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Opioid-Sparing Effects of Cannabinoids on Morphine Analgesia: Participation of CB1 and CB2 Receptors

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Abstract
Background and Purpose: Much of the opioid epidemic arose from abuse of prescription opioid drugs. This study sought to determine if the combination of a cannabinoid with an opioid could produce additive or synergistic effects on pain, allowing reduction in the opioid dose needed for maximal analgesia.
Experimental Approach: Pain was assayed using the formalin test in mice and the carrageenan assay in rats. Morphine and two synthetic cannabinoids were tested: WIN55,212-2 (WIN), which binds to both CB1 and CB2 receptors, and possibly TrpV1 receptors; and GP1a, which has activity at CB2 receptors and is reported to inhibit fatty acid amide hydrolase (FAAH), thus raising endogenous cannabinoids.
Key Results: Morphine in combination with WIN in the formalin test gave synergistic analgesia. Studies with selective antagonists showed that WIN was acting through the CB1 receptor. Morphine in combination with GP1a in the formalin test was sub-additive. In the carrageenan test, WIN had no added effect when combined with morphine, but GP1a with morphine showed enhanced analgesia. Both WIN and Gp1a used alone had analgesic activity in the formalin pain test, but not in the carrageenan pain test.
Conclusions and Implications: The ability of a cannabinoid to produce an additive or synergistic effect on analgesia when combined with morphine varies with the pain assay and may be mediated by CB1 or CB2 receptors. These results hold the promise of using cannabinoids to reduce the dose of opioids for analgesia in certain pain conditions.

Abbreviations:
Δ^9-THC: Δ^9-tetrahydrocannabinol
CB1: Cannabinoid receptor 1
CB2: Cannabinoid receptor 2
CCL: C-C chemokine ligand
CCR: C-C chemokine receptor
CXCL: C-X-C chemokine ligand
CXCR: C-X-C chemokine receptor
MPA: Maximal possible analgesia
G-CSF: Granulocyte-colony stimulating factor
GM-CSF: Granulocyte-macrophage-colony stimulating factor
LTB: Lymphotoxin beta
WIN: WIN55,212-2

Keywords: Opioid; Cannabinoid; Synergy; Pain; Analgesia; Drug Combinations; Inflammation

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Hyperlinks:

Ligands:

WIN55,212-2:
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=733

Resources:

Taconic Biosciences:

https://www.taconic.com/

Nomenclature of Targets and Ligands:
Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Bullet Point Summary

What is already known?

- Combinations of some cannabinoids plus morphine give enhanced analgesia over either drug alone.
- Cannabinoids alone can mediate analgesia through CB1 or CB2 receptors.

What this study adds:

- Additive/synergistic analgesic effects of cannabinoids, when combined with morphine, vary with the pain assay.

- CB1 or CB2 receptors can mediate additive/synergistic analgesic effects of cannabinoids combined with morphine.

Clinical significance:

- Combinations of cannabinoids with opioids may be an effective way of reducing opioid doses.

Introduction

The U.S. is currently in the midst of an opioid epidemic that has been declared a "public health emergency" by the Department of Health and Human Services (https://www.hhs.gov/about/news/2017/10/26/hhs-acting-secretary-declares-public-health-emergency-address-national-opioid-crisis.html). The United Kingdom has also seen a significant rise in prescription opioid abuse (Giraudon *et al.*, 2013) (https://www.nytimes.com/2018/02/04/world/europe/uk-fentanyl-opioid-addiction.html). The National Survey on Drug Use and Health carried out by the Substance Abuse and Mental Health Services Administration (SAMHSA) in 2016 found that 11.5 million Americans misused prescription opioids, and 62.3% gave as the reason for their use, relief of pain (Ahrnsbrak *et al.*, 2017). It is estimated that 30% of Americans suffer from acute or chronic pain (Volkow and McLellan, 2016; Volkow *et al.*, 2011), leading to the use of prescribed or illicit opioids for pain relief. One potential mitigating strategy for decreasing opioid use is to employ combination therapies, with the objective of producing equal analgesia using a lower dose of opioid. In animal models, cannabinoids have documented analgesic activity (Pertwee, 2001; Walker and Hohmann, 2005) as well as anti-inflammatory effects (Eisenstein and Meissler, 2015; Klein, 2005).

There are complexities to the cannabinoid system, as two cannabinoid receptors, designated CB1 and CB2 have been identified (Matsuda *et al.*, 1990; Munro *et al.*, 1993). CB1 is highly expressed on neurons in the central nervous system (Herkenham *et al.*, 1991) and to a lesser extent on cells of the immune system (Daaka *et al.*, 1996; Galiegue *et al.*, 1995). CB2 is primarily expressed on cells of the immune system (Daaka *et al.*, 1996; Galiegue *et al.*, 1995; Carlisle *et al.*, 2002), as well as on activated microglia (Carlisle *et al.*, 2002). CB2 has been detected on neurons in the central nervous system, but levels of expression of this receptor are low compared to CB1 (Gong *et al.*, 2006; Van Sickle *et al.*, 2005).

Both endogenous cannabinoid system ligands and exogenous cannabinoid receptor ligands have been shown to mitigate pain (Woodhams *et al.*, 2017). Δ^9-THC has activity at both CB1 and CB2 receptors, and exerts analgesic activity through both receptors (Agarwal *et al.*, 2007; Elikottil *et al.*, 2009; Craft *et al*., 2013; Thapa *et al*., 2018). CB2 receptors have also been shown to modulate pain (Deng *et al.*, 2015; Guindon and Hohmann, 2008; Kinsey *et al.*, 2011; Brownjohn and Ashton, 2012; Gutierrez *et al.*, 2011). Using CB2 knock-out mice, as well as CB2-overexpressing transgenic mice, it was shown that activation of this receptor decreases sciatic nerve injury pain (Racz *et al.*, 2008). A synthetic CB2-selective agonist was shown to have analgesic activity in a model of chemotherapy-induced neuropathic pain in mice, which correlated with a reduction in mRNA for selected pro-inflammatory cytokines and chemokines (Deng *et al.*, 2015). Further, a synergistic combination between morphine and a selective CB2 agonist, JWH015, was also shown in rodent models of post-surgery and neuropathic pain (Grenald *et al*., 2017). Contributions of CB1 and CB2 receptors were reported to participate in synergistic combinations of cannabinoids with morphine in a mouse model of cancer pain (Khasabova *et al.,* 2011). Activation of the CB2 receptor on cells of the immune system results mainly in immunosuppression (Eisenstein and Meissler, 2015; Klein, 2005). Use of a CB2-

selective agonist would, therefore, be predicted to reduce inflammation, which plays a major role in many types of pain.

There is a pre-clinical literature showing that combinations of cannabinoids and opioids have additive or synergistic analgesic effects (Cichewicz, 2004; Nielsen *et al.*, 2017; Welch, 2009). Most of these studies did not provide information on which cannabinoid receptor was mediating the opioid-sparing effect. The hypothesis being tested in the present studies is that a combination of a cannabinoid with a sub-analgesic dose of an opioid can achieve a level of pain relief observed with an optimal dose of the opioid alone. Such combinations, which would permit use of opioids at lower doses, could have the added advantage of reducing unwanted adverse effects of opioids, such as nausea, vomiting, constipation, sedation, respiratory depression, and pruritus, as well as potential development of tolerance and dependence. The present project was undertaken to investigate the feasibility of using morphine in combination with two different cannabinoids, one of which (WIN55,212-2 [WIN]) binds to both CB1 and CB2 receptors, and the other (GP1a), which has CB2 agonist activity in *in vivo* studies (Franklin and Carrasco, 2013; Kong *et al.*, 2014). We also used cannabinoid receptor antagonists SR141716A (CB1), and SR144528 (CB2) to understand which cannabinoid receptor mediates additive or synergistic analgesic effects with morphine. We chose WIN since there is a history of its use in animals for pain studies (Martin *et al*., 1999). WIN has been reported to have activity at the CB1, CB2, and TrpV1 receptors (Lowin *et al.*, 2016). In the present study, a TrpV1 antagonist, SB366791, was also studied using the cannabinoid and opioid combination to investigate possible TrpV1 receptor involvement. Kong *et al*. (2014) reported that GP1a, treatment decreased demyelination and axonal loss, as well as reduced clinical scores and facilitated recovery in experimental autoimmune encephalomyelitis in mice. Analgesic effects of the compounds alone and in combination with morphine were tested in two different pain assays. Formalin-induced nociception was used in mice as a tonic/chronic pain model (Murray *et al*., 1988) and carrageenan-induced inflammation was used in rats as a model of inflammatory pain (Kocher *et al*., 1987).

Methods:

Animals: Outbred Swiss-Webster male mice (20-25 g) and outbred male Sprague-Dawley rats (120-150 g), were purchased from Taconic Biosciences (Albany,NY). Animals were housed in the Central Animal Facility of the Medical School in ventilated micro-isolator cages under a schedule of 12 hr of light and 12 hr of darkness. They had access to food and water *ad libitum*. All experiments were carried out under protocols approved by the University IACUC. In all experiments, the N = 6-10 animals/group, as designated in the individual figures.

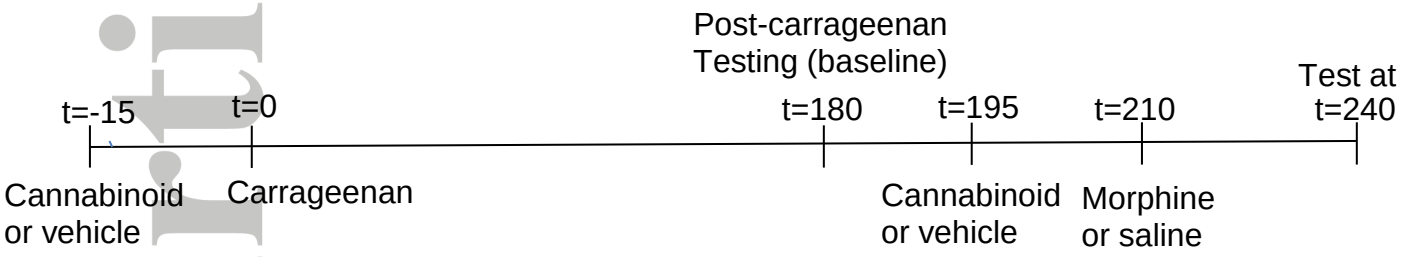
Reagents: Cannabinoids and their antagonists were purchased from Tocris (Minneapolis, MN). WIN55,212-2 (WIN) was dissolved in 5% DMSO when used in

mice, and in 10% DMSO when used in rats. GP1a was dissolved in 5% DMSO. The cannabinoid antagonists, SR141716A (CB1), SR144528 (CB2), and the TrpV1 antagonist, SB366791, were dissolved in 5% DMSO. Morphine sulfate (doses expressed as the salt) was obtained from NIDA and dissolved in saline.

