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RESEARCH PAPER

Single and combined effects of Δ^9-tetrahydrocannabinol and cannabidiol in a mouse model of chemotherapy-induced neuropathic pain

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BACKGROUND AND PURPOSE

The non-psychoactive phytocannabinoid cannabidiol (CBD) can affect the pharmacological effects of Δ^9-tetrahydrocannabinol (THC). We tested the possible synergy between CBD and THC in decreasing mechanical sensitivity in a mouse model of paclitaxel-induced neuropathic pain. We also tested the effects of CBD on oxaliplatin- and vincristine-induced mechanical sensitivity.

EXPERIMENTAL APPROACH

Paclitaxel-treated mice (8.0 mg·kg^{-1} i.p., days 1, 3, 5 and 7) were pretreated with CBD (0.625–20.0 mg·kg^{-1} i.p.), THC (0.625–20.0 mg·kg^{-1} i.p.) or CBD + THC (0.04 + 0.04–20.0 + 20.0 mg·kg^{-1} i.p.), and mechanical sensitivity was assessed on days 9, 14 and 21. Oxaliplatin-treated (6.0 mg·kg^{-1} i.p., day 1) or vincristine-treated mice (0.1 mg·kg^{-1} i.p. days 1–7) were pretreated with CBD (1.25–10.0 mg·kg^{-1} i.p.), THC (10.0 mg·kg^{-1} i.p.) or THC + CBD (0.16 mg·kg^{-1} THC + 0.16 mg·kg^{-1} CBD i.p.).

KEY RESULTS

Both CBD and THC alone attenuated mechanical allodynia in mice treated with paclitaxel. Very low ineffective doses of CBD and THC were synergistic when given in combination. CBD also attenuated oxaliplatin- but not vincristine-induced mechanical sensitivity, while THC significantly attenuated vincristine- but not oxaliplatin-induced mechanical sensitivity. The low dose combination significantly attenuated oxaliplatin- but not vincristine-induced mechanical sensitivity.

CONCLUSIONS AND IMPLICATIONS

CBD may be potent and effective at preventing the development of chemotherapy-induced peripheral neuropathy, and its clinical use may be enhanced by co-administration of low doses of THC. These treatment strategies would increase the therapeutic window of cannabis-based pharmacotherapies.

Abbreviations

CB_1, cannabinoid 1 receptor subtype; CB_2, cannabinoid 2 receptor subtype; CBD, cannabidiol; CIPN, chemotherapy-induced peripheral neuropathy; E_{add}, predicted additive effect level; E_{obs}, actual effect level observed; THC, δ^9-tetrahydrocannabinol

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Tables of Links

TARGETS	
GPCRs[a]	**Voltage–gated ion channels**[b]
CB_1 receptor	TRPV1 channels
CB_2 receptor	
5-HT_{1A} receptor	

LIGANDS	
Cannabidiol	SR144528
Cisplatin	Δ^9-Tetrahydrocannabinol
Paclitaxel	Vincristine
SR141716	WIN55,212

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a serious dose-limiting adverse effect associated with several commonly used chemotherapeutic agents, including taxanes, platinum agents and vinca alkaloids. The exact mechanism of CIPN has not been fully elucidated and can differ across classes of chemotherapeutic agents. In general, these agents probably initiate toxicity by affecting cellular microtubules, disrupting mitochondrial function and/or impairing DNA synthesis. These assaults on peripheral nerves can lead to peripheral and central nitroxidative stress and inflammation, sensitization and spontaneous activity of peripheral nerve fibres and hyperexcitability in the dorsal column of the spinal cord (Hu and McLachlan, 2002), leading to ascending pain pathway sensitization (Peters *et al.*, 2007). In most cases, CIPN is only partly reversible with cessation of treatment and, in the worst cases, damage can be permanent. To date, no one drug or drug class is considered to be safe and effective for treatment of CIPN (Lynch *et al.*, 2004). It is therefore necessary to identify novel therapies to prevent or treat CIPN.

Cannabinoids suppress neuropathic pain induced by traumatic nerve injury, toxic insults and metabolic changes (Guindon and Hohmann, 2008). We have recently reported that the non-psychoactive phytocannabinoid cannabidiol (CBD) prevents the development of paclitaxel-induced mechanical sensitivity in mice (Ward *et al.*, 2011; Ward *et al.*, 2014). The mixed CB_1/ CB_2 receptor agonist WIN55,212 also attenuates paclitaxel- and vincristine-induced neuropathic pain through both CB_1 and CB_2 receptor mechanisms (Pascual *et al.*, 2005; Rahn *et al.*, 2007), while selective CB_2 receptor agonism has also been shown to attenuate cisplatin- and paclitaxel-induced neuropathic pain (Deng *et al.*, 2012). While several of CBD's overt effects are similar to cannabinoids acting at CB_1 and/or CB_2 receptors, such as Δ^9-tetrahydrocannabinol (THC), CBD has very low affinity for these receptors. Instead, CBD interacts with several other receptors and intracellular messengers that have been identified as targets for its pharmacological effects. For example, we demonstrated that agonism at the 5-HT_{1A} receptor may underlie CBD's protective effect against development of paclitaxel-induced mechanical sensitivity, as its protective effect was lost in the presence of the 5-HT_{1A} receptor antagonist WAY100635, but not the CB_1 receptor antagonist SR141716 or the CB_2 receptor antagonist SR144528 (Ward *et al.*, 2014). Others have identified a role for the activation of TRPV1 channels (Comelli *et al.*, 2008) and suppression of ROS and other inflammatory mediators (Costa *et al.*, 2007) in the attenuation of CIPN by CBD, in rodent models.

