to increased nociceptive processing. Similar effects of cisplatin in the two rodent species add confidence to conclusions about the underlying pathophysiology of the drug, however it is difficult to interpret differences in the data between the two species. It is noteworthy that the differences in data are more frequently “no change” in the rat compared to “decrease” in mouse which may reflect differences between the species in the magnitude of changes as well as differences in normalization of data. Importantly, the 2-fold increase in MGL mRNA in paw supports the expectation of active enzyme for inhibition by JZL184

4.2 JZL184 blocked mechanical hyperalgesia in cisplatin-treated mice

Co-administration of a low dose of JZL184 (equivalent to 0.33 mg/kg) into one hind paw in mice suppressed the mechanical hyperalgesia that developed with daily injections of cisplatin. Daily administration of JZL184 over 1 week resulted in a systemic effect, in that mechanical hyperalgesia was also suppressed in the hind paw contralateral to the injection. In contrast, acute administration of the same dose of JZL184 was restricted to an ipsilateral effect and was only half as effective as the 7 day treatment. Although JZL184 has an estimated half-life of 7 h, inhibition of MGL persists for 24 h [22]. Thus, it is likely that summation of enzyme inhibition contributed to the effect observed in the contralateral hind paw and greater efficacy observed with daily injections. The suppression of mechanical hyperalgesia in cisplatin-treated mice by a cumulative dose of JZL184 (< 2.3 mg/kg) approximates the dose of 8 mg/kg that was required to suppress mechanical allodynia in cisplatin-treated rats [12]. The dose, however, is 10-fold less than the dose used to suppress mechanical allodynia in a murine model of neuropathic pain [27] indicating efficacy of this drug with repeated administration at very low doses. Importantly, CB1 receptor expression and activity is not altered by daily administration of a dose of 4 mg/kg for six days [27]; a change that does occur at a 10-fold higher dose of JZL184 [36].

Cold allodynia is also reported by patients treated with platinum-based agents [37]. Hypersensitivity to cooling occurred in cisplatin-treated mice (25°-15° C, Uhelski and Simone, unpublished observation) but the effect of inhibitors of endocannabinoid metabolism on sensitivity to cooling has not been determined. JZL184 suppression cold allodynia (increased response to acetone) in cisplatin-treated rats was blocked by either a CB1 or CB2 receptor antagonist in parallel with mechanical allodynia [12], suggesting that the mechanisms underlying the anti-allodynic effects of JZL184 in cisplatin-treated rats are similar.

4.3 Endogenous 2-AG contributed to the effect of JZL184

Anatomical, biochemical and physiological evidence support the conclusion that 2-AG contributed to the anti-hyperalgesic effect of JZL184 in the present study. First, intraplantar injection of this dose of JZL184 acutely increased the level of 2-AG in paw skin 4-fold

without altering the level of AEA [34]. Second, intraplantar injection of 2-AG mimicked the acute effect of intraplantar injection of JZL184. Third, repeated injection of JZL184 normalized the level of 2-AG in DRGs of cisplatin treated mice. It is curious that the level of 2-AG in the skin was not increased in the same subjects, but it is possible that a large increase in MGL in skin masked an effect. Moreover, the absence of a measureable change does not exclude an effective change in the microenvironment of the nociceptor terminal. Coupling an increase in 2-AG in DRGs with the anti-hyperalgesic effect of JZL184 in the same animals from which samples were taken for biochemistry indicates that autocrine endocannabinoid signaling in DRG neurons mediates the effect of the drug. This conclusion is supported by evidence that MGL mRNA occurred in DRGs, and the protein co-localizes with calcitonin gene-related peptide and isolectin B4 in sensory neurons that project to nociceptive areas in the dorsal horn of the spinal cord [38].

In light of evidence that cisplatin enhances release of prostaglandin E2 (PGE2) from satellite cells of trigeminal ganglia *in vitro* [39], it is relevant to consider whether the anti-hyperalgesic effect of JZL184 was mediated by reducing levels of arachidonic acid formed by MGL hydrolysis of 2-AG [40]. Arachidonic acid is a substrate for prostaglandin synthesis, and activation of prostaglandin receptors on nociceptors facilitates hyperalgesia by increasing their excitability (reviewed by Petho and Reeh [41]). However, it is likely that the anti-hyperalgesic effect of JZL184 in cisplatin-treated mice is independent of decreased production of prostaglandins because a cannabinoid receptor antagonist blocked the effect of JZL184.

