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RESEARCH PAPER

Actions of the dual FAAH/MAGL inhibitor JZL195 in a murine neuropathic pain model

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BACKGROUND AND PURPOSE
While cannabinoids have been proposed as a potential treatment for neuropathic pain, they have limitations. Cannabinoid receptor agonists have good efficacy in animal models of neuropathic pain; they have a poor therapeutic window. Conversely, selective fatty acid amide hydrolase (FAAH) inhibitors that enhance the endocannabinoid system have a better therapeutic window, but lesser efficacy. We examined whether JZL195, a dual inhibitor of FAAH and monacylglycerol lipase (MAGL), could overcome these limitations.

EXPERIMENTAL APPROACH
C57BL/6 mice underwent the chronic constriction injury (CCI) model of neuropathic pain. Mechanical and cold allodynia, plus cannabinoid side effects, were assessed in response to systemic drug application.

KEY RESULTS
JZL195 and the cannabinoid receptor agonist WIN55212 produced dose-dependent reductions in CCI-induced mechanical and cold allodynia, plus side effects including motor incoordination, catalepsy and sedation. JZL195 reduced allodynia with an ED_{50} at least four times less than that at which it produced side effects. By contrast, WIN55212 reduced allodynia and produce side effects with similar ED50s. The maximal anti-allodynic effect of JZL195 was greater than that produced by selective FAAH, or MAGL inhibitors. The JZL195-induced anti-allodynia was maintained during repeated treatment.

CONCLUSIONS AND IMPLICATIONS
These findings suggest that JZL195 has greater anti-allodynic efficacy than selective FAAH, or MAGL inhibitors, plus a greater therapeutic window than a cannabinoid receptor agonist. Thus, dual FAAH/MAGL inhibition may have greater potential in alleviating neuropathic pain, compared with selective FAAH and MAGL inhibitors, or cannabinoid receptor agonists.

Abbreviations
CCI, chronic constriction injury; FAAH, fatty acid amide hydrolase; JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl (hydroxy)methyl)piperidine-1-carboxylate; JZL195, 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate; MAGL, monacylglycerol lipase; URB597, 3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate; (+)-WIN55212 mesylate, [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate

Tables of Links

TARGETS
GPCRs[a]
CB_1 receptors
CB_2 receptors
Enzymes[b]
FAAH, fatty acid amide hydrolase
MAGL, monoacylglycerol lipase

LIGANDS
2-AG, 2-arachidonoylglycerol
Anandamide
JZL 184
JZL 195
PF04457845
URB597
WIN55212-2

This table lists protein targets and ligands that 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 the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Neuropathic pain is a persistent, severe and debilitating form of chronic pain induced by physical trauma, or disease that results in damage to peripheral nerves, the spinal cord and brain (Jensen *et al.*, 2011). The currently recommended pharmacological treatments for neuropathic pain have poor effectiveness and commonly produce a range of side effects that can render these treatment options intolerable to patients (Dworkin *et al.*, 2010). Thus, there is a need to identify new therapeutic targets that have improved effectiveness and a better therapeutic window between pain relief and side effects.

Cannabinoids have long been suggested as a potential treatment for neuropathic pain. The psychoactive component of *Cannabis sativa*, Δ9-tetrahydrocannabinol (THC) and synthetic non-selective cannabinoid receptor agonists displays high efficacy in reducing the allodynia associated with animal models of neuropathic pain (e.g. Herzberg *et al.*, 1997; Fox *et al.*, 2001; Lim *et al.*, 2003; Costa *et al.*, 2004; De Vry *et al.*, 2004; Scott *et al.*, 2004). These cannabinoid agonists, however, also produce a range of side effects such as motor incoordination, catalepsy and immobility, plus cognitive impairment. The therapeutic window between pain relief and side effects of systemically delivered non-selective cannabinoid agonists is poor in a rat neuropathic pain model (Fox *et al.*, 2001).

In order to improve the therapeutic window of cannabinoids, it has been suggested that modulation of endogenous cannabinoid (endocannabinoid) levels might provide an alternative approach to direct agonist activation of cannabinoid receptors (Petrosino and Di Marzo, 2010; Roques *et al.*, 2012). The two main endocannabinoids, N-arachidonyl ethanolamide (anandamide) and 2-arachidonyl glycerol (2-AG), play an important role in pain via their actions at cannabinoid CB1 and CB2 receptors, plus other targets (Bradshaw and Walker, 2005; Pacher *et al.*, 2006; Hill *et al.*, 2009; Alexander *et al.*, 2011). Anandamide and 2-AG are metabolized by the enzymes fatty acid amide hydrolase (FAAH) and monacylglycerol lipase (MAGL) respectively (Piomelli, 2003; Di Marzo *et al.*, 2005). A number of agents that potently and selectively inhibit FAAH (e.g. URB597, PF3485 and PF04457845) and MAGL (e.g. JZL184 and KML29) have been characterized, which elevate brain/peripheral levels of anandamide and 2-AG, respectively, when delivered systemically (e.g. Kathuria *et al.*, 2003; Long *et al.*, 2009a). Systemic delivery of selective FAAH and MAGL inhibitors reduces the allodynia associated with nerve injury models of neuropathic pain (Russo *et al.*, 2007; Kinsey *et al.*, 2009; Kinsey *et al.*, 2010; Kinsey *et al.*, 2013; Ignatowska-Jankowska *et al.*, 2014). These inhibitors are thought to produce cannabinoid side effects only at higher doses in neuropathic pain models (Jayamanne *et al.*, 2006; Comelli *et al.*, 2007; Ahn *et al.*, 2011). Unfortunately, selective FAAH and MAGL inhibitors display lower efficacy compared with cannabinoid agonists in neuropathic pain models (Kinsey *et al.*, 2009; Kinsey *et al.*, 2010). In this regard, the FAAH inhibitor PF04457845 had no significant effect in a recent clinical trial on osteoarthritic pain (Huggins *et al.*, 2012), although clinical effectiveness in neuropathic pain states remains to be established.