Therefore, while CBD and cannabinoid receptor agonists both attenuate chemotherapy-induced neuropathic pain in rodent models, these agents are likely to work through different sites of action. This suggests that when co-administered, these compounds may interact in a manner that deviates from additive, supporting the commonly held 'entourage effect' concept that components of the marijuana plant interact synergistically in producing there pharmacological effects. Such potential interactions between the phytocannabinoids CBD and THC have been investigated since the 1970s in a range of animal models and with mixed results. By and large, these studies have focused on the 'tetrad' ofcannabinoid effects (catalepsy, locomotor activity, hypothermia and antinociception). These are classical CB1 receptor-mediated behavioural pharmacological effects, reliably measured in rodents (Smith *et al.*, 1994) that are typically not observed after administration of CBD. Therefore, these experiments are characterizing whether ineffective doses of CBD have no effect, increase or decrease, the effects of THC on these behaviours. Moderate to high doses of CBD, which are ineffective on their own, had no effect (Jones and Pertwee, 1972; Ham and De Jong, 1975), increased (Varvel *et al.*, 2006; Hayakawa *et al.*, 2008) or decreased (Welburn *et al.*, 1976; Formukong *et al.*, 1988) tetrad behaviours with little consistency between studies. Most pertinent to the present experiments, moderate to high (10–50 $mg \cdot kg^{-1}$) doses of CBD, lacking antinociceptive effects on their own, did increase the antinociceptive potency of THC, while lower doses (e.g. 3 $mg \cdot kg^{-1}$) did not (Karniol and Carlini, 1973; Varvel *et al.*, 2006; Hayakawa *et al.*, 2008). However, to our knowledge, no published studies have determined whether effective anti-neuropathic doses of CBD and THC alone interact in an additive, synergistic or subadditive manner when combined. The drug Sativex (Nabixomols), a defined extract of the *Cannabis* plant that contains THC, CBD and a range

of non-cannabinoid chemicals native to the plant, was developed by GWPharma based on the tenet that the combination of THC, CBD and other chemicals in cannabis-based medicinal extracts may produce a therapeutic effect greater than the sum of its parts. This is an exciting notion that needs to be empirically tested.

In the present set of experiments, we examined if the mixed CB_1/CB_2 receptor agonist THC was as potent and efficacious as CBD in preventing paclitaxel-induced mechanical sensitivity alone and if the combination of CBD and THC produced synergistic effects. We also tested the efficacy of selected doses of CBD, THC and in combination, to prevent mechanical sensitivity following oxaliplatin or vincristine administration.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the Temple University Institutional Animal Care and Use Committee and were as humane as possible. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015) and US NIH guidelines for reporting experiments involving animals. The total number of animals used was 344.

Male C57Bl6 mice weighing 18–20 g (Taconic Farms, Cranbury NJ, USA) were acclimatized to the temperature- and humidity-controlled vivarium and housed in groups of four for at least 5 days before initiation of the study. Mice were housed under a reverse 12 h light/dark cycle (lights off 9:00 h), with access to food and water *ad libitum*. Following experimentation, all mice were killed by CO_2 asphyxiation.

Mechanical allodynia

Paclitaxel study. Baseline sensitivity to mechanical stimuli was assessed prior to drug treatment using von Frey monofilaments (0.07–2.0 g) and the up-down method, as described previously (Ward *et al.*, 2011; Ward *et al.*, 2014). Chemotherapeutic agents, cannabinoid compounds and/or vehicle were then administered on experimental days 1, 3, 5 and 7. Paclitaxel was given at a dose of 8.0 $mg{\cdot}kg^{-1}$ per injection. Fifteen minutes prior to paclitaxel injection, mice were first pretreated with vehicle or a range of CBD (0.625–20 $mg{\cdot}kg^{-1}$), THC (0.625–20 $mg{\cdot}kg^{-1}$) or CBD + THC combination (0.04 + 0.04–20.0 + 20.0 $mg{\cdot}kg^{-1}$) doses. Mechanical sensitivity was reassessed on days 9, 14 and 21. Three paclitaxel + vehicle groups were tested throughout the duration of the study: one alongside the CBD dose–response groups, one alongside the THC dose–response groups and one alongside the combination groups, to ensure that the effect of paclitaxel remained consistent throughout the course of the study.

Oxaliplatin study. Baseline sensitivity to mechanical stimuli was assessed prior to drug treatment in mice as described above. A dose of 6 $mg{\cdot}kg^{-1}$ oxaliplatin was administered once. CBD (1.25–10.0 $mg{\cdot}kg^{-1}$), THC (10.0 $mg{\cdot}kg^{-1}$) or THC + CBD (0.16 $mg{\cdot}kg^{-1}$ THC + 0.16 $mg{\cdot}kg^{-1}$ CBD) was administered 15 min prior to the single oxaliplatin injection. Mechanical sensitivity was reassessed on days 2, 4, 7 and 10.

Vincristine study. Baseline sensitivity to mechanical stimuli was assessed prior to drug treatment in mice as described above. A dose of 0.1 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ vincristine was administered once daily for 7 days. CBD (1.25–10.0 $mg{\cdot}kg^{-1}$), THC (10.0 $mg{\cdot}kg^{-1}$) or THC + CBD (0.16 $mg{\cdot}kg^{-1}$ THC + 0.16 $mg{\cdot}kg^{-1}$ CBD) was administered 15 min prior to each vincristine injection. Mechanical sensitivity was reassessed on days 5, 10, 15 and 22.

