THE EFFECT OF PHYTOCANNABINOIDS ON AIRWAY HYPERRESPONSIVENESS, AIRWAY INFLAMMATION AND COUGH

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ABBREVIATIONS

Δ⁹-THC, Δ⁹-tetrahydrocannabinol, (−)-(6aR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol

AM251, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide

BAL, bronchoalveolar lavage

CBC, cannabichromene, 2-Methyl-2-(4-methylpent-3-enyl)-7-pentyl-5-chromenol

CBD, cannabidiol, 2-[(1R,6R)-6-isopropenyl-3-methylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol

CBG, cannabigerol, 2-[(2E)-3,7-dimethylocta-2,6-dienyl]-5-pentyl-benzene-1,3-diol

CB, cannabinoid

CBDA, cannabidiolic acid, (1'R,2'R)-2,6-dihydroxy-5'-methyl-4-pentyl-2'-(prop-1-en-2-yl)-1',2',3',4'-tetrahydro-[1,1'-biphenyl]-3-carboxylic acid

CP55940, 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol

EFS, electrical field stimulation

DMEM, Dulbecco’s modified Eagle’s medium

JTE907 N-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide

LPS, lipopolysaccharide

THCV, tetrahydrocannabivarin, 6,6,9-Trimethyl-3-propyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol

TNF–α, tumour necrosis factor
WIN 55212-2, (R)-(+)\-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6yl]-1-naphtalenylmethanone mesylate, WIN 55212-2

RECOMMENDED MANUSCRIPT SECTION ASSIGNMENT

Inflammation, Immunopharmacology, and Asthma
Cannabis has been demonstrated to have bronchodilator, anti-inflammatory and anti-tussive activity in the airways, but, information on the active cannabinoids, their receptors and the mechanisms for these effects are limited. We compared the effects of Δ⁹-tetrahydrocannabinol, cannabidiol, cannabigerol, cannabichromene, cannabidiolic acid and tetrahydrocannabivarin on contractions of the guinea-pig isolated trachea and bronchoconstriction induced by nerve stimulation or methacholine in anaesthetised guinea-pigs following exposure to saline or the pro-inflammatory cytokine, TNF-α. CP55940, a synthetic cannabinoid agonist was also investigated in vitro. The cannabinoids were also evaluated on TNF−α- and LPS-induced leucocyte infiltration into the lungs and citric acid-induced cough responses in guinea-pigs. TNF−α, but not saline, augmented tracheal contractility and bronchoconstriction induced by nerve stimulation, but not methacholine. Δ⁹-tetrahydrocannabinol and CP55940 reduced TNF−α-enhanced nerve-evoked contractions in vitro to the magnitude of saline-incubated trachea. This effect was antagonised by the CB₁ and CB₂ receptor antagonist AM251 and JTE907, respectively. Tetrahydrocannabivarin partially inhibited the TNF−α-enhanced nerve-evoked contractions whilst the other cannabinoids were without effect. The effect of cannabidiol and Δ⁹-tetrahydrocannabinol together did not differ from that of the latter alone. Only Δ⁹-tetrahydrocannabinol inhibited TNF−α enhanced vagal-induced bronchoconstriction, neutrophil recruitment to the airways and citric acid-induced cough responses. TNF−α potentiated contractions of airway smooth muscle to nerve stimulation by enhancing postganglionic acetylcholine release. Δ⁹-THC and CP55940 inhibited the TNF−α-enhanced acetylcholine release, hence contraction and bronchoconstriction, through activation of pre-synaptic CB₁ and CB₂ receptors. The other cannabinoids did not influence cholinergic transmission and only Δ⁹-THC demonstrated...
effects on airway hyperresponsiveness, anti-inflammatory activity and antitussive activity in the airways.
INTRODUCTION

The Cannabis sativa plant has been valued since ancient times for its medicinal and psychotropic properties. Despite its present classification as a controlled substance in the majority of the Western World, reports on the use of herbal preparations of the plant for recreational and self-medication purposes have implicated the effectiveness of extracts of the plant in the treatment of a variety of ailments, including asthma and cough (O’Shaughnessy, 1842; Iversen, 2000; van Hoozen & Cross, 1997). Botanical preparations of the cannabis plant and certain plant-derived cannabinoids have been evaluated for their bronchodilator (Grassin-Delyle et al., 2004; Vachon et al., 1973; Tashkin et al., 1974; 1975; 1977; Williams et al., 1976; Hartley et al., 1978), anti-inflammatory (Jan et al., 2003) and anti-tussive (Gordon et al., 1976) activity in subjects with asthma and in experimental animals. However, because of local irritation of the airways caused by constituents of the cannabis smoke, the poor bioavailability of the plant components following oral administration and the intoxication caused by Δ9-tetrahydrocannabinol (Δ9-THC), the major pharmacologically active component of cannabis, the medicinal use of cannabis for treatment of inflammatory and respiratory diseases has been hampered (Shapiro et al., 1977; Tashkin et al., 1977). In contrast to the extensive research that has been carried out on the respiratory consequences of cannabis smoking e.g. pulmonary carcinoma and inflammation (van Hoozen & Cross, 1997), very limited information is available with respect to the mechanism by which the pharmacologically active cannabinoids of the plant may influence the underlying phenotypic changes of the airways observed in patients with asthma.

Recent studies in airways have primarily focussed on the effects of the synthetic cannabinoid receptor agonist WIN 55212-2, and to a lesser extent on the endogenous cannabinoids, e.g.
anadamide (Calignano et al., 2000; Jan et al., 2003). For example, WIN 55212-2 has been demonstrated to suppress the bronchospasm in guinea-pigs *in vivo* evoked by capsaicin (Calignano et al., 2000), and *in vitro* this cannabinoid has been found to inhibit the cholinergic and non-adrenergic non-cholinergic constriction of the human, rat and guinea-pig airway smooth muscle evoked by electrical field stimulation (EFS) (Grassin-Delyle et al., 2014; Yoshihara et al., 2004; 2005a,b; Yousif & Oriowo, 1999), the airway hyperresponsiveness induced by nerve growth factor (de Vries et al., 2001) and extravasation of plasma proteins into the airways induced by ovalbumin in allergic guinea-pigs (Fukuda et al., 2003). Furthermore, WIN 55212-2 has also been shown to inhibit capsaicin-evoked sensory nerve depolarisation in the human and guinea-pig isolated vagus nerve (Patel et al., 2003) and suppress capsaicin- and citric acid- induced cough responses in guinea-pigs *in vivo* (Calignano et al., 2000; Morita and Kamei, 2003; Patel et al., 2003).

All these protective effects appear to involve a down-regulation of sensory nerve excitability through a putative action at either the CB₁ or CB₂ receptors, but not both, depending on the experimental model employed. Although these data are invaluable, our understanding of the pulmonary pharmacological effects of the large number of phytocannabinoids that are present in the cannabis plant is limited.

Therefore, we have sought to evaluate six of the most abundant cannabinoids in the cannabis plant i.e. Δ⁹-THC, cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), cannabidiolic acid (CBDA) and tetrahydrocannabivarin (THCV) for their ability to influence airway hyperresponsiveness, airways inflammation and cough in guinea-pigs exposed to the pro-inflammatory cytokine TNF-α or to the pro-tussive agent citric acid using methodology described elsewhere (Makwana et al., 2012a; Venkatasamy et al., 2010). To aid in the
interpretation of data obtained with the plant-derived cannabinoids, some experiments were also performed using the non-selective synthetic cannabinoid receptor agonist CP55940.

