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RESEARCH PAPER

Combined inhibition of monoacylglycerol lipase and cyclooxygenases synergistically reduces neuropathic pain in mice

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BACKGROUND AND PURPOSE

Neuropathic pain is commonly treated with GABA analogues, steroids or non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit one or more COX isozymes but chronic COX inhibition paradoxically increases gastrointestinal inflammation and risk of unwanted cardiovascular events. The cannabinoids also have analgesic and anti-inflammatory properties and reduce neuropathic pain in animal models. The present study investigated the analgesic effects of inhibiting both monoacylglycerol lipase (MAGL) and COX enzymes, using low doses of both inhibitors.

EXPERIMENTAL APPROACH

Mice subjected to chronic constriction injury (CCI) were tested for mechanical and cold allodynia after administration of the MAGL inhibitor, JZL184, or the non-selective COX inhibitor diclofenac. Then, both drugs were co-administered at fixed dose proportions of 1:3, 1:1 and 3:1, based on their ED_{50} values. PGs, endocannabinoids and related lipids were quantified in lumbar spinal cord.

KEY RESULTS

Combining low doses of JZL184 and diclofenac synergistically attenuated mechanical allodynia and additively reduced cold allodynia. The cannabinoid CB_1 receptor antagonist, rimonabant, but not the CB_2 receptor antagonist, SR144528, blocked the analgesic effects of the JZL184 and diclofenac combination on mechanical allodynia, implying that CB_1 receptors were primarily responsible for the anti-allodynia. Diclofenac alone and with JZL184 significantly reduced PGE_2 and $PGF_{2\alpha}$ in lumbar spinal cord tissue, whereas JZL184 alone caused significant increases in the endocannabinoid metabolite, *N*-arachidonoyl glycine.

CONCLUSIONS AND IMPLICATIONS

Combining COX and MAGL inhibition is a promising therapeutic approach for reducing neuropathic pain with minimal side effects.

Abbreviations

2-AG, 2-arachidonoylglycerol; anandamide, *N*-arachidonoylethanolamine; CCI, chronic constriction injury; JZL184, 4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate; MAGL, monoacylglycerol lipase; KML29, 1,1,1,3,3,3-hexafluoropropan-2-yl 4-(*bis*(benzo[d][1,3]dioxol-5-yl)(hydroxy)methyl)piperidine-1-carboxylate; PF-3845, N-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide; SR144528, N-[(1S)-endo-1,3,3,-trimethylbicyclo(2.2.1)heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; THC, Δ^9-tetrahydrocannabinol

Tables of Links

TARGETS
Enzymes[a]
MAGL, monoacylglycerol lipase;
COX, cyclooxygenase
FAAH, fatty acid amide hydrolase
GPCRs[b]
CB_1 receptor
CB_2 receptor
Ion channels[c]
TRPA1 channels
TRPM8 channels

LIGANDS
2-AG, 2-arachidonoylglycerol
Anandamide, *N*-arachidonoylethanolamine
Diclofenac
Gabapentin
JZL184
NAGly, N-arachidonoylglycine
PF-3845
PGE_2
$PGF_{2\alpha}$
Rimonabant
SR144528
THC, Δ9-tetrahydrocannabinol

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Neuropathic pain decreases quality of life and is co-morbid with decreased physical function as well as depression, anxiety, sleep disturbances and social withdrawal (Jensen *et al.*, 2007; Sofaer-Bennett *et al.*, 2007). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used analgesics that decrease pain and inflammation by inhibiting COX enzyme activity, thus blocking PG synthesis. PGs are lipids that are produced throughout the body and modulate physiological processes including inflammation. COX-1 is the constitutive isoform, provides homeostatic functions and regulates the gastric mucosa (Rouzer and Marnett, 2009), whereas COX-2 is induced by tissue injury and is involved in regulating inflammation (Viegas *et al.*, 2011). Although inhibiting COX-1 has acute anti-inflammatory effects, it paradoxically causes gastrointestinal inflammation by altering regulatory PG levels. For example, causing gastric haemorrhages by one or more mechanisms can result in hindered gastric endothelial cell renewal and delayed healing of the stomach (Musumba *et al.*, 2009). The severity of side effects increases with age and can be predicted to some degree by the patient's medical history (Raffa, 2001).

Given the intractable nature of neuropathic pain and the side effects of current treatments, some patients self-medicate with cannabinoids (Trafton *et al.*, 2004). In order to avoid the cognitive side effects of exogenous cannabinoids and also to better understand the biology underlying cannabinoid-mediated analgesia, current research focuses on the endogenous cannabinoid or endocannabinoid system. The levels of the endocannabinoids are tightly regulated *in vivo* by synthetic and metabolic enzymes. Monoacylglycerol lipase (MAGL) (Blankman *et al.*, 2007) and fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996) are catabolic enzymes that inactivate the two major endocannabinoids, 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamine (anandamide) respectively. Arachidonic acid is a primary metabolite of both 2-AG (with glycerol) and anandamide (with ethanolamine), although other bioactive metabolites, such as *N*-arachidonoyl glycine (NAGly), have also been shown to be formed from anandamide (Bradshaw *et al.*, 2009). Exogenous administration of the endocannabinoids is pharmacologically ineffective due to their rapid degradation by MAGL and FAAH *in vivo*. However, the genetic deletion or pharmacological inhibition of MAGL or FAAH increases brain levels of 2-AG or anandamide and causes analgesia (Lichtman *et al.*, 2004; Kinsey *et al.*, 2009; Long *et al.*, 2009).

Animal models of neuropathic pain are used to better understand the neurobiological aetiology of the disease states as well as possible therapeutic treatments. The best-characterized mouse model of neuropathic pain uses partial ligation of the sciatic nerve, chronic constriction injury (CCI). After CCI, immune cells are recruited to the injury site and glia in the dorsal root ganglia of the spinal cord ipsilateral to the injury and produce proinflammatory cytokines, which then activate and recruit additional immune cells to permeate the injured area (McMahon *et al.*, 2005). The inflammatory response initiated by CCI causes mechanical (i.e. noxious response to tactile stimuli) and cold allodynia (i.e. noxious response to innocuous, cold stimuli) in the paw ipsilateral to the nerve injury. In experimental animal models of pain, allodynia is operationally defined by various behavioural responses to paw stimulation that involve the mouse lifting its paw off the testing table. Similarly, patients suffering from neuropathic pain report an increase in touch and cold sensitivity, thus increasing the clinical relevance of CCI as an experimental animal model of neuropathic pain (Attal *et al.*, 2006).

As detailed earlier, manipulation of the endocannabinoids *in vivo* is possible via selective inhibition of their catabolic enzymes. For example, JZL184 and PF-3845 are syn-

thetic compounds that inhibit MAGL and FAAH respectively (Ahn *et al.*, 2009; Long *et al.*, 2009). MAGL or FAAH inhibition prevents the breakdown of 2-AG or anandamide, which increases brain levels of the corresponding endocannabinoid. MAGL or FAAH inhibition decreases mechanical and cold allodynia in mice subjected to CCI (Russo *et al.*, 2007; Kinsey *et al.*, 2009; 2011; Sasso *et al.*, 2012). Unlike the exogenous cannabinoid, Δ^9-tetrahydrocannabinol (THC), JZL184 and PF-3845 have little or no effect on locomotor activity, indicating that their analgesic effects are not due to sedation (Kinsey *et al.*, 2011).