4.4 The anti-hyperalgesic effect of JZL184 was mediated by AEA activation of CB1 receptors

Whether JZL184 was given daily for 7 days or as a single dose, the CB1 receptor antagonist acutely reversed the anti-hyperalgesic effect of JZL184 in cisplatin-treated mice. The CB2 receptor antagonist had no effect on either repeated or acute administration of JZL184. This result differs from that observed in cisplatin-treated rats where CB1 and CB2 receptor antagonists each blocked the anti-allodynic effect of a 4-fold higher dose of JZL184 [12]. An underlying contribution of CB2 receptors to the results in rat is supported by evidence that the CB2 receptor agonist AM1241 was also anti-hyperalgesic in cisplatin-treated rats [42]. Thus, differences in the results with JZL184 may reflect differences in species, drug dosages and disposition, as well as the underlying pathophysiology. The level of CB2 receptors was decreased in skin and spinal cord in cisplatin-treated mice; a comparable measure was not made in cisplatin-treated rats [12]. It is noteworthy that there is considerable variability in the literature concerning whether CB1 or CB2 receptors mediate the effect of inhibitors of MGL. Anti-hyperalgesic effects mediated by activation of CB2 receptors are expected in pathophysiological models of pain that involve cells that express high levels of CB2 receptors, such as inflammation [23,25] and tumor cells [34,43]. However, in a murine model of neuropathic pain, the anti-hyperalgesic effect of JZL184 is due to CB1 receptors [26,44], suggesting that the underlying pathophysiology contributes significantly to the results. Therefore we explored cellular mechanisms that could account for the CB1 receptor-dependent effect of JZL184.

Inhibition of AEA uptake by DRG neurons suppresses hyperalgesia by increasing the availability of AEA for activation of CB1 receptors on nociceptors [32]. AEA uptake occurs along a concentration gradient determined largely by its hydrolysis and transport proteins that move AEA into and through the cytoplasm (reviewed by Fowler [45]). JZL184 was used at a concentration of 1 μ M in biochemical studies because an earlier report confirmed inhibition of MGL at this concentration with no effect on FAAH activity [22]. At this concentration, JZL184 reduced uptake of [3 H]AEA by DRG cells. Evidence that 2-AG mimicked this effect is consistent with the expectation that JZL184 inhibited the degradation of endogenous 2-AG. However, JZL184 also significantly reduced hydrolysis of AEA in brain by 90% at this concentration; a much greater effect than expected given its reported FAAH IC₅₀ of 4 μ M [22]. Whether the effect of JZL184 on AEA hydrolysis is due to inhibition of FAAH or a variant expressed in this strain of mice is unclear. Regardless, suppression of AEA uptake and its activation of CB1 receptors on nociceptors can account for the pharmacology of JZL184 observed *in vivo* in cisplatin-treated mice where we have already demonstrated the efficacy of a FAAH inhibitor [5].

4.5 Substrates of MGL do not modulate the threshold for mechanical nociception

The intraplantar dose of JZL184 that reduced mechanical hyperalgesia in cisplatin- and tumor-evoked [34] models of pain had no effect on mechanical sensitivity in naive mice indicating that substrates for monoglycerol lipase in the periphery do not suppress the transduction of noxious mechanical stimuli in naive mice. This observation is consistent with an earlier report using a larger systemic dose in naïve rats (8 mg/kg, [12]). In contrast, JZL184 produced mechanical and thermal analgesia in mice with a much higher dose (40 mg/kg, [36]). Evidence that this high dose increased 2-AG in brain suggests the analgesic effect in this instance was mediated by central mechanisms. The intraplantar dose of JZL184 used in the present study increased the local level of 2-AG [34], and 2-AG is a full-agonist at CB2 receptors [46]. Evidence that neither systemic injection of a low dose of the CB2 agonist AM1241 [47] nor intraplantar injection of a CB2 receptor antagonist [34] affected mechanical sensitivity in naive mice adds further support to the conclusion that CB2 receptors do not modulate the threshold for mechanical nociception under normal conditions. However, evidence that systemic administration of AM1241 attenuates thermal nociception [15,47] indicates that CB2 receptors selectively modulate the threshold for thermal nociception.