Preliminary accounts of some of these data have been communicated to the American Thoracic Society (Makwana et al., 2012b).
MATERIALS AND METHODS

Animals

All animal care and experiments complied with the requirements of the UK Animals (Scientific Procedures) Act 1986 and were approved by the King’s College London ethics committee. Male adult Dunkin-Hartley guinea-pigs (250 - 350 g) were used from stock originating at B&K Laboratories (Hull, UK). The animals were housed in rooms with a controlled temperature (22 ± 1°C), humidity (55 ± 10 %) and 12 h light-dark cycle. Food and water were available ad libitum.

Reagents

Citric acid monohydrate salt, Dulbecco’s Modified Eagle’s Medium, Ketamine, E.coli Lipopolysachharide, Methacholine, Penicillin, Sodium Pentobarbital, Streptomycin, Suxamethonium, Urethane and Xylazine were all obtained from Sigma-Aldrich (Dorset, UK). CP55940, AM251 and JTE907 were purchased from Tocris Biosciences (Bristol. UK). Recombinant human TNF-α was purchased from First Link (Birmingham, UK). Solutol HS-15, was a gift from BASF (Derby, UK). All other chemicals were purchased from Fisher Scientific (Loughborough, UK). The phytocannabinoids were supplied by GW Pharmaceuticals, (London, UK). All solutions were prepared fresh on the day of the experiment. Cannabinoids were dissolved in a solutol HS-15 as a 10% v/v solution of solutol in saline. In cough experiments, a 10 mg/ml solution of a cannabinoid in 10% v/v solutol could be prepared and nebulised without difficulty. Concentrations of solutol greater than 10% v/v were noticeably viscous and did not nebulise effectively.
Tracheal contraction experiments

For a detailed description of the methodology please refer to Makwana et al., (2012a). Briefly, guinea-pigs were humanely killed by cervical dislocation followed by exsanguination. The tracheae were rapidly removed and immersed into sterile Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4500 mg.L⁻¹ D-glucose, 110 mg.L⁻¹ sodium pyruvate, 584 mg.L⁻¹ L-glutamine supplemented with 100 U.mL⁻¹ penicillin and 100 μg.mL⁻¹ streptomycin.

The tracheae were subsequently divided into four segments and either used immediately after dissection (fresh) or placed individually in DMEM for culture. Under sterile tissue culture conditions, the tracheal rings were incubated at 37 °C in a humidified incubator circulated with 5% CO₂ in air for 4 days with either saline or TNF-α (100 ng.mL⁻¹) in 300 μL DMEM in 96 well plates. Segments were moved into a new well containing fresh media and TNF-α where appropriate every day.

Tracheal smooth muscle contractions were measured in 4 mL organ baths containing Krebs-Henseleit solution (composition in mM; NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, D-glucose 11.1, CaCl₂ 2.5) supplemented with the cyclooxygenase inhibitor indomethacin (10 μM). The Krebs-Henseleit solution was gassed with 95 % O₂ and 5 % CO₂ and maintained at 37 °C. using a previously described method (Makwana et al., 2012a).

Tissues were exposed to either a cannabinoid (1 μM) or its vehicle 30 min prior to eliciting contractions in response to EFS (1 - 30 Hz frequency, 5 sec trains every 2 min at, 0.5 ms pulse width and a voltage that was 10% greater than the voltage required to elicit maximal contractions) and then to cumulative additions of methacholine.
In some experiments, the saline- and TNF-α- incubated tracheal segments were subjected to
EFS over the entire duration of the experiment with trains of pulses of 5 Hz frequency for 5
sec every 100 sec, 0.5 ms pulse width and 110 % voltage. Only one concentration-response
curve was constructed for Δ⁹-THC or CP55940 (0.1 nM – 10 μM) per tissue because these
cannabinoids could not be washed from the tissue by renewing the bath with drug-free Krebs-
Henseleit solution. When competition studies were performed, AM251 (3 nM), a CB₁
receptor antagonist, or JTE907 (30 nM), a CB₂ receptor antagonist, was administered 30 min
prior to the addition of the cannabinoid receptor agonist. All experiments were performed in
parallel with relevant vehicle treated and time controls.

**Lung function experiments**
A detailed description of the methodology has been published previously (Makwana et al.,
2012a). Briefly, guinea-pigs were exposed to an aerosolised solution of either 10% v.v⁻¹
solutol or 10% v.v⁻¹ solutol containing 10mg.mL⁻¹ of Δ⁹-THC, CBD, THCV, CBC, CBDA or
CBG for 20 min using an ultrasonic nebuliser (DeVilbiss Ultra Neb 99, PA, USA). Thirty
minutes later, the animals were anaesthetised with an intramuscular injection of ketamine (40
mg.kg⁻¹) and xylazine (8 mg.kg⁻¹) to enable instillation of 50 μL saline or TNF-α (2 μg)
dissolved in 50 μL saline into the airways using a Hamilton syringe.. Six hours after the
instillation, the animals were injected with urethane (1.5 g.kg⁻¹ i.p.) to induce surgical
anaesthesia and instrumented as described previously (Makwana et al., 2012a) for measuring
the transpulmonary pressure and calculation of total airway resistance (R_L; cm.H₂O s.L⁻¹) as
an index of airways obstruction.
The jugular vein and carotid artery were cannulated for intravenous administration of drug and measurement of mean arterial blood pressure, respectively. Bilateral vagotomy was also performed on all animals to enable the vagi to be threaded through a pair of platinum electrodes for vagal electrical stimulation using a Grass S88 stimulator (Grass Instruments, MA, USA).

Following stable baseline recording of total lung resistance, heart rate and mean arterial blood pressure, the vagi were stimulated with 5 sec trains every minute at 1 - 30 Hz frequency, 0.5 ms pulse width and 40 V. This was followed by construction of a dose-response curve to intravenous methacholine in the vagotomised animals. Prior to terminating the experiment, the animal was euthanised with an overdose of sodium pentobarbital (65 mg.kg⁻¹ i.v.) and a lavage of the lungs performed using 5 mL of 0.9 % saline.

**Lipopolysaccharide (LPS) -induced inflammatory cell infiltration into the airways**

Guinea-pigs were exposed to an aerosolised solution of either saline, 10% v.v⁻¹ solutol or 10% v.v⁻¹ solutol containing either 10 mg mL⁻¹ or 50 mg mL⁻¹ of the phytocannabinoids for 20 min. Following a 30 min rest, the animals were exposed to an aerosolised solution of LPS (100 μg mL⁻¹) for 20 min. Four hours after the exposure to LPS, the animals were euthanised with an injection of sodium pentobarbital (1 g kg⁻¹ i.p.) followed by a BAL using 5 mL of 0.9 % saline.

**Inflammatory cell count in the lung lavage fluid**

A 100 μL sample of the lavage fluid from the above animals was added to an equal volume of 50 % v.v⁻¹ Turk’s stain solution for determining the concentration of cells.mL⁻¹ present in the lavage sample using a Neubauer haemocytometer. A differential cell count was performed
after using the REASTAIN Quick-Diff stains on cytospins of the lavage fluid to identify the
different types of cells present in the lavage fluid as macrophages, neutrophils and
eosinophils,

**Cough experiments**

A detailed description of the method for inducing and recording the cough response has been
described elsewhere (Venkatasamy et al., 2010). Briefly, guinea-pigs were placed
individually in Perspex cylindrical exposure chambers with an air flow of 1.5 l/min and
allowed to acclimatise for 10 min during which a baseline recording of the pressure and
auditory signals inside the chamber was performed using a differential pressure transducer
and a microphone, respectively, connected a personal computer through an EMKA IOX
(version 1.8) data acquisition system.