In rats, CCI increases the expression of the cannabinoid CB_1 receptor in the spinal cord (Lim *et al.*, 2003), providing further support to the idea that pain modulation by endocannabinoids is a promising treatment of pain perception. Antagonists of CB_1 and CB_2 receptors reverse the anti-allodynic effects of FAAH inhibitors in the CCI model in mice (Russo *et al.*, 2007; La Rana *et al.*, 2008; Kinsey *et al.*, 2009). On the other hand, the anti-allodynic effects of JZL184 are blocked by the CB_1 receptor antagonist rimonabant (SR141716A) or genetic deletion, but not by CB_2 receptor antagonism or genetic deletion, indicating that MAGL inhibition reduces CCI-induced allodynia via a mechanism involving CB_1, but not CB_2, receptors (Kinsey *et al.*, 2009; 2010). Notably, JZL184 (≥4 mg·kg^{-1}) also increases whole brain levels of 2-AG after oral administration in mice (Long *et al.*, 2009), increasing the possible clinical relevance of MAGL inhibition.

Combining drugs with different mechanisms of action can produce greater pain attenuation than individually administered analgesics (Raffa, 2001). This dual administration approach may allow for sub-effective doses of each drug, thereby lessening the side effects of each compound while maintaining adequate pain relief (Guindon *et al.*, 2007). This type of broader coverage of multiple mechanisms is used successfully in the treatment of diseases such as HIV, diabetes and hypertension and, thus, pair dosing may also be effective for neuropathic pain treatments (Raffa, 2001; Tallarida, 2011). Previous research has evaluated synergy in the combined administration of NSAIDs and anandamide modulators. Co-administration of the NSAID diclofenac and the FAAH inhibitor URB597 in equipotent, low doses, synergistically attenuated acetic acid-induced stretching, an acute visceral pain model (Naidu *et al.*, 2009). Local administration of exogenous anandamide combined with a COX inhibitor (non-selective and COX-2 selective inhibitors were used) reduced thermal hyperalgesia and mechanical allodynia induced by partial sciatic nerve ligation in rats (Guindon and Beaulieu, 2006). The selective and peripherally restricted FAAH inhibitor, URB937, was combined with the NSAID indomethacin to synergistically reduce mechanical and thermal hyperalgesia as well as mechanical allodynia in mice (Sasso *et al.*, 2012). Although there is evidence that combined inhibition of FAAH and COX synergistically attenuates inflammatory and neuropathic pain, the analgesic effects of a similar combination of MAGL and COX inhibition have not been assessed.

The present study investigated the anti-allodynic effects of combined administration of the MAGL inhibitor JZL184 and the non-selective COX inhibitor diclofenac. To this end, a range of doses of either JZL184 or diclofenac were administered to mice with CCI, and then tested for mechanical and cold allodynia. Next, different ratios of both drugs were administered in order to test the hypothesis that coadministration of diclofenac and JZL184 enhanced the analgesic effects of either compound, attenuating CCI-induced mechanical and cold allodynia. Third, CB_1 and CB_2 receptor antagonists were administered to evaluate the necessity of either cannabinoid receptor for the observed synergy. Finally, we measured lumbar spinal cord levels of endocannabinoids, PGs and other fatty acid precursors to elucidate the mechanism(s) involved in the analgesic effect of combining JZL184 and diclofenac.

Methods

Animals

All animal care and experimental procedures were in accordance with ARRIVE guidelines and were approved by the Animal Care and Use Committee at West Virginia University. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 64 animals were used in the experiments described here. Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were approximately 20 weeks old at the start of the experiments. Mice were housed two to five per cage in a temperature- (20–22°C) and humidity-controlled environment with *ad libitum* access to food and water.

CCI

Surgery was performed as described previously (Russo *et al.*, 2007). Briefly, mice were anaesthetized with inhaled isoflurane (Phoenix Pharmaceuticals, Burlingame, CA, USA) in oxygen. The right hind leg was shaved and cleaned with three alternating wipes of Betadine solution followed by ethanol. An incision was made on the skin over the posterior femur. After separating the muscle, the sciatic nerve was isolated with forceps and partially ligated with one 5-0 silk suture that was knotted four times. The muscle and skin were then closed with two or more 6-0 nylon sutures. Mice recovered in clean, heated cages and were observed for absence of ataxia before being returned to the vivarium. Mice were treated with the NSAID ketoprofen (5 mg·kg^{-1}, i.p.) for 3 days as a post-operative analgesic.

Behaviour assessments

The mice were tested for allodynia starting 7 days after CCI surgery. The mice were brought into the testing room, weighed, injected, then placed inside ventilated polycarbonate chambers on an aluminium mesh table and allowed to acclimatise for 60 min before testing. Mice were injected with diclofenac or vehicle 60 min before testing. For experiments using JZL184 alone and in combination with diclofenac, mice were injected with drug or vehicle 120 min before testing (Long *et al.*, 2009). In the antagonist studies, rimonabant (3 mg·kg^{-1}), SR144528 (3 mg·kg^{-1}) or vehicle was given 15 min before administering the JZL184/diclofenac combination. The injured paw of the vehicle-treated mice acted as the control in that the ipsilateral paw responses from the drug

treatment groups were compared with the ipsilateral vehicle paw responses. Mice were randomly assigned, and the experimental assessments were made without knowledge of the treatments.

Mechanical allodynia test. Mechanical allodynia was tested using von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) using the 'up-down' method (Chaplan *et al.*, 1994; Kinsey *et al.*, 2009). The plantar surface of each hind paw was stimulated with each filament, ranging from a minimum of 0.16 g to a maximum 6.0 g, starting with the 0.6 g filament, five times at a frequency of ~2 Hz (i.e. two stimulations per second). The filaments were tested in ascending order until the mouse lifted its paw after three out of the five stimulations (this was considered to be a positive response). Once a positive response occurred, the filaments were tested in descending order until a positive response was not detected, thus establishing a sensory threshold.

Acetone-induced cold allodynia test. Immediately following the von Frey test (approximately 30 min from the start of von Frey testing), 10 µL of acetone (99% HPLC grade; Thermo Fisher Scientific, Waltham, MA, USA) was propelled via a 100 µL pipette (USA Scientific, Ocala, FL, USA) onto the plantar surface of each hind paw to test cold allodynia (Choi *et al.*, 1994; Decosterd and Woolf, 2000). Acetone was applied from below the testing table via air burst by 'expressing' the pipette, thereby avoiding mechanical stimulation of the paw with the pipette tip. Cold allodynia was operationally defined as total time lifting or clutching each hind paw, which included paw lifting when walking or grooming. A maximum cut-off time of 20 s was used (Decosterd and Woolf, 2000).