5. CONCLUSIONS

Co-administration of a low dose of JZL184 inhibited mechanical allodynia in a murine model of cisplatin-induced peripheral neuropathy through a CB1 receptor-dependent mechanism. JZL184 did not appear to be neuroprotective because hyperalgesia was precipitated acutely by systemic injection of a CB1 receptor antagonist. AEA activation of CB1 receptors is the downstream effect of JZL184 inhibition of MGL and FAAH. Although the level of CB2 receptors in the periphery was decreased in cisplatin-treated mice, the data do not exclude the possibility of CB2 receptor modulation of thermosensation because that modality was not tested in the present experiments. In addition, while peripheral targets are sufficient to account for the effect of a low dose of JZL184, repeated administration of

higher doses of the drug may engage targets in the central nervous system that suppress nociception.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AEA	anandamide
2-AG	2-arachidonoyl- <i>sn</i> -glycerol
CB	Cannabinoid
d	deuterated
DRG	dorsal root ganglion
EC₅₀	concentration of drug that evokes 50% of a maximum response
FAAH	fatty acid amide hydrolase
IC₅₀	concentration of drug that reduces the standard response by 50%
L	lumbar
MGL	monoacylglycerol lipase
i.pl.	intraplantar
PEA	palmitoylethanolamide

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Figure 1.

JZL184 attenuated development of cisplatin-induced hyperalgesia in a CB1 receptor-dependent manner. Data are the mean $\pm$ SEM of the average withdrawal frequency of the hind paws to a mechanical stimulus (3.9 mN, see text for details). **A.** The baseline (BL) measures of withdrawal frequency were made prior to drug treatment. Mechanical hyperalgesia developed in mice during treatment with cisplatin (1mg/kg of body weight, daily for 7 d, i.p., n=10). There was no change in the mechanical sensitivity of vehicle-treated mice during this period [n=6; vehicle was DMSO:Tween-80:saline,12:1:87 (v:v:v)].

Co-administration of JZL184 (10 µg, s.c., dorsal hind paw; n=14) with cisplatin attenuated the development of mechanical hyperalgesia *Different from vehicle at $p<0.05$, #different from cisplatin-treated mice at $p<0.05$ (Two-way ANOVA with Bonferroni test). **B.** The anti-hyperalgesic effect of JZL184 was mediated by CB1 receptors. AM281 (0.4mg/kg, i.p.) blocked the effect of JZL184, but AM630 (1mg/kg, i.p.) did not. *Significantly different from vehicle-treated group at $p<0.02$, Two-way ANOVA with Bonferroni t test, N=7-9 mice/group.

Figure 2.

Local increases in 2-AG signaling attenuated the mechanical hyperalgesia produced by cisplatin. Data are the mean $\pm$ SEM of the average withdrawal frequency of the hind paws to a mechanical stimulus (3.9 mN, see text for details). A single injection of JZL184 (10 μ g, i.pl., **A**) or 2-AG (18 μ g, i.pl., **B**) into the hind paw transiently attenuated the cisplatin-evoked hyperalgesia ipsilateral, but not contralateral (CL) to drug injection. The anti-hyperalgesic effect of each drug was blocked by the CB1 receptor antagonist, AM281 (10 μ g, i.pl.) but not the CB2 receptor antagonist AM630 (4 μ g, i.pl.). *Significantly different from BL at $p<0.05$, Two-way ANOVA with Bonferroni t test, N=6-7 mice/group, #Significantly different from JZL184 or 2-AG-treated groups at $p<0.05$, Two-way ANOVA with Bonferroni test, N=6-7 mice/group.

Figure 3.

Effect of enzyme inhibitors in naive mice. Data are the mean $\pm$ SEM of the withdrawal frequency of the hind paw ipsilateral to the injection to a mechanical stimulus (3.9 mN). **A.** The dose of JZL184 (10 μ g, i.pl.) that suppressed hyperalgesia in cisplatin-treated mice at 1 h following administration had no effect on mechanical sensitivity in naive mice ($p=0.54$, two-way ANOVA, $n=5$ mice/group). The vehicle was DMSO:Tween-80:saline, 12:1:87 (v:v:v). **B.** In contrast, the FAAH inhibitor URB597 (9 μ g, i.pl.) decreased mechanical sensitivity in naive mice at 1 h following injection. *Different from vehicle (18% ethanol in saline) at $p<0.001$ (Two-way ANOVA, $n=5$ mice/group).

Figure 4.

Activation of 2-AG signaling reduced [^3H]AEA uptake by DRG cells. **A.** Co-incubation of [^3H]AEA with 2-AG dose-dependently decreased uptake of [^3H]AEA. V=vehicle (0.4% ethanol in HEPES buffer). **B.** Pretreatment with JZL184 (1 μM) decreased uptake of [^3H]AEA in a time-dependent manner. The open circle represents the vehicle control for the 60 min pretreatment. V=vehicle (0.0025% DMSO in HEPES buffer). Data represent the mean $\pm$ SEM of values generated in 3 independent experiments. *Different from control within each experiment at $p<0.05$; **different at $p<0.001$ (One-way ANOVA with Bonferroni t test).