Formalin pain assay: In this assay, the analgesic effects of morphine alone, a cannabinoid alone, or a combination of morphine plus a cannabinoid, were tested (n=7-8). Five minutes before formalin injection (t = -5), animals were placed into a transparent chamber connected to an isoflurane vaporizer, and briefly anesthetized. At t = 0, morphine or saline vehicle, or a cannabinoid (WIN or Gp1a), or 5% DMSO alone, was injected subcutaneously (s.c.) into the dorsal flank area of mice. In combination experiments, opposite sides of the dorsal flank were used. Immediately after drug or vehicle injections, the dorsal side of the left hind paw was injected s.c. with 20 µl of a 5% formalin solution diluted in saline. In experiments in which an antagonist was used, it was given i.p. 30 min before agonists and formalin. After receiving the formalin injection, animals were placed into a large glass jar where their actions could be monitored. Formalin injection causes intense licking of the injected paw in two phases. The late phase of licking, between 20 and 35 min after administration of formalin, represents a combination of peripheral inflammatory- and spinal-mediated pain (Tjolsen *et al.*, 1992) and thus total licking time between 20 and 35 min was chosen for measurement of the response. Licking was scored for each animal as the number of sec of licking that occurred during that 15-min period (20 to 35 min after formalin injection). Animals were randomly assigned to groups, and experiments were carried out with the observer/recorder of the behavior blinded to the treatment given to the animal.

Carrageenan pain assay: The carrageen test has two parameters, pain and swelling (edema) of rat paws following injection of the irritant, carrageenan. To carry out the test, unrestrained rats were placed inside a clear plastic chamber (10 cm w x 21 cm deep x 13 cm high) with a glass floor (32^0 C) that is part of the Hargreaves' Plantar Test Apparatus (Model 400, IITC Life Science, Woodland Hills, CA). After 60 min of habituation, all animals were exposed on the plantar surface of the left hind paw to a beam of radiant heat (intensity=45) through the glass floor. They were tested 3 times, with 5-min intervals between each stimulus. The latency (sec) to paw withdrawal was used as the antinociceptive index and was automatically scored by the apparatus. Edema was measured using a digital plethysmometer. To carry out the pain assay, rats were injected i.p. with the desired cannabinoid, WIN or GP1a, or the vehicle (10% DMSO), 15 min before the carrageenan injection (t = -15). At t = 0, rats were injected into the plantar side of the left hind paw with 0.1 ml of a 2% carrageenan solution (FMC, Philadelphia, PA), freshly prepared in saline. All animals were tested by exposure to the heat beam 3 times at 5-min intervals to establish the baseline pain value 180 min after carrageenan injection. Fifteen min after the post-carrageenan baseline, the animals received a second injection of cannabinoid or vehicle. At t = +210 min (15 min after second cannabinoid or vehicle injection), they received an injection of morphine (3.0 mg/kg) or saline s.c. into the dorsal flank area. Latency of paw withdrawal to radiant heat and edema was measured at 240 min (30 min after morphine or saline injection) and expressed as a percentage change from post-carrageenan baseline. The percent of maximal possible antinociception (%MPA) for each animal at each time was calculated using the following formula: %MPA = [(test latency at t = +240 min – baseline latency at t = +180 min)/(22

second- baseline latency at t = +180 min)] x 100. A cutoff limit of 22 seconds was set to avoid damage to the paw. To measure edema induced by the carrageenan and the effect of cannabinoids, morphine, or the combination of the two compounds, swelling of the left paw was measured at t = 0 (just after carrageenan injection) 180, and 240 min post carrageenan administration. To quantitate edema, the following formula was used: Volume difference of edema (ml) = Volume at t=+240 min– Volume at t=+180 min. Experiments were carried out with the observer/recorder of the behavior blinded to the treatment given to the animal. The timeline for the carrageenan experiments is shown below:



mRNA levels of immune mediators:
For determining mRNA levels, the draining popliteal lymph nodes from 2 animals in each group, as well as from 2 animals that received no carrageenan or drug (to obtain baseline values), were collected at t = 35 minutes from mice in the formalin test and at t = 4 hr from rats in the carrageenan test. The lymph nodes were then extracted by the RNeasy® Microarray Tissue Mini Kit (Qiagen, Gaithersburg, MD). These RNAs were used to generate cDNA using the RT_2 First Strand Kit (Qiagen). The cDNAs from the two duplicate animals were pooled and assayed using the RT_2 Profiler® PCR Arrays for Rat or Mouse Inflammatory Cytokines and Receptors (Qiagen), with the RT_2 SYBR® Green ROX® qPCR Mastermix (Qiagen). The rat and mouse arrays have probes for mRNA for 84 immune mediators. The PCR arrays were run on an ABI StepOne Plus® qPCR thermocycler (Applied Biosystems, Foster City, CA), using the cycling conditions given in the protocol supplied with the RT_2 Array. Data were processed by the online GeneGlobe Data Analysis Center (Qiagen). Results are presented as heat maps showing mRNA for cytokines and chemokines in the array of the treated groups compared with mRNA level expression in the baseline, untreated control group.

Statistical Methods
For dose-response curves where multiple doses were tested, a one-way ANOVA was performed followed by Sidak's multiple comparison test when comparing drug-treated animals to a control group. Sidak's test was used for comparisons between groups treated with different drugs or different drug dosages. $p<0.05$ was considered to be a statistically significant difference.
Isobolographic analysis was used to determine if there were additive or synergistic effects when morphine was combined with a cannabinoid, where both drugs demonstrated analgesic activity. The ED_{50} values of the individual drugs were plotted on the x and y axes and connected by an intersecting line. The intersecting line

represents points along the line that show additivity. A point above the line represents sub-additivity, and a point below the line represents synergy. The new ED_{50} value of the combinations can be plotted to determine if the combinations are additive, sub-additive or synergistic.
In the case where one drug in the combination was effective (morphine), and the other drug was ineffective or only slightly effective, precluding calculation of its ED_{50}, the dose equivalence method (Tallarida, 2006) was used to determine if the combinations were different from either drug alone. In this method, the value for the second drug was converted to the equally effective dose of morphine, and expected effects were calculated. To evaluate if the two drugs interact, these expected effects were compared to the effects observed. If the observed effects were below what was expected, the interaction was classified as sub-additive; if the effects were equal to the expected effect, the interaction was classified as additive; and if the observed effects were greater than what was expected, the interaction was classified as synergistic.

Results:

Analgesia induced by WIN alone, morphine alone, or a combination treatment in the formalin assay.
Initial experiments were undertaken to assess the analgesic activity of morphine (0.5-10 mg/kg, s.c.) alone, WIN (0.5-5 mg/kg, s.c.) alone, or the two drugs (morphine: WIN, 1:1 ratio, 0.1-1 mg/kg, s.c.) in combination. Figure 1A shows that morphine significantly decreases formalin-induced licking in a dose-related manner compared to vehicle ($p<0.0001$, one-way ANOVA followed by Dunnett's multiple comparison). Figure 1B shows that WIN also significantly decreases licking time in a dose-related manner, compared to the vehicle group ($p<0.0001$, one-way ANOVA followed by Sidak's multiple comparison). Thus, both the opioid and the cannabinoid gave strong, dose-related antinociceptive effects in the formalin test. Combination experiments were then carried out using these two compounds. The doses used in the combination studies were derived from the data gleaned from the individual dose-response curves, with attention to the range within which an increase or decrease in analgesic effect could be observed. Figure 2A shows that the licking time in response to formalin injected into the paw significantly decreased in a dose-related manner in the animals given the combination of morphine and WIN. At the 0.5 and the 1.0 mg/kg doses, morphine alone and WIN alone produced significantly reduced licking in response to the formalin injection, which was even further reduced when the drugs were combined at those doses. Using isobolographic analysis, the drug interactions were found to be highly synergistic. The results demonstrated the predicted increase in antinociceptive effect of the combination over each individual drug alone (Fig 2B).

To examine the receptor through which WIN (1 mg/kg) in combination with morphine (1 mg/kg), produced enhanced analgesia in the formalin test, antagonists for CB1, CB2 and TrpV1 receptors were used. SR141716A (CB1, 5 mg/kg, s.c.), SR144528 (CB2, 5 mg/kg, s.c.), and SB366791 (TrpV1, 1 mg/kg, s.c.) were administered 20 min before the test compounds and formalin administration. Figure 2C shows that the synergistic activity of WIN and morphine in the formalin test is through the CB1 receptor, as the CB1 antagonist (SR141716A) returned licking time to that seen with vehicle alone (*$p<0.05$, one-way ANOVA followed by Sidak's multiple comparison).

Neither the CB2 antagonist, nor the TrpV1 antagonist, had an effect on the analgesic activity of the WIN plus morphine combination. Also, none of the three antagonists had any effect on licking by themselves (Fig. 2C).

Analgesia induced by GP1a alone, morphine alone, or a combination treatment in the formalin assay.

Figure 1C shows the dose-response effect for GP1a alone in the formalin assay. Gp1a significantly reduced (around 30%-50%) licking time at doses ranging between 1.0 mg/kg and 50.0 mg/kg in a non-dose-related manner (***p<0.01: one-way ANOVA followed by Sidak's multiple comparison test). The next experiments tested the effect of the combination of GP1a with morphine, using the morphine dose-response curve shown in Fig. 1A as a reference. Figures **3**A, 3B and 3C show the effect of GP1a alone (1.0, 5.0 or 10.0 mg/kg), or a combination of these three different doses of GP1a with two different doses (0.5 and 1 mg/kg) of morphine. Using dose equivalence analysis, as explained in the section on statistical analysis in the Methods, the combinations of GP1a and morphine were sub-additive.

Analgesia in the carrageenan assay induced by the cannabinoids, WIN or GP1a each alone, morphine alone, or combination treatments.

Figure 4A shows the analgesic dose-response for morphine (0.5-10 mg/kg, s.c.), and figures 4B and 4C present the respective dose-responses for WIN (1-5 mg/kg, s.c.) and GP1a (0.5-10 mg/kg, s.c.) in the carrageenan test. Morphine produced significant analgesia in a dose-dependent manner (**p<0.01, ***p<0.001, one-way ANOVA followed by Sidak's multiple comparison), but had no effect on edema (Supp Fig. 1A). Neither WIN nor GP1a had significant analgesic activity in this assay (Figs. 4B and 4C), and were also without effect on paw edema (Supp. Figs. 1B and 1C). The animals that received only vehicle (10% DMSO) in panels 4A, 4B, and 4C appeared to show hyperalgesia, but it was not statistically significant due to the large standard errors. Also, no trend towards an increase in pain with vehicle was found in a subsequent experiment (see Fig. 5A and 5B). Combination experiments of morphine with a cannabinoid were carried out using the carrageenan test. As shown in Figure 5A, WIN (1-5 mg/kg) did not enhance the analgesia induced by a suboptimal dose of morphine (3.0 mg/kg) in this test, nor did the WIN-morphine combination result in a reduction in edema (Supp. Fig. 1C). Thus, in contrast to the formalin assay in mice, in the carrageenan assay in rats, WIN showed no additive or synergistic effect with morphine. Figure 5B presents data on the combination of morphine at a suboptimal dose (3.0 mg/kg) given with GP1a (5.0 mg/kg). An increased analgesic effect (*p<0.05, one-way ANOVA followed by Sidak's multiple comparison) was observed using this combination of GP1a plus morphine. No drug interactions were seen in reducing swelling of the paw (Supp. Fig. **1D**). Thus, GP1a showed an increased analgesic response with morphine in the carrageenan test, but in the formalin test this drug combination was no better than either compound alone.