Lipid extraction

Lipid extractions and partial purification of the tissue was performed as previously described (Stuart *et al.*, 2013). In brief, frozen tissue was placed in 40:1 volumes of methanol and 100 pmol of deuterium-labelled PGE_2 and *N*-arachidonoyl glycine were added to each solution, as internal standards. The tubes were covered with parafilm and left on ice and in darkness for approximately 2 h. Remaining on ice, the samples were homogenized using a Polytron for approximately 1 min on each sample. The samples were centrifuged at 19 000× *g* at 24°C for 20 min. The supernatants were collected and placed in polypropylene tubes (15 or 50 mL) and HPLC-grade water was added making the final supernatant/water solution 25% organic. To isolate the compounds of interest partial purification of the 25% solution was performed on a Preppy apparatus (Sigma-Aldrich) assembled with 500 mg C18 solid phase extraction columns (Agilent Technologies, Santa Clara, CA, USA). The columns were conditioned with 5 mL of HPLC-grade methanol immediately followed by 2.5 mL of HPLC-grade water. The supernatant/water solution was then loaded onto the C18 column and washed with 2.5 mL of HPLC-grade water followed by 1.5 mL of 40% methanol. The PGs were collected with a 1.5 mL elution of 70% methanol, NAGly with a 1.5 mL elution of 85% methanol and the *N*-acyl ethanolamides with a 1.5 mL elution of 100% methanol. All were collected in individual autosampler vials and then stored in a –80°C freezer until analysis by mass spectrometry.

LC/MS/MS analysis and quantification. Samples were removed from the –80°C freezer and allowed to warm to room temperature (~15 min) then vortexed for approximately 1 min before being placed into the autosampler and held at 24°C (Agilent 1100 series autosampler, Palo Alto, CA, USA) for LC/MS/MS analysis. Twenty microlitre of eluants was injected separately for each sample to be rapidly separated using a C18 Zorbax reversed-phase analytical column (Agilent Technologies) to scan for individual compounds (mobile phase A: 20% HPLC methanol, 80% HPLC water, 1 mM ammonium acetate; mobile phase B: 100% HPLC methanol, 1 mM ammonium acetate). Gradient elution (200 $\mu L \cdot min^{-1}$) was carried out with the pressure created by two Shimadzu 10AdVP pumps (Columbia, MD, USA). Next, electrospray ionization was accomplished using an Applied Biosystems/MDS Sciex (Foster City, CA, USA) API3000 triple quadruple mass spectrometer. A multiple reaction monitoring (MRM) setting on the LC/MS/MS was used to analyse levels of each compound present in the sample injection. Synthetic standards were used to generate optimized MRM methods and standard curves for analysis.

The amount of analyte in each sample was calculated using a combination of calibration curves of the synthetic standards and percent recovery adjustments from the levels of deuterium-labelled internal standards obtained from the Analyst software (Applied Biosystems-MDS Sciex, Framingham, MA, USA). The standards provided a reference for the retention times by which the analytes could be compared. They also helped to identify the specific precursor ion and fragment ion for each analyte that enabled their isolation. These processes provide confidence in the claim that the compounds measured were, in fact, the compound of interest. The amount of each compound in each tissue was then converted to moles per gram tissue and statistically analysed. In the event that a sample had no chromatographic peak of an analyte (e.g. $PGF_{2\alpha}$) and was therefore below detection limit, a value of '0' was given in order to analyse any differences among groups.

Data analyses

The results of the dose–response curves of JZL184, diclofenac and the combination ratios are presented as means ± SEM. Pre- versus post-surgery paw sensitivity data were analysed using a two-way mixed design ANOVA (time point and paw as within subjects) followed by Bonferroni *post hoc* tests. Dose–response data were analysed using one-way ANOVA followed by Dunnett's *post hoc* test. The antagonist studies were analysed by two-way (combo vs. antagonist) between-subject ANOVA followed by Bonferroni *post hoc* tests.

For the mechanical allodynia assay, raw data from the paw threshold assays was expressed as percent maximum possible effect (%MPE) using the equation %MPE = (*Test Threshold/Max Threshold*) × 100, where *Max Threshold* was the assay's maximum filament (i.e. 6 g) and *Test Threshold* was the paw's established filament threshold. For the cold allodynia assay, raw seconds the paw was lifted was expressed as %MPE using the equation %MPE = [(*Max Cutoff* – *Test Time*)/(*Max Cutoff*)] × 100, where *Max Cutoff* was the assay's maximum cut-off point (i.e. 20 s) and *Test Time* was the time (s) the paw was lifted off the testing table. The ED_{50} values were calculated by interpolation when only two data points were available (one below and one above 50% MPE) or by standard

linear regression analysis when at least three data points were available on the linear portion of the dose–effect curve. To determine synergistic, additive or subadditive interactions, the theoretical additive ED_{50} value of the combined drugs was calculated from the individual dose–response curves. The combination is assumed to equal the sum of the effects of each drug.

For dose addition analysis (Tallarida, 2006; Naidu *et al.*, 2009), the ED_{50} of diclofenac was plotted on the abscissa (*x*-axis) and the isoeffective dose of JZL184 was plotted on the ordinate (*y*-axis). A line connecting the two points represents the theoretical additive effect of JZL184 and diclofenac dose combinations. The drug mixture ED_{50} value was determined by linear regression as the overall mixture dose (JZL184 and diclofenac sodium doses were summed). The experimentally derived ED_{50} values (Z_{mix}) from the dose–response curves of the ratios were compared with the predicted additive ED_{50} values (Z_{add}). If the ED_{50} values of the Z_{mix} are below those of Z_{add} and the confidence intervals (CI) do not overlap, then the interaction is considered synergistic. The difference between the theoretical additivity ED_{50} value and the experimental ED_{50} value was analysed using a Fisher's exact test.

Materials

Diclofenac sodium and gabapentin were purchased from Sigma-Aldrich (St. Louis, MO, USA). JZL184 was synthesized as described previously (Long *et al.*, 2009). KML29 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Rimonabant (SR141716; CB_1 receptor antagonist) and SR144528 (CB_2 receptor antagonist) were generously provided by the National Institute on Drug Abuse (Bethesda, MD, USA) Drug Supply Program. All compounds were dissolved in a vehicle consisting of ethanol, Cremophor (Sigma-Aldrich) and normal saline in a ratio of 1:1:18 parts (Pinto *et al.*, 2010). All solutions were warmed to room temperature and injected i.p. at a volume of 10 $\mu L \cdot g^{-1}$ body mass.

Results

CCI induces mechanical and cold allodynia

Mice were tested for allodynia, prior to and 1 week after CCI surgery. As reported previously (Benett and Xie, 1988; Starowicz *et al.*, 2012), CCI induced mechanical [(M = 0.93 SE = 0.138), $F(1,19) = 99.50$, $P < 0.01$] and cold allodynia [(M = 12.05 SE = 0.98), $F(1,19) = 50.91$, $P < 0.01$] (data not shown). *Post hoc* analyses revealed that this interaction was driven by paws ipsilateral to the nerve injury. CCI had no effect on paws contralateral to the nerve injury (mechanical allodynia $P = 0.56$, cold allodynia $P = 0.42$). The ipsilateral paws were also significantly different from contralateral paws (mechanical allodynia $P < 0.01$; cold allodynia $P < 0.01$) after the CCI surgery (data not shown).