The aforementioned evidence indicates that current cannabinoid agents have limitations for the treatment of chronic neuropathic pain. Cannabinoid receptor agonists have good efficacy against neuropathic pain, but have a poor therapeutic window, while selective FAAH and MAGL inhibitors have a good therapeutic window, but lesser efficacy against neuropathic pain symptoms compared with cannabinoid agonists. One way to improve the efficacy of endocannabinoid modulators could be to inhibit multiple endocannabinoid degradation enzymes. Recently, the compound JZL195 has been described which potently inhibits both FAAH and MAGL, with IC_{50}s of 2 and 4 nM respectively (Long *et al.*, 2009b). Systemic delivery of JZL195 increases the brain levels of anandamide and 2-AG, with similar dose dependency, and produces efficacious antinociception (Long *et al.*, 2009b). We have recently shown that JZL195 has good anti-allodynic efficacy and therapeutic window in an inflammatory pain model (Anderson *et al.*, 2014). Given the problematic nature of neuropathic pain treatment, we therefore examined the pain relieving efficacy and therapeutic window of JZL195 in a mouse model of this chronic pain state. The characteristics of JZL195 were compared with those of a cannabinoid receptor agonist and selective FAAH and MAGL inhibitors. We also examined whether it maintained efficacy during repeated administration.

Methods

Neuropathic pain model

Animals underwent surgery for the chronic constriction injury (CCI) model of neuropathic pain. To do this, the left common sciatic nerve was exposed at mid-thigh level, and two 7-0 chromic gut sutures were loosely tied around the nerve. The incision was closed in layers, and the animal recovered. In some experiments, animals underwent sham surgery in which the left sciatic nerve was exposed, but not ligated.

Behavioural and pain testing

To assess neuropathic pain, mechanical and cold allodynia were measured. Mice were placed in elevated perspex cages (15 × 10 × 10 cm) with a wire mesh floor and allowed to acclimatize for 10–30 min prior to testing. To assess mechanical allodynia, the paw withdrawal threshold (PWT) to mechanical stimulation of the left hind paw was assessed using von Frey hairs (North Coast Medical, San Jose, USA). A series of von Frey hairs (0.2–8.5 g) were pressed perpendicularly (for 2 s) onto the plantar surface of the hind paw (four times for each hair), and the mechanical PWT was calculated using a threshold tracking algorithm (Chaplan *et al.*, 1994). To assess cold allodynia, 20 μL of acetone was sprayed onto the plantar surface of the left hind paw to induce evaporative cooling. The number of acetone-induced left hind limb lifts, shakes and/or licks was counted over a 2 min period.

A number of side effects were also assessed. Motor impairment was measured using a rotarod device (Ugo Basile, Comerio, Italy) that gradually accelerated (0–30 r.p.m.) over a period of 300 s. Rotarod latency was measured as the time in which the mouse fell off the rotarod, or just held onto the cylinder for two consecutive rotations, with a cut-off of 300 s. Catalepsy was assessed with the bar test by placing the animal's forepaws on a bar 4.5 cm off the ground. Bar latency was the time taken to remove both forepaws from this position, with a cut-off of 120 s. Spontaneous locomotor activity was assessed using a dark open field. Each animal was placed in an open topped, opaque, Perspex enclosure (40 × 40 × 40 cm) and video recorded for 2 min. For analysis, the area was divided into a 4 × 4 grid, and the number of complete forepaw grid crossings counted. The aforementioned tests were carried out in low-level white light, except for the open field test that was carried out in low-level red light (both <3 lux).

Drugs and administration

The JZL184 (4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate), JZL195 (4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate), (+)-WIN55212 mesylate ([(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate) and URB597 ((3′-(aminocarbonyl) [1,1′-biphenyl]-3-yl)-cyclohexylcarbamate) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions of all drugs were prepared in a vehicle solution that comprised 2% randomly methylated β-cyclodextrin, 15% dimethylsulfoxide and 5% Tween-80 in saline. The s.c. injections were made in the back of the neck at a volume of 0.1–0.12 mL per 10 g body wt. Solutions of all agents were made up immediately prior to administration.

Experimental protocols

All animals were initially acclimatized, in batches of four, to their holding cages and testing devices (except for the open field) over a 4–5 day period. Separate groups of animals then underwent the following series of experiments. For series 1 experiments, we examined the effect of the nerve injury model on all pain/behaviour measures by comparing CCI and sham-operated animals. In these experiments, animals underwent baseline pre-surgery pain/side-effect testing (all tests, except the open field). On the following day, animals underwent CCI, or sham surgery. On day 7 post-CCI/sham surgery, animals underwent pain/side-effect testing (all tests).

For series 2 experiments we examined the time course of drug action following a single systemic injection in CCI-operated animals. In these experiments, animals underwent baseline pre-CCI pain/side-effect testing. Only mechanical PWT and rotarod were assessed due to shorter inter-test intervals. On the following day, animals underwent CCI surgery. On day 7 post-CCI surgery, mechanical PWT and rotarod testing were carried out twice over a 45 min period; the animal then received a single drug injection, and testing was repeated at 0.5, 1, 2, 4 and 6 h post-injection.

For series 3 experiments, we examined drug dose dependence following a single systemic injection in CCI-operated animals. In these experiments, animals underwent pain/side-effect testing (all tests except open field). On the following day, animals underwent CCI surgery. On day 7 post-CCI surgery, pain/side-effect testing (all tests except open field) was carried out twice over a 45 min period; the animal then received a single drug injection, and testing was repeated at 1 and 2 h post-injection (all tests, except open field that was only performed at 2 h).

For series 4 experiments, we examined the effect of repeated systemic drug injection in CCI-operated animals. In these experiments, animals underwent pain/side-effect testing (all tests). On the following day, animals underwent CCI surgery. Starting at day 7 post-CCI surgery, pain/side-effect testing (all tests except open field) was carried out twice over a 45 min period; the animal then received a single drug injection, and testing was repeated at 1 and 2 h post-injection (all tests, except open field only at 2 h). This was repeated on days 8–11 post-CCI surgery.

Analysis

For the sham versus CCI and drug time course experiments, comparisons were made using two-way repeated measures ANOVA, and *post hoc* comparisons were made using Tukey's adjustment (Prism, GraphPad Software, La Jolla, CA, USA). For the dose-response experiments, data (except for the open field) were averaged over the 1 and 2 h post-injection time points and normalized as a percentage of the maximum possible effect (MPE), as we have performed previously (Anderson *et al.*, 2014). For mechanical PWT and the bar latency, this was calculated as [100 * (post-drug − pre-drug) / (cut-off)], with cut-off values of 8.5 g and 120 s respectively.