Immune mediators, receptors and other molecules.

Preliminary data were obtained on the effect of opioid and cannabinoid treatment on production of inflammatory mediators using mRNA arrays. Data are displayed as heat maps. Figure 6A, shows the results for the formalin test in mice, using pooled mRNA extracts of popliteal lymph nodes of 2 mice in each group. Animals in every group received a formalin injection in addition to the treatments noted at the bottom of each column. The left-hand column represents the levels of mediators induced by

the formalin injection alone plus saline and DMSO vehicles. Qualitatively, one can observe that many of the analytes were elevated (darker red color). Morphine or WIN given alone each had a moderate suppressive effect on mediator expression. The morphine plus WIN combination dampened the chemokine/cytokine mRNA profile, as seen by the increase in the number of green bars between formalin alone (2 bars) and the combination treatment (7 bars). The CB2 antagonist, but not the CB1 antagonist, returned the mediator intensity closer to that observed with formalin alone. Interestingly, the Trvp-1 antagonist given with morphine and WIN resulted in highly depressed levels of mRNA for many mediators when compared to morphine plus WIN, even though it did not inhibit the analgesia induced by this combination. Figure 6B shows a similar analysis for the combination of morphine plus the CB2-selective agonist, GP1a, on the average mRNA levels of tissue extracted from 2 individual rats in each treatment group. Carrageenan plus vehicles (left most column) induced a broad inflammatory response. The combination of morphine plus Gp1a in the carrageenan test was more suppressive than either morphine alone or GP1a alone.

Discussion and Conclusions:

Two different cannabinoids, WIN and GP1a were tested in combination with morphine in two different pain tests, the formalin assay and the carrageenan assay. Morphine, as expected, gave strong dose-dependent analgesia in both tests (Figs. 1A and 4A). It was found that WIN produced analgesia in the formalin test (Fig. 1B), and when combined with morphine had a synergistic interaction in reducing pain (Figs. 2A and B). Use of receptor-selective antagonists, SR141716 (CB1), SR144528 (CB2) and SB366791 (TrpV1), showed that the synergistic interaction on the analgesic activity produced by WIN was mediated through the CB1 receptor (Fig. 2C). The other cannabinoid, GP1a, produced statistically significant analgesia in the formalin test at doses ranging between 1.0 mg/kg and 50.0 mg/kg, without a clear dose-response effect in that range (Fig. 1C). Combinations of three different doses of GP1a with two different doses of morphine did not result in additive or synergistic interactions in this assay (Figs. 3A, B, and C). In contrast, in the carrageenan assay, neither WIN nor GP1a produced analgesia when given alone over a range of doses (Figs. 4B, and C). Unlike the results with formalin, in the carrageenan test WIN did not give statistically significant interactive effects when combined with morphine (Fig. 5A), while the combination of GP1a and morphine resulted in a greater analgesic effect than with morphine alone at one dose combination (Fig. 5B). Thus, the results for the combinations of the cannabinoids with morphine yielded opposite results in the two tests. These results are summarized in Table 1. Similarly, previous report from our laboratory (Inan et al., 2018) showed that combining sub-analgesic doses of morphine with chemokine receptor antagonists could provide maximal analgesia in a rat model of incisional pain.

Much of the literature on interactions between opioids and cannabinoids relating to analgesia, in both preclinical models and clinical studies, has reported on use of Δ^9-tetrahydrocannabinol (Δ^9-THC). In rhesus monkeys, the dose-response curves for morphine and other opioids (fentanyl, etorphine, and buprenorphine, but not nalbuphine) in the warm water tail-flick test were significantly shifted leftward by Δ^9-

THC and also by the synthetic cannabinoid, CP55,940 (Maguire and France, 2014). CP55,940, like Δ^9-THC and WIN, is active at both the CB1 and CB2 receptor. Synergy was also found between Δ^9-THC and morphine administered intrathecally,i.c.v., s.c. or p.o. in inducing analgesia measured using the tail-flick test in mice (Smith *et al.*, 1998; Welch and Stevens, 1992; Welch *et al.*, 1995). Antagonist studies showed that Δ^9-THC was acting via the CB1 receptor (Smith *et al.*, 1998). Experiments using genetic deletion of the peripheral CB1 receptor in mice showed that the analgesia mediated by WIN in an assay of neuropathic pain was mainly through the CB1 receptor (Agarwal *et al.*, 2007). There is also a report that Δ^9-THC used in combination with morphine in arthritic rats has synergistic analgesic interactions that were mediated via the CB2 receptor (Cox *et al.*, 2007b; Cox *et al.*, 2007a). The experiments carried out in the present studies using WIN, with and without antagonists selective for CB1 and CB2, show that in the mouse formalin test, WIN was active via the CB1 receptor. The results reveal very strong synergy of WIN with non-analgesic doses of morphine (0.1-1 mg/kg, Fig. 2).

There is currently marked interest in the analgesic activity of Δ^9-THC, as marijuana has been legalized for medicinal purposes in 24 states and the District of Columbia, including approval for use for many conditions that have pain as a primary symptom. There are reports that availability of medical marijuana has decreased prescriptions for other FDA-approved medications to treat pain (Bradford and Bradford, 2016). Δ^9-THC has the disadvantage of being psychoactive and potentially leading to tolerance and dependence. The nonselective cannabinoid agonists like WIN and CP55,940 also bind to CB1 receptors with the same potentially negative side effects. The development of selective CB2 agonists raises the possibility of utilizing cannabinoids devoid of psychoactive activity as analgesics. In fact, CB2 agonists given acutely or chronically have been reported to have analgesic activity against neuropathic pain in mice (Deng *et al.*, 2015; Lin *et al.*, 2018). Further, CB2 knock-out mice have enhanced nociception in a mouse model of sciatic nerve injury (Racz *et al.*, 2008).

The current experiments show that GP1a has an increased analgesic effect with morphine in the carrageenan test in rats, but did not enhance morphine analgesia in the formalin test in mice. GP1a was promoted as a selective CB2 agonist and found active on CB2 receptors in *in vivo* studies (Franklin and Carrasco, 2013; Kong *et al*., 2014) until it was reported recently as an inverse agonist by Soethoudt *et al*. (2017). Also, in the same study, it was stated that GP1a showed partial inhibition (30-40%) of fatty acid amide hydrolase (FAAH), an enzyme that breaks down endogenous anandamide. Inhibition of this enzyme will result in an increase in endogenous anandamide levels. Recently, inhibitors of FAAH have been investigated as potential therapeutic targets for pain and CNS disorders (Ahn *et al*., 2009; Deutsch, 2016). Our results show that GP1a has analgesic activity on chronic/tonic pain by itself, as well as increasing morphine's effect on inflammatory pain. GP1a might have analgesic activity through enhancing endogenous anandamide levels by blocking FAAH. A recent study (Slivicki *et al.,* 2018) shows synergistic effects of FAAH inhibitors on morphine-induced analgesia against chemotherapy-induced neuropathic pain in mice. Further, and critically, Slivicki et al. (2018) also reported that this synergy was not shown on morphine-induced reduction of gastrointestinal transit. Yuill et al. (2017) noted an additive interaction between the CB2-selective agonist, JWH-133, and morphine in the mouse formalin test at a single dose ratio. If GP1a is working by increasing endogenous levels of anandamide, it could be

hypothesized that its effect may not have been robust enough to produce synergism when combined with morphine in the formalin test, but was sufficient to increase the analgesic effect at one dose in the carrageenan test. Lin *et al*. (2018) reported that a CB2 agonist used chronically to treat paclitaxel-induced neuropathy can block development of tolerance to the analgesic effects of morphine when it is given post cannabinoid treatment. In the present experiments, all of the drugs were given acutely and the effect of drug combinations on analgesic activity was only evaluated in an acute time frame.

The present studies show that there are differing results for analgesia evoked by two cannabinoids, each acting on distinct receptors, and also by combinations of opioids and cannabinoids in different pain assays. These results point to the conclusion that there are divergent mechanisms involved in pain induction, transmission or perception in the two assays. Formalin causes a biphasic response with a rapid onset of pain that lasts for 3-5 min, followed by a later phase starting 15-20 min after the formalin injection, which has an inflammatory component (Tjolsen *et al.*, 1992). Injection of carrageenan into the footpad results in pain and swelling that increases over a period of 4 hr, the endpoint when measurements were taken. Time-course studies showed that pain persisted for at least 5 hr. Thus, the pain induced by carrageenan is longer lasting and is accompanied by a clear inflammatory component, which is manifest in measurable, marked paw edema. Since GP1a and CB2-selective agonists have been shown to have suppressive effects on the immune system (Eisenstein and Meissler, 2015), one would have predicted that this cannabinoid would have had efficacy in the carrageenan assay. In spite of a lack of activity by itself in the carrageenan assay, GP1a increased morphine's antinociceptive effect at one dose.

The immune assays were undertaken to determine if morphine alone, the cannabinoids alone, or the combination of the two classes of drugs would show suppressive effects on levels of inflammatory mediators that might correlate with reductions in pain. The results presented are preliminary as only a limited number of samples were tested. Results in Figure 6A represent the pooled mRNA extracted from 2 mice, and the results in Figure 6B represent the average of results from 2 arrays from 2 rats. From this preliminary data it can be observed that suboptimal analgesic levels of morphine alone in the formalin and carrageenan assays moderately suppressed mRNA for a broad panel of mediators induced by the respective painful insults, which is consonant with the literature showing that morphine is immunosuppressive (Eisenstein *et al.*, 2006; Ninković and Roy, 2013). Overall, these preliminary data suggest that the combination treatment of morphine plus a cannabinoid was superior in reducing inflammatory mediators compared to morphine alone or a cannabinoid alone in two different pain tests in two different rodent species, which was consonant with a greater analgesic effect of the combinations in these two assays. The assays would need to be repeated with more animals to reach a firm conclusion. The mechanisms underlying these observations will require further investigation. Welch proposed that additive or synergistic interactions between cannabinoid and opioid receptors might be due to release of endogenous opioids by cannabinoids (Welch, 2009), for which there is evidence, and possibly also through formation of opioid-cannabinoid heterodimers (Rios *et al.*, 2006).

In summary, the present studies demonstrate that synthetic cannabinoids, when combined with morphine, can exert synergistic or increased analgesic effects in rodents in two pain models. These results provide a rationale for the use of such drug combinations in the treatment of pain. In a small clinical study, Abrams et al. (2011) reported that vaporized cannabis enhanced analgesia in chronic pain patients on sustained-release morphine or oxycodone. Combinations of cannabinoids with opioids may be an effective way of reducing opioid doses, with the potential for reducing opioid-induced side effects and subsequent opioid physical dependence.