Either JZL184 or diclofenac sodium attenuates allodynia

JZL184 or diclofenac given alone attenuated CCI-induced allodynia. Administration of the MAGL inhibitor JZL184 significantly reduced mechanical allodynia [$F(5,72) = 9.00$, $P < 0.01$; Figure 1A] and cold allodynia [$F(5,72) = 27.52$, $P < 0.01$; Figure 1B]. *Post hoc* analyses revealed that JZL184 significantly attenuated mechanical allodynia at $\geq 8\ mg \cdot kg^{-1}$ and cold allodynia at $\geq 4\ mg \cdot kg^{-1}$. Diclofenac also attenuated mechanical allodynia [$F(6,102) = 4.47$, $P < 0.01$; Figure 1A] and cold allodynia [$F(6,102) = 5.12$, $P < 0.01$; Figure 1B]. *Post hoc* analyses revealed that diclofenac significantly attenuated mechanical allodynia at $\geq 50\ mg \cdot kg^{-1}$ and cold allodynia at $\geq 75\ mg \cdot kg^{-1}$. In a separate group of mice with CCI, diclofenac (11 and 75 $mg \cdot kg^{-1}$, i.p.) or vehicle was administered either 1 or 2 h before testing, and no difference was found between pretreatment times for mechanical ($P = 0.61$; data not shown) or cold allodynia ($P = 0.16$; data not shown).

The ED_{50} for JZL184 was 8.04 $mg \cdot kg^{-1}$ (CL 95% = 4.49–14.4 $mg \cdot kg^{-1}$) for mechanical allodynia and 4.13 $mg \cdot kg^{-1}$ (CL

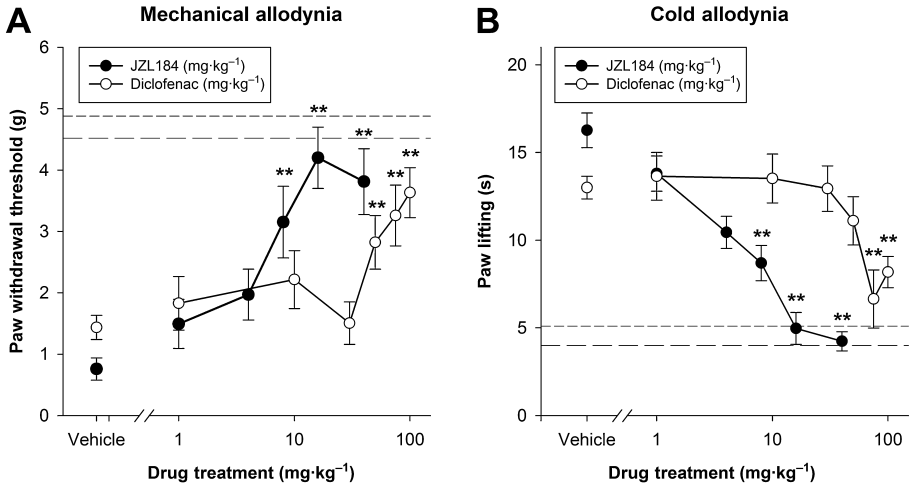

Figure 1

The MAGL inhibitor, JZL184, or the COX inhibitor, diclofenac, attenuated mechanical and cold allodynia induced by CCI of the sciatic nerve. Mice were subjected to CCI and then tested for mechanical (A) and acetone-induced cold allodynia (B). Data are expressed as mean ± SEM ($n = 10$–18). Mean contralateral paws for the JZL184-treated cohort (long dashed line) and diclofenac-treated cohort (short dashed line). $^{**}P < 0.01$ versus vehicle.

95% = 3.07–5.56 mg·kg^{-1}) for cold allodynia. The ED_{50} for diclofenac was 76.3 mg·kg^{-1} (CL 95% = 24.3–240 mg·kg^{-1}) for mechanical allodynia and 53.5 mg·kg^{-1} (CL 95% = 21.5–133 mg·kg^{-1}) for cold allodynia.

Augmented anti-allodynic effects of JZL184 and diclofenac sodium mixtures

Dose–effect correlations were determined for JZL184 and diclofenac mixtures using fixed-dose ratios of 1:3, 1:1 or 3:1 (Figure 2). The doses for each of these ratios were determined based on the respective ED_{50} value of either compound. To find the overall ED_{50} for JZL184, the ED_{50}s for mechanical and cold allodynia, 8.04 and 4.13 mg·kg^{-1}, respectively, were averaged (mean), resulting in an overall ED_{50} of 6 mg·kg^{-1}. For the overall ED_{50} of diclofenac, the ED_{50}s for mechanical and cold allodynia, 76.3 and 53.5 mg·kg^{-1}, respectively, were averaged, resulting in an overall ED_{50} of 65 mg·kg^{-1}. Using the overall ED_{50}s for JZL184 and diclofenac, the 1:1 ratio reflects one part JZL184 to 11 parts diclofenac, the 1:3 ratio reflects one part JZL184 to 33 parts diclofenac, and the 3:1 ratio reflects one part JZL to 3.7 parts diclofenac. The Z_{mix} in each ratio in the mechanical allodynia test was significantly less than the Z_{add} without CI overlap, indicating that the interaction was synergistic (Table 1). The Z_{mix} in each ratio in the cold allodynia test was less than the Z_{add}; however, there was some CI overlap, and thus the interaction was considered additive (Table 1).

The Z_{mix} results from the dose-response data for each ratio were plotted on an isobologram and compared with the predicted values (i.e. theoretical line of additivity) based on the individual ED_{50} values of either drug. Because the experimental points of the collective mechanical allodynia tests lie significantly below the line of additivity, the interaction was synergistic (Figure 3A). The experimental points of the collective cold allodynia tests do not differ significantly from the