For the acetone responses and rotarod latency, this was calculated as [100 * (pre-drug – post-drug) / (pre-drug)]. Raw data were used for open field crossings as these were only assessed at 2 h post-injection. Data in all figures are presented as mean ± SEM. Dose response curves were constructed by fitting a sigmoidal curve with variable slope and constrained by cut-off values where appropriate (Prism). From the curve fits, the estimated ED_{50} and maximal values were compared between groups using a sums-of-squares *F*-test (Prism). Other comparisons of treatment groups were made using one-way ANOVA (Prism), and *post hoc* comparisons were made using Tukey's adjustment.

Results

In the first series of experiments, we examined the effect of nerve injury on the pain and side-effect assays. At 7 days following surgery, CCI-operated animals displayed a decrease in mechanical PWT and an increase in acetone responses compared with pre-surgery values (Figure 1A,B; $P < 0.0001$, $n = 6$). Matched sham-operated animals did not display a difference in mechanical PWT, or in acetone responses between pre-surgery and post-surgery values (Figure 1A,B; $P > 0.05$, $n = 6$). Both CCI and sham-operated animals did not display a difference in rotarod, or bar latency between pre-surgery and post-surgery values (Figure 1C,D; $P = 0.15$, $F(1,10) = 2.48$ and $P = 0.23$, $F(1,10) = 1.60$, $n = 6$ each). CCI and sham-operated animals did not differ in the number of open field crossings at 7 days post-surgery (79 ± 4 and 89 ± 11 crossing for CCI and sham animals, $P = 0.5$ *t*-test, $n = 6$ each).

Time course of action of JZL195 and WIN55212

In the second series of experiments, we examined the time course of action of the dual FAAH/MAGL inhibitor JZL195 (18 mg·kg^{-1}) and the pan cannabinoid receptor agonist WIN55212 (3 mg·kg^{-1}), at doses we have previously shown to be near maximal in an inflammatory pain model (Anderson *et al.*, 2014). At 7 days following CCI surgery, the effect of s.c. injection of JZL195, WIN55212 and vehicle on mechanical PWT ($F_{10,75} = 5.2$, $P < 0.0001$) and rotarod latency ($F_{10,75} = 10.9$, $P < 0.0001$) differed over time (Figure 2, $n = 6$ per treatment group).

Both JZL195 and WIN55212 produced an increase in mechanical PWT that plateaued within 1–2 h (Figure 2A). The JZL195-induced increase in mechanical PWT was significantly greater than vehicle at 0.5–6 h post-injection ($P < 0.0001$–0.01). The WIN55212-induced increase in mechanical PWT was significantly greater than vehicle at 0.5–4 h post-injection ($P < 0.01$–0.0001). Both JZL195 and WIN55212 also produced a decrease in rotarod latency that plateaued at 1–2 h (Figure 2B). The JZL195-induced decrease in rotarod latency was significantly greater than vehicle at 1–2 h post-injection ($P < 0.01$–0.05). The WIN55212-induced decrease in rotarod latency was significantly greater than vehicle at 0.5–2 h post-injection ($P < 0.0001$). For the rest of the study, we measured allodynia and side effects at 1–2 h after drug/vehicle injection.

Effect of JZL195

In the third series of experiments, we examined the effect of a range of doses of JZL195 (0.1–30 mg·kg^{-1}) on pain behaviours and side effects at 7 days following CCI surgery ($n = 6$ per dose). JZL195 produced a dose-dependent reversal of the

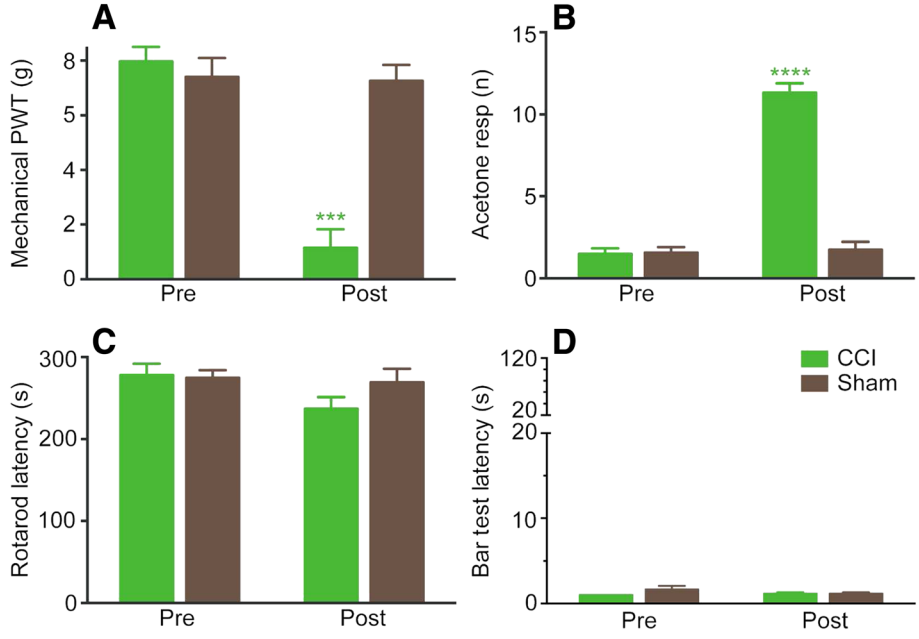

Figure 1

Effect of CCI nerve injury on behavioural measures. Bar charts showing the effect of CCI versus sham surgery on raw values of (A) mechanical PWT, (B) acetone responses (Resp), (C) rotarod latency and (D) bar latency. *** and **** denote $P < 0.001$, 0.0001 for pre-surgery versus post-surgery.