Competing Interests Statement: None

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Declaration of transparency and scientific rigour:

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Opioid-Sparing Effects of Cannabinoids on Morphine Analgesia: Participation of CB1 and CB2 Receptors

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Abstract

Background and Purpose: Much of the opioid epidemic arose from abuse of prescription opioid drugs. This study sought to determine if the combination of a cannabinoid with an opioid could produce additive or synergistic effects on pain, allowing reduction in the opioid dose needed for maximal analgesia.
Experimental Approach: Pain was assayed using the formalin test in mice and the carrageenan assay in rats. Morphine and two synthetic cannabinoids were tested: WIN55,212-2 (WIN), which binds to both CB1 and CB2 receptors, and possibly TrpV1 receptors; and GP1a, which has activity at CB2 receptors and is reported to inhibit fatty acid amide hydrolase (FAAH), thus raising endogenous cannabinoids.
Key Results: Morphine in combination with WIN in the formalin test gave synergistic analgesia. Studies with selective antagonists showed that WIN was acting through the CB1 receptor. Morphine in combination with GP1a in the formalin test was sub-additive. In the carrageenan test, WIN had no added effect when combined with

morphine, but GP1a with morphine showed enhanced analgesia. Both WIN and Gp1a used alone had analgesic activity in the formalin pain test, but not in the carrageenan pain test.
Conclusions and Implications: The ability of a cannabinoid to produce an additive or synergistic effect on analgesia when combined with morphine varies with the pain assay and may be mediated by CB1 or CB2 receptors. These results hold the promise of using cannabinoids to reduce the dose of opioids for analgesia in certain pain conditions.

Abbreviations:
Δ^9-THC: Δ^9-tetrahydrocannabinol
CB1: Cannabinoid receptor 1
CB2: Cannabinoid receptor 2
CCL: C-C chemokine ligand
CCR: C-C chemokine receptor
CXCL: C-X-C chemokine ligand
CXCR: C-X-C chemokine receptor
MPA: Maximal possible analgesia
G-CSF: Granulocyte-colony stimulating factor
GM-CSF: Granulocyte-macrophage-colony stimulating factor
LTB: Lymphotoxin beta
WIN: WIN55,212-2

Keywords: Opioid; Cannabinoid; Synergy; Pain; Analgesia; Drug Combinations; Inflammation

Bullet Point Summary

What is already known?

- Combinations of some cannabinoids plus morphine give enhanced analgesia over either drug alone.
- Cannabinoids alone can mediate analgesia through CB1 or CB2 receptors.

What this study adds:

- Additive/synergistic analgesic effects of cannabinoids, when combined with morphine, vary with the pain assay.

- CB1 or CB2 receptors can mediate additive/synergistic analgesic effects of cannabinoids combined with morphine.

Clinical significance:

• Combinations of cannabinoids with opioids may be an effective way of reducing opioid doses.

Introduction

The U.S. is currently in the midst of an opioid epidemic that has been declared a "public health emergency" by the Department of Health and Human Services (https://www.hhs.gov/about/news/2017/10/26/hhs-acting-secretary-declares-public-health-emergency-address-national-opioid-crisis.html). The United Kingdom has also seen a significant rise in prescription opioid abuse (Giraudon *et al.*, 2013) (https://www.nytimes.com/2018/02/04/world/europe/uk-fentanyl-opioid-addiction.html). The National Survey on Drug Use and Health carried out by the Substance Abuse and Mental Health Services Administration (SAMHSA) in 2016 found that 11.5 million Americans misused prescription opioids, and 62.3% gave as the reason for their use, relief of pain (Ahrnsbrak *et al.*, 2017). It is estimated that 30% of Americans suffer from acute or chronic pain (Volkow and McLellan, 2016; Volkow *et al.*, 2011), leading to the use of prescribed or illicit opioids for pain relief. One potential mitigating strategy for decreasing opioid use is to employ combination therapies, with the objective of producing equal analgesia using a lower dose of opioid. In animal models, cannabinoids have documented analgesic activity (Pertwee, 2001; Walker and Hohmann, 2005) as well as anti-inflammatory effects (Eisenstein and Meissler, 2015; Klein, 2005).

There are complexities to the cannabinoid system, as two cannabinoid receptors, designated CB1 and CB2 have been identified (Matsuda *et al.*, 1990; Munro *et al.*,

1993). CB1 is highly expressed on neurons in the central nervous system (Herkenham *et al.*, 1991) and to a lesser extent on cells of the immune system (Daaka *et al.*, 1996; Galiegue *et al.*, 1995). CB2 is primarily expressed on cells of the immune system (Daaka *et al.*, 1996; Galiegue *et al.*, 1995; Carlisle *et al.*, 2002), as well as on activated microglia (Carlisle *et al.*, 2002). CB2 has been detected on neurons in the central nervous system, but levels of expression of this receptor are low compared to CB1 (Gong *et al.*, 2006; Van Sickle *et al.*, 2005).

Both endogenous cannabinoid system ligands and exogenous cannabinoid receptor ligands have been shown to mitigate pain (Woodhams *et al.*, 2017). Δ^9-THC has activity at both CB1 and CB2 receptors, and exerts analgesic activity through both receptors (Agarwal *et al.*, 2007; Elikottil *et al.*, 2009; Craft *et al*., 2013; Thapa *et al*., 2018). CB2 receptors have also been shown to modulate pain (Deng *et al.*, 2015; Guindon and Hohmann, 2008; Kinsey *et al.*, 2011; Brownjohn and Ashton, 2012; Gutierrez *et al.*, 2011). Using CB2 knock-out mice, as well as CB2-overexpressing transgenic mice, it was shown that activation of this receptor decreases sciatic nerve injury pain (Racz *et al.*, 2008). A synthetic CB2-selective agonist was shown to have analgesic activity in a model of chemotherapy-induced neuropathic pain in mice, which correlated with a reduction in mRNA for selected pro-inflammatory cytokines and chemokines (Deng *et al.*, 2015). Further, a synergistic combination between morphine and a selective CB2 agonist, JWH015, was also shown in rodent models of post-surgery and neuropathic pain (Grenald *et al*., 2017). Contributions of CB1 and CB2 receptors were reported to participate in synergistic combinations of cannabinoids with morphine in a mouse model of cancer pain (Khasabova *et al.,* 2011). Activation of the CB2 receptor on cells of the immune system results mainly in immunosuppression (Eisenstein and Meissler, 2015; Klein, 2005). Use of a CB2-selective agonist would, therefore, be predicted to reduce inflammation, which plays a major role in many types of pain.

There is a pre-clinical literature showing that combinations of cannabinoids and opioids have additive or synergistic analgesic effects (Cichewicz, 2004; Nielsen *et al.*, 2017; Welch, 2009). Most of these studies did not provide information on which cannabinoid receptor was mediating the opioid-sparing effect. The hypothesis being tested in the present studies is that a combination of a cannabinoid with a sub-analgesic dose of an opioid can achieve a level of pain relief observed with an optimal dose of the opioid alone. Such combinations, which would permit use of opioids at lower doses, could have the added advantage of reducing unwanted adverse effects of opioids, such as nausea, vomiting, constipation, sedation, respiratory depression, and pruritus, as well as potential development of tolerance and dependence. The present project was undertaken to investigate the feasibility of using morphine in combination with two different cannabinoids, one of which (WIN55,212-2 [WIN]) binds to both CB1 and CB2 receptors, and the other (GP1a), which has CB2 agonist activity in *in vivo* studies (Franklin and Carrasco, 2013; Kong *et al*., 2014). We also used cannabinoid receptor antagonists SR141716A (CB1), and SR144528 (CB2) to understand which cannabinoid receptor mediates additive or synergistic analgesic effects with morphine. We chose WIN since there is a history of its use in animals for pain studies (Martin *et al*., 1999). WIN has been reported to have activity at the CB1, CB2, and TrpV1 receptors (Lowin *et al.*, 2016). In the present study, a TrpV1 antagonist, SB366791, was also studied using the cannabinoid and opioid combination to investigate possible TrpV1 receptor

involvement. Kong *et al*. (2014) reported that GP1a, treatment decreased demyelination and axonal loss, as well as reduced clinical scores and facilitated recovery in experimental autoimmune encephalomyelitis in mice. Analgesic effects of the compounds alone and in combination with morphine were tested in two different pain assays. Formalin-induced nociception was used in mice as a tonic/chronic pain model (Murray *et al*., 1988) and carrageenan-induced inflammation was used in rats as a model of inflammatory pain (Kocher *et al*., 1987).

Methods:

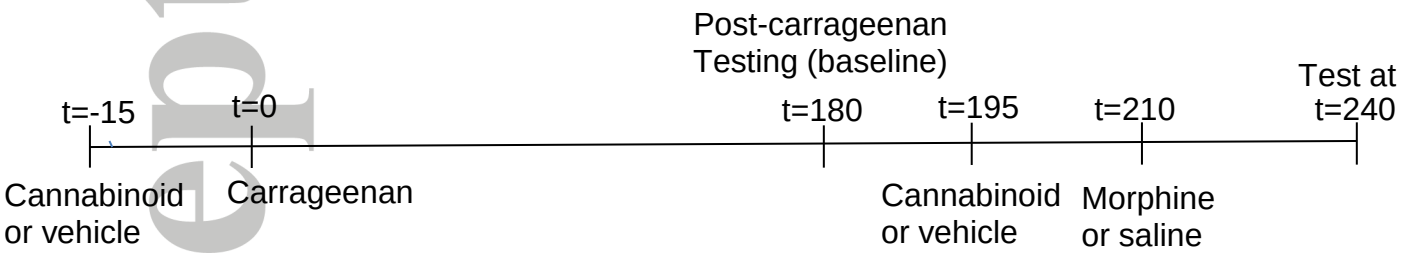
Animals: Outbred Swiss-Webster male mice (20-25 g) and outbred male Sprague-Dawley rats (120-150 g), were purchased from Taconic Biosciences (Albany,NY). Animals were housed in the Central Animal Facility of the Medical School in ventilated micro-isolator cages under a schedule of 12 hr of light and 12 hr of darkness. They had access to food and water *ad libitum*. All experiments were carried out under protocols approved by the University IACUC. In all experiments, the N = 6-10 animals/group, as designated in the individual figures.

Reagents: Cannabinoids and their antagonists were purchased from Tocris (Minneapolis, MN). WIN55,212-2 (WIN) was dissolved in 5% DMSO when used in mice, and in 10% DMSO when used in rats. GP1a was dissolved in 5% DMSO. The cannabinoid antagonists, SR141716A (CB1), SR144528 (CB2), and the TrpV1 antagonist, SB366791, were dissolved in 5% DMSO. Morphine sulfate (doses expressed as the salt) was obtained from NIDA and dissolved in saline.