Guinea-pigs were exposed for 5 min to a mist of 0.3M citric acid generated by an ultrasonic
nebuliser (Ultra-Neb®99 Devilbiss, PA, USA) at a rate of 1ml/min. Coughs were recorded
throughout the 5 min exposure period and thereafter for a further 10 min, (a total 15 min
period). Animals were removed from the exposure chamber and allowed to recover before
being returned to the Biological Services Unit.

Four days after the citric acid exposure, guinea-pigs were randomised to receive either
aerosolised saline, 10% v/v solutol or a cannabinoid (10mg/ml in 10% v/v solutol) for 20
min. Twenty minutes later, the animals were placed in the cough recording chambers and a
baseline record of the pressure and auditory signals was obtained for 10 min followed by an
exposure for a second time to 0.3M citric acid for 5 min. The coughs were recorded
throughout the 5 min exposure period and a further 10 min. Animals were removed from the
exposure chamber and allowed to recover before being returned to the Biological Services Unit. This was repeated 4 days later but with animals crossed over to treatment.

**Data analysis**

Individual agonist concentration-response curves obtained from the organ bath studies in the absence and presence of competing ligands were fitted by non-linear regression to a four-parameter Hill equation described previously (Makwana et al., 2012a) using GraphPad PRISM 5.0 for Windows (GraphPad Software, CA., U.S.A). The concentration-response data were plotted as the mean ± s.e.m. In competition studies, shifts of an agonist concentration-response curve by the presence of a competing ligand e.g. antagonist were compared by a one-way ANOVA followed by a Dunnett’s *post hoc* test for multiple comparisons, or a Student’s unpaired *t*-test for comparisons of individual means as described previously (Makwana et al., 2012a). The concentration ratio for the rightward shift of the agonist concentration-response curve in the presence of a competing ligand was determined as the ratio of concentrations corresponding to the 50 % equieffective agonist response level of the curves. The antagonist potency (pA₂) was calculated from the Gaddum-Schild equation (Schild, 1949).

Data obtained from the lung function experiments were calculated in percentage terms as changes from the baseline value determined immediately prior to administration of an electrical stimulus or spasmogen and reported as the mean ± s.e.m. with n values representing the number of animals used per experiment. For cough experiments, repeated measures ANOVA (and means tests, two-tailed) were used to test for differences with respect to time, cross over design, repeated measures and use of the data from the initial screening procedure as a covariate. All other data were analysed using a Student’s unpaired *t*-test for comparisons.
of individual means. The probability $P < 0.05$ was taken to be statistically significant. Receptor nomenclature follows the guide to receptors and channels of the International Union of Basic and Clinical Pharmacology (NC-IUPHAR) and Alexander et al., (2011).
RESULTS

Tracheal contraction experiments

Contractions of freshly isolated tracheal rings in the absence of any drug in response to each frequency of EFS and concentration of methacholine were abolished by atropine (1 μM), but only the contractions in response to EFS were sensitive to tetrodotoxin (1 μM). Moreover, the contractions to both EFS and methacholine were refractory to hexamethonium (100 μM, Makwana et al., 2012a). Administration of ∆9-THC, CBD, THCV, CBC, CBDA, CBG, CP55940, AM251 or JTE907 (all at 10 μM) to the freshly isolated tracheal rings did not alter the resting basal tension over a 30 min duration period or contractions in response to EFS and methacholine.

Fully defined frequency- and concentration-response curves were obtained to EFS and methacholine, respectively, for all segments cultured for 4 days in the presence of saline and TNF-α (100 ng.mL−1, Figure 1). There was no significant difference in the maximal contraction or the sensitivity of the saline-incubated and freshly dissected tracheal rings to each frequency of EFS and methacholine (data not shown, see Makwana et al., 2012a).

However, treatment of the segments for 4 days with TNF-α (100 ng.mL−1) resulted in significantly larger contractions at each frequency of EFS compared to the contractions of tracheal segments incubated with saline (Figure 1a,b and 2). For example, stimulation at the highest frequency of EFS i.e. 30Hz resulted in significantly larger 130.9 ± 23.5 % (P< 0.05, n = 6) contractions of tracheal segments incubated with TNF-α than saline. In contrast, the contractions in response to methacholine were not different between the saline and TNF-α treatment (Figure 2b).
A 30 min incubation with $\Delta_9^9$-THC, CP55940 or $\Delta_9^9$-THC together with CBD abolished the EFS-evoked contractions augmented by the incubation with TNF-$\alpha$ without causing a change in the amplitude of contractions of saline-treated segments (Figure 2a). However, the contractions of the saline- or TNF-$\alpha$- incubated tracheal segments in response to methacholine were not altered by these cannabinoids (Figure 2b). Figures 1d and 2a show an experimental recording and the data showing the effect of $\Delta_9^9$-THC, respectively. At the highest frequency of EFS i.e. 30Hz, THCV caused a partial but significant $19.6 \pm 6.1 \% (P < 0.05$, paired $t$-test, $n = 6$) inhibition of the TNF-$\alpha$-augmented EFS-evoked contractions only. THCV did not alter the contractions in response to methacholine. The other phytocannabinoids or selective cannabinoid receptor antagonists were without effect on the contractions of saline- or TNF-$\alpha$- incubated tracheal segments in response to EFS or methacholine (Figure 2 and 3).

Stimulation of tracheal segments incubated for 4 days with saline or TNF-$\alpha$ with trains of EFS pulses of 5 Hz frequency for 5 sec every 100 sec resulted in uniformed sized contractions of $8.8 \pm 3.1$ and $18.6 \pm 5.4$ mN ($n = 6$ for each), respectively. Cumulative additions of $\Delta_9^9$-THC (Figure 4) or CP55940 had no significant effect on the contractions of the tracheal segments incubated with saline, but caused a concentration-related inhibition of the enhanced contractions of the TNF-$\alpha$-incubated segments to the amplitude of the contractions of the saline-treated segment. Figure 5 shows that the pEC$_{50}$ values for the inhibition of the TNF-$\alpha$ augmented contractions by $\Delta_9^9$-THC and CP55940 was $7.7 \pm 0.1$ and $8.3 \pm 0.1$ respectively ($n = 6$ for each). Additionally, both $\Delta_9^9$-THC and CP55940 caused an equivalent maximal inhibition of the TNF-$\alpha$ potentiated contractions of $86.9 \pm 2.7$ and $86.2 \pm$
2.1 % ($n = 6$ for each), respectively. A comparison of the pEC$_{50}$ values indicated that CP55940 was approximately 4 times more potent ($P < 0.05$, paired $t$-test) at inhibiting the contractions than $\Delta^9$-THC.

The presence of AM251 (3 nM) or JTE907 (30 nM) caused a significant ($P < 0.05$, ANOVA and Dunnett’s test) parallel rightward shift of the $\Delta^9$-THC and CP55940 concentration-response curves with no significant ($P < 0.05$, paired $t$-test) effect on the maximum effect induced by each agonist (Figure 5). The pA$_2$ value of AM251 for the antagonism of the $\Delta^9$-THC and CP55940 induced inhibition of the TNF-$\alpha$ potentiated contractions was $8.6 \pm 0.3$ and $8.5 \pm 0.4$ ($n = 6$ for each) respectively. These values were not significantly different against both agonists ($P < 0.05$, paired $t$-test). The pA$_2$ value of JTE907 for the antagonism of the $\Delta^9$-THC and CP55940 was $7.6 \pm 0.3$ and $7.8 \pm 0.4$ ($n = 6$ for each) respectively, and these values were not significantly different against both agonists ($P < 0.05$, paired $t$-test).

AM251 (3 nM) or JTE907 (30 nM) had no significant ($P > 0.05$, paired $t$-test) effect on the amplitude of the TNF-$\alpha$-potentiated contractions or contractions of saline-treated segments.