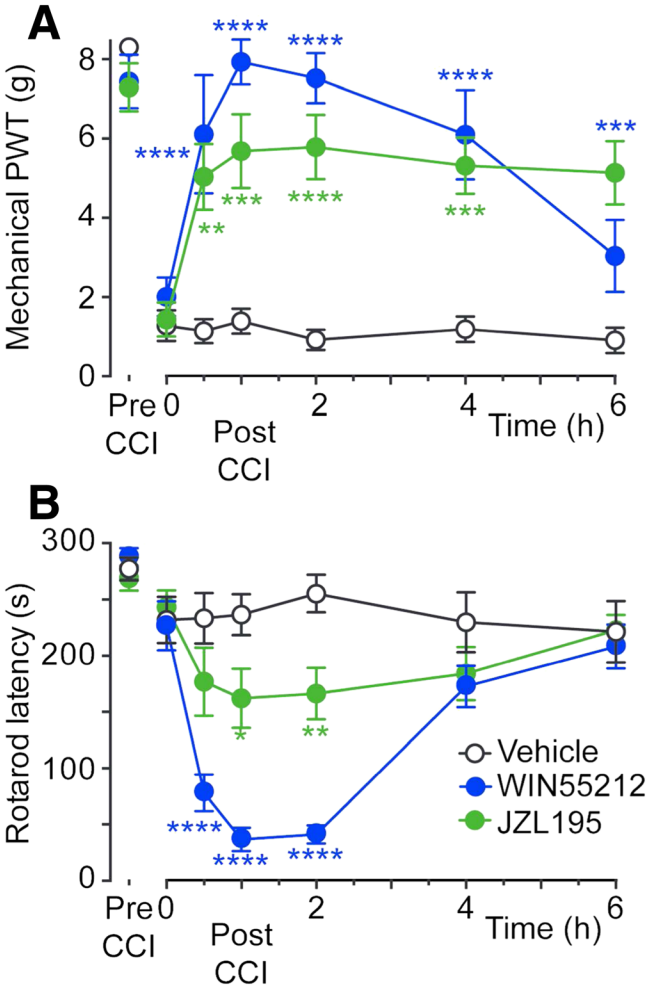

Figure 2

Time course of WIN55212 and JZL195-induced anti-allodynia. Time plots of the effect of JZL195 (18 mg·kg^{-1}), WIN55212 (3 mg·kg^{-1}) and matched vehicle on (A) mechanical PWT and (B) rotarod latency. Animals received an s.c. injection at time 0 h, 7 days after CCI surgery (post-CCI). The data for JZL195, WIN55212 and vehicle are also shown prior to CCI surgery (pre-CCI). *, **, *** and **** denote $P < 0.05$, 0.01, 0.001 and 0.0001 compared with vehicle at the corresponding time points.

CCI-induced reduction in mechanical PWT and of the CCI-induced increase in acetone responses (Figure 3A). The maximal effect of JZL195 on acetone responses was greater than that for mechanical PWT (Figure 3A, $P = 0.01$). The ED_{50} of JZL195 for mechanical PWT and the acetone responses was 2.1 and 1.5 mg·kg^{-1} respectively (Table 1). These ED_{50} values were not significantly different ($P = 0.2$).

In these animals, JZL195 produced an increase in bar latency and a reduction in rotarod latency only at the 18, or 30 mg·kg^{-1} dose (Figure 3B, Table 1). It might be noted that JZL195 produced pop-corning, observed as hyper-reactivity and jumping that occurred when the animal was placed on the bar test and rotarod devices (but not open field, or allodynia testing chambers), as observed previously (Long *et al.*, 2009b; Anderson *et al.*, 2014). Pop-corning was detected in 1/6 and 3/6 animals at the 18 and 30 mg·kg^{-1} doses respectively. This pop-corning led to a reduction in bar and rotarod latency at the higher doses, and precluded ED_{50} estimation as full sigmoidal dose response curves could not be obtained. JZL195 produced a dose-dependent decrease in open field crossings with an ED_{50} of 10.3 mg·kg^{-1} (Figure 3C, Table 1). The ED_{50}s of JZL195 for mechanical PWT and acetone responses were both less than that for the open field (Table 1, $P = 0.04$, 0.01). Thus, JZL195 produced side effects with an ED_{50} that was at least 4.9 times greater than that at which it reduced allodynia (Table 1).

Effect of WIN55212

As a comparison, we examined the effect of a range of doses of WIN55212 (0.1–10 mg·kg^{-1}) at 7 days following CCI surgery ($n = 6$ per dose). WIN55212 produced a dose-dependent reversal of the CCI-induced reduction in mechanical PWT and of the CCI-induced increase in acetone responses (Figure 4A). The ED_{50} of WIN55212 for the acetone responses (0.86 mg·kg^{-1}) was less than that for mechanical PWT (2.0 mg·kg^{-1}) (Table 1, $P = 0.002$).

In these animals, WIN55212 produced dose-dependent increases in the bar and rotarod latencies and a dose-dependent decrease in open field crossings (Figure 4B,C). The ED_{50}s of WIN55212 for the bar test, rotarod and open field were 1.9, 1.4 and 1.2 mg·kg^{-1} (Table 1). The ED_{50} of WIN55212 for mechanical PWT was not significantly different to that for the bar and rotarod latencies ($P = 0.9$, 0.2), but was greater than that for the open field crossing ($P = 0.005$). By contrast, the ED_{50} of WIN55212 for acetone responses was less than that for the bar latency, rotarod latency and open field crossings ($P = 0.003$, 0.046, 0.02). Thus, WIN55212 produced side effects with an ED_{50} that was 0.6–2.2 times that at which it reduced allodynia (Table 1).

Contribution of FAAH and MAGL to the actions of JZL195

We also compared the efficacy of JZL195 (18 mg·kg^{-1}) to that of the FAAH and MAGL inhibitors, URB597 (18 mg·kg^{-1}) and JZL184 (18 mg·kg^{-1}), and to WIN55212 (3 mg·kg^{-1}) by testing doses of each agent that had a maximal anti-allodynic effect (Figures 3 and 4 and Kinsey *et al.*, 2009; Kinsey *et al.*, 2013) ($n = 6$ each group). At these doses, URB597, but not JZL184 produced an increase in mechanical PWT that was greater than that produced by vehicle (Figure 5A, $P < 0.05$ and >0.05). The effect of JZL195 on mechanical PWT was greater than that for both URB597 and JZL184 ($P < 0.05$, 0.01), but was less than that for WIN55212 ($P < 0.05$) (Figure 5A). Both URB597 and JZL184 produced a decrease in acetone responses, which was greater than that produced by vehicle (Figure 5A, $P < 0.01$, 0.05). The effect of JZL195 on acetone responses was greater than that for both URB597 and JZL184 ($P < 0.01$, 0.001), but was similar to that for WIN55212 ($P > 0.05$) (Figure 5A).