Table 1

Levels of endocannabinoids in tissues of cisplatin-treated mice

Tissue	Treatment	2AG	AEA	PEA
Skin	<i>units</i>	<i>nmol/g lipid</i>	<i>pmol/g lipid</i>	<i>nmol/g lipid</i>
	Vehicle	12.6 ± 0.8 (5)	140 ± 16 (5)	49.6 ± 4.1 (5)
	Cisplatin	6.6 ± 0.8 (5) *	81 ± 9 (5) *	50.8 ± 9.8 (5)
	Cisplatin+JZL184	7.3 ± 1.4 (5) *	73 ± 17 (5) *	33.0 ± 1.9 (5)
DRGs	<i>units</i>	<i>pmol/sample^a</i>	<i>pmol/sample^a</i>	<i>pmol/sample</i>
	Vehicle	76 [69-92] (6)	1.12 [0.87-1.77] (6)	76 ± 9 (6)
	Cisplatin	53 [46-57] (5) #	0.45 [0.42-0.53] (5) #	94 ± 11 (5)
	Cisplatin+JZL184	83 [60-145] (5) ###	0.49 [0.41-0.54] (5) #	107 ± 10 (5)
Spinal Cord	<i>units</i>	<i>nmol/g lipid</i>	<i>nmol/g lipid</i>	<i>nmol/g lipid</i>
	Vehicle	72 ± 7 (6)	0.436 ± 0.048 (6)	33.0 ± 3.9 (6)
	Cisplatin	217 ± 32 (5) *	0.807 ± 0.052 (5) *	95.7 ± 13.1 (5) *
	Cisplatin+JZL184	201 ± 30 (5) *	0.978 ± 0.061 (5) *	78.8 ± 12.5 (5) *
Midbrain	<i>units</i>	<i>nmol/g lipid</i>	<i>nmol/g lipid</i>	<i>nmol/g lipid</i>
	Vehicle	408 ± 66 (6)	1.14 ± 0.11 (6)	68.1 ± 2.1 (6)
	Cisplatin	401 ± 54 (5)	1.04 ± 0.05 (5)	66.7 ± 6.8 (5)
	Cisplatin+JZL184	636 ± 222 (5)	0.72 ± 0.16 (4)	70.2 ± 8.5 (5)

Mice were treated with saline or cisplatin (1 mg/kg) for 7 days prior to tissue collection. Data represent the mean ± SEM unless otherwise noted (^a).

^a Data represent the median [25th-75th percentile range]. Numbers in parentheses indicate the sample size.

* different from vehicle within the same lipid at p<0.05, one-way ANOVA with Bonferroni *t*-test

different from vehicle

different from cisplatin treatment within the same lipid at p<0.05, Kruskal-Wallis One Way ANOVA on ranks with Dunn's test.

Table 2

Effect of cisplatin treatment on levels of mRNA and protein related to 2-AG and AEA signaling.

	Skin				DRG			
	Vehicle	(n)	Cisplatin	(n)	Vehicle	(n)	Cisplatin	(n)
mRNA								
MGL	100 ± 18	(8)	190 ± 24*	(14)	100 ± 6	(8)	50 ± 10.4**	(14)
FAAH	100 ± 18	(8)	100 ± 15	(14)	100 ± 8	(8)	71 ± 5.6**	(14)
CB1	100 ± 20	(4)	124 ± 34*	(7)	100 ± 5	(8)	99 ± 7.4	(14)
Protein								
CB2	0.424 ± 0.126	(6)	0.100 ± 0.05*	(7)	n.d.		n.d.	

Mice were treated with cisplatin (1 mg/kg) or saline for 7 days. DRGs L3-L5 and plantar hind paw skin from both sides of one mouse were pooled to make one sample of each tissue type at the time of euthanasia. Data represent the mean ± SEM. mRNA for CB1 receptors in paw skin was measured only in a subset of mice.

#Different from vehicle at p<0.05

n.d.=not done.

** different from vehicle at p<0.01 (Student's *t* test)

Table 3

Effect of JZL184 on FAAH activity.

	DRG	Brain
Vehicle	5.6 ± 0.4 (6)	32.5 ± 1.1 (6)
JZL184	1.4 ± 0.6 * (6)	3.3 ± 0.9 * (6)
URB597	< 0 (6)	< 0 (5)

FAAH activity was measured in primary cultures of DRGs maintained *in vitro* for 24 h from 2 independent experiments (~3 DRGs/sample). Vehicle was 0.5% DMSO. Data are the mean ± SEM of the % conversion of [³H]AEA in each sample. See methods for additional details.

* Different from vehicle at $p < 0.001$ (one-way ANOVA with Bonferroni t test).