**Lung function experiments**

Intratracheal instillation of saline or TNF-$\alpha$ into guinea-pigs did not result in breathing difficulties or a permanent effect on the baseline total lung resistance to airflow. Additionally, there was no statistically significant difference in the resting mean arterial blood pressure and heart rate between the two treatment groups as observed previously (Makwana et al., 2012a).
EFS of the vagus nerves or intravenous administration of methacholine elicited a rapid and transient frequency- and dose-dependent bronchoconstriction in all animals, respectively. The bronchoconstriction to vagal EFS was significantly augmented in animals challenged with TNF-α compared with saline (Figure 6b). For instance, the bronchoconstriction in the TNF-α-treated animals at the highest frequency of EFS i.e. 30 Hz was 83.4 ± 11.5 % (P < 0.05, unpaired t-test, n = 6) greater than that of saline-challenged animals. Moreover, the frequency of vagal EFS eliciting an increase in total lung resistance by 200 % above baseline in the saline- and TNF-α-challenged animals was 15 ± 3 Hz and 9 ± 2 Hz, respectively (P < 0.05, unpaired t-test, n = 6, Figure 7a). In contrast to the stimulation with EFS, the bronchoconstriction in response to methacholine was not different between the vagotomised animals challenged with either saline or TNF-α (Figure 7b). The bronchoconstriction to vagal EFS and methacholine in both saline- and TNF-α- instilled animals was abolished by intravenous administration of atropine (10 μg kg⁻¹) as shown previously (Makwana et al., 2012a).

The bronchoconstrictor response of the Δ⁹-THC–exposed, saline-challenged animals in response to vagal EFS or intravenous methacholine was not different to that of solutol-exposed, saline-treated control animals (Figure 6a,c, 7b). However, the enhanced bronchoconstriction of animals exposed to TNF-α in response to vagal stimulation was abolished by Δ⁹-THC (Figure 6d, 7c). However, Δ⁹-THC did not alter the bronchoconstriction in response to methacholine in animals treated with TNF-α (Figure 8d). Table A shows that none of the phytocannabinoids including Δ⁹-THC or a combination of Δ⁹-THC and CBD significantly altered bronchoconstriction in response to methacholine in animals treated with TNF-α (Figure 7d, Table A, see Supplementary data ).
**Cell count in the lavage fluid 6h post intratracheal administration of TNF-α in vivo**

A total of $47.6 \pm 4.2 \times 10^4$ and $86.0 \pm 9.6 \times 10^4$ leukocytes ml$^{-1}$ ($P < 0.05, n = 6$) were present in the BAL fluid recovered from guinea-pigs instilled with saline or TNF-α, respectively (Figure 8). These data indicated that exposure to TNF-α resulted in an 84.3 % ($P < 0.05, n = 6$) increase in cell recruitment to the lungs. A differential cell count showed that the greater total number of cells in the in BAL fluid of animals exposed to TNF-α was attributed to an increase of $18.0 \pm 2.9 \times 10^4$ neutrophils ml$^{-1}$. Eosinophils were largely absent from the BAL fluid obtained from either saline- or TNF-α exposed animals. The exposure to Δ⁹-THC caused a significant 84 and 82 % ($P < 0.05$, unpaired t-test, $n = 6$) inhibition of the TNF-α induced migration of total leukocytes and neutrophils into the lungs respectively, without affecting ($P > 0.05$, unpaired t-test, $n = 6$) the numbers of these cells in animals exposed to saline. Exposure to aerosolized cannabinoid significantly reduced neutrophil recruitment to the airways (treatment x stimulus interaction $F[6,70] = 3.576, P = 0.004$). This was reflected by a significant suppression (mean difference vs solutol treatment, 95 % Confidence interval) of neutrophil migration (cells mL$^{-1}$) in Δ⁹-THC (17 (7.3-26.7) x $10^4$, $P < 0.05$), CBG (14 (4.3-23.7) x $10^4$, $P < 0.05$), CBC (12 (2.3-21.7) x $10^4$, $P = 0.05$) treated animals. In terms of percentage inhibition, this amounted to a 70 ± 8 %, 58 ± 10 % and 50 ± 9 % ($n = 6$ per group) reduction (Table 1) compared to mean neutrophil numbers in solutol treated animals. Whilst THCV and CBDA caused a decrease in cell number (37 + 12 %, 39 + 15 %, respectively) compared to solutol treated animals, this did not achieve statistical significance (9.2 (-0.5-18.8) x $10^4$, $P > 0.05$, 9.5 (-0.185 - 19.2) x $10^4$, $P > 0.05$). The combination of Δ⁹-THC with CBD did not result in an inhibitory effect different to that achieved with Δ⁹-THC alone.
Cell count in the lavage fluid 4h post aerosol administration of LPS in vivo

A total of $61.1 \pm 10.0 \times 10^4$ and $200.9 \pm 12.6 \times 10^4$ leukocytes mL$^{-1}$ ($P < 0.001, n = 14, 29$ respectively) were present in the BAL fluid recovered from guinea-pigs exposed to saline and LPS, respectively. A differential cell count showed that the increase in the total number of leucocytes was predominantly attributed to $153.3 \pm 10.9 \times 10^4$ neutrophils mL$^{-1}$ in the BAL fluid ($P < 0.001$) and represented $75 \pm 4\%$ of the total cell count in LPS-treated guinea-pigs (Table B, see Supplementary data). THCV (50 mg kg$^{-1}$) attenuated the recruitment of neutrophils to the lung with a mean difference of $68.4 \pm 23.9 \times 10^4$ leukocytes mL$^{-1}$ and amounting to a $42 \pm 12\%$ decrease in cell recruitment ($P = 0.035$). The remaining cannabinoids caused a 10-30\% reduction in neutrophil recruitment to the airways, but this did not achieve statistical significance (Table B, see Supplementary data).

Cough experiments

Exposure to aerosolised citric acid immediately provoked powerful coughs that were observed as sudden transient changes in the exposure chamber pressure (Figure A, see Supplemental data). An average of $27 \pm 2$ coughs ($n = 62$) were recorded during the 15 min recording period. We have previously shown that a reproducible number of cough responses are produced by three consecutive citric acid challenges spaced 4 days apart with no statistical difference between the time points (Venkatasamy et al., 2010).

Treatment with aerosolised saline or solutol did not result in a significant difference in the number of citric acid-induced coughs compared to those recorded 4 days earlier in the absence of these vehicles (Figure 9a, Table 2). Moreover, there was no statistical difference in the number of coughs in the sequence in which the treatments were given.
Cough in response to citric acid was measured in 8 animals randomised to receive either 10% solutol or Δ⁹-THC (10 mg/ml). Δ⁹-THC significantly inhibited the cough response compared to exposure to solutol in 7 out of 8 animals. A comparison of the mean number of coughs for the entire group of animals indicated that Δ⁹-THC caused a 68.7% decrease in the number of coughs compared to solutol (Figure 9b, Table 2). The mean difference (± SEM) between solutol and Δ⁹-THC-treated animals was 12.2 ± 3.8 coughs, (df = 9, P < 0.05), or mean (confidence interval) of 12 coughs (2.8 – 22). In animals exposed to Δ⁹-THC first, the antitussive effect did not influence the coughing following exposure to solutol 4 days later.

CBD significantly (P < 0.05) suppressed cough in only 3 out of 8 animals. Nevertheless, a t-test analysis on the total mean number of coughs of all the CBD versus solutol exposed animals indicated that CBD had no significant effect compared to solutol (Figure 9c, Table 2). For the 3 out 8 animals that responded to CBD, the mean difference (± SEM) between solutol and CBD treated animals was 26.0 ± 1.8 cough, (df = 4, P = 0.0182), or mean (confidence interval) of 24 coughs (19.4 – 32.5).