URB597 and JZL184 did not have a significantly different effect on bar and rotarod latency compared with that produced by vehicle (Figure 5B, $P > 0.05$). The effect of JZL195 on bar latency was not significantly different to that for URB597 and JZL184 ($P > 0.05$), but was less than that for WIN55212 ($P < 0.0001$) (Figure 5B). The effect of JZL195 on rotarod latency was greater than that for URB597 ($P < 0.05$),

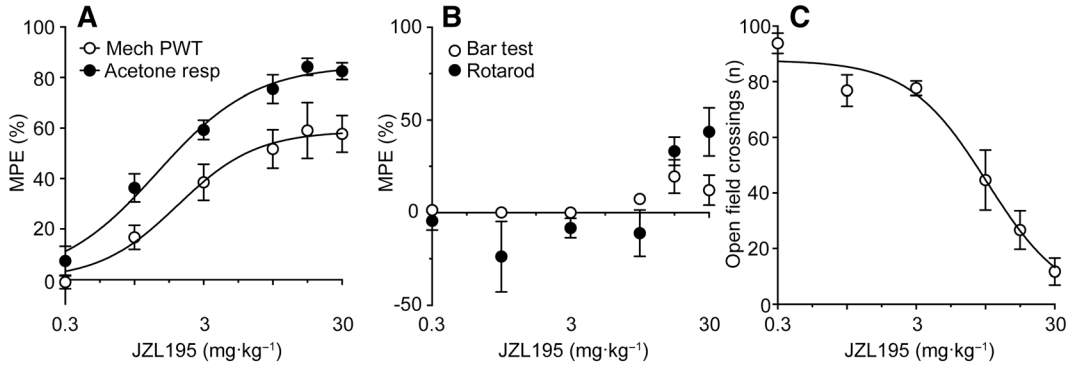

Figure 3

JZL195 reduces allodynia at doses below those at which it produces side effects. Dose response curves for the effect of JZL195 on (A) mechanical (Mech) PWT and acetone responses, (B) bar and rotarod latency, and (C) open field crossings. All data are expressed as a percentage of the maximum possible effect (% MPE), except open fields crossings for which raw data are shown (number of crossing over 2 min). The lines in (A) and (C) represent sigmoidal curve fits to the data.

Table 1

ED_{50} and therapeutic window for JZL195 and WIN55212 in behavioural assays

	JZL195		WIN55212	
	ED_{50} (mg·kg^{-1})	TI (Mechanical PWT, acetone response)	ED_{50} (mg·kg^{-1})	TI (Mechanical PWT, acetone response)
Mechanical PWT	2.1 (1.3–3.4)		2.0 (1.4–2.6)	
Acetone response	1.5 (0.9–2.4)		0.86 (0.57–1.3)	
Bar test	[a]	–	1.9 (1.3–2.6)	1.0, 2.2
Rotarod	[a]	–	1.4 (1.0–2.1)	0.7, 1.7
Open field	10.3 (7.2–14.7)	4.9, 6.9	1.2 (0.91–1.5)	0.6, 1.4

ED_{50}s are shown as the mean (95% confidence interval). TI, therapeutic index.
[a]Full dose response and ED_{50} not determined. The therapeutic window was calculated as the ratio of the ED_{50}s for mechanical PWT, or acetone responses versus those for the bar test, rotarod and open field.

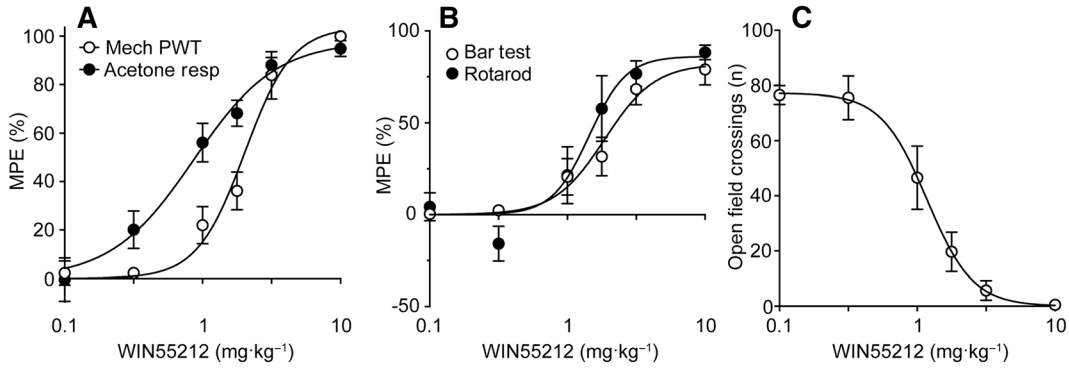

Figure 4

WIN55212 reduces allodynia at similar doses to those at which it produces side effects. Dose response curves for the effect of WIN55212 on (A) mechanical (Mech) PWT and acetone responses, (B) bar and rotarod latency and (C) open field crossings. The lines in (A–C) represent sigmoidal curve fits to the data.

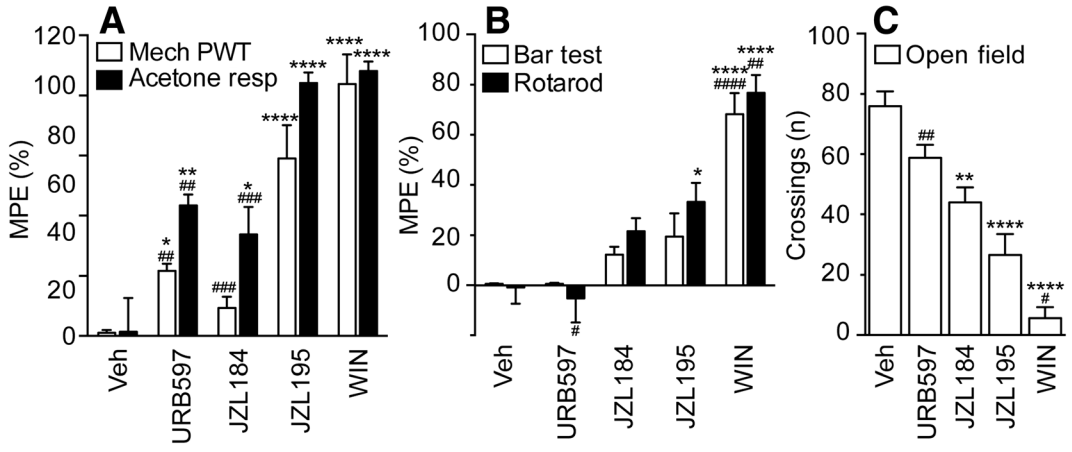

Figure 5

Comparison of the effect of maximal doses of endocannabinoid degradation inhibitors. Bar charts showing the effect of systemic administration of vehicle, or doses of URB597 (18 mg·kg^{-1}), JZL184 (18 mg·kg^{-1}) and JZL195 (18 mg·kg^{-1}) and WIN55212 (3 mg·kg^{-1}), which produced a maximal reduction in allodynia. Data are shown for (A) mechanical (Mech) PWT and acetones responses (Resp) (% MPE), (B) bar latency and rotarod latency (% MPE) and (C) open field crossings (number of grid crossing over 2 min). *, ** and **** denote $P < 0.05$, 0.01 and 0.0001 compared with vehicle; #, ##, ### and #### denote $P < 0.05$, 0.01, 0.001 and 0.0001 compared with JZL195.