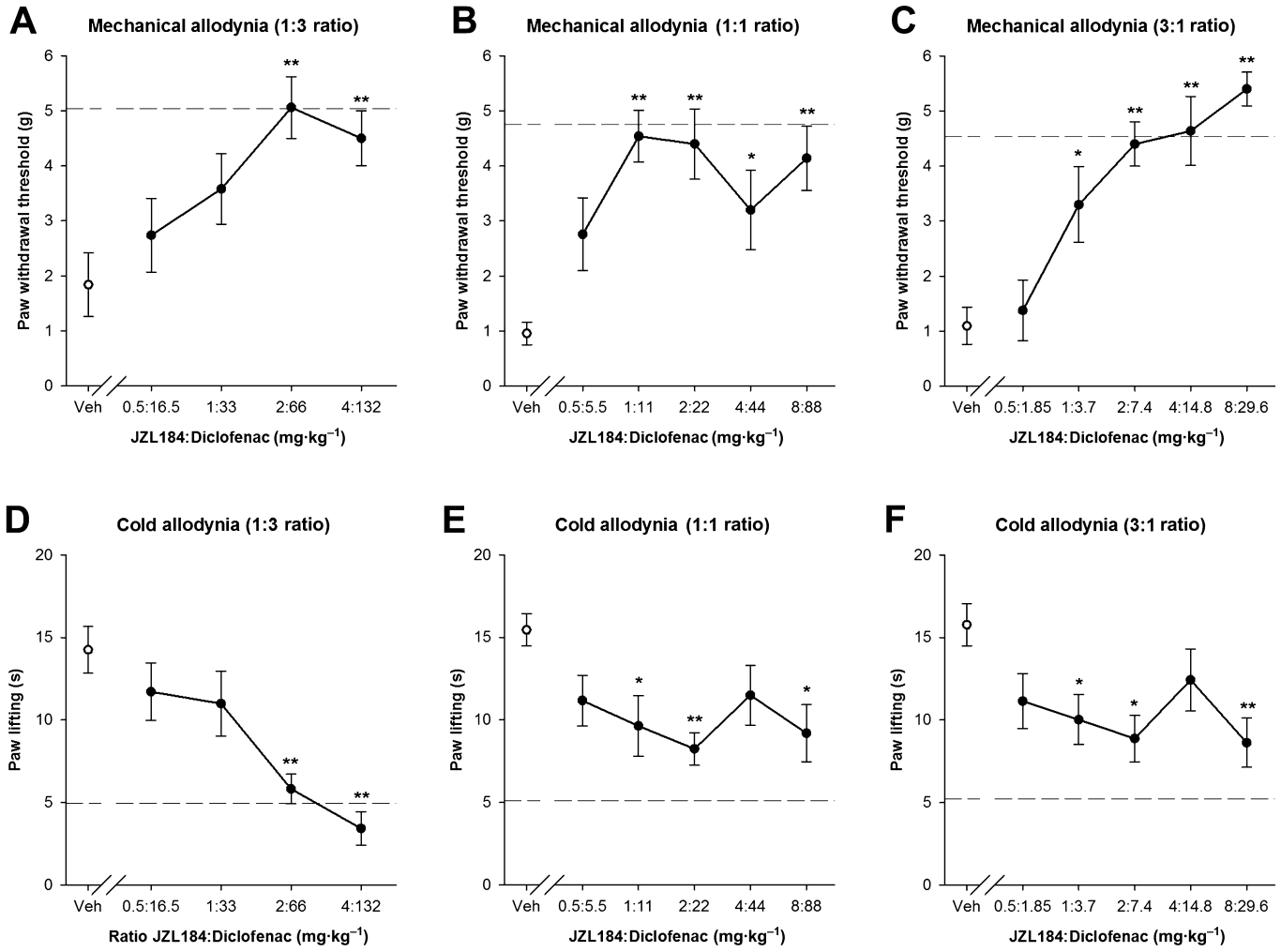

Figure 2

Coadministration of JZL184 and diclofenac elicits synergistically attenuated mechanical allodynia test and additively reduced cold allodynia in mice with CCI. Doses of JZL184 and diclofenac were administered in ratios of 1:3 (A,D), 1:1 (B,E) or 3:1 (C,F) parts of JZL184:diclofenac. The multiple doses for these ratios were determined based on the ED_{50} values of either compound. Data are expressed as mean ± SEM ($n = 10$). *$P < 0.05$ versus vehicle, **$P < 0.01$ versus vehicle. Dashed line represents the mean contralateral paw response.

Table 1

ED_{50} values of combinations of JZL184 and diclofenac

Combination (doses in mg·kg^{-1})	Combination ratio		ED_{50} mg·kg^{-1} i.p. (95% confidence limit) JZL184:diclofenac	
			Z_{add} (theoretical)	Z_{mix} (experimental)
JZL184:diclofenac 0.5:5.5 1:11 2:22 4:44 8:88	1:1	Mechanical allodynia Cold allodynia	35.99 (26.46–45.53) 29.03 (11.15–40.19)	8.85 (5.09–15.40)* 22.88 (14.60–35.85)
JZL184:diclofenac 0.5:1.85 1:3.7 2:7.4 4:14.8 8:29.6	3:1	Mechanical allodynia Cold allodynia	21.41 (16.31–26.51) 16.94 (12.41–29.35)	4.68 (3.14–6.96)* 10.24 (6.55–16.02)
JZL184:diclofenac 0.5:16.5 1:33 2:66 4:132	1:3	Mechanical allodynia Cold allodynia	50.58 (36.39–64.76) 41.12 (27.74–68.86)	19.48 (11.30–33.56)* 30.59 (21.70–43.13)
KML29:diclofenac 1:3 4.44:13.3 13.33:40 30:90 40:120	1:1	Mechanical allodynia Cold allodynia	41.31 (31.43–51.20) 39.25 (29.89–48.96)	11.51 (7.03–18.88)* 26.55 (16.63–42.36)

The Z_{add} and Z_{mix} values reflect the total amount of both drugs combined, where diclofenac and JZL184 were summed for each combination. Experimentally determined Z_{mix} values and predicted Z_{add} values (95% CL) for mixtures of JZL184 and diclofenac in mechanical and cold allodynia assays. Synergistic interactions are shown by significant differences between Z_{mix} and Z_{add} values. *$P < 0.05$, compared with Z_{add} using the Fisher test; non-overlapping 95% CL between Z_{mix} and Z_{add} values.

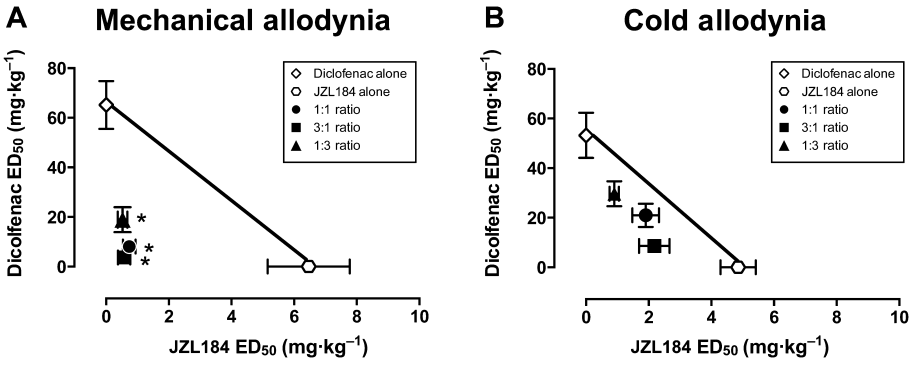

Figure 3

Isobologram of the interactions between JZL184 and diclofenac in the CCI-induced allodynia model. The ED_{50} values for JZL184 and diclofenac are shown on the *x*- and *y*-axes respectively. The isobole of additivity is shown as a solid line connecting the ED_{50} values of JZL184 and diclofenac and providing the theoretical line of additivity. (A) The experimental points of the collective mechanical allodynia tests lie significantly below the line of additivity indicating a synergistic interaction. (B) The experimental points of the collective cold allodynia tests do not differ significantly from the theoretical line of additivity, indicating an additive interaction. Test values are reported in Table 1. *$P < 0.05$ ($n = 10$ mice per treatment group).

theoretical line of additivity, and thus the interaction was additive (Figure 3B).

Anti-allodynia effects of KML29 alone and in combination with diclofenac sodium

In order to determine that the anti-allodynic effects of combined MAGL and COX inhibition were not specific to the MAGL inhibitor JZL184, another selective MAGL inhibitor, KML29, was also tested alone and in the presence of diclofenac. KML29 significantly reduced mechanical allodynia [$F(4,67) = 39.92$, $P < 0.01$; Figure 4A] and cold allodynia [$F(4,67) = 11.83$, $P < 0.01$; Figure 4D]. *Post hoc* analyses revealed that KML29 attenuated mechanical allodynia at ≥30 mg·kg^{-1} and cold allodynia at ≥30 mg·kg^{-1}. In addition, the GABA analogue gabapentin (50 mg·kg^{-1}, i.p.) was tested as a positive anti-allodynic control.

To find the overall ED_{50} for KML29, the ED_{50}s for mechanical and cold allodynia, 16.6 and 27.3 mg·kg^{-1}, respectively, were averaged, resulting in an overall ED_{50} of 22 mg·kg^{-1}. For the overall ED_{50} of diclofenac, the ED_{50}s for mechanical and cold allodynia, 76.3 and 53.5 mg·kg^{-1}, respectively, were averaged, resulting in an overall ED_{50} of 65 mg·kg^{-1}. Using the overall ED_{50}s for KML29 and diclofenac, the 1:1 ratio reflects one part KML29 to three parts diclofenac (Figure 4B, 4E). The Z_{mix} in the 1:1 ratio in the mechanical allodynia test was significantly less than the Z_{add} without CI overlap, indicating that the interaction was synergistic (Table 1). The Z_{mix} in the 1:1 ratio in the cold allodynia test was less than the Z_{add}; however, there was some CI overlap, and thus the interaction was considered additive (Table 1).