An exposure to equivalent concentrations of 10 mg/ml of both Δ⁹-THC and CBD produced a significant (P < 0.05) inhibitory effect on the cough response (Figure 9d, Table 2). Whilst the 46.7% inhibition did not appear significantly different in magnitude to that caused by Δ⁹-THC alone, further analysis of these data taking into account sequence, period and treatment effects, and using baseline screen as a covariate indicated a significant effect. The mean difference (± SEM) between solutol and the combined Δ⁹-THC and CBD treatment animals was 18.0 ± 3.6 cough, (df = 6, P = 0.0182), mean (confidence interval) of 18 coughs (9 – 27). Therefore, at present, it cannot be concluded that the combination treatment did not cause a superior effect to Δ⁹-THC alone as the effect of lower doses of these substances were not tested.
The citric acid-induced cough response was refractory to treatment with THCV, CBG, CBDA and CBC (Table 2).
DISCUSSION

A range of cannabinoids at concentrations which elicit a robust effect in most isolated tissue bioassays (Izzo et al., 2009; Pertwee et al., 2010; Russo, 2011) did not alter baseline tension of airway smooth muscle or contraction induced by EFS or methacholine. Hence, these cannabinoids lack bronchodilator activity suggesting that cognate receptors were either absent or poorly coupled to their effector pathways in airway smooth muscle. Similarly, CP55940 does not alter basal muscle tone, relax ACh pre-contracted trachea or modulate EFS-evoked contractions in this preparation consistent with a lack of [3H]-CP55940 binding sites (Spicuzza et al., 2000). However, these receptors might be localized to other cell types. For example, localisation of CB1 receptors has been observed on nerve fibres distributed among bronchial and bronchiolar smooth muscle cells where axons are packed together into glial capsules (Calignano et al., 2000). Recently, human bronchial tissue was found to express CB1 receptors that were functionally coupled to inhibition of EFS-evoked cholinergic contractions, but not basal tone (Grassin-Delyle et al., 2014).

TNF-α, potentiated contraction in response to EFS but not methacholine, as a result of an enhanced release of ACh from post-ganglionic nerves rather than an increased potency of ACh at the smooth muscle M3 muscarinic receptors (Makwana et al., 2012a). This potentiation of the EFS-evoked contraction was abolished by Δ9-THC, CP55940 and Δ9-THC combined with CBD, but only partially attenuated by THCV. The other cannabinoids were without effect. Our findings point to a pre-synaptic inhibition of ACh release from post-ganglionic nerve terminals and not antagonism of post-junctional muscarinic M3 receptors. Emerging evidence suggests that synergy exists between the activities of different cannabinoids, encompassing a potentiation or antagonism of the beneficial or adverse effects of one phytocannabinoid by another. For example, despite having negligible affinity, CBD
antagonises cannabinoid receptors to modulate $\Delta^9$-THC-associated adverse events such as anxiety, tachycardia, hunger and sedation in rats and humans (Nicholson et al., 2004; Murillo-Rodriguez et al., 2006; Russo and Guy, 2006). However, we did not find any evidence of such an interaction in the guinea-pig trachea.

The inhibitory effect of $\Delta^9$-THC and CP55940 on TNF-$\alpha$-potentiated EFS-evoked contractions was equal in magnitude, and their pEC$_{50}$ and rank order of potency consistent with those reported at guinea pig, human, mouse and rat CB$_1$ and CB$_2$ receptors in other bioassays (Pertwee and Fernando, 1996; Pertwee et al., 1996; Makwana et al., 2010a). That AM251 and JTE907 produced an agonist-independent, parallel and surmountable dextral shift of the $\Delta^9$-THC and CP55940 concentration-response curve with pA$_2$ values within the range of pK$_{B}/pK_i$ values of AM251 at the CB$_1$ receptor (8.12 to 8.57, Pertwee, 2005) and of JTE907 at the CB$_2$ receptor (7.21 to 7.44, Marini et al., 2013; Iwamura et al., 2001), revealed that both agonists were full agonists at these receptors and their interaction with the antagonists was simple competition.

The mechanism for the selective inhibition of the EFS-evoked contractions of TNF-$\alpha$-incubated trachea by $\Delta^9$-THC and CP55940 is not clear. Perhaps exposure to TNF-$\alpha$ induced or enhanced the expression and stimulus-response coupling capacity of cannabinoid receptors at post-ganglionic nerve terminals. By elevating receptor density to create a receptor reserve or enhancing intracellular signalling, the efficacy to an agonist would be increased. Induction of receptor expression, upregulation and functional effects of both cannabinoid receptor subtypes has been shown to occur in response to inflammatory insults in the intestinal tract (Izzo et al., 2000; Duncan et al., 2008).
In addition to their antagonist activity, AM251 and JTE907 can elicit effects that are invariably opposite in direction from those produced by agonists acting at their respective cannabinoid receptor (Marini et al., 2013; Iwamura et al., 2001). Such effects occur through the unmasking of constitutive receptor activity via inverse agonism, or the antagonism of tonically-released endocannabinoid agonists at the receptors. Neither antagonist potentiated the effect of TNF-α on EFS-evoked contractions suggesting the absence of a constitutive cannabinoid receptor activity or inadequate endocannabinoid agonist stimulation (Jia et al., 2002). The modest inhibition afforded by THCV was not examined further. Whether this occurred through partial agonism at both cannabinoid receptors is unknown given its protean activity i.e. the ability to concurrently display agonist and antagonist activity at the same receptors depending on the intracellular effector pathways or tissue used (Thomas et al., 2005; Pertwee et al., 2007, Pertwee, 2008).

Intratracheal instillation of TNF-α into guinea-pigs potentiated the bronchoconstriction in response to vagal stimulation but not methacholine (Makwana et al., 2012a). Only ∆⁹-THC and ∆⁹-THC combined with CBD attenuated this enhanced response to vagal stimulation whilst none of the cannabinoids altered methacholine-evoked bronchoconstriction in the vagotomised guinea-pigs. The absence of an interaction between ∆⁹-THC and CBD observed on the isolated trachea was confirmed in vivo. THCV also lacked inhibitory activity in vivo. Bradycardia and hypotension induced by vagal stimulation or methacholine was refractory to TNF-α or cannabinoid treatment implying a localised pulmonary site of action.

We have confirmed that TNF-α induced an airway neutrophilia that is known to be dependent on the release of chemokines like IL-8 and expression of adhesion molecules on pulmonary endothelium and leucocytes (Horgan et al., 1993; Kuo et al., 1997). Only ∆⁹-THC inhibited
this response, an effect suggestive of CB2 receptor action (Galiegue et al., 1995; Ihenetu et al., 2003). We also confirmed that LPS was able to induce a pulmonary neutrophilia, but in contrast, this was more refractory to the actions of cannabinoids as only THCV produced a significant inhibitory effect. The pulmonary inflammation induced by LPS involves both TNFα dependent and independent mechanisms (Smith et al., 1998; Schnyder-Candrian et al., 2005), and activation of multiple signalling pathways (Nick et al., 1999; De Stefano et al., 2013). Activation of different inflammatory pathways by TNF-α and LPS is a likely explanation for the differences we have observed in the anti-inflammatory actions of the phytocannabinoids in these experiments.