was not significantly different to that for JZL184 ($P > 0.05$) and was less than that for WIN55212 ($P < 0.01$) (Figure 5B). JZL184 ($P < 0.01$), but not URB597 ($P > 0.05$) produced a decrease in open field crossings, which was greater than that produced by vehicle (Figure 5C). The effect of JZL195 on open field crossings was greater than that for URB597 ($P < 0.01$), was not significantly different to that for JZL184 ($P > 0.05$) and was less than that for WIN55212 ($P < 0.05$) (Figure 5C).

Repeated administration of JZL195

In the fourth series of experiments, we examined the effect of repeated systemic administration of JZL195 at doses that produced approximately 50 and 100% of MPE reductions in acetone responses (3, 18 mg·kg^{-1}). To do this, vehicle, or JZL195 was injected for five consecutive days starting at 7 days post-CCI ($n = 6$ for each treatment group). On each treatment day, all assays were measured before and 1–2 h after drug administration, except the open field that only measured 2 h after drug administration. On treatment days 1 and 5, vehicle injection had no effect on mechanical PWT, acetone responses, bar latency and rotarod latency (Figure 6A–D, $P > 0.05$). In addition, the number of open field crossings following vehicle injection on treatment days 1 and 5 was not significantly different to that prior to CCI surgery (Figure 6E, $P > 0.05$).

JZL195 produced a significant increase in mechanical PWT on treatment days 1 and 5 at the 18 mg·kg^{-1}, but not at the 3 mg·kg^{-1} dose (Figure 6A, post-injection versus pre-injection: $P > 0.05$ for 3 mg·kg^{-1} and $P < 0.01$, 0.001 for 18 mg·kg^{-1}). JZL195 produced a significant decrease in acetone responses on treatment days 1 and 5 at both doses (Figure 6B, post-injection versus pre-injection: $P < 0.01$ for 3 mg·kg^{-1} and $P < 0.0001$ for 18 mg·kg^{-1}). Both the pre-injection mechanical PWT and acetone responses did not differ significantly between treatment days 1 and 5, at either dose of JZL195 (Figure 6A,B, day 5 versus day 1: $P > 0.05$ for 3 and 18 mg·kg^{-1}). Similarly, the post-injection mechanical PWT and acetone responses did not differ significantly between treatment days 1 and 5, at either dose of JZL195 (Figure 6A,B, day 5 versus day 1: $P > 0.05$ for 3 and 18 mg·kg^{-1}).

JZL195 produced a significant increase in bar latency on treatment days 1 and 5 at the 18 mg·kg^{-1}, but not at the 3 mg·kg^{-1} dose (Figure 6C, post-injection versus pre-injection: $P > 0.05$ for 3 mg·kg^{-1} and $P < 0.05$ for 18 mg·kg^{-1}). It might be noted, however, that this increase in bar latency was less than 5% of MPE (Figure 6C). JZL195 did not have a significant effect on rotarod latency on treatment days 1 and 5 at both doses (Figure 6D, post-injection versus pre-injection: $P > 0.05$ for 3 and 18 mg·kg^{-1}). The pre-injection bar and rotarod latencies did not differ significantly between treatment days 1 and 5, at either dose of JZL195 (Figure 6C,D, day 5 versus day 1: $P > 0.05$ for 3 and 18 mg·kg^{-1}). The JZL195 post-injection bar latency did not differ significantly between treatment days 1 and 5, at either dose of JZL195 (Figure 6C, day 5 versus day 1: $P > 0.05$ for 3 and 18 mg·kg^{-1}). While the JZL195 post-injection rotarod latency did not differ significantly between treatment days 1 and 5, at the 3 mg·kg^{-1} dose, it was significantly greater on treatment day 5 compared with day 1, at the 19 mg·kg^{-1} dose (Figure 6C,D, day 5 versus day 1: $P > 0.05$ for 3 mg·kg^{-1} and $P < 0.05$ for mg·kg^{-1}).

The number of open field crossings after injection of 3 mg·kg^{-1} JZL195 was not significantly different to that prior to CCI nerve surgery on treatment days 1 and 5 (Figure 6E, post-injection versus pre-CCI: $P > 0.05$). By contrast, the number of open field crossing after injection of 18 mg·kg^{-1} JZL195 was significantly less than that prior to CCI nerve surgery on treatment day 5, but not treatment day 1 (Figure 6E, post-injection versus pre-CCI: $P > 0.05$ for day 1 and $P < 0.001$ for day 5). It might be noted, however, that the number of open field crossing after injection of both doses of JZL195 did not differ significantly between days 1 and 5 (Figure 6E, post-injection day 1 versus day 5: $P > 0.05$ for 3 and 18 mg·kg^{-1}).

