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Corresponding Author: Dr. Matteo Marti, PhD

Corresponding Author's Institution: University of Ferrara

First Author: Andrea Ossato

Order of Authors: Andrea Ossato; Isabella Canazza; Claudio Trapella; Fabrizio Vincenzi; Maria Antonietta De Luca; Claudia Rimondo; Katia Varani; Pier Andrea Borea; Giovanni Serpelloni; Matteo Marti, PhD

Abstract: JWH-250 and JWH-073 are two synthetic cannabinoid agonists with nanomolar affinity at CB1 and CB2 receptors. They are illegally marketed within "herbal blend" for theirs psychoactive effects greater than those produced by Cannabis. Recently, we analyzed an "herbal" preparation containing a mixture of both JWH-250 and JWH-073. The present study was aimed at investigating the in vitro and in vivo pharmacological activity of JWH-250 and JWH-073 in male CD-1 mice. In vitro competition binding experiments performed on mouse and human CB1 and CB2 receptors revealed a nanomolar affinity and potency of the JWH-250 and JWH-073. In vivo studies showed that JWH-250 and JWH-073, administered separately, induced a marked hypothermia, increased pain threshold to both noxious mechanical and thermal stimuli, caused catalepsy, reduced motor activity, impaired sensorimotor responses (visual, acoustic and tactile), caused seizures, myoclonia, hyperreflexia and promote aggressiveness in mice. Moreover, microdialysis study in freely moving mice showed that systemic administration of JWH-250 and JWH-073 stimulated dopamine release in the nucleus accumbens in a dose-dependent manner. Behavioral, neurological and neurochemical effects were fully prevented by the selective CB1 receptor antagonist/inverse agonist AM 251. Co-administration of ineffective doses of JWH-250 and JWH-073 synergistically impaired visual sensorimotor responses, improved mechanical pain threshold and stimulated mesolimbic DA transmission in mice, living unchanged all others behavioral and physiological parameters. For the first time the present study demonstrates the overall pharmacological effects induced by the administration of JWH-250 and JWH-073 in mice and it reveals their synergistic action suggesting that co-administration of different synthetic cannabinoids may potentiate the detrimental effects of individual compounds increasing their dangerousness and abuse potential.

Suggested Reviewers: Jenny L Wiley
Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, USA
jwiley@rti.org

She is an expert in the behavioral pharmacology of synthetic cannabinoids (in particular of the JWH-Like compounds)

Marilyn Ann Huestis
Chemistry and Drug Metabolism, National Institute on Drug Abuse, Intramural Research Program, Baltimore, MD 21224, USA
mhuestis@intra.nida.nih.gov
Her current research efforts are focused on toxicology and mechanisms of action of cannabinoid agonists and antagonists

Daniela Parolaro
DiSTA, Biomedical Research Division, Neuroscience Center, University of Insubria, Italy
daniela.parolaro@uninsubria.it

Opposed Reviewers:

ETHICAL STATEMENT:

Authors declare that all animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC.

Moreover, Authors also certify that experimental protocols were approved by Italian Ministry of Health and by the Ethical Committee of the University of Ferrara. Adequate measures were taken to minimize the number of animals used, their pain and discomfort.

Effect of JWH-250, JWH-073 and their interaction on "tetrad", sensorimotor, neurological and neurochemical responses in mice.

Andrea Ossato[a], Isabella Canazza[a], Claudio Trapella[c], Fabrizio Vincenzi [g], Maria Antonietta De Luca[f], Claudia Rimondo[e], Katia Varani [g], Pier Andrea Borea[g], Giovanni Serpelloni[d] and Matteo Marti[a,b*]

[a]*Department of Life Sciences and Biotechnology (SVeB), University of Ferrara, Italy*
[b]*Center for Neuroscience and Istituto Nazionale di Neuroscienze, Italy*
[c]*Department of Chemistry and Pharmaceutical Sciences, University of Ferrara, Italy*
[d]*Department of Neuroscience, Psychology, Medicine and Child Health (NEUROFARBA), University of Florence, Italy*
[e]*Department of Public Health and Community Medicine, University of Verona, Italy*
[f]*Department of Biomedical Sciences, University of Cagliari, Italy*
[g]*Department of Medical Sciences, University of Ferrara, Italy*