Formalin pain assay: In this assay, the analgesic effects of morphine alone, a cannabinoid alone, or a combination of morphine plus a cannabinoid, were tested (n=7-8). Five minutes before formalin injection (t = -5), animals were placed into a transparent chamber connected to an isoflurane vaporizer, and briefly anesthetized. At t = 0, morphine or saline vehicle, or a cannabinoid (WIN or Gp1a), or 5% DMSO alone, was injected subcutaneously (s.c.) into the dorsal flank area of mice. In combination experiments, opposite sides of the dorsal flank were used. Immediately after drug or vehicle injections, the dorsal side of the left hind paw was injected s.c. with 20 µl of a 5% formalin solution diluted in saline. In experiments in which an antagonist was used, it was given i.p. 30 min before agonists and formalin. After receiving the formalin injection, animals were placed into a large glass jar where their actions could be monitored. Formalin injection causes intense licking of the injected paw in two phases. The late phase of licking, between 20 and 35 min after administration of formalin, represents a combination of peripheral inflammatory- and spinal-mediated pain (Tjolsen *et al.*, 1992) and thus total licking time between 20 and 35 min was chosen for measurement of the response. Licking was scored for each animal as the number of sec of licking that occurred during that 15-min period (20 to 35 min after formalin injection). Animals were randomly assigned to groups, and experiments were carried out with the observer/recorder of the behavior blinded to the treatment given to the animal.

Carrageenan pain assay: The carrageen test has two parameters, pain and swelling (edema) of rat paws following injection of the irritant, carrageenan. To carry out the test, unrestrained rats were placed inside a clear plastic chamber (10 cm w x 21 cm

deep x 13 cm high) with a glass floor (32^0 C) that is part of the Hargreaves' Plantar Test Apparatus (Model 400, IITC Life Science, Woodland Hills, CA). After 60 min of habituation, all animals were exposed on the plantar surface of the left hind paw to a beam of radiant heat (intensity=45) through the glass floor. They were tested 3 times, with 5-min intervals between each stimulus. The latency (sec) to paw withdrawal was used as the antinociceptive index and was automatically scored by the apparatus. Edema was measured using a digital plethysmometer. To carry out the pain assay, rats were injected i.p. with the desired cannabinoid, WIN or GP1a, or the vehicle (10% DMSO), 15 min before the carrageenan injection (t = -15). At t = 0, rats were injected into the plantar side of the left hind paw with 0.1 ml of a 2% carrageenan solution (FMC, Philadelphia, PA), freshly prepared in saline. All animals were tested by exposure to the heat beam 3 times at 5-min intervals to establish the baseline pain value 180 min after carrageenan injection. Fifteen min after the post-carrageenan baseline, the animals received a second injection of cannabinoid or vehicle. At t = +210 min (15 min after second cannabinoid or vehicle injection), they received an injection of morphine (3.0 mg/kg) or saline s.c. into the dorsal flank area. Latency of paw withdrawal to radiant heat and edema was measured at 240 min (30 min after morphine or saline injection) and expressed as a percentage change from post-carrageenan baseline. The percent of maximal possible antinociception (%MPA) for each animal at each time was calculated using the following formula: %MPA = [(test latency at t = +240 min – baseline latency at t = +180 min)/(22 second- baseline latency at t = +180 min)] x 100. A cutoff limit of 22 seconds was set to avoid damage to the paw. To measure edema induced by the carrageenan and the effect of cannabinoids, morphine, or the combination of the two compounds, swelling of the left paw was measured at t = 0 (just after carrageenan injection) 180, and 240 min post carrageenan administration. To quantitate edema, the following formula was used: Volume difference of edema (ml) = Volume at t=+240 min– Volume at t=+180 min. Experiments were carried out with the observer/recorder of the behavior blinded to the treatment given to the animal. The timeline for the carrageenan experiments is shown below:



<u>mRNA levels of immune mediators</u>:
For determining mRNA levels, the draining popliteal lymph nodes from 2 animals in each group, as well as from 2 animals that received no carrageenan or drug (to obtain baseline values), were collected at t = 35 minutes from mice in the formalin test and at t = 4 hr from rats in the carrageenan test. The lymph nodes were then extracted by the RNeasy® Microarray Tissue Mini Kit (Qiagen, Gaithersburg, MD). These RNAs were used to generate cDNA using the RT_2 First Strand Kit (Qiagen). The cDNAs from the two duplicate animals were pooled and assayed using the RT_2 Profiler® PCR Arrays for Rat or Mouse Inflammatory Cytokines and Receptors (Qiagen), with the RT_2 SYBR® Green ROX® qPCR Mastermix (Qiagen). The rat and

mouse arrays have probes for mRNA for 84 immune mediators. The PCR arrays were run on an ABI StepOne Plus® qPCR thermocycler (Applied Biosystems, Foster City, CA), using the cycling conditions given in the protocol supplied with the RT_2 Array. Data were processed by the online GeneGlobe Data Analysis Center (Qiagen). Results are presented as heat maps showing mRNA for cytokines and chemokines in the array of the treated groups compared with mRNA level expression in the baseline, untreated control group.

Statistical Methods

For dose-response curves where multiple doses were tested, a one-way ANOVA was performed followed by Sidak's multiple comparison test when comparing drug-treated animals to a control group. Sidak's test was used for comparisons between groups treated with different drugs or different drug dosages. $p<0.05$ was considered to be a statistically significant difference.

Isobolographic analysis was used to determine if there were additive or synergistic effects when morphine was combined with a cannabinoid, where both drugs demonstrated analgesic activity. The ED_{50} values of the individual drugs were plotted on the x and y axes and connected by an intersecting line. The intersecting line represents points along the line that show additivity. A point above the line represents sub-additivity, and a point below the line represents synergy. The new ED_{50} value of the combinations can be plotted to determine if the combinations are additive, sub-additive or synergistic.

In the case where one drug in the combination was effective (morphine), and the other drug was ineffective or only slightly effective, precluding calculation of its ED_{50}, the dose equivalence method (Tallarida, 2006) was used to determine if the combinations were different from either drug alone. In this method, the value for the second drug was converted to the equally effective dose of morphine, and expected effects were calculated. To evaluate if the two drugs interact, these expected effects were compared to the effects observed. If the observed effects were below what was expected, the interaction was classified as sub-additive; if the effects were equal to the expected effect, the interaction was classified as additive; and if the observed effects were greater than what was expected, the interaction was classified as synergistic.

Results:

Analgesia induced by WIN alone, morphine alone, or a combination treatment in the formalin assay.

Initial experiments were undertaken to assess the analgesic activity of morphine (0.5-10 mg/kg, s.c.) alone, WIN (0.5-5 mg/kg, s.c.) alone, or the two drugs (morphine: WIN, 1:1 ratio, 0.1-1 mg/kg, s.c.) in combination. Figure 1A shows that morphine significantly decreases formalin-induced licking in a dose-related manner compared to vehicle ($p<0.0001$, one-way ANOVA followed by Dunnett's multiple comparison). Figure 1B shows that WIN also significantly decreases licking time in a dose-related manner, compared to the vehicle group ($p<0.0001$, one-way ANOVA followed by Sidak's multiple comparison). Thus, both the opioid and the cannabinoid gave strong, dose-related antinociceptive effects in the formalin test. Combination experiments were then carried out using these two compounds. The doses used in the combination studies were derived from the data gleaned from the individual dose-response curves, with attention to the range within which an increase or

decrease in analgesic effect could be observed. Figure 2A shows that the licking time in response to formalin injected into the paw significantly decreased in a dose-related manner in the animals given the combination of morphine and WIN. At the 0.5 and the 1.0 mg/kg doses, morphine alone and WIN alone produced significantly reduced licking in response to the formalin injection, which was even further reduced when the drugs were combined at those doses. Using isobolographic analysis, the drug interactions were found to be highly synergistic. The results demonstrated the predicted increase in antinociceptive effect of the combination over each individual drug alone (Fig 2B).

To examine the receptor through which WIN (1 mg/kg) in combination with morphine (1 mg/kg), produced enhanced analgesia in the formalin test, antagonists for CB1, CB2 and TrpV1 receptors were used. SR141716A (CB1, 5 mg/kg, s.c.), SR144528 (CB2, 5 mg/kg, s.c.), and SB366791 (TrpV1, 1 mg/kg, s.c.) were administered 20 min before the test compounds and formalin administration. Figure 2C shows that the synergistic activity of WIN and morphine in the formalin test is through the CB1 receptor, as the CB1 antagonist (SR141716A) returned licking time to that seen with vehicle alone (*$p<0.05$, one-way ANOVA followed by Sidak's multiple comparison). Neither the CB2 antagonist, nor the TrpV1 antagonist, had an effect on the analgesic activity of the WIN plus morphine combination. Also, none of the three antagonists had any effect on licking by themselves (Fig. 2C).

<u>Analgesia induced by GP1a alone, morphine alone, or a combination treatment in the formalin assay</u>.
Figure 1C shows the dose-response effect for GP1a alone in the formalin assay. Gp1a significantly reduced (around 30%-50%) licking time at doses ranging between 1.0 mg/kg and 50.0 mg/kg in a non-dose-related manner (***$p<0.01$: one-way ANOVA followed by Sidak's multiple comparison test). The next experiments tested the effect of the combination of GP1a with morphine, using the morphine dose-response curve shown in Fig. 1A as a reference. Figures **3**A, 3B and 3C show the effect of GP1a alone (1.0, 5.0 or 10.0 mg/kg), or a combination of these three different doses of GP1a with two different doses (0.5 and 1 mg/kg) of morphine. Using dose equivalence analysis, as explained in the section on statistical analysis in the Methods, the combinations of GP1a and morphine were sub-additive.

<u>Analgesia in the carrageenan assay induced by the cannabinoids, WIN or GP1a each alone, morphine alone, or combination treatments.</u>
Figure 4A shows the analgesic dose-response for morphine (0.5-10 mg/kg, s.c.), and figures 4B and 4C present the respective dose-responses for WIN (1-5 mg/kg, s.c.) and GP1a (0.5-10 mg/kg, s.c.) in the carrageenan test. Morphine produced significant analgesia in a dose-dependent manner (**$p<0.01$, ***$p<0.001$, one-way ANOVA followed by Sidak's multiple comparison), but had no effect on edema (Supp Fig. 1A). Neither WIN nor GP1a had significant analgesic activity in this assay (Figs. 4B and 4C), and were also without effect on paw edema (Supp. Figs. 1B and 1C). The animals that received only vehicle (10% DMSO) in panels 4A, 4B, and 4C appeared to show hyperalgesia, but it was not statistically significant due to the large standard errors. Also, no trend towards an increase in pain with vehicle was found in a subsequent experiment (see Fig. 5A and 5B). Combination experiments of morphine with a cannabinoid were carried out using the carrageenan test. As shown in Figure 5A, WIN (1-5 mg/kg) did not enhance the analgesia induced by a

suboptimal dose of morphine (3.0 mg/kg) in this test, nor did the WIN-morphine combination result in a reduction in edema (Supp. Fig. 1C). Thus, in contrast to the formalin assay in mice, in the carrageenan assay in rats, WIN showed no additive or synergistic effect with morphine. Figure 5B presents data on the combination of morphine at a suboptimal dose (3.0 mg/kg) given with GP1a (5.0 mg/kg). An increased analgesic effect (*$p<0.05$, one-way ANOVA followed by Sidak's multiple comparison) was observed using this combination of GP1a plus morphine. No drug interactions were seen in reducing swelling of the paw (Supp. Fig. **1D**). Thus, GP1a showed an increased analgesic response with morphine in the carrageenan test, but in the formalin test this drug combination was no better than either compound alone.