Mice were randomized to their treatment groups, and all behavioural experimenters were blinded to the treatment conditions of the mice during behavioural testing. One experimenter placed the mice in the allodynia testing cubbies for habituation, while the other experimenter conducted allodynia testing procedure outside the room. Mice are used because they are a common species used for this model, and we have used them in our CIPN research for the past decade. Sample sizes were $n = 6$–12 per group for all studies ($n = 12$ for combination experiment due to higher variability in the combination data), based on our and others' past results. Dosing regimens for each chemotherapeutic agent differ based on the optimal dosing regimens to produce reliable and robust mechanical sensitivity based on published data from rodent models.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). For statistical analyses and graphical presentation of data, a percent baseline sensitivity value for each mouse is calculated and the mean percent baseline sensitivity (±SEM) was determined for each treatment group. Baseline sensitivity for each mouse was calculated as their sensitivity threshold value on test day divided by their sensitivity threshold value at baseline multiplied by 100. Therefore, 100% baseline sensitivity represents no development of mechanical sensitivity following treatment, with lower % values equating to the development of increasing sensitivity to mechanical stimulation. One-way repeated measures ANOVAs and Dunnett's *post hoc* comparisons (GraphPad Prism 6) were used to determine doses significantly different from vehicle treatment. $P < 0.05$ was used as the limit of statistical significance.

Additionally, in order to determine dose-effect levels of the ascending limb of the CBD and THC dose-effect curves, as well as the CBD + THC dose combinations, for dose-equivalence analyses (see below), paclitaxel data from day 14 for each animal were also transformed into percent maximum possible effect (%MPE ±SEM). To determine %MPE for each mouse, the relevant paclitaxel group's percent baseline value is subtracted from the mouse's percent baseline value, as the paclitaxel effect will represent 0% MPE. In addition, a scaling multiplier is calculated by subtracting the paclitaxel group's percent baseline value from 100% and multiplying dividing this number into 100. The final calculation for each mouse is as follows: (percent baseline value − paclitaxel percent baseline value) × (100/100-paclitaxel baseline value). For example, on day 14, the first-run paclitaxel percent baseline average was 39.8%. The percent baseline sensitivity of a mouse in the

2.5 mg·kg^{-1} CBD group on day 14 was 71.4%. The above formula was used to determine the %MPE for that mouse (52.4%) when the paclitaxel value was set to 0%. Derived values exceeding 100% were capped at 100%, and derived values in the negative were assigned 0%. Linear regression analysis (GraphPad Prism 6) was then used to determine the ED_{50} values for the ascending limb of the CBD, THC and CBD + THC dose–response curves.

Dose-equivalence analysis was used to predict the combined effects of CBD and THC, administered in a 1:1 ratio, based on their effects alone on the ascending limbs of their dose response curves. Briefly, in dose-equivalence analysis, for each CBD dose, an effect-equivalent dose of THC is identified. This matched THC dose is added to the actual THC dose in each combination to be tested so that the sum is the predicted effective dose for that combination. The test for significance for the difference between the predicted additive effect level (E_{add}) and the actual effect level observed (E_{obs}) is based on Student's *t* distribution (see Tallarida, 2000, Tallarida, 2012) for details).

Materials

Paclitaxel solution (Teva Parenteral Medicines, dissolved in 1:1 mixture of alcohol and Cremophor), oxaliplatin (Teva Parenteral Medicines) and vincristine (Hospira Worldwide) were purchased from the Temple University Pharmacy (Philadelphia, PA, USA). Paclitaxel was then diluted with sterile saline to 0.8 mg·mL^{-1}. Oxaliplatin was dissolved in water to a concentration of 0.6 mg·mL^{-1}. Vincristine was dissolved in saline to a concentration of 0.01 mg·mL^{-1}. Laboratory-synthesized CBD was provided by INSYS Therapeutics, Inc. (Austin, TX) and THC was provided by the National Institute on Drug Abuse drug supply programme (Bethesda, MD, USA). CBD and THC were dissolved in a mixture of ethyl alcohol, Cremophor and saline; 1:1:18, v/v. All agents were injected i.p. in a volume of 10 μL·g^{-1} of body weight. For combination testing, CBD and THC were dissolved together into a single solution and administered by a single injection.

Results

Reproducibility of paclitaxel-induced mechanical sensitivity

As we have reported previously, administration of paclitaxel (8.0 mg·kg^{-1} per injection, administered on experimental days 1, 3, 5 and 7) produced robust and reliable mechanical sensitivity in C57Bl/6 mice (Figure 1). This effect lasted for at least 21 days post-initiation of administration with peak allodynic effects occurring between days 9 and 14. Because of the long duration of the study, multiple paclitaxel + vehicle groups were run to determine that paclitaxel administration was producing a consistent level of mechanical sensitivity across all phases of CBD and THC alone and combination testing.

Dose-response effects of CBD or THC on paclitaxel-induced mechanical sensitivity

Treatment with either CBD (Figure 2A) or THC (Figure 2B) produced a biphasic, dose-related attenuation of paclitaxel-induced mechanical sensitivity. For CBD treated groups, analysis of the results from day 9 with one-way ANOVA indicated a significant effect of CBD treatment [$F_{(8,\ 62)}$ = 4.566]. Dunnett's multiple comparisons test indicated significant effects of the 1.0 and 20 mg·kg^{-1} doses of CBD. For the results from day 14, one-way ANOVA indicated a significant effect of CBD treatment [$F_{(8,\ 62)}$ = 2.693] Dunnett's multiple comparisons test indicated significant effects of the 1.0 and 20 mg·kg^{-1} doses of CBD. Analysis of the results from day 21 indicated no significant effect of CBD treatment [one-way ANOVA; $F_{(8,\ 62)} < 1.0$].

For the THC treated groups, analysis of the results from day 9 indicated a significant effect of THC treatment [one-way ANOVA ; $F_{(7,\ 56)}$ = 3.819]. Dunnett's multiple comparisons test indicated significant effect of the 2.5 and 10 mg·kg^{-1} doses of THC. Results from day 14 indicated a significant effect of THC treatment [one-way ANOVA $F_{(7,\ 56)}$ = 2.810] and Dunnett's multiple comparisons test indicated significant effect of the 10 mg·kg^{-1} dose of THC. For Day 21, one-way ANOVA indicated a nearly significant effect of THC treatment [$F_{(7,\ 56)}$ = 2.104, P = 0.058].