[*]Corresponding Author:
Department of Life Sciences and Biotechnology (SVeB), University of Ferrara
via Fossato di Mortara 17-19, 44121 Ferrara Italy
phone +39 0532 455208, fax +39 0532 455205
email: m.marti@unife.it

Abbreviations

AM 251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide
DA	dopamine
NAc shell	Nucleus Accumbens shell
JWH-250	1-pentyl-3-(2-methoxyphenylacetyl)-indole
JWH-073	1-butyl-3-(1-naphthoyl)indole
JWH-018	1-pentyl-3-(1-naphthoyl)indole
JWH-018-R	JWH-018, JWH-018Cl and JWH-018Br
Δ^9-THC	(-)-Δ^9-THC or Dronabinol®

Abstract

JWH-250 and JWH-073 are two synthetic cannabinoid agonists with nanomolar affinity at CB_1 and CB_2 receptors. They are illegally marketed within ”herbal blend” for theirs psychoactive effects greater than those produced by Cannabis. Recently, we analyzed an “herbal” preparation containing a mixture of both JWH-250 and JWH-073. The present study was aimed at investigating the in vitro and in vivo pharmacological activity of JWH-250 and JWH-073 in male CD-1 mice. In vitro competition binding experiments performed on mouse and human CB_1 and CB_2 receptors revealed a nanomolar affinity and potency of the JWH-250 and JWH-073. In vivo studies showed that JWH-250 and JWH-073, administered separately, induced a marked hypothermia, increased pain threshold to both noxious mechanical and thermal stimuli, caused catalepsy, reduced motor activity, impaired sensorimotor responses (visual, acoustic and tactile), caused seizures, myoclonia, hyperreflexia and promote aggressiveness in mice. Moreover, microdialysis study in freely moving mice showed that systemic administration of JWH-250 and JWH-073 stimulated dopamine release in the nucleus accumbens in a dose-dependent manner. Behavioral, neurological and neurochemical effects were fully prevented by the selective CB_1 receptor antagonist/inverse agonist AM 251. Co-administration of ineffective doses of JWH-250 and JWH-073 synergistically impaired visual sensorimotor responses, improved mechanical pain threshold and stimulated mesolimbic DA transmission in mice, living unchanged all others behavioral and physiological parameters. For the first time the present study demonstrates the overall pharmacological effects induced by the administration of JWH-250 and JWH-073 in mice and it reveals their synergistic action suggesting that co-administration of different synthetic cannabinoids may potentiate the detrimental effects of individual compounds increasing their dangerousness and abuse potential.

Keywords: Δ^9-THC; JWH-073; JWH-250; JWH-018; microdialysis

1.Introduction

Synthetic cannabinoids (SCBs) are a large family of chemically distinct compounds functionally similar to delta-9-tetrahydrocannabinol (Δ^9-THC) which bind with high affinity at central and peripheral CB_1 and CB_2 cannabinoid receptors (Showalter et al., 1996; Grotenhermen, 2003; Wintermeyer et al., 2010). In the course of the past few years several types of SCBs have appeared on the drug marked worldwide. These compounds are mixed in "herbal incense" preparations and are sold with an attractive packaging under exotic brand names such as "Spice", "Amazonas", "Forest green", "Jamaican spirits", "K2" and others (Schifano et al., 2009; Uchiyama et al., 2011). SCBs became popular for their powerful psychoactive and euphoric cannabis-like effects and also for their ability to escape detection by standard cannabinoid screening tests (Fattore and Fratta, 2011; Fantegrossi et al., 2014). Among others, the synthetic cannabinoids JWH-250 [(1-pentyl-3-2-methoxyphenylacetyl-indole)] and JWH-073 [1-butyl-3-(1-naphthoyl)indole], either alone or mixed with others SCBs, were frequently detected in Spice (Dresen et al., 2010; Uchiyama et al., 2011; Penn et al., 2011; Gottardo et al., 2012). JWH-250 is a member of the JWH phenylacetylindole family that was synthesized in the 2005 (Huffman et al. , 2005) and first identified in May 2009 by the German Federal Criminal Police (EMCDDA, 2009) as ingredients of herbal smoking mixtures (Uchiyama et al., 2011). JWH-250 possesses a high binding affinity towards the central CB_1 (11±2 nM) and the peripheral CB_2 receptor (33±3 nM; (Huffman et al., 2005) and it is rapidly biotransformed in nineteen metabolites in human and eleven metabolites in rats (Grigoryev et al., 2011). Whereas JWH-073 is a member of the JWH naphthoylindoles family structurally similar to JWH-018 except for a butylic lateral chain on the nitrogen of the indole ring (Huffman et al., 1994). JWH-073 has a high binding affinity towards central CB_1 (Ki that ranging from 8.9±1.8 and 12.9±3.4 nM) and peripheral CB_2 receptor (38±24 nM) (Wiley et al., 1998; Brents et al., 2012; Aung et al., 2000) and it is biotransformed in vivo into monohydroxylated metabolites that retain significant affinity and activity at CB_1 receptors (Brents et al., 2012).