Because the experimental points of the collective mechanical allodynia tests lie significantly below the line of

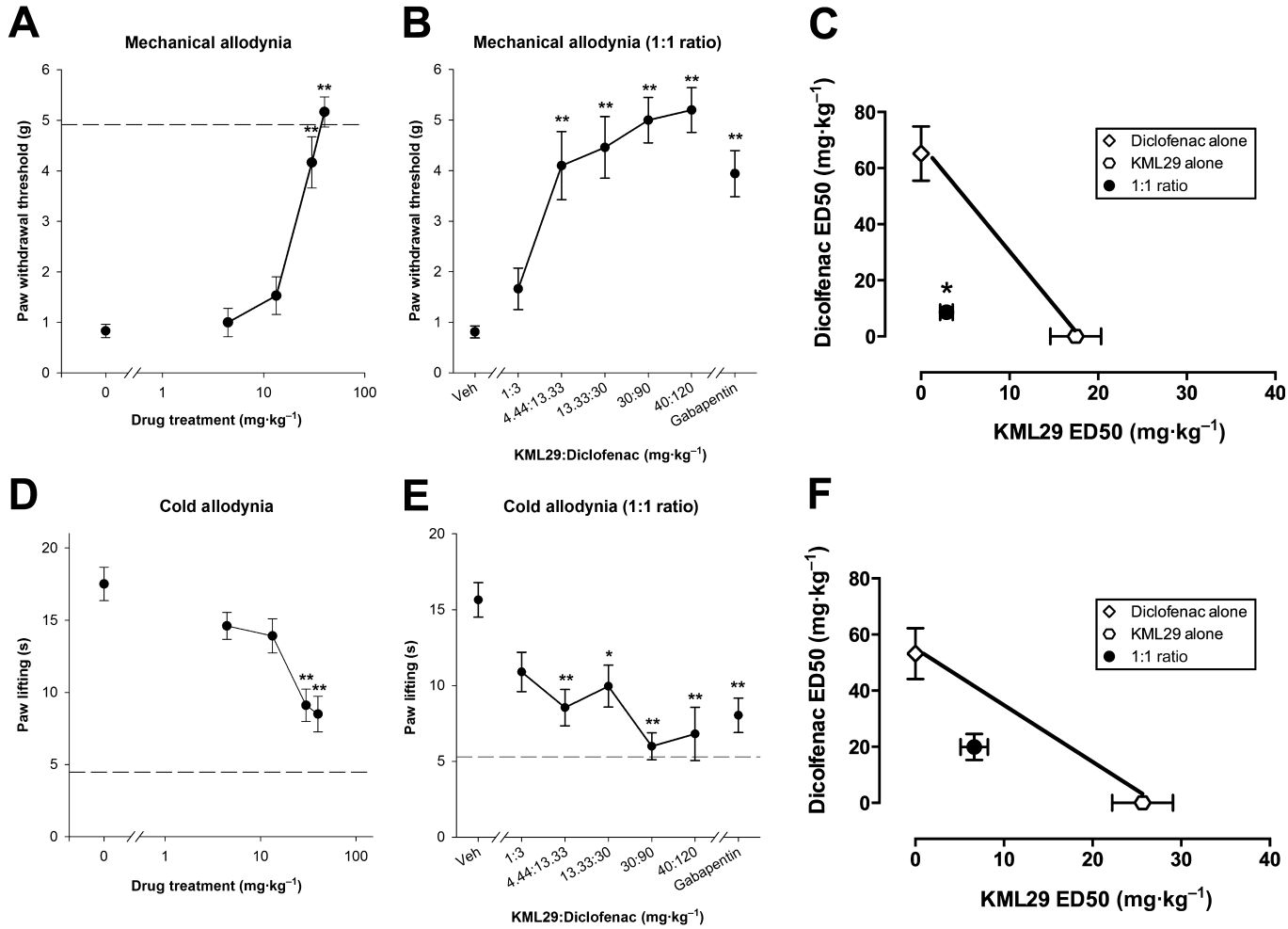

Figure 4

Coadministration of the selective MAGL inhibitor KML29 and diclofenac synergistically attenuated mechanical allodynia and additively reduced cold allodynia in mice with CCI. A separate group of mice with CCI were administered KML29 or vehicle and tested for mechanical (A) and cold allodynia (D). Then, KML29 was coadministered with diclofenac and mechanical (B) and cold allodynia (E) were assessed. The isobole of additivity is shown as a solid line connecting the ED_{50} values of KML29 and diclofenac and depicting the theoretical line of additivity. (C) The experimental points of the collective mechanical allodynia tests lie significantly below the line of additivity indicating a synergistic interaction. (F) The experimental points of the collective cold allodynia tests do not differ significantly from the theoretical line of additivity, indicating an additive interaction. *$P < 0.05$; **$P < 0.01$ versus vehicle (n = 10–16 mice per treatment group).

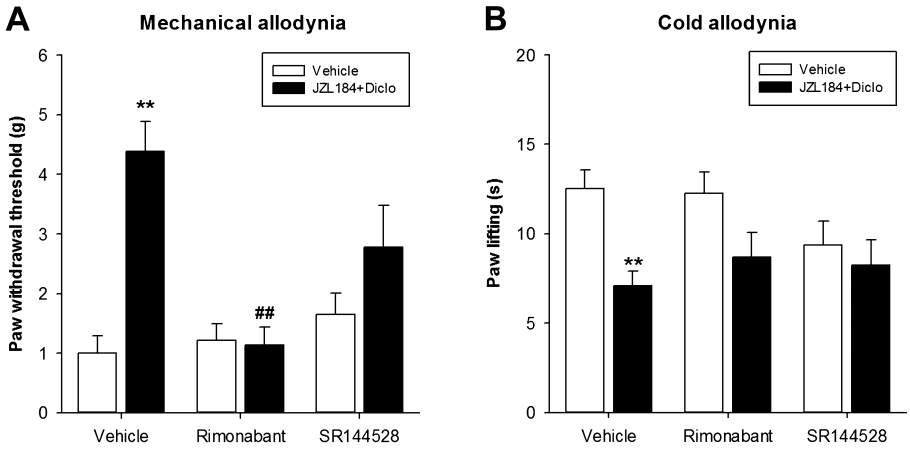

Figure 5

Pretreatment with the CB_1 receptor antagonist, rimonabant (3 mg·kg^{-1} i.p.), but not the CB_2 antagonist, SR144528 (3 mg·kg^{-1} i.p.), blocked the analgesic effects of JZL184:diclofenac (1:11 mg·kg^{-1} i.p.) administration on mechanical allodynia. Data are expressed as mean ± SEM (n = 12). **P < 0.01 versus vehicle, ##P < 0.01 versus JZL184:diclofenac.

additivity, the interaction was synergistic (Figure 4C). The experimental points of the collective cold allodynia tests do not differ significantly from the theoretical line of additivity, and thus the interaction was additive (Figure 4F).