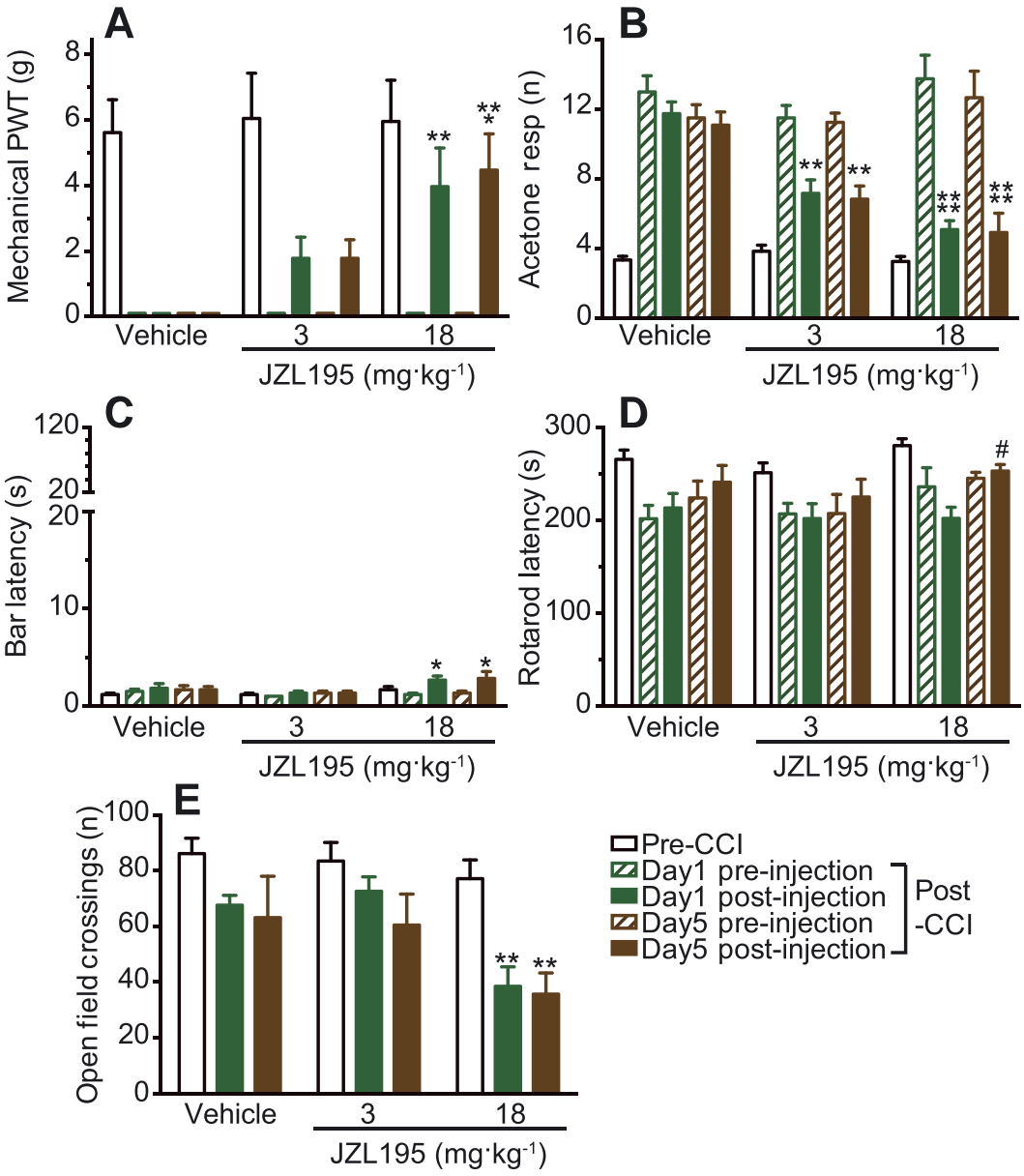

Figure 6

The JZL195-induced reduction in allodynia is maintained with repeated treatment. The effect of systemic injection of JZL195 (3, 18 mg·kg^{-1}) and vehicle on (A) mechanical PWT, (B) acetones responses (Resp), (C) bar latency, (D) rotarod latency and (E) open field crossing. Treatment with JZL195, or vehicle commenced at 7 days post-CCI nerve injury, and was repeated for five consecutive days. In (A–D), data are shown for pre-CCI nerve injury and pre-injection and post-injection on treatment days 1 and 5. In (E), raw open field data are shown for pre-CCI nerve injury and post-injection only on treatment days 1 and 50. In (A–D) *, **, *** and **** denote $P < 0.05$, 0.01, 0.001 and 0.0001 for pre-drug versus post-drug injection on days 1 and 5 of treatment; # denotes $P < 0.05$ for post-injection day 5 versus day 1. In (E), ** denotes $P < 0.01$ for pre-CCI versus post-drug injection.

Discussion and conclusions

The present results demonstrate that systemic administration of the dual FAAH/MAGL inhibitor JZL195 reduced the mechanical and cold allodynia induced by a model of neuropathic pain with greater efficacy than selective FAAH, or MAGL inhibitors. JZL195 also displayed a greater therapeutic window between anti-allodynia and side effects than the pan-cannabinoid receptor agonist WIN55212. Finally, JZL195 retained anti-allodynic efficacy during repeated treatment.

In the present study, JZL195 produced a dose-dependent reduction in the mechanical and cold allodynia induced by the CCI model of neuropathic pain in mice. This anti-allodynia was observed as a reversal of the CCI-induced decrease in mechanical PWT and increase in acetone responses in the operated hind limb. The cannabinoid

receptor agonist WIN55212 also had a dose-dependent anti-allodynic effect as observed previously for a range of non-selective cannabinoid receptor agonists (Herzberg *et al.,* 1997; Fox *et al.,* 2001; Lim *et al.,* 2003; Costa *et al.,* 2004; De Vry *et al.,* 2004; Scott *et al.,* 2004). While JZL195 had a lesser maximal effect on mechanical allodynia than WIN55212, both agents had similar maximal effects on cold allodynia. The differences between the effects of these agents on mechanical and cold allodynia could be due to divergent actions on these pain modalities, or to the assessment of threshold for a mechanical stimulus versus response magnitude for a cold stimulus. While the effect of JZL195 on neuropathic pain has not been examined previously, the reduction in mechanical allodynia was similar to that we have previously observed in an inflammatory pain model (Anderson *et al.,* 2014).

Like WIN55212, JZL195 produced cannabinoid-like side effects, including impaired motor coordination in the rotarod test, catalepsy in the bar test and reduced locomotion (sedation) in the dark open field, as observed previously in naïve mice (Long *et al.,* 2009b; Wise *et al.,* 2012). The dose response profiles for these JZL195-induced side effects were similar to those we have previously observed in mice, which have undergone an inflammatory pain model (Anderson *et al.,* 2014). JZL195 had a greater effect on mobility in the open field test compared with the other side-effect assays. This was likely to be due to enhanced reactivity, or 'popcorning', induced by the higher doses of JZL195, which interfered with assessment in the bar and rotarod tests, and is consistent with JZL195 producing context-dependent cataleptic responses (Long *et al.,* 2009b). These observations stress the importance of using multiple assays and doses to asses side effects. Nonetheless, other side effects would need to be explored in future studies, including abuse liability because JZL195 substitutes for THC in drug discrimination assays, and physical withdrawal that is observed following repeated high-dose JZL184 administration (Long *et al.,* 2009b; Kinsey *et al.,* 2013).