The effect of phytocannabinoids on various indices of the inflammatory response have been investigated. For example, paw oedema following exposure to LPS is suppressed by Δ9-THC (CB1 receptor dependent) and CBC (CB1 and CB2 receptor independent) (DeLong et al., 2010) and cell migration was attenuated in CB1 receptor knockout mice (Marquart et al., 2010). Δ9-THC and CBD appear to suppress myeloperoxidase activity in rat colon following an inflammatory stimulus (Jamottt et al., 2010; Schicho et al., 2012) and decrease neutrophil recruitment to the airways in acute lung injury (Ribeiro et al., 2012). THCV suppressed paw oedema due to carrageenan that was reversed by antagonism of CB2 receptors, although the effect of this cannabinoid on inflammatory cell recruitment was not investigated (Bolognini et al., 2010). The phytocannabinoids used in this study can also activate non CB1 and CB2-receptor dependent pathways including TRPV1 and TRPA1 (De Petrocellis et al., 2011). However, the genetic ablation of TRPV1 appears to either promote (Helyes et al., 2007; Wang et al., 2013) or suppress (Szitter et al., 2010) neutrophilic inflammation, whilst genetic ablation of TRPA1 was without effect (Bonet et al., 2013; Meseguer et al., 2014) suggesting a very complex interaction of mechanisms.
Citric acid evokes cough in guinea-pigs via stimulation of acid sensing ion channels and TRPV1 receptors on bronchopulmonary vagal afferent Aδ and C-fibres (Watanabe et al., 2005; Kollarik et al., 2007). Δ⁹-THC suppressed the tussive effect of citric acid consistent with an earlier study documenting inhibition of cough elicited by EFS of the laryngeal nerve and mechanical stimulation of the trachea (Gordon et al., 1976). The absence of [³H]-CP55940 binding in the rat brainstem (Herkenham et al., 1990; Malilleux & Vanderhaeghan, 1992), the localization of CB₁ receptors in the airways that have been implicated in the antitussive action of anandamide (Calignano et al., 2000), the lack of respiratory depression and low lethality of a cannabis overdose in man (Howlett et al., 2002; Iversen, 2001) may point to a peripheral locus of anti-tussive action of Δ⁹-THC. However, the recent demonstration of functional CB₁ receptors in the brainstem suggests that a centrally-mediated antitussive action of Δ⁹-THC is also possible (Fawley et al., 2014). Additionally, JWH133 inhibited depolarisation of the guinea-pig and human vagus nerve evoked by hypertonic saline, capsaicin and prostaglandin E₂ through CB₂ receptor activation and inhibited citric acid-induced cough in guinea-pigs following systemic administration (Patel et al., 2003). Therefore, a possible peripheral CB₂ receptor-mediated anti-tussive effect of Δ⁹-THC is plausible. The presence of CB₂ receptors in the rat brain stem and their functional control of emesis (van Sickle et al., 2005) suggests that brainstem CB₂ receptors may also modulate the cough reflex and could serve as a non-psychotropic cannabinoid target for treating cough. Thus, to date, the precise location of antitussive action of Δ⁹-THC remains to be established.
Other phytocannabinoids evaluated for anti-tussive activity were not effective at the dose studied. However, despite having negligible affinity for CB₁ and CB₂ receptors, these cannabinoids can exhibit pharmacological activity through the modulation of certain other receptor types implicated in cough e.g. TRPV₁, 5HT₃ (Izzo et al., 2009; Pertwee 2008). Furthermore, the phytocannabinoids CBC, CBD and CBDA can activate TRPV₁ (Izzo et al., 2009, Pertwee 2008). However, the lack of effect of these cannabinoids on the citric acid-induced cough could be attributed to their low affinity, potency or poor receptor-stimulus coupling capacity. The similar anti-tussive effect of Δ⁹-THC with that of Δ⁹-THC and CBD together was indicative of a lack of interaction between these cannabinoids on the cough reflex.

Taken together, only Δ⁹-THC attenuated the TNF-α-augmented neuronal cholinergic transmission via activation of CB₁ and CB₂ receptors and possessed modest inflammatory and anti-tussive activity suggestive of a broad range of beneficial pharmacological effects of this cannabinoid in the airways.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Makwana, Spina, Page.
Contributed new reagents or analytic tools: Page
Performed experimental work: Makwana
Performed data analysis: Makwana, Spina
Participated in cough and LPS experiments: Venkatasamy
Wrote or contributed to the writing of the manuscript: Makwana, Spina, Page
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potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *Br J Pharmacol.* 
150(5):613-23.


FIGURE LEGENDS

Figure 1: Isometric contractions of the guinea-pig isolated trachea in response to electrical field stimulation (EFS; 1-30 Hz) after incubation for 4 days with (A) saline and (B) TNFα (100 ng mL⁻¹) in the presence of vehicle (solutol; A and B, respectively) and Δ⁹-THC (10⁻⁶M, C and D, respectively). EFS parameters: 1 min trains every 2 min at 1 - 30 Hz frequency, 0.5 ms pulse width and a voltage 10 % greater than the voltage required to evoke maximal contractions.

Figure 2: Effect of cannabinoids CP 55940 (●), Δ⁹-THC (▲), CBD (♦), CBDA (▼), THCV (◇), CBG (●), CBC (×) and Δ⁹-THC with CBD (●) on contractions of the guinea-pig isolated trachea in response to (a) electrical field stimulation and (b) methacholine following 4 days incubation with TNF-α (☐, 100 ng.mL⁻¹). The cannabinoids were used at a concentration of 10⁻⁶ M. The contractions of the saline-incubated (■) tracheal segments are shown for reference. Each point represents the mean ± SEM (n = 6). *P < 0.05 indicates a significant difference between contractions of the TNF-α-incubated tissue in the absence (☐) and presence of CP 55940(●), Δ⁹-THC(#), Δ⁹-THC + CBD (π) and THCV ( φ).

Figure 3: Lack of effect of cannabinoids AM 251 (3x10⁻⁹M, ◊) and JTE 907 (3x10⁻⁸ M, ●) on contractions of the guinea-pig isolated trachea in response to (a) electrical field stimulation and (b) methacholine following 4 days incubation with TNF-α (☐, 100 ng.mL⁻¹) or saline (■). Each point represents the mean ± SEM (n = 6).
Figure 4: Representative traces of the electrical field stimulation (EFS)-evoked contractions of the guinea-pig isolated trachea in the absence and presence of Δ⁹-THC. The tracheal rings were previously incubated for 4 days with saline (top) or TNF-α (100 ng mL⁻¹, bottom). Note in the bottom trace that the contractions inhibited by Δ⁹-THC were not restored to the original height in the absence of Δ⁹-THC by a washout. Atropine (10⁻⁶ M) administered at the end of the experiment abolished the contractions of both types of tissues. EFS parameters: 5 sec trains every 100 sec at 5 Hz frequency, 0.5 ms pulse width and a voltage 10 % greater than the voltage required to evoke maximal contractions.

Figure 5: Concentration–response curves for the inhibition of the electrical field stimulation (EFS)-evoked contractions of the guinea-pig isolated trachea incubated for 4 days with TNF-α (100 ng mL⁻¹) by (a) Δ⁹-THC and (b) CP 55,940 in the presence of ethanol (vehicle), AM251 (3x10⁻⁹ M) or JTE 907 (3x10⁻⁸ M). EFS parameters: 5 sec trains every 100 sec at 5 Hz frequency, 0.5 ms pulse width and a voltage 10 % greater than the voltage required to evoke maximal contractions. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the contractions measured immediately before the addition of any drug to the organ bath. Vertical lines indicate SEM, n = 6 for each curve. AM251 or ethanol was added 20 min before the first addition of the agonist.