Anti-allodynic effects of JZL184 were blocked by CB_1 receptor antagonists

The CB_1 antagonist rimonabant or the CB_2 antagonist SR144528 was used to evaluate the underlying receptor mechanism of the antinociceptive effects of combining JZL184 (1 mg·kg^{-1}) and diclofenac (11 mg·kg^{-1}). Rimonabant (3 mg·kg^{-1}, i.p.), but not SR144528 (3 mg·kg^{-1}, i.p.), significantly blocked the analgesic effect in the mechanical allodynia test [$F(2,66)$ = 8.34, P < 0.05], indicating a primarily CB_1 receptor mechanism of action (Figure 5A). *Post hoc* analyses revealed that rimonabant with the combination of JZL184+diclofenac was significantly different from the control JZL184+diclofenac combination (P < 0.01), whereas SR144528 with JZL184+diclofenac did not differ from the control JZL184+diclofenac combination (P = 0.16). In the acetone-induced cold allodynia test, either rimonabant or SR144528 partially blocked the anti-allodynic effects of the JZL184+diclofenac combination [$F(2,66)$ = 1.63, P = 0.20] (Figure 5B).

Diclofenac reduced PG, but not endocannabinoid, levels

Diclofenac alone (11 mg·kg^{-1}), or in combination with JZL184 (1 mg·kg^{-1}), significantly reduced spinal cord levels of PGE_2 [$F(3,20)$ = 6.27, P < 0.01] and $PGF_{2\alpha}$ [$F(3,20)$ = 7.77, P < 0.01]. Administration of JZL184 alone increased levels of NAGly [$F(3,20)$ = 4.73, P < 0.05]. Spinal cord levels of anandamide (P = 0.90) and 2-AG (P = 0.25) did not differ between treatment groups. These data are summarized in Table 2.

Discussion and conclusions

The goal of this study was to test the hypothesis that combined inhibition of MAGL and COX would synergistically decrease nociceptive responses in mice with CCI. The selective MAGL inhibitors JZL184 and KML29 reduced mechanical and cold allodynia as previously reported (Kinsey *et al.*, 2009; 2013; Ignatowska-Jankowska *et al.*, 2014). Diclofenac also significantly reduced mechanical and cold allodynia. Fixed dose proportions of JZL184 or KML29 and diclofenac synergistically reduced mechanical allodynia but not cold allodynia in mice with CCI. The combination of JZL184 or KML29 and diclofenac and additively reduced cold allodynia while having no effect on control paws in either allodynia test. Thus, combined inhibition of COX and MAGL reduced the dose of either drug to the extent that significant reductions in allodynia were achieved at subthreshold doses.

The second objective of this study was to determine cannabinoid receptor involvement in the antinociceptive effects of the combination treatment. Rimonabant, but not SR144528, blocked the analgesic effect in mechanical allodynia, indicating that the anti-allodynic effects were primarily mediated by CB_1 receptors. However, neither rimonabant nor SR144528 prevented analgesia in cold allodynia. This may be attributed to a non-cannabinoid mechanism. Cold allodynia may be mediated by the thermosensitive receptors TRPA1 and/or TRPM8, which act as cold sensors and contribute to temperature thresholds (Bandell *et al.*, 2004; Xing *et al.*, 2007; Karashima *et al.*, 2009).

We next evaluated spinal cord levels of endocannabinoids and PGs after administration of JZL184 (1 mg·kg^{-1}), diclofenac (11 mg·kg^{-1}), the combination treatment [JZL184 (1 mg·kg^{-1})+diclofenac (11 mg·kg^{-1})] or vehicle. Although MAGL inhibition decreases brain levels of free arachidonic acid (Nomura *et al.*, 2011) and COX inhibition prevents free arachidonic acid from being converted into PGs, in our

Table 2

Levels of eicosanoids, endocannabinoids and related fatty acid derivatives in extracts of spinal cord, after inhibition of MAGL and COX

	JZL184 (1 mg·kg^{-1})	Diclofenac (11 mg·kg^{-1})	JZL184:diclofenac (1:11 mg·kg^{-1})	Vehicle
Arachidonic acid	45 985.3	36 432.4	36 245.1	40 654.3
	(5237.9)	(2587.2)	(4567.2)	(5353.7)
Linoleic acid	2387.0	3643.1	2345.0	1950.5
	(504.0)	(1115.7)	(511.7)	(276.3)
PGE_2	73.6	7.6*	9.4*	74.8
	(21.7)	(2.0)	(2.0)	(21.0)
$PGF_{2\alpha}$	30.4	0.0**	0.0**	38.3
	(9.7)	(0.0)	(0.0)	(10.6)
N-arachidonoyl-glycine	28.13*	17.29	20.52	18.22
	(2.24)	(2.45)	(2.23)	(2.13)
N-docosahexaenoyl-glycine	32.35	30.61	28.94	29.48
	(2.54)	(2.98)	(5.23)	(3.57)
N-linoleoyl-glycine	4.3	5.5	6.4	4.2
	(1.0)	(0.9)	(1.6)	(0.5)
N-oleoyl-glycine	19.4	15.5	17.5	17.7
	(1.5)	(1.7)	(2.5)	(1.6)
N-palmitoyl-glycine	58.7	53.4	58.7	54.2
	(4.2)	(3.3)	(6.6)	(6.3)
N-stearoyl-glycine	21.7	17.2	22.6	18.3
	(1.5)	(1.7)	(3.4)	(2.9)
2-Arachidonoylglycerol	3810.7	2792.2	3173.7	3415.0
	(234.1)	(216.5)	(445.6)	(432.2)
2-Linoleoylglycerol	716.7	553.3	564.9	623.5
	(25.2)	(83.4)	(40.3)	(60.4)
2-Oleoylglycerol	38 258.5	33 345.0	32 803.8	32 803.8
	(2722.7)	(5172.3)	(4833.3)	(4833.3)
N-arachidonoyl ethanolamine	5.3	5.2	4.7	4.8
	(0.4)	(0.6)	(1.0)	(0.6)
N-docosahexaenoyl ethanolamine	32.4	30.6	28.9	29.5
	(2.5)	(3.0)	(5.2)	(3.6)
N-linoleoyl ethanolamine	19.9	19.2	15.5	16.2
	(2.7)	(2.1)	(2.8)	(2.2)
N-oleoyl ethanolamine	309.0	255.2	210.1	240.8
	(49.9)	(23.5)	(33.0)	(37.0)
N-palmitoyl ethanolamine	353.9	282.5	262.8	281.7
	(46.0)	(26.6)	(38.8)	(35.6)
N-stearoyl ethanolamine	431.0	333.4	316.4	332.8
	(61.1)	(33.2)	(42.0)	(49.3)

Spinal cord levels are expressed as mean (SEM) in pmol·g^{-1}. Tissue was collected from mice with CCI 2 h after drug administration. *$P < 0.05$, **$P < 0.01$ versus vehicle, Dunnett's *post hoc* test.

experiments, there were no changes in levels of free arachidonic acid after any of the treatments. However, administration of diclofenac and the combination treatment significantly reduced levels of PGE_2 and $PGF_{2\alpha}$, although the combination did not further reduce these levels, indicating diclofenac inhibition of COX drove the reduction in these PGs, indicating that there is likely a 'ceiling effect' with the level of diclofenac used here.