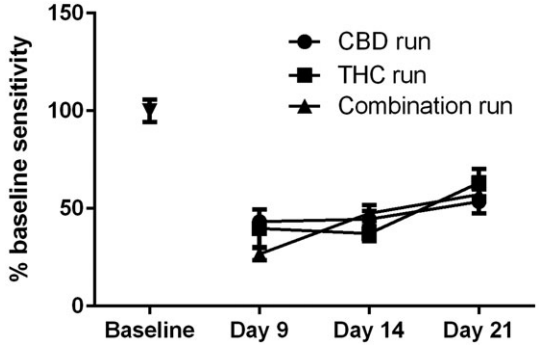

Figure 1

Effect of paclitaxel on mechanical sensitivity in three separate groups of mice on different days. Paclitaxel (8.0 mg·kg^{-1} per injection) is administered on experimental days 1, 3, 5 and 7 following baseline measurement of mechanical sensitivity. Three separate groups of paclitaxel-treated mice were run in order to provide appropriate non-cannabinoid treated control data along the length of the study. n = 8 per group.

Dose-response effects of CBD + THC combination on paclitaxel-induced mechanical sensitivity

Treatment with CBD + THC in a 1:1 ratio based on dose produced a biphasic, dose-related attenuation of paclitaxel-induced mechanical sensitivity (Figure 3). This effect was more potent than either agent given alone. Analysis of the results from CBD + THC treatment on day 14, indicated a significant effect of CBD + THC treatment [one-way ANOVA; $F_{(10,\ 113)}$ = 2.576,] and Dunnett's multiple comparisons test indicated a statistically significant effect of the combined 0.31 mg·kg^{-1} (0.16 mg·kg^{-1} CBD + 0.16 mg·kg^{-1} THC) dose compared with paclitaxel treatment alone. We focused on the day 14 combination effect because this is the time point of peak allodynia, but the combination treatments at days 9

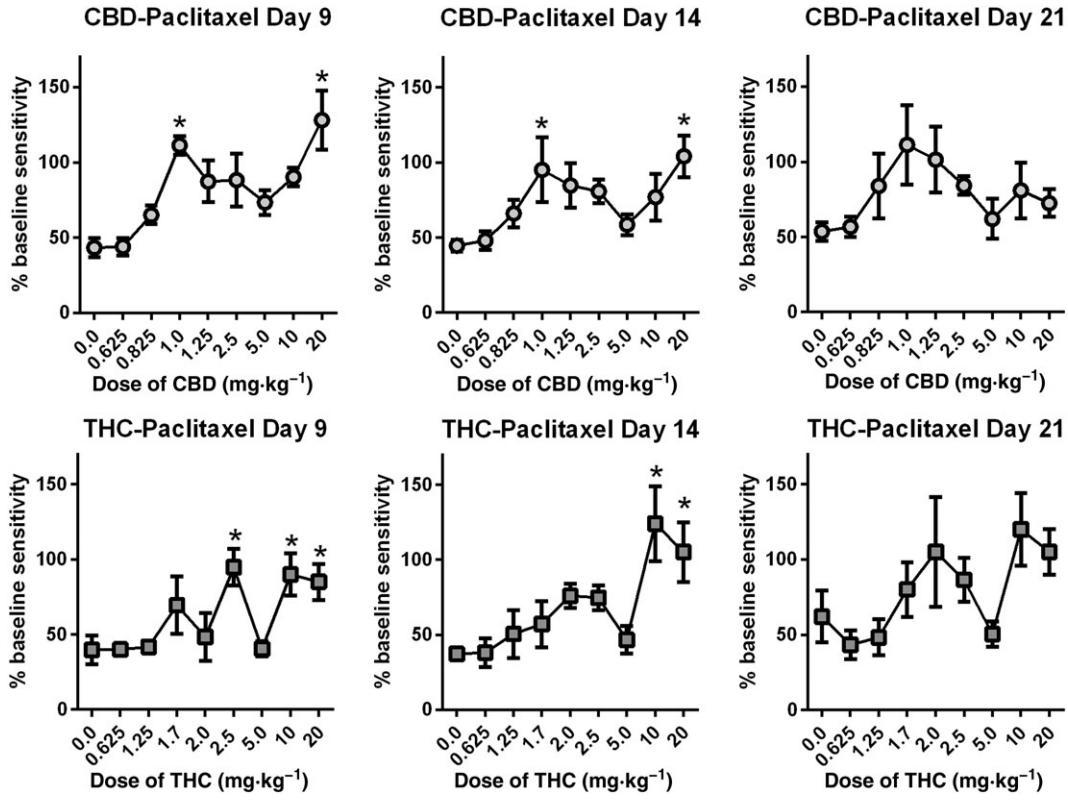

Figure 2

Effect of CBD or THC on paclitaxel-induced mechanical sensitivity 9, 14 and 21 days after the start of treatment. Paclitaxel alone (8.0 mg·kg^{-1} per injection) or following pretreatment with CBD (A) or THC (B) (0.625–20 mg·kg^{-1} i.p.) was administered on experimental days 1, 3, 5 and 7, following baseline measurement of mechanical sensitivity. Mechanical sensitivity was tested 9, 14 and 21 days following initiation of treatment. $n = 8$ per group, with the exception of the 10 mg·kg^{-1} CBD group which was $n = 7$ due to one mouse found dead in home cage. *$P < 0.05$, significantly different from ??? ; Dunnett's multiple comparisons test.