Immune mediators, receptors and other molecules.

Preliminary data were obtained on the effect of opioid and cannabinoid treatment on production of inflammatory mediators using mRNA arrays. Data are displayed as heat maps. Figure 6A, shows the results for the formalin test in mice, using pooled mRNA extracts of popliteal lymph nodes of 2 mice in each group. Animals in every group received a formalin injection in addition to the treatments noted at the bottom of each column. The left-hand column represents the levels of mediators induced by the formalin injection alone plus saline and DMSO vehicles. Qualitatively, one can observe that many of the analytes were elevated (darker red color). Morphine or WIN given alone each had a moderate suppressive effect on mediator expression. The morphine plus WIN combination dampened the chemokine/cytokine mRNA profile, as seen by the increase in the number of green bars between formalin alone (2 bars) and the combination treatment (7 bars). The CB2 antagonist, but not the CB1 antagonist, returned the mediator intensity closer to that observed with formalin alone. Interestingly, the Trvp-1 antagonist given with morphine and WIN resulted in highly depressed levels of mRNA for many mediators when compared to morphine plus WIN, even though it did not inhibit the analgesia induced by this combination. Figure 6B shows a similar analysis for the combination of morphine plus the CB2-selective agonist, GP1a, on the average mRNA levels of tissue extracted from 2 individual rats in each treatment group. Carrageenan plus vehicles (left most column) induced a broad inflammatory response. The combination of morphine plus Gp1a in the carrageenan test was more suppressive than either morphine alone or GP1a alone.

Discussion and Conclusions:

Two different cannabinoids, WIN and GP1a were tested in combination with morphine in two different pain tests, the formalin assay and the carrageenan assay. Morphine, as expected, gave strong dose-dependent analgesia in both tests (Figs. 1A and 4A). It was found that WIN produced analgesia in the formalin test (Fig. 1B), and when combined with morphine had a synergistic interaction in reducing pain (Figs. 2A and B). Use of receptor-selective antagonists, SR141716 (CB1), SR144528 (CB2) and SB366791 (TrpV1), showed that the synergistic interaction on the analgesic activity produced by WIN was mediated through the CB1 receptor (Fig. 2C). The other cannabinoid, GP1a, produced statistically significant analgesia in the formalin test at doses ranging between 1.0 mg/kg and 50.0 mg/kg, without a clear dose-response effect in that range (Fig. 1C). Combinations of three different doses

of GP1a with two different doses of morphine did not result in additive or synergistic interactions in this assay (Figs. 3A, B, and C). In contrast, in the carrageenan assay, neither WIN nor GP1a produced analgesia when given alone over a range of doses (Figs. 4B, and C). Unlike the results with formalin, in the carrageenan test WIN did not give statistically significant interactive effects when combined with morphine (Fig. 5A), while the combination of GP1a and morphine resulted in a greater analgesic effect than with morphine alone at one dose combination (Fig. 5B). Thus, the results for the combinations of the cannabinoids with morphine yielded opposite results in the two tests. These results are summarized in Table 1. Similarly, previous report from our laboratory (Inan et al., 2018) showed that combining sub-analgesic doses of morphine with chemokine receptor antagonists could provide maximal analgesia in a rat model of incisional pain.

Much of the literature on interactions between opioids and cannabinoids relating to analgesia, in both preclinical models and clinical studies, has reported on use of Δ^9-tetrahydrocannabinol (Δ^9-THC). In rhesus monkeys, the dose-response curves for morphine and other opioids (fentanyl, etorphine, and buprenorphine, but not nalbuphine) in the warm water tail-flick test were significantly shifted leftward by Δ^9-THC and also by the synthetic cannabinoid, CP55,940 (Maguire and France, 2014). CP55,940, like Δ^9-THC and WIN, is active at both the CB1 and CB2 receptor. Synergy was also found between Δ^9-THC and morphine administered intrathecally,i.c.v., s.c. or p.o. in inducing analgesia measured using the tail-flick test in mice (Smith *et al.*, 1998; Welch and Stevens, 1992; Welch *et al.*, 1995). Antagonist studies showed that Δ^9-THC was acting via the CB1 receptor (Smith *et al.*, 1998). Experiments using genetic deletion of the peripheral CB1 receptor in mice showed that the analgesia mediated by WIN in an assay of neuropathic pain was mainly through the CB1 receptor (Agarwal *et al.*, 2007). There is also a report that Δ^9-THC used in combination with morphine in arthritic rats has synergistic analgesic interactions that were mediated via the CB2 receptor (Cox *et al.*, 2007b; Cox *et al.*, 2007a). The experiments carried out in the present studies using WIN, with and without antagonists selective for CB1 and CB2, show that in the mouse formalin test, WIN was active via the CB1 receptor. The results reveal very strong synergy of WIN with non-analgesic doses of morphine (0.1-1 mg/kg, Fig. 2).

There is currently marked interest in the analgesic activity of Δ^9-THC, as marijuana has been legalized for medicinal purposes in 24 states and the District of Columbia, including approval for use for many conditions that have pain as a primary symptom. There are reports that availability of medical marijuana has decreased prescriptions for other FDA-approved medications to treat pain (Bradford and Bradford, 2016). Δ^9-THC has the disadvantage of being psychoactive and potentially leading to tolerance and dependence. The nonselective cannabinoid agonists like WIN and CP55,940 also bind to CB1 receptors with the same potentially negative side effects. The development of selective CB2 agonists raises the possibility of utilizing cannabinoids devoid of psychoactive activity as analgesics. In fact, CB2 agonists given acutely or chronically have been reported to have analgesic activity against neuropathic pain in mice (Deng *et al.*, 2015; Lin *et al.*, 2018). Further, CB2 knock-out mice have enhanced nociception in a mouse model of sciatic nerve injury (Racz *et al.*, 2008).

The current experiments show that GP1a has an increased analgesic effect with morphine in the carrageenan test in rats, but did not enhance morphine analgesia in

the formalin test in mice. GP1a was promoted as a selective CB2 agonist and found active on CB2 receptors in *in vivo* studies (Franklin and Carrasco, 2013; Kong *et al*., 2014) until it was reported recently as an inverse agonist by Soethoudt *et al*. (2017). Also, in the same study, it was stated that GP1a showed partial inhibition (30-40%) of fatty acid amide hydrolase (FAAH), an enzyme that breaks down endogenous anandamide. Inhibition of this enzyme will result in an increase in endogenous anandamide levels. Recently, inhibitors of FAAH have been investigated as potential therapeutic targets for pain and CNS disorders (Ahn *et al*., 2009; Deutsch, 2016). Our results show that GP1a has analgesic activity on chronic/tonic pain by itself, as well as increasing morphine's effect on inflammatory pain. GP1a might have analgesic activity through enhancing endogenous anandamide levels by blocking FAAH. A recent study (Slivicki *et al.,* 2018) shows synergistic effects of FAAH inhibitors on morphine-induced analgesia against chemotherapy-induced neuropathic pain in mice. Further, and critically, Slivicki et al. (2018) also reported that this synergy was not shown on morphine-induced reduction of gastrointestinal transit. Yuill et al. (2017) noted an additive interaction between the CB2-selective agonist, JWH-133, and morphine in the mouse formalin test at a single dose ratio. If GP1a is working by increasing endogenous levels of anandamide, it could be hypothesized that its effect may not have been robust enough to produce synergism when combined with morphine in the formalin test, but was sufficient to increase the analgesic effect at one dose in the carrageenan test. Lin *et al*. (2018) reported that a CB2 agonist used chronically to treat paclitaxel-induced neuropathy can block development of tolerance to the analgesic effects of morphine when it is given post cannabinoid treatment. In the present experiments, all of the drugs were given acutely and the effect of drug combinations on analgesic activity was only evaluated in an acute time frame.

The present studies show that there are differing results for analgesia evoked by two cannabinoids, each acting on distinct receptors, and also by combinations of opioids and cannabinoids in different pain assays. These results point to the conclusion that there are divergent mechanisms involved in pain induction, transmission or perception in the two assays. Formalin causes a biphasic response with a rapid onset of pain that lasts for 3-5 min, followed by a later phase starting 15-20 min after the formalin injection, which has an inflammatory component (Tjolsen *et al.*, 1992). Injection of carrageenan into the footpad results in pain and swelling that increases over a period of 4 hr, the endpoint when measurements were taken. Time-course studies showed that pain persisted for at least 5 hr. Thus, the pain induced by carrageenan is longer lasting and is accompanied by a clear inflammatory component, which is manifest in measurable, marked paw edema. Since GP1a and CB2-selective agonists have been shown to have suppressive effects on the immune system (Eisenstein and Meissler, 2015), one would have predicted that this cannabinoid would have had efficacy in the carrageenan assay. In spite of a lack of activity by itself in the carrageenan assay, GP1a increased morphine's antinociceptive effect at one dose.

The immune assays were undertaken to determine if morphine alone, the cannabinoids alone, or the combination of the two classes of drugs would show suppressive effects on levels of inflammatory mediators that might correlate with reductions in pain. The results presented are preliminary as only a limited number of samples were tested. Results in Figure 6A represent the pooled mRNA extracted

from 2 mice, and the results in Figure 6B represent the average of results from 2 arrays from 2 rats. From this preliminary data it can be observed that suboptimal analgesic levels of morphine alone in the formalin and carrageenan assays moderately suppressed mRNA for a broad panel of mediators induced by the respective painful insults, which is consonant with the literature showing that morphine is immunosuppressive (Eisenstein *et al.*, 2006; Ninković and Roy, 2013). Overall, these preliminary data suggest that the combination treatment of morphine plus a cannabinoid was superior in reducing inflammatory mediators compared to morphine alone or a cannabinoid alone in two different pain tests in two different rodent species, which was consonant with a greater analgesic effect of the combinations in these two assays. The assays would need to be repeated with more animals to reach a firm conclusion. The mechanisms underlying these observations will require further investigation. Welch proposed that additive or synergistic interactions between cannabinoid and opioid receptors might be due to release of endogenous opioids by cannabinoids (Welch, 2009), for which there is evidence, and possibly also through formation of opioid-cannabinoid heterodimers (Rios *et al.*, 2006).

In summary, the present studies demonstrate that synthetic cannabinoids, when combined with morphine, can exert synergistic or increased analgesic effects in rodents in two pain models. These results provide a rationale for the use of such drug combinations in the treatment of pain. In a small clinical study, Abrams et al. (2011) reported that vaporized cannabis enhanced analgesia in chronic pain patients on sustained-release morphine or oxycodone. Combinations of cannabinoids with opioids may be an effective way of reducing opioid doses, with the potential for reducing opioid-induced side effects and subsequent opioid physical dependence.