The therapeutic window for JZL195, measured as the ratio of ED_{50}s for the side effects versus anti-allodynia, ranged between 4.2 and 7.0. This was similar to that previously observed in an inflammatory pain model (Anderson *et al.,* 2014). By contrast, the therapeutic window for WIN55212 ranged between 0.8 and 2.2 and was similar to that previously reported for cannabinoid receptor agonists in the rat partial sciatic nerve ligation and spinal nerve ligation models, using a variety of pain and side-effect assays (Fox *et al.,* 2001; Scott *et al.,* 2004). To put this into context, it might be noted that conotoxin MVIIA, which is approved for use in chronic pain, has a therapeutic window of 0.7–2.1 in rodent neuropathic pain models when delivered intrathecally (Scott *et al.,* 2002; Jayamanne *et al.,* 2013). The greater therapeutic window of JZL195 compared with WIN55212, in conjunction with its higher anti-allodynic efficacy compared with URB597 and JZL184, suggests that JZL195 may represent an improved cannabinoid strategy for treating neuropathic pain states compared with both cannabinoid receptor agonists and selective FAAH/MAGL inhibitors.

It was also observed that the reduction in cold and mechanical allodynia produced by systemic JZL195 was maintained during repeated administration, at both EC_{50} and near-maximally effective doses (3 and 18 $mg \cdot kg^{-1}$). This lack of tolerance is similar to that previously reported for the anti-allodynic actions of URB597 and the putative anandamide transport blocker AM404 over a similar treatment period to that used in the present study (La Rana *et al.,* 2006; Russo *et al.,* 2007). By contrast, other studies have observed that high doses of the MAGL inhibitor JZL184 display anti-allodynic tolerance (Schlosburg *et al.,* 2010; Kinsey *et al.,* 2013). The lack of tolerance observed during repeated treatment in the present study may have been due to the use of JZL195 doses that were likely to produce submaximal increases in anandamide and 2-AG (Figure 2 in Long *et al.,* 2009b); it is likely that anti-allodynic tolerance would be observed at higher doses of JZL195. Repeated treatment with JZL195 did not unmask motor side effects in the rotarod assay, nor did it enhance the cataleptic and sedative effects (in bar and open field tests), even at the higher dose of JZL195. This suggests that JZL195 maintains near-maximal anti-allodynic effects in the absence of enhanced side effects during chronic treatment, at least for the behavioural assays and doses used in the present study.

In the present neuropathic pain model, FAAH and MAGL inhibition were likely to make differing contributions to the anti-allodynic actions and side effects of JZL195. The anti-allodynic effect of a maximal dose of JZL195 was greater than that for the selective FAAH and MAGL inhibitors, URB597 and JZL184. This was similar to our prior study of JZL195 in an inflammatory pain model, and for the antinociceptive effects of JZL184 in FAAH knockout mice (Anderson *et al.,* 2014; Schlosburg *et al.,* 2014). The JZL195-induced anti-allodynia was therefore likely to be due to inhibition of both FAAH and MAGL. It might be noted that this differs to chronic pain induced by stress and nerve GF in which combined FAAH/MAGL inhibition does not have a greater anti-hyperalgesic effect than either alone (Lomazzo *et al.,* 2015). It remains to be determined whether combined FAAH and MAGL blockade reduces allodynia in an additive, or synergistic manner, as has been demonstrated for FAAH or MAGL with COX synergistically reduces nerve injury induced allodynia (Grim *et al.,* 2014; Crowe *et al.,* 2015). In contrast to its anti-allodynic actions, the JZL195-induced catalepsy (bar test), motor impairment (rotarod) and sedation (open field) were greater than that for URB597, but similar to that for JZL184. This suggests that the observed side effects were largely mediated by inhibition of MAGL, although a role for FAAH cannot be excluded.

The observed effects of JZL195 were likely to be mediated by inhibition of both FAAH and MAGL and a subsequent elevation in the levels of the endocannabinoids anandamide and 2-AG (Long *et al.,* 2009b). Anandamide and 2-AG are known to act via a range of partially overlapping targets, including cannabinoid CB1 and 2 receptors, TRPV1 (Bradshaw and Walker, 2005; Pacher *et al.,* 2006; Hill *et al.,* 2009). Although not examined in the present study, the anti-allodynic effects of JZL195 are mediated by both cannabinoid CB1 and CB2 receptors in an inflammatory pain model, while its side effects are largely CB1-mediated (Long *et al.,* 2009b; Anderson *et al.,* 2014). In addition to elevating endocannabinoid levels, it might be noted that both JZL195, like JZL184, produces a reduction in arachidonic acid and consequently prostaglandin levels, which could also contribute to its anti-allodynic effects (Nomura *et al.,* 2011).

While cannabinoids have been mooted as a potential treatment for neuropathic pain, current experimental agents

remain problematic. Cannabinoid receptor agonists have good anti-allodynic efficacy, but a poor therapeutic window in animal neuropathic pain models. On the other hand, selective FAAH and MAGL inhibitors have a better therapeutic window, but lesser efficacy in these pain models. Indeed, the FAAH inhibitor PF04457845 has not progressed through human chronic pain studies because of poor efficacy (Huggins *et al.*, 2012). The present study has demonstrated that the dual FAAH/MAGL inhibitor JZL195 reduces the allodynia associated with a murine neuropathic pain model with efficacy approaching that of cannabinoid receptor agonists, while retaining a good therapeutic window. It is therefore possible that the balance of efficacy and therapeutic window of dual FAAH/MAGL inhibition may provide an improved cannabinoid treatment for chronic neuropathic pain.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contribution

N. S. A. B. performed the study, analysed the data and contributed to writing the manuscript. V. A. M. performed the study, analysed the data and contributed to writing the manuscript. N. P. K. performed the study, analysed the data and contributed to writing the manuscript. C. W. V. conceived and designed the study, performed the study, analysed the data and contributed to writing the manuscript.

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