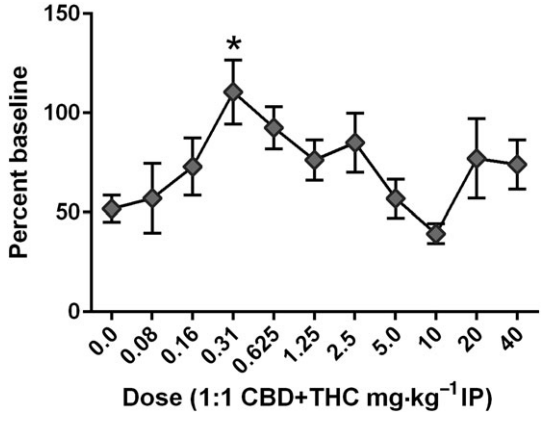

Figure 3

Effect of CBD + THC on paclitaxel-induced mechanical sensitivity 14 days after the start of treatment. Paclitaxel alone (8.0 mg·kg^{-1} per injection) or following pretreatment with CBD + THC (combined doses 0.08–40.0 mg·kg^{-1} per injection i.p.) is administered on experimental days 1, 3, 5 and 7 following baseline measurement of mechanical sensitivity. n = 12 per group, with the xexception of the 10 mg·kg^{-1} dose, where two mice were removed from the study due to skin lesions from a dominant cage mate. *$P < 0.05$, significantly different from paclitaxel alone; one-way ANOVA with Dunnett's multiple comparisons test.

and 21 showed a similar leftward shift of the dose-response curve (data not shown).

Comparison of single, compared with combined, phytocannabinoid treatment on paclitaxel-induced mechanical sensitivity

To determine whether the 1:1 CBD + THC combination produced protective effects in the CIPN model that deviated from simple additivity, predicted effect levels for these dose combinations were first determined from the dose effects of each agent alone. To do this, raw data for each animal from the initial ascending limbs of the CBD and THC dose-response curves (0.625–2.5 mg·kg^{-1}) were transformed into %MPE values, as described in the Methods. Linear regression analysis of the ascending limb indicated an ED_{50} for CBD of 1.014 mg·kg^{-1} and for THC of 1.8 mg·kg^{-1} (Figure 4A, B). To determine the predicted additive effect of doses of CBD and THC given in a 1:1 ratio, effect levels of each dose/drug in the combination were determined using the following dose effect equations derived for each for each drug: $E = \frac{78.47D^{2.5}}{D^{2.5}+0.497}$ for CBD and $E = \frac{80D^3}{D^3+3.44}$ for THC. These effect levels were added together to determine the E_{add} for each dose combination tested (Figure 4C). The E_{add}s were then compared graphically and statistically to the actual

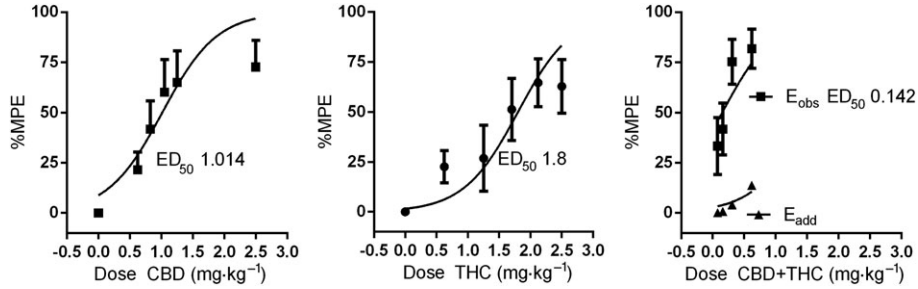

Figure 4

Linear regression analysis of ascending limbs of the observed single and combined agent dose–response curves and predictive additive curve. Data were transformed to %MPE (see text for details) in order to determine relative effective dose levels. E_{obs} is the observed dose effect levels of CBD + THC combinations along the ascending limb of the combination dose response curve. E_{add} is the predicted additive dose effect levels of those same doses based on the ascending limb of the single agent dose response curves.

effect levels determined by the experiment (E_{obs}, Figure 4C). Student's *t*-test indicated a significant difference between the predicted additive and observed effect levels.

Effect of CBD on oxaliplatin-induced mechanical sensitivity

The effect of CBD (1.25–10.0 mg·kg^{-1} i.p.) on oxaliplatin-induced mechanical sensitivity was tested in 40 male C57Bl/6 mice. Two-way ANOVA indicated a significant effect of time [$F_{(3,\ 140)}$ = 7.505] and treatment [$F_{(4140)}$ = 4.260,]. There was no significant interaction between time and treatment [$F_{(12,\ 140)}$ = 1.239 (Figure 5A) and Dunnett's post test indicated no specific significant differences for any dose of CBD, compared with oxaliplatin alone. The effect of 10.0 mg·kg^{-1} THC was also tested in a separate group of animals, and two-way ANOVA indicated a significant effect of time [$F_{(3,\ 48)}$ = 5.780] and a non-significant attenuated mechanical sensitivity [$F_{(1,\ 48)}$ = 2.912] (Figure 5B). The effect of the low dose combination 0.16 mg·kg^{-1} THC + 0.16 mg·kg^{-1} CBD produced a significant effect of treatment [$F_{(1,\ 48)}$ = 7.180] and of time [$F_{(3,\ 48)}$ = 7.155] (Figure 5C).

Effect of CBD on vincristine-induced mechanical sensitivity

The effect of CBD (1.25–10.0 mg·kg^{-1} ip) on vincristine-induced mechanical sensitivity was tested in 40 additional male C57Bl/6 mice. Two-way ANOVA indicated a significant effect of time [$F_{(3,\ 140)}$ = 32.00] but not of treatment [$F_{(4,\ 140)}$ = 1.163] (Figure 5D). The effect of 10.0 mg·kg^{-1} THC was also tested in a separate group of animals, and two-way ANOVA indicated a significant effect of treatment [$F_{(1,\ 56)}$ = 18.82] (Figure 5E). The low dose combination (0.16 mg·kg^{-1} THC + 0.16 mg·kg^{-1} CBD) did not attenuate vincristine-induced mechanical sensitivity [$F_{(1,56)}$ = 1.550] (Figure 5F).