Competing Interests Statement: None

Author Contributions: X.C., M.W.A., T.K.E., A.C., S.M.R., S.I., and E.B.G. contributed to the design of the experiments. X.C., S.I, and M.N.W. performed the in vivo experiments. J.J.M. performed the RT-PCR arrays. R.J.T. and C.S.T. performed data analysis. X.C., S.I., T.K.E., A.C., S.M.R., J.J.M., and E.B.G contributed to the writing of the manuscript. All authors reviewed the final draft of the manuscript.

Declaration of transparency and scientific rigour:

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure Legends

Figure 1. Dose-response curves for morphine, WIN, and GP1a on formalin-induced nociception in mice. Morphine (Panel A), WIN (Panel B) or GP1a (Panel **C)** were given s.c. immediately before injection of 20 µl of 5% formalin into the dorsal side of the left hind paw. Formalin control mice received saline s.c. (vehicle for morphine) or 5% DMSO (vehicle for WIN and GP1a). Total licking time was scored between 20 and 35 min post formalin injection. Each point represents the mean ± SEM paw licking time (seconds). ****p<0.0001, ***p<0.001 vs control, as determined using a one-way ANOVA followed by Sidak's multiple comparisons test.

Figure 2. Effect of morphine alone, WIN alone, and the combination of WIN plus morphine on formalin-induced nociception in mice. Mice received a formalin injection into the paw and either s.c. morphine, WIN, or combinations of morphine plus WIN. Formalin control mice received s.c. saline (vehicle for morphine) or 5% DMSO (vehicle for WIN), or a saline injection and a 5% DMSO injection. Values for all 3 vehicle controls were averaged. Panel A: Data are the mean paw licking time (seconds) ± SEM of treated mice. Panel B: Isobolographic analysis to determine synergism between morphine and WIN. The 1:1 ratio of WIN: morphine produced the half maximal effect shown here as point P. The expected additive point is shown as Q. Panel C: Effect of antagonists (SR141716A (CB1 antagonist), SR144528 (CB2 antagonist), and SB366791 (Trpv1 antagonist)) on licking time produced by the morphine plus WIN combination. D = DMSO; S = saline. *p<0.05 by one-way ANOVA followed by Sidak's multiple comparison test between the groups.

Figure 3. Effect of different doses of GP1a (1, 5 or 10 mg/kg) in combination with different doses of morphine in the formalin test. Panel A: GP1a = 1 mg/kg. Panel B: GP1a = 5 mg/kg. Panel C: GP1a = 10 mg/kg. D+S = DMSO plus saline. Dose equivalence analysis determined that the effect of combinations on analgesia was sub-additive.

Figure 4. Dose-response curves for morphine, WIN, and GP1a in the rat carrageenan test. Rats received a carrageenan injection into the paw. WIN or GP1a was injected i.p. 15 min before and 195 min after the carrageenan injection. Morphine was injected at 210 min post carrageenan s.c. into the dorsal flank. Analgesia was assessed 30 min after morphine injection using the Hargreaves' apparatus with a radiant heat beam. Panel A: Morphine: percentage of maximal analgesia. One-way ANOVA followed by Sidak's multiple comparison test showed significance at 1, 3, 5 and 10 mg/kg of morphine compared to vehicle; **$p<0.01$, ***$p<0.001$. Panel B: WIN: percentage of maximal analgesia. Panel C: GP1a: percentage of maximal analgesia. Panels B and C: Not significant by one-way ANOVA compared to vehicle.

Figure 5. Effect of morphine, WIN, or GP1a alone, or in combination with morphine, in the rat carrageenan test. Panel A: Morphine plus WIN, percentage of maximal analgesia. Effect of the combination was not significant by one-way ANOVA compared to morphine alone. Panel B: Morphine plus GP1a. *$p<0.05$ by one-way ANOVA with Sidak's multiple comparison test. D = DMSO; S = saline.

Figure 6: Change in mRNA expression in popliteal lymph nodes of mice receiving formalin or rats receiving carrageenan in the foot pad, with and without morphine or cannabinoid treatments. Heat maps show the changes in mRNA expression for panels of chemokines and cytokines, with or without treatments, compared with untreated, baseline popliteal lymph nodes. The scale is shown to the right of the graphs. Red indicates up-regulation compared to baseline. Black represents no change. Green indicates down-regulation of mediators. Panel A. Samples of tissue from mice injected with formalin into the foot-pad and given different treatments. Each bar represents relative amounts of mRNA in a pooled extract of 2 mice/group. Panel B: Samples of tissue from rats injected with carrageenan into the foot-pad and given different treatments. Each bar represents relative amounts of mRNA averaged from 2 arrays performed on samples from 2 different rats in each group.

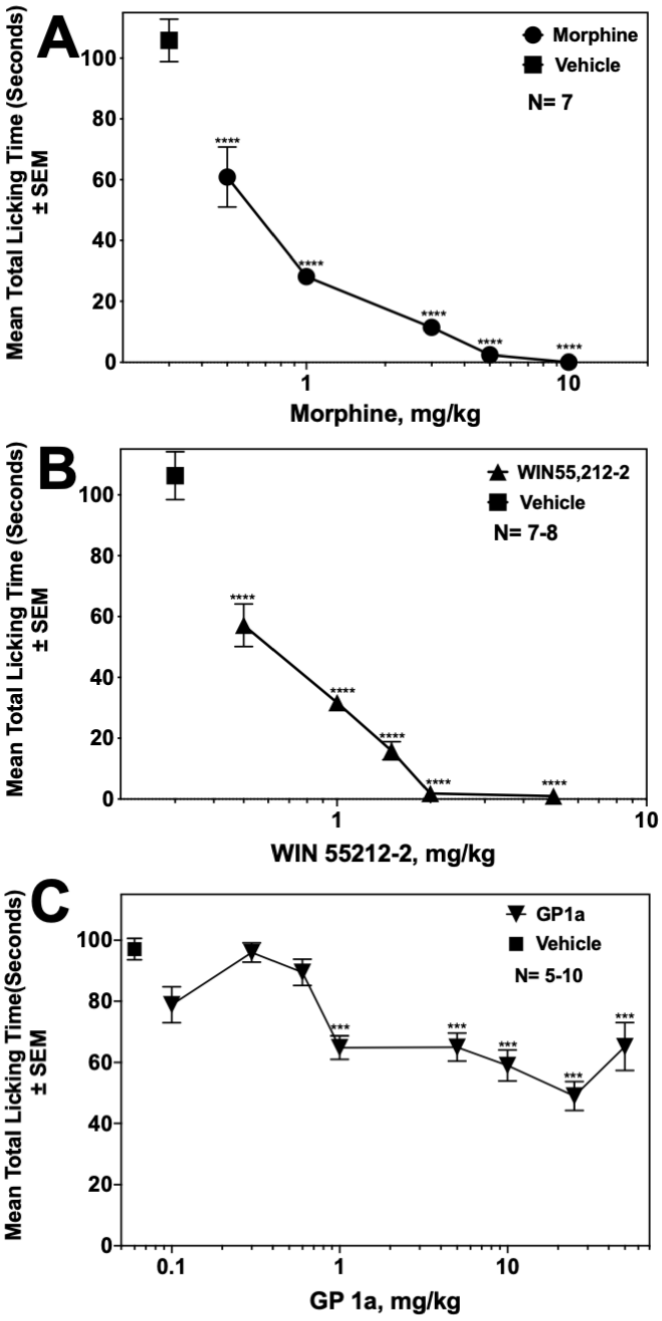

Figure 1

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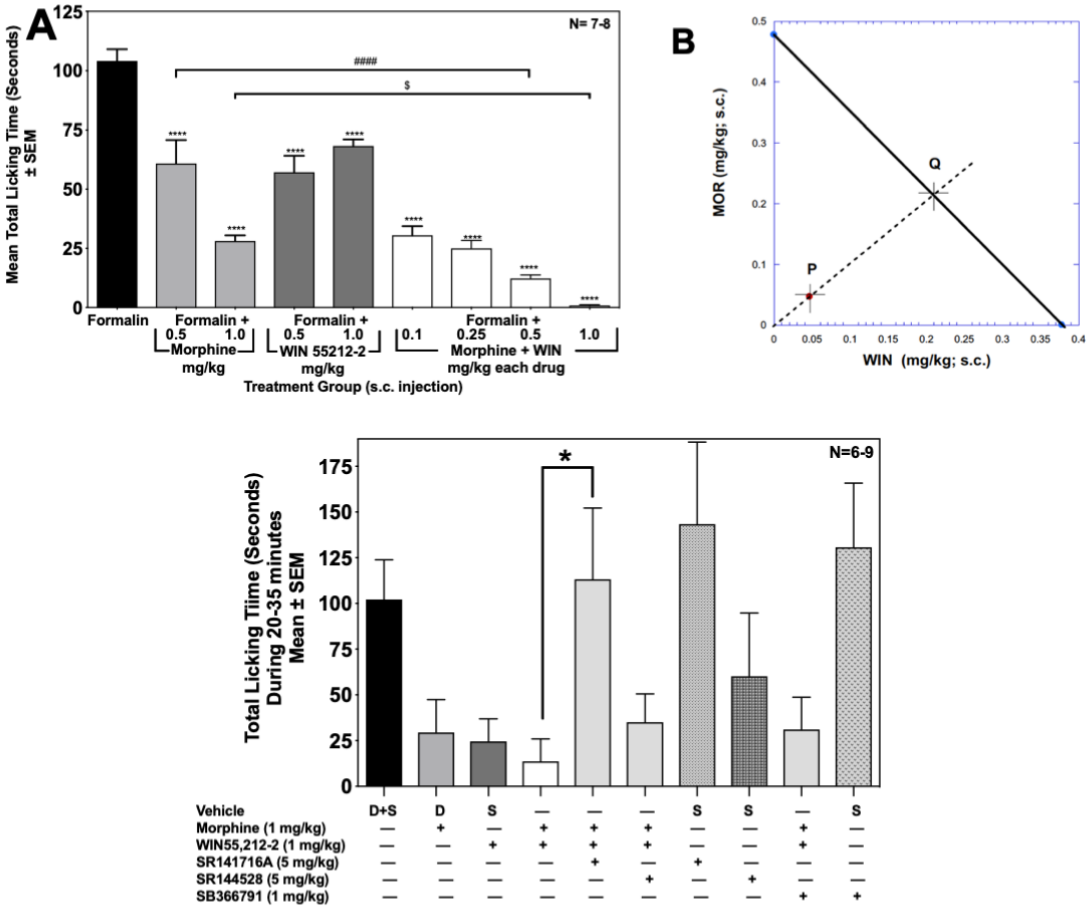