Figure 6: Increases in airway lung resistance, as an index of bronchoconstriction, in anaesthetised guinea-pigs in response to electrical field stimulation (EFS) of the vagus nerves 6 h after intratracheal instillation of (a) saline and (b) TNFα (2 μg) and a 20 min aerosol pre-exposure with 10% solutol (a and b) or Δ⁹-THC (10 mg mL⁻¹; c and d). EFS parameters: 5 s
trains every minute at 1–30 Hz frequency, 0.5 ms pulse width and a voltage 10% greater than
the voltage required to evoke maximal bronchoconstriction.

Figure 7: Increases in airway resistance of anaesthetised guinea-pigs to vagal electrical field
stimulation (EFS) and methacholine 6 h after intra-tracheal instillation of either saline (■) or
2 μg human TNF-α (□). Each guinea-pig was previously exposed to an aerosolised solution
of (a and b) solutol 10% or (c and d) Δ⁹-THC (10 mg.mL⁻¹) for 20 min. Each point represents
the mean ± SEM (n = 6). *P < 0.05 indicates a significant difference between the
bronchoconstriction of the saline- and TNF-α- treated animals to vagal EFS at 30 Hz.

Figure 8: (a) Total and (b) differential count of the leucocytes (cells x 10⁴ mL⁻¹) in the lumen
of the guinea-pig airways 6 hours after intra-tracheal instillation of 50 μl of either saline (□)
or 2 μg of human TNF-α (■) following 20 min aerosol of 10% solutol. In (b), m, n and e
represent monocytes, neutrophils and eosinophils, respectively whereas S and T represent
Saline and TNF-α treatment, respectively. * signifies a significant difference (P < 0.05)
relative to the corresponding saline-treated (□) animal bar. Each point represents the mean ±
S.E.M (n = 6).

Figure 9: Number of coughs in response to aerosolized citric acid over a 15 min duration
following an exposure to (a) solutol (10% in saline) or (b) Δ⁹-THC (10mg/ml) (c) CBD
(10mg/ml) and (d) Δ⁹-THC and CBD together (both at 10mg/ml) for 20 mins and a 30 min
rest period. Vertical lines indicate mean ± sem. n = 8. * represents a significant difference (P
< 0.05) compared to solutol treatment.
Table 1: Effect of aerosolised cannabinoids on the total number of inflammatory cells and neutrophils recovered from bronchoalveolar lavage fluid in guinea-pigs 6 h after an intra-tracheal administration of saline or + TNF-α.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>+ TNF-α</th>
<th>% increase</th>
<th>Saline</th>
<th>+ TNF-α</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solutol</td>
<td>47.7 ± 4.3</td>
<td>86.3 ± 3.9</td>
<td>80.9 ± 9.4</td>
<td>6.2 ± 1.6</td>
<td>24.2 ± 2.7</td>
<td>290.3 ± 24.2</td>
</tr>
<tr>
<td>△⁹-THC</td>
<td>39.7 ± 3.4</td>
<td>44.8 ± 2.4</td>
<td>12.8 ± 4.3</td>
<td>4.8 ± 1.2</td>
<td>7.2 ± 1.9</td>
<td>50.0 ± 5.6</td>
</tr>
<tr>
<td>CBD</td>
<td>39.2 ± 4.8</td>
<td>80.3 ± 5.8</td>
<td>104.8 ± 2.1</td>
<td>5.8 ± 1.4</td>
<td>20.5 ± 1.3</td>
<td>253.5 ± 21.3</td>
</tr>
<tr>
<td>△⁹-THC + CBD</td>
<td>40.3 ± 3.5</td>
<td>43.3 ± 3.5</td>
<td>7.4 ± 3.6</td>
<td>4.6 ± 2.4</td>
<td>8.6 ± 3.2</td>
<td>86.9 ± 11.4</td>
</tr>
<tr>
<td>CBG</td>
<td>39.5 ± 2.4</td>
<td>81.1 ± 4.3</td>
<td>105.3 ± 5.2</td>
<td>4.8 ± 1.1</td>
<td>11.1 ± 2.4</td>
<td>131.3 ± 32.2</td>
</tr>
<tr>
<td>CBDA</td>
<td>55.2 ± 3.7</td>
<td>88.1 ± 1.9</td>
<td>59.6 ± 5.2</td>
<td>9.1 ± 3.7</td>
<td>23.6 ± 3.5</td>
<td>159.3 ± 41.2</td>
</tr>
<tr>
<td>CBC</td>
<td>42.5 ± 4.4</td>
<td>81.6 ± 3.7</td>
<td>92.0 ± 3.5</td>
<td>6.0 ± 1.2</td>
<td>12.2 ± 2.2</td>
<td>103.3 ± 19.3</td>
</tr>
<tr>
<td>THCV</td>
<td>44.2 ± 2.4</td>
<td>87.2 ± 3.2</td>
<td>97.3 ± 6.3</td>
<td>6.5 ± 0.8</td>
<td>15.3 ± 0.8</td>
<td>135.4 ± 23.6</td>
</tr>
</tbody>
</table>

Comparison of the effect of cannabinoids on the increases in total count of leukocytes and neutrophils (cells x 10⁴ mL⁻¹) in the lavage fluid from guinea-pig lungs 6 h after intra-tracheal instillation of either 50µL of saline or 2 µg human TNF-α. Each animal was exposed to an aerosolised solution of either a cannabinoid (10 mg.mL⁻¹) or its vehicle, solutol, for 20 min 1 h before the intra-tracheal challenge with saline or TNF-α. Each value represents the mean ± SEM (n = 6).
Table 2: Effect of aerosolised cannabinoids on the total number of coughs of guinea-pigs in response to aerosolised citric acid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of coughs</th>
<th>Treatment</th>
<th>No. of coughs</th>
<th>Treatment</th>
<th>No. of coughs</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>Saline</td>
<td>27.7 ± 6.3</td>
<td>+ Saline</td>
<td>20.8 ± 5.0</td>
<td>+ Solutol</td>
</tr>
<tr>
<td>ii</td>
<td>Screen</td>
<td>24.8 ± 2.9</td>
<td>+ Δ⁹-THC</td>
<td>7.8 ± 4.6</td>
<td>* + Solutol</td>
</tr>
<tr>
<td>iii</td>
<td>Screen</td>
<td>16.6 ± 3.5</td>
<td>+ CBD</td>
<td>14.5 ± 3.2</td>
<td>+ Solutol</td>
</tr>
<tr>
<td>iv</td>
<td>Screen</td>
<td>26.8 ± 5.01</td>
<td>+Δ⁹-THC &amp; CBD</td>
<td>14.3 ± 3.6*</td>
<td>+ Solutol</td>
</tr>
<tr>
<td>v</td>
<td>Screen</td>
<td>29.3 ± 3.2</td>
<td>+ CBG</td>
<td>25.1 ± 2.6</td>
<td>+ Solutol</td>
</tr>
<tr>
<td>vi</td>
<td>Screen</td>
<td>32.6 ± 2.6</td>
<td>+ CBDA</td>
<td>29.0 ± 3.6</td>
<td>+ Solutol</td>
</tr>
<tr>
<td>vii</td>
<td>Screen</td>
<td>30.6 ± 6.1</td>
<td>+ CBC</td>
<td>24.9 ± 4.9</td>
<td>+ Solutol</td>
</tr>
<tr>
<td>viii</td>
<td>Screen</td>
<td>29.4 ± 3.7</td>
<td>+ THCV</td>
<td>27.3 ± 4.9</td>
<td>+ Solutol</td>
</tr>
</tbody>
</table>