Discussion

Previously, we have already demonstrated that pretreatment with the phytocannabinoid CBD prevented the development of behavioural symptoms of CIPN in a mouse mode (Ward *et al.*, 2011, 2014). The main objectives of the present study were to demonstrate whether the primary phytocannabinoid THC shows similar potency and efficacy in the mouse CIPN model and to determine if CBD and THC interact in a manner deviating from additivity in this model. This is important because, while the entourage effects of cannabinoids working together as more than a sum of their parts have been anecdotally discussed at length, few rigorous studies have been executed to demonstrate such a phenomenon. These data in the present study were subjected to equivalence analyses to quantitatively determine whether the two dominant phytocannabinoids can work synergistically to prevent the development of neuropathic pain in a mouse model of CIPN.

Initially, it was critical to determine the relative potency of each compound alone to produce comparable anti-allodynic effects. As shown in Figure 2, both phytocannabinoids show very similar triphasic dose–response curves with two apparent peaks in efficacy, one within a dose range of 1.0–2.5 mg·kg^{-1} and the other within the 10–20 mg·kg^{-1} range. Biphasic effects of cannabinoids have been reported (Grisham and Ferraro, 1972; Kwiatkowska *et al.*, 2004; Margulies and Hammer, 1991), and one study has reported triphasic effects of THC on locomotor activity across a wide range of doses (Sanudo-Pena *et al.*, 2000). In female mice dosed daily with CBD for 14 days, paclitaxel-induced mechanical sensitivity was attenuated at the 10 mg·kg^{-1} dose but not the 5.0 mg·kg^{-1} dose (Ward *et al.*, 2011). In a follow-up experiment, female mice were treated with CBD (2.5 or 5.0 mg·kg^{-1}) only prior to each paclitaxel injection (days 1, 3, 5 and 7), and both CBD doses attenuated the development of paclitaxel-induced mechanical sensitivity, but through a 5-HT_{1A} but not a CB_1 or CB_2 receptor-mediated mechanism (Ward *et al.*, 2014). Our present results with synthetic CBD (manufactured by INSYS Rx) showed that doses as low as 1.0 mg·kg^{-1} i.p. fully blocked the development of mechanical sensitivity, following paclitaxel administration in male mice. Taken together, potency differences we have obtained with CBD in the CIPN model may be dependent on sex as has been determined with THC (Tseng *et al.*, 2004) or on source of compound. In the present study, efficacy was again observed at 10 and 20 mg·kg^{-1} i.p., showing a triphasic dose–response curve.