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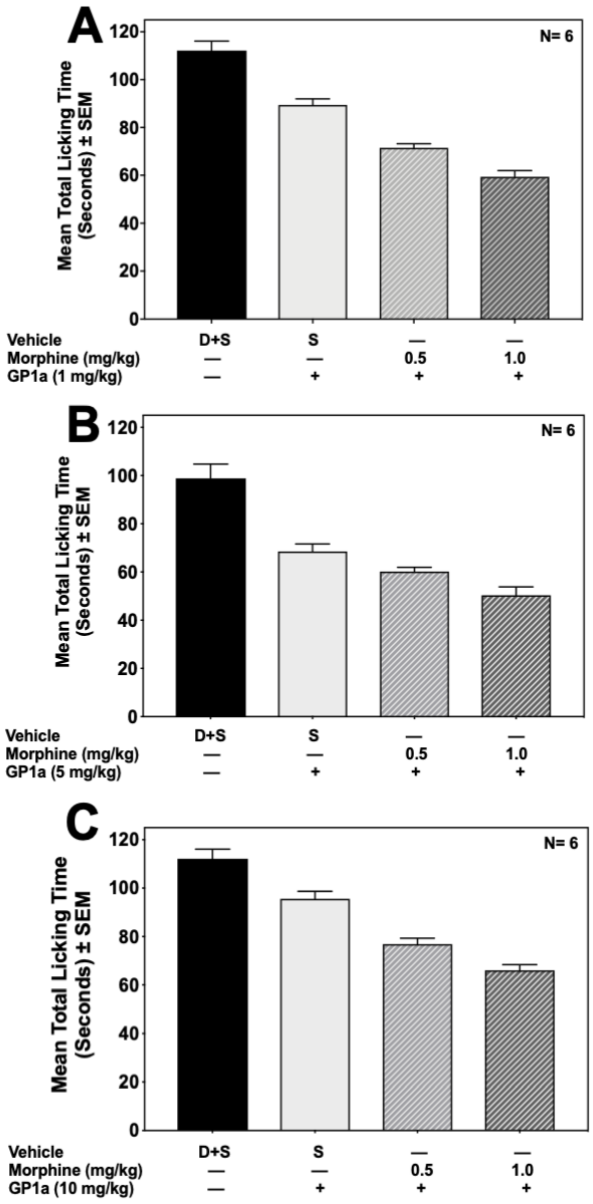

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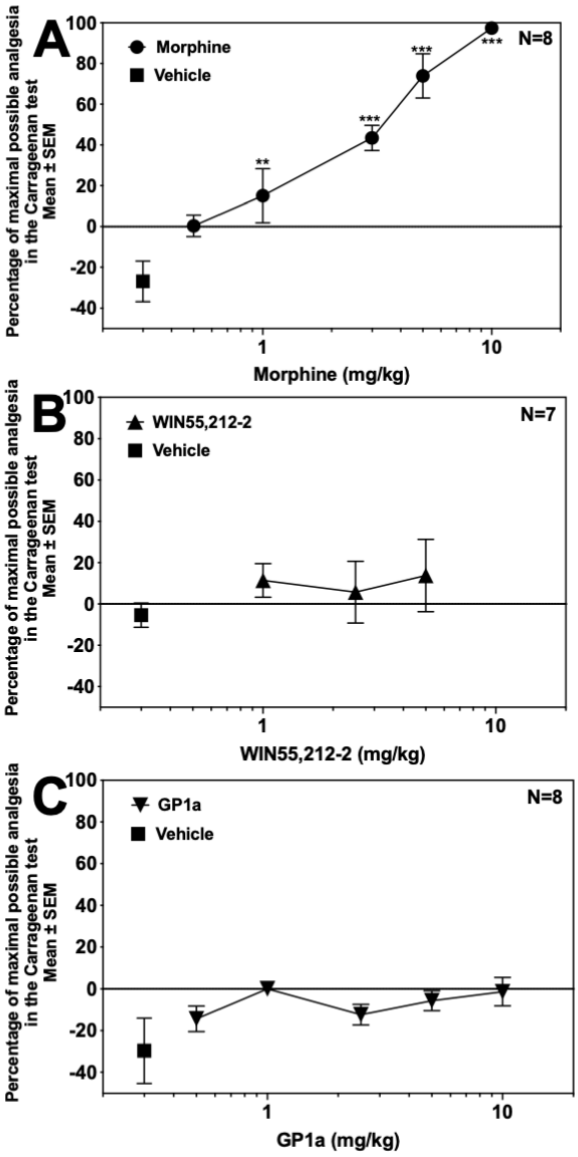

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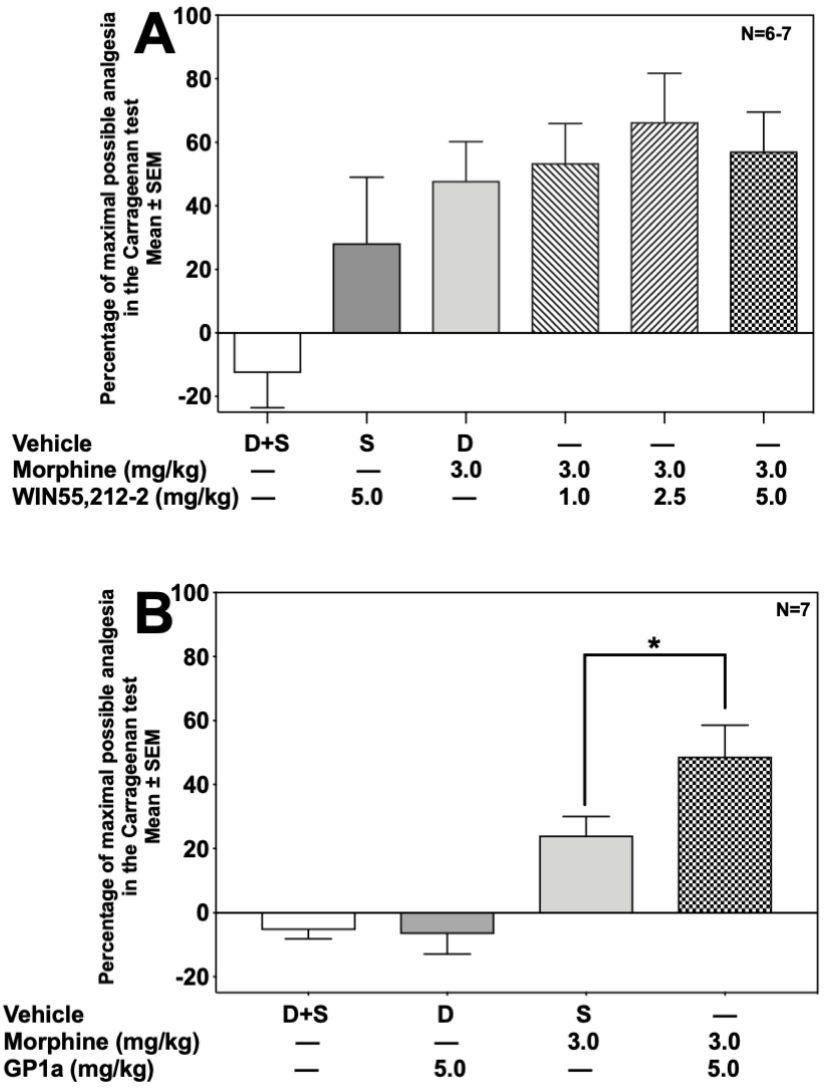

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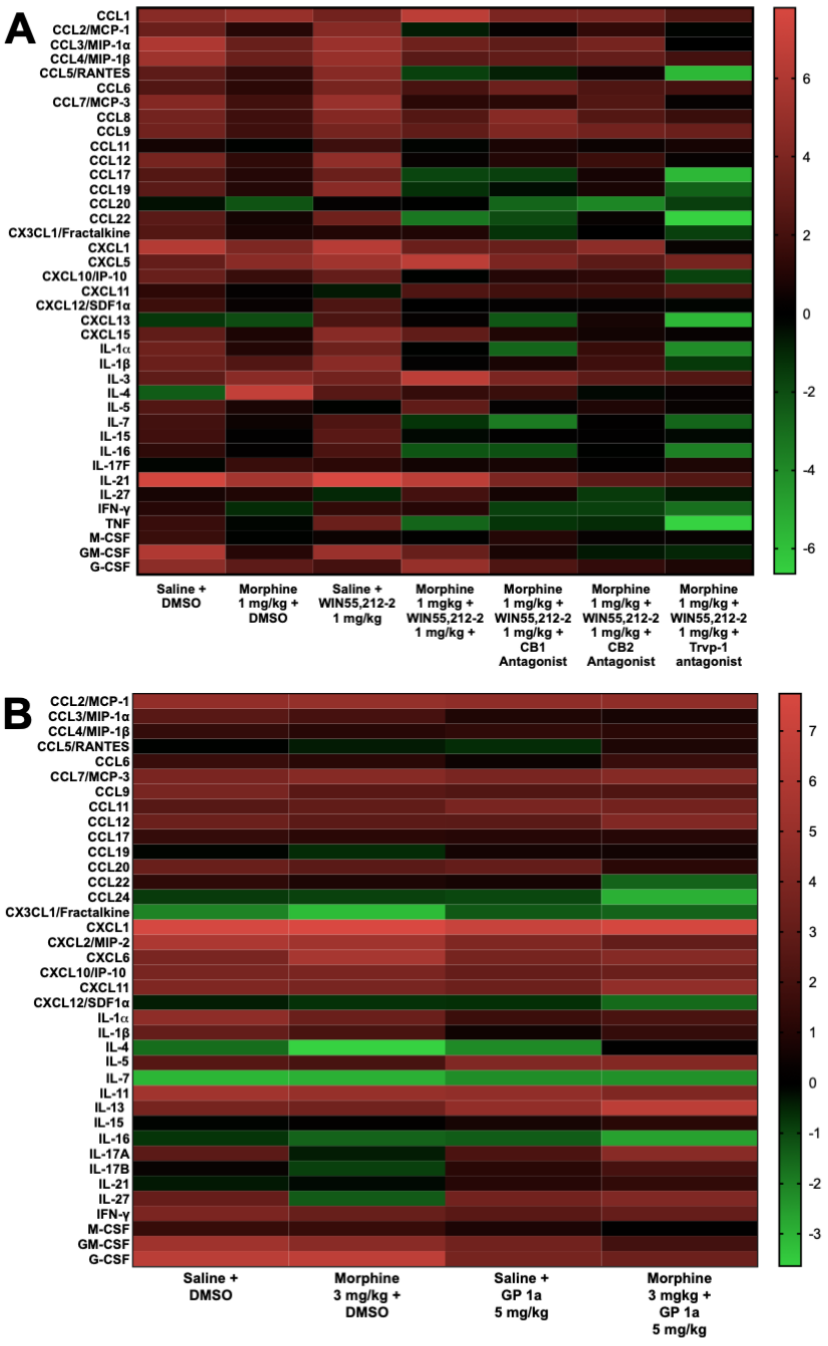

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Table 1. Summary of cannabinoid and opioid interactions in two pain assays.

	WIN alone	WIN + morphine	GP1a alone	GP1a + morphine
Formalin test	+[1]	+ synergy[4]	+	–[2] sub-additive[3]
Carrageenan test	–	–	–	+ enhanced analgesia

[1] + means it produced analgesia
[2] – means there was no analgesia
[3] "Sub-additive" means that the combination was not as efficacious as would be predicted from the sum of the individual drugs alone.
[4] "Synergy" means that the effect of the combined drugs was greater than an additive effect of the two drugs. "Synergy" can also be called "a super-additive effect".