Each animal was initially exposed to aerosolised citric acid (0.3M) for 15 min to determine the number of coughs elicited in the absence of test substances (Screen). After 4 days half of the animals in a group were either exposed to an aerosolised cannabinoid (10 mg.mL⁻¹) or its vehicle, solutol, for 20 min. 30 min later the animals challenged with citric acid (0.3M) and number of coughs counted. This was repeated 4 days later but with animals crossed over to treatment. Each value represents the mean ± SEM (n = 8 animals). * represents a significant difference P < 0.05
FIGURE 1

a) Saline / Vehicle

b) TNF-α / Vehicle

c) Saline / Δ9-THC

d) TNF-α / Δ9-THC

H: 1  5  10  15  20  25  30
FIGURE 2

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FIGURE 4

Saline incubated

$\Delta^9$-THC (M)  $10^{-10}$  $10^{-9}$  $10^{-8}$  $10^{-7}$  $10^{-6}$  $10^{-5}$  Wash  Atropine $10^{-6}$ M

TNF-α incubated

10mN  20 min
Log $\Delta^9$ -THC (M) vs. % inhibition of TNFα-augmented EFS-evoked contraction

Log [CP 55940] (M) vs. % inhibition of TNFα-augmented EFS-evoked contraction

+ Ethanol  + AM 251  + JTE 907
FIGURE 6

a) Saline

b) TNF-α

c) Saline / Δ⁹-THC

d) TNF-α / Δ⁹-THC

Hz: 1  5  10  15  20  25  30

1000 cm H₂O s.L

1 min
FIGURE 7

a) % Increase in baseline lung resistance vs. Frequency (Hz)

b) % Increase in baseline lung resistance vs. MCh (µg.kg⁻¹)

c) % Increase in baseline lung resistance vs. Frequency (Hz)

d) % Increase in baseline lung resistance vs. MCh (µg.kg⁻¹)

Saline + TNF-α
Total number of cells x 10^4 ml^-1

FIGURE 8

a) b)
THE EFFECT OF PHYTOCANNABINOIDS ON AIRWAY HYPERRESPONSIVENESS, AIRWAY INFLAMMATION AND COUGH

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Table A: Comparison of the effect of aerosolised cannabinoids or their vehicle (solutol) on the maximal increase in airway resistance of saline- and TNF-α- treated guinea-pigs in response to vagal nerve electrical stimulation or methacholine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vagal EFS-evoked increase in airway resistance above baseline ( cm.H₂O s.L⁻¹) at maximal frequency (30Hz)</th>
<th>MCh-evoked increase in airway resistance above baseline ( cm.H₂O s.L⁻¹) at maximal dose (32μg.kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% increase in vagal-evoked contractions by TNF-α</td>
<td>% increase in MCh-evoked contractions by TNF-α</td>
</tr>
<tr>
<td>Saline + TNF-α</td>
<td>365.7 ± 15.7</td>
<td>869.5 ± 93.2</td>
</tr>
<tr>
<td>Solutol</td>
<td>654.2 ± 16.7</td>
<td>871.6 ± 27.3</td>
</tr>
<tr>
<td>365.7 ± 15.7</td>
<td>78.8</td>
<td>0.2</td>
</tr>
<tr>
<td>+ TNF-α</td>
<td>443.9 ± 24.7</td>
<td>802.2 ± 16.9</td>
</tr>
<tr>
<td>22.2*</td>
<td>791.8 ± 19.1</td>
<td>1.3</td>
</tr>
<tr>
<td>CBD</td>
<td>401.7 ± 30.9</td>
<td>790.6 ± 23.8</td>
</tr>
<tr>
<td>597.5 ± 21.9</td>
<td>48.7</td>
<td>785.3 ± 19.7</td>
</tr>
<tr>
<td>455.2 ± 14.7</td>
<td>27.4</td>
<td>-0.6</td>
</tr>
<tr>
<td>357.1 ± 20.2</td>
<td>821.2 ± 16.4</td>
<td>797.3 ± 16.9</td>
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<tr>
<td>455.2 ± 14.7</td>
<td>27.4</td>
<td>-2.9</td>
</tr>
<tr>
<td>372.1 ± 26.0</td>
<td>805.4 ± 21.4</td>
<td>816.8 ± 23.3</td>
</tr>
<tr>
<td>660.4 ± 56.63</td>
<td>77.4</td>
<td>1.4</td>
</tr>
<tr>
<td>CBG</td>
<td>379.2 ± 19.3</td>
<td>729.6 ± 20.8</td>
</tr>
<tr>
<td>690.7 ± 30.7</td>
<td>82.1</td>
<td>753.0 ± 15.4</td>
</tr>
<tr>
<td>701.2 ± 34.2</td>
<td>68.9</td>
<td>3.2</td>
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<tr>
<td>CBDA</td>
<td>415.0 ± 25.5</td>
<td>795.1 ± 16.1</td>
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<td>748.4 ± 56.0</td>
<td>71.3</td>
<td>5.9</td>
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<tr>
<td>CBC</td>
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<td>787.3 ± 15.7</td>
</tr>
<tr>
<td>436.8 ± 24.5</td>
<td>78.8</td>
<td>0.1</td>
</tr>
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</table>

Comparison of the effect of cannabinoids on the maximal increases in guinea-pig airway resistance above baseline in response to vagal electrical field stimulation (EFS) and methacholine 6 h after intra-tracheal instillation of either saline or 2 μg human TNF-α. Each guinea-pig was exposed to an aerosolised solution of a cannabinoid (10 mg.mL⁻¹) for 20 min. Each point represents the mean ± SEM (n = 6). *P < 0.05 indicates a significant (P < 0.05)
inhibition of TNF-α-induced enhancement of the increases in airway resistance in response to vagal EFS at 30 Hz by a cannabinoid treatment.
Table B: Comparison of the total number of inflammatory cells and neutrophils recovered from BAL fluid in guinea-pigs 4 h following aerosol exposure to LPS after systemic administration of a cannabinoid.

<table>
<thead>
<tr>
<th></th>
<th>Total cells (10^4 \text{ mL}^{-1})</th>
<th>Neutrophils (10^4 \text{ mL}^{-1})</th>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Solutol</td>
</tr>
<tr>
<td>Saline</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>(10)</td>
<td>61.1 ± 10</td>
<td></td>
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<tr>
<td>LPS</td>
<td>(29)</td>
<td>200.9 ± 12.6</td>
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<td>(\Delta^9)-THC</td>
<td>(4, 4)</td>
<td>252.3 ± 94.7</td>
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<tr>
<td>CBD</td>
<td>(6, 6)</td>
<td>159.3 ± 22.0</td>
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<tr>
<td>CBG</td>
<td>(6, 6)</td>
<td>184.5 ± 22.3</td>
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<tr>
<td>CBDA</td>
<td>(5, 5)</td>
<td>165.2 ± 42.5</td>
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<td>CBC</td>
<td>(9, 8)</td>
<td>164.6 ± 14.6</td>
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<tr>
<td>THCV</td>
<td>(6, 6)</td>
<td>165.0 ± 26.0</td>
</tr>
</tbody>
</table>

Comparison of the effect of cannabinoids on the increases in total count of leukocytes and neutrophils (cells x 10^4 mL\(^{-1}\)) in the lavage fluid from guinea-pig lungs 4 h after an aerosol challenge with either LPS (100 \(\mu\)g mL\(^{-1}\)) or saline for 20 min. Each guinea-pig injected with a cannabinoid (10 or 50 mg kg\(^{-1}\)) or its vehicle, solutol 1h prior to the LPS exposure. Each
value represents the mean ± SEM and values in parenthesis indicate animal number per treatment group. *$P < 0.05$ cf LPS; †$P = 0.06$ cf LPS.

Figure A: Experimental recording of the changes in chamber pressure (upper panel) and sound (lower panel) during a cough (C) and sneeze (S) in response to aerosolised citric acid 0.3 M.