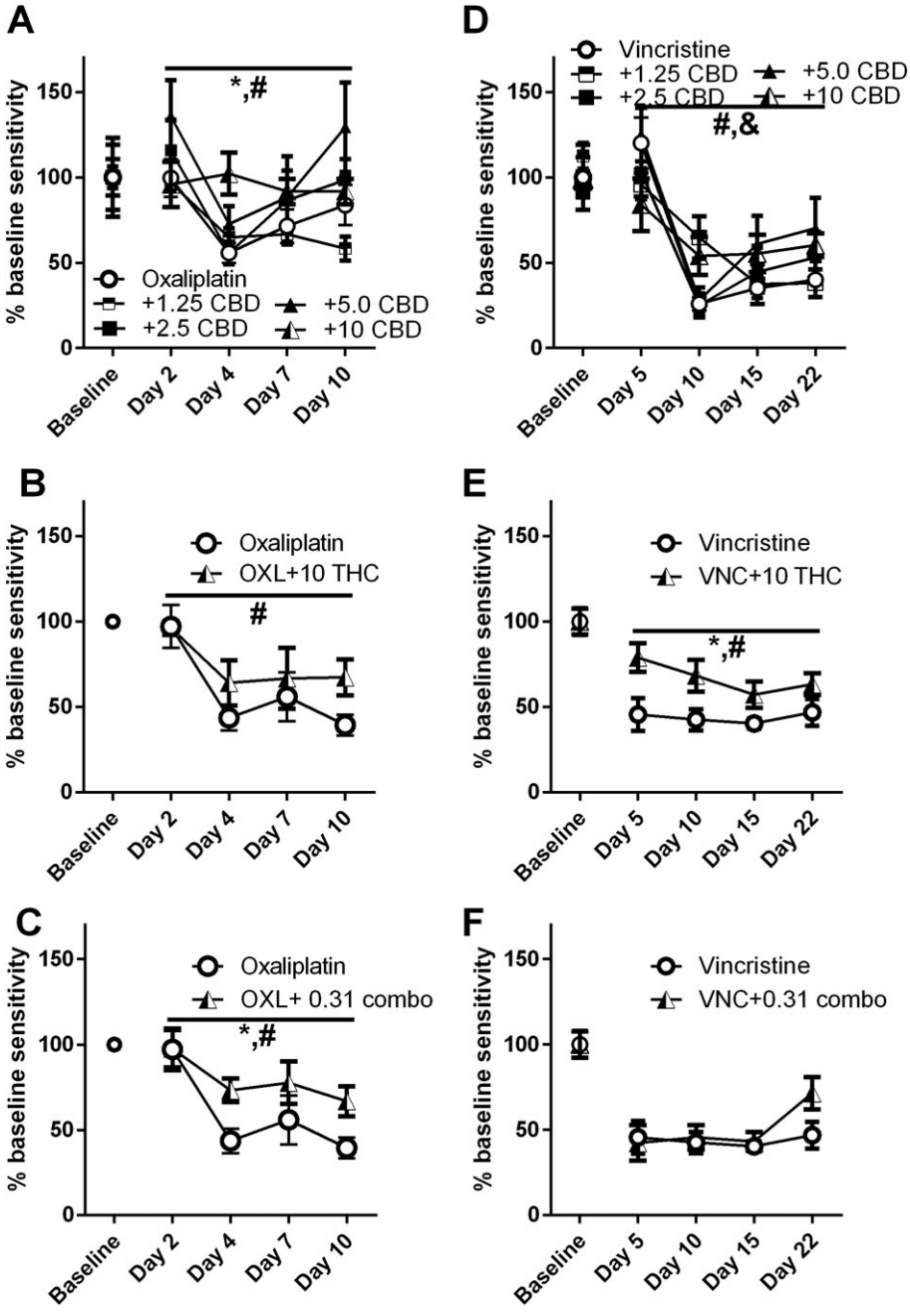

Figure 5

Effect of CBD, THC and their combination on oxaliplatin- or vincristine-induced mechanical sensitivity. Oxaliplatin (6.0 mg·kg^{-1} i.p.) alone or following pretreatment with CBD (doses 1.25–10.0 mg·kg^{-1} per injection i.p.), THC (10.0 mg·kg^{-1} i.p.) or THC + CBD (0.16 mg·kg^{-1} THC + 0.16 mg·kg^{-1} CBD i.p.) was administered on experimental day 1 following baseline measurement of mechanical sensitivity. Vincristine (0.1 mg·kg^{-1} i.p.) alone or following pretreatment with CBD (doses 1.25–10.0 mg·kg^{-1} per injection i.p.), THC (10.0 mg·kg^{-1} i.p.) or THC + CBD (0.16 mg·kg^{-1} THC + 0.16 mg·kg^{-1} CBD i.p.) was administered on experimental days 1–7 following baseline measurement of mechanical sensitivity. n = 8 per group, with the exception of the oxaliplatin group in B and C, which is n = 6 due to two mice being killed because of urinary blockage. # $P < 0.05$, significant main effect of time; * $P < 0.05$, significant main effect of treatment; $^{\&}$ $P < 0.05$, significant interaction.

Such effects of these higher doses have also been reported in other pain (Costa *et al.,* 2004; Ward *et al.,* 2011) and CNS injury models (Kwiatkoski *et al.,* 2012). The reason(s) underlying these triphasic effects need to be further examined. One possibility, made more plausible by the fact that CBD has such a range of putative therapeutic sites of action, is that the two ascending limbs of the CBD dose–response curve represent the compound working by two distinct

mechanisms. For example, we have previously demonstrated that the anti-neuropathic effects of CBD (5.0 mg·kg^{-1} every other day for 4 days) may be mediated by agonist activity at the 5-HT$_{1A}$ receptor, while Comelli *et al.* (2008) reported that these effects of CBD (10.0 mg·kg^{-1} daily for 14 days) may be mediated by the TRPV1 cation channel.

In the present model, THC produced triphasic effects, comparable to those observed for CBD. The 2.5 mg·kg^{-1} dose of THC fully blocked the development of mechanical sensitivity following paclitaxel administration, as did higher doses of 10 and 20 mg·kg^{-1}. It is unlikely that motor impairment was responsible for these high dose effects of THC, as the final cannabinoid doses were given on day 7 and anti-allodynic responses were measured up to day 21. We are unaware of a comprehensive preclinical study demonstrating dose–response effects of the partial CB$_1$/CB$_2$ receptor agonist THC in a rodent model of neuropathic pain, which is surprising given that targeting CB$_1$ and CB$_2$ receptors to produce antinociception and anti-inflammation, respectively, has been investigated extensively in animal models. Previous studies demonstrated that full agonists at CB$_1$ (Vera *et al.,* 2013) and CB$_2$ receptors (Deng *et al.,* 2015) can attenuate chemotherapy-induced neuropathic pain, but the primary mechanism of action of the partial mixed CB$_1$/ CB$_2$ receptor agonist THC in this model remains to be determined. Taken together, these experiments reveal that CBD and THC are roughly equipotent and equi-effective at preventing the development of mechanical sensitivity induced by paclitaxel administration.