Combining these two drugs leads to simultaneous reduction of PG synthesis and activation of CB_1 receptors. Some

NSAIDs can inhibit FAAH when given in very high doses, thereby increasing anandamide brain levels (Holt *et al.*, 2007). However, there were no differences in levels of anandamide after any treatment, in the present study, indicating FAAH was not inhibited by either diclofenac or JZL184 at the chosen doses. The lack of treatment-related differences in the assay of endocannabinoids, at the level of whole tissue, does not necessarily preclude the existence of altered endocannabinoid or PG levels in the extracellular space. Future studies using microdialysis, as well as targeting more discrete anatomical structures, may provide higher resolution readouts of biomarker changes, and thus, a better understanding of the mechanisms contributing to the observed synergistic analgesia.

The present data are the first to report a synergistic interaction between MAGL and COX inhibition. However, our data parallel previous published data that inhibition of another cannabinoid catabolic enzyme, FAAH, potentiated the effects of an NSAID. Many of these reports are based on *in vitro* studies (Fowler *et al.*, 1997; 1999; Cipriano *et al.*, 2013) as well as structural modelling that reveal that COX inhibitors are also capable of inhibiting FAAH (Bertolacci *et al.*, 2013). *In vivo*, the FAAH inhibitors URB597 and URB937 synergistically reduce visceral and neuropathic pain (Naidu *et al.*, 2009) in mouse models (Sasso *et al.*, 2012). Similarly, a recently published report also indicates that the FAAH inhibitor PF-3845 potentiated the anti-allodynic effects of diclofenac *in vivo* using both the CCI model as well as the carrageenan model of inflammatory pain (Grim *et al.*, 2014).The gastrointestinal side effects of high-dose NSAID administration are attenuated by inhibiting MAGL (Kinsey *et al.*, 2013; Ignatowska-Jankowska *et al.*, 2014) or FAAH (Naidu *et al.*, 2009; Sasso *et al.*, 2012), or administering THC (Kinsey and Cole, 2013; Kinsey *et al.*, 2013). However, the molecular mechanism of action through which this gastroprotection occurs is unknown. The interaction of combined administration of an NSAID and a MAGL inhibitor may further diminish unwanted NSAID side effects through changes in lipid biochemistry. Another advantage of using lower doses of enzyme inhibitors to reduce neuropathic pain is a reduced risk of drug tolerance. For example, repeated administration of high-dose JZL184 (≥16 mg·kg^{-1}) causes down-regulation and desensitization of CB_1 receptors (Schlosburg *et al.*, 2010; Kinsey *et al.*, 2013). This is likely to result in a reversal of the antinociceptive and gastroprotective effects of JZL184. However, repeated administration of low-dose JZL184 (e.g. ≤8 mg·kg^{-1}) did not alter receptor expression or function (Kinsey *et al.*, 2013). In other words, at lower doses, repeated administration of JZL184 maintains analgesic and gastroprotective effects. These results support a potential therapeutic treatment of neuropathic pain with low doses of inhibitors of endocannabinoid catabolic enzymes.

Reducing the required analgesic NSAID dose also reduces its side effects. Selective COX-2 inhibitors were developed to circumvent the detrimental gastrointestinal side effects of COX-1 inhibition but patients using these drugs had a significant increase of negative cardiovascular events. These results led to many coxibs, in particular rofecoxib, being removed from the market. Non-selective COX inhibitors (i.e. NSAIDs) also produce an increased risk of a cardiovascular event, especially with high doses (Ong *et al.*, 2013). The risk of cardiovascular event doubled for some NSAIDs at higher doses, while lower doses were nearly risk free for certain NSAIDs (McGettigan and Henry, 2011). These findings support the feasibility of a pain treatment using lower doses of NSAIDs to avoid adverse side effects.

The ultimate goal of this research is to help identify targets for future pharmacological treatments for patients experiencing neuropathic pain and to understand the basic molecular mechanisms through which analgesia occurs. Patients with neuropathic pain tend to report higher pain severity scores and an increased use of analgesics, compared with other forms of chronic pain (Torrance *et al.*, 2006), again emphasizing the need to develop new effective therapeutic treatments. Thus, a possible future direction is to examine the effectiveness of a monotherapy using a MAGL inhibitor in clinical trials in humans. Then, trials using combined administration of an NSAID and a MAGL inhibitor may be feasible. Although MAGL inhibition has not been tested in humans, a recent clinical trial tested PF-04457845, a selective FAAH inhibitor, in osteoarthritis patients, for pain relief. PF-04457845 inhibited FAAH activity in the patients, but it was indistinguishable from placebo in regard to analgesia (Huggins *et al.*, 2012). Huggins *et al.* (2012) speculated that, in addition to FAAH inhibition, COX inhibition may also be necessary for pain relief in humans. Considering the negative side effects of high-dose NSAIDs (e.g. gastrointestinal ulcers, cardiovascular and renal failure), using a low dose of an NSAID may be ideal. However, the lower dose NSAID alone may not sufficiently produce analgesia.

It is possible that combined administration of a MAGL inhibitor and an NSAID could be effective in other models of pain, such as inflammatory or postsurgical hyperalgesia. Because the combined administration of these two classes of drugs yields significant analgesia at low doses, this strategy may be used effectively in a wide range of conditions. For example, as mentioned earlier, combined FAAH/COX inhibition reduced acute visceral pain (Naidu *et al.*, 2009), although MAGL inhibitors have not been tested in this paradigm. Similarly, although it has been reported previously that chronic low-dose JZL184 does not cause functional CB_1 receptor loss (Kinsey *et al.*, 2013), chronic dual administration may have unknown effects that may or may not be limited to CB_1 receptors. Thus, exploring the long-term effects of combined JZL184 and diclofenac administration is warranted.

In addition to these behavioural studies, it would be informative to examine the lipid biochemistry of the spinal cord and brain with a wider range of dual drug combinations and time points. It is possible that the lack of effect on levels of 2-AG with JZL184 treatment was that the time course examined here was too narrow. It is not obvious that the dose (i.e. 1 mg·kg^{-1} JZL184) was lower than that previously shown to increase brain levels of 2-AG. However, there may be an acute spike in 2-AG directly after the drug delivery, not measured here, that accounts for some of the cascade of biochemical cellular responses that set the stage for later neurophysiological responses. The levels of NAGly were significantly increased at this later time point with JZL184 and not diclofenac, illustrating that there are shifts in the lipidome with each of these drugs that are still being discovered and are likely to ultimately play a role in behaviour.

In summary, coadministration of JZL184 and diclofenac synergistically reduced CCI-induced mechanical allodynia and additively reduced cold allodynia in mice. Moreover, the synergistic analgesia in the mechanical allodynia test was mediated by CB_1 receptors. The combined inhibition of MAGL and COX enzymes may be beneficial in maximizing analgesia while reducing NSAID side effects, in particular gastrointestinal ulcers and negative cardiovascular events.

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Author contributions

M. S. C., M. L. B., H. B. B. and S. G. K. participated in research design. M. S. C., E. L. and S. G. K. conducted experiments. M. S. C., E. L., M. L. B., H. B. B. and S. G. K. performed data analysis. M. S. C., E. L., A. M., M. L. B., H. B. B. and S. G. K. wrote or contributed to the writing of the manuscript. R. G. and A. M. contributed experimental compounds.

Conflict of interest

The authors state no conflict of interest.

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