For the next phase of the study, a dose-equivalence analysis experimental design (where combinations are paired by dose), as opposed to a dose-addition analysis design (where combinations are paired by effect level), was implemented to model the cannabis extract Sativex, which contains nearly equal amounts of THC (2.7 mg) and CBD (2.5 mg) per spray delivery. When equal dose pairs of THC and CBD were administered to mice receiving paclitaxel, the cannabinoid combination treatment was more potent than either CBD or THC treatment alone. A combined dose of 0.32 mg·kg^{-1} (0.16 mg·kg^{-1} THC: 0.16 mg·kg^{-1} CBD) was fully effective at preventing paclitaxel-induced mechanical sensitivity 14 days after initiation of drug administration. Linear regression analyses revealed an ED$_{50}$ of 1.014 mg·kg^{-1} for CBD and for THC, an ED$_{50}$ of 1.8 mg·kg^{-1} and, for the combination THC + CBD, a much decreased ED$_{50}$ of 0.142 mg·kg^{-1}. Interestingly, the combined dose-effect curve also maintained its triphasic dose response function. These results reveal a significant interaction between THC and CBD and demonstrate for the first time quantitatively that these phytocannabinoids act in a synergistic manner to exert shared physiological effects, in this case to prevent the development of neuropathic pain in a rodent model. As mentioned in the Introduction, several previous studies have investigated the effect of predominantly ineffective doses of CBD on established behavioural pharmacological effects of THC. In mice alone, results have pointed to a lack of interaction, a potentiation of THC effects or an attenuation of THC effects across studies, even on the same behavioural endpoint [e.g. catalepsy (Jones and Pertwee, 1972; Karniol and Carlini, 1973; Ham and De Jong, 1975; Formukong *et al.,* 1988; Hayakawa *et al.,* 2008)]. Specifically regarding pain, interaction studies have been carried out again in rodent models, showing that CBD was ineffective, with findings that CBD can increase the antinociceptive potency of THC (Varvel *et al.,* 2006) but it decreased the attenuation of formic acid-induced writhing by THC (Welburn *et al.,* 1976). Because previous reports have focused on relatively high doses of CBD in animal models, where CBD is typically ineffective, it is difficult to compare the present degree of observed synergy with these earlier studies. Overall, our present synergistic results are critical because they demonstrate and reinforce several key points. Firstly, the effects of the interactions between phytocannabinoids, be they additive, synergistic or sub-additive, are highly dependent on the physiological endpoint. Secondly, ultra-low doses of these phytocannabinoids may be effective in clinical populations for the treatment of neuropathic pain, allowing for a more favourable therapeutic index with reduced adverse effects. Finally, THC and CBD are likely to be working through separate, facilitative mechanisms of action which, if elucidated, could lead to the development of a wider class of safe and effective anti-neuropathic pharmacotherapies. It is generally recognized now that the initial receptor sites of action of these two compounds are distinct. As mentioned, the effects of THC in this model could be mediated by CB$_1$, CB$_2$ or both receptor subtypes. Regarding CBD, a role for 5-HT$_{1A}$ receptors (Ward *et al.,* 2014), glycine channels (Xiong *et al.,* 2014) or TRPV1 channels (Costa *et al.,* 2004),among others, have been shown to be involved in the anti-neuropathic actions of CBD. The present synergistic results suggest that co-activation by CND and THC of these distinct sites leads either to increased potency of these compounds to stimulate their intracellular signalling pathways or to the stimulation of alternative pathways that are not activated by either compound alone. Moreover, these interactions could occur by CBD and THC activating separate receptors on the same cell types, or by co-modulating distinct cell types. The cell types primarily responsible for these anti-neuropathic and anti-inflammatory effects include not only neurons, but also microglia, astrocytes and/or other cell types associated with the immune system. These possibilities provide for a range of experimental approaches and directions in order to elucidate possible mechanisms at the cellular, receptor and signalling levels. Lastly, changes in pharmacokinetics of each compound may also play a role in the increased potency of the combination, as some have found that CBD can decrease THC metabolism and increase plasma and brain THC levels (Jones and Pertwee, 1972; Bornheim *et al.,* 1995).

To further our understanding of the anti-allodynic efficacy of CBD, THC and their combination, we examined whether CBD treatment would lead to protection from mechanical sensitivity resulting from two additional chemotherapeutic agents, oxaliplatin and vincristine. This suggestion of selectivity is also important to consider given the different mechanisms of action of oxaliplatin, vincristine and paclitaxel in their chemotherapeutic and chemotoxic effects. Differential effects of cannabinoids on these various induced neuropathies might speak to the generalization of a treatment class across chemotoxic neuropathies and to more distinct mechanisms of action of CBD, THC and their

combination, as they relate to specific chemotoxic neuropathies. Our results to date suggest that CBD is effective at preventing oxaliplatin-induced mechanical sensitivity, but the dose–response relationship is shifted to the right, again suggesting a different mechanism of action of CBD (and perhaps oxaliplatin) in this model. One reported difference between these chemotherapeutic agents in the preclinical models is the involvement of non-neuronal cell types in CIPN. For example, there are reports that paclitaxel administration is associated with microglial activation in the dorsal horn of the spinal cord (Pevida *et al.* 2013) and infiltration of T-cells into the dorsal root ganglia, whereas oxaliplatin activated spinal astrocytes but not microglia (Robinson *et al.,* 2014).THC at 10 $mg{\cdot}kg^{-1}$ produced a non-significant attenuation of oxaliplatin-induced mechanical sensitivity, and perhaps more importantly, we again observed a very potent, significant effect of the low dose combination 0.16 $mg{\cdot}kg^{-1}$ THC + 0.16 $mg{\cdot}kg^{-1}$ CBD. Interestingly, CBD was ineffective at preventing vincristine-induced mechanical sensitivity. It should be noted that in the vincristine/CBD experiment, vincristine induced a much more profound percent baseline effect on mechanical allodynia than that observed with paclitaxel and oxaliplatin, so the lack of CBD efficacy after vincristine may be due to an insurmountable level of neuropathy in these mice. THC at 10 $mg{\cdot}kg^{-1}$ was also effective at preventing vincristine-induced mechanical sensitivity, while in this case, the low dose combination (0.16 $mg{\cdot}kg^{-1}$ THC + 0.16 $mg{\cdot}kg^{-1}$ CBD) did not attenuate it. This may again be due to different underlying mechanisms for sensory sensitivity induced by the different chemotherapeutic agents, with some mechanistic targets being less sensitive to CBD or combined treatment than others.

In summary, these experiments demonstrate quantitatively for the first time that CBD and THC work synergistically to prevent the development of neuropathic pain using a mouse model of chemotherapy-induced peripheral neuropathy. We and others identified more than one non-cannabinoid receptor-mediated mechanisms underlying the efficacy of CBD in this model, while related data suggest that THC is likely to act through CB_1 and CB_2 receptors in this assay. Further work is warranted to determine the mechanism of synergy between THC and CBD, from the cellular to receptor to intracellular signalling level, to enhance our ability to develop novel safe and effective treatment strategies for CIPN and other neuropathic pain disorders.

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Author contributions

K.K., A.M. and A.S.-M.performed behavioural testing and was involved in statistical analysis and critical review of the manuscript; R.F.T. and R.J.T. assisted in the conception and design of the work and in critical review of the manuscript; S.J.W. was responsible for the conception and design of the work. She also was responsible for the majority of the statistical analyses and the drafting of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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 recommended by funding agencies, publishers and other organisations engaged with supporting research.

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