The consumption of herbal blend that contain JWH-250 and/or JWH-073 (Uchiyama et al., 2011) in addition to the "desired" psychoactive effects induces significant psychiatric and physical adverse effects in consumers. The most common psychiatric effects reported were agitation/anxiety, restlessness, acute psychosis, hallucinations, hypersensitivity to light and external stimuli, unconsciousness, panic, confusion, drowsiness and alterations in cognitive abilities (Papanti et al., 2013; Auwarter et al., 2009; Hermanns-Clausen et al., 2013; Zawilska and Wojcieszak, 2014). In particular, an overview of the literature focusing on the psychopathological issues associated with JWH-250 and JWH-073 intake showing that their misuse could be considered as a relevant factor in precipitating and/or perpetuating psychosis in vulnerable individuals (Papanti et al., 2013). While

physical effects ranging in severity from blurred vision, unsteady gait, loss of balance, light headedness, nausea, sedation to more serious sympathomimetic-like symptoms such as psychomotor agitation, diaphoresis, palpitations, tachycardia, tachyarrhythmia, hyperreflexia, and generalized convulsions (Papanti et al., 2013; Auwarter et al., 2009; Hermanns-Clausen et al., 2013; Gurney et al., 2014).

In vivo animal studies report that JWH-073 reproduces the typical "tetrad" effects of Δ^9-THC such as hypothermia, analgesia, hypolocomotion, akinesia (Wiley et al., 1998; Brents et al., 2012; Marshell et al., 2014), it affectes drug discrimination paradigm, place preference in rodents (Wiley et al., 1998); (Marshell et al., 2014; Cha et al., 2014) and in monkeys (Ginsburg et al., 2012). Otherwise its effects on sensorimotor functions and on mesoaccumbal dopaminergic transmission are still unknowns. Conversely, no preclinical investigations were reported for JWH-250.

Recently we have obtained from a seizure an herbal preparation that was used by a group of teens in the context of "magical-spiritual" meetings in a wood to get a psychoactive/hallucinatory effect while performing the ritual. The analysis of the herbaceous material (by HR-LC-MS analysis) revealed the presence of both synthetic cannabinoids JWH-250 and JWH-073. This aspect is of considerable importance since it is known that the presence of two or more SCBs in the same package of "spice" may determine the possible potentiation of effects induced by the individual substances (Brents et al., 2013).

Therefore, the present study was aimed at investigating firstly the acute effect of JWH-250 and JWH-073 on body temperature, acute mechanical and thermal analgesia, catalepsy, motor activity, sensorimotor responses (to visual, acoustic and tactile stimulation), neurological changes (convulsion, hyperreflexia, and myoclonia), aggressive response and modulation of dopaminergic release in mesoaccumbal pathway in mice. Secondly, since the two cannabinoids have been found in the same "herbal preparation", we studied the effect of co-administration of ineffective doses of JWH-250 and JWH-073 to highlight the presence of a synergistic or additive effect (Brents et al., 2013). Moreover, to better understand the behavioral effects of the JWH-250 and JWH-073, their actions were compared with those of JWH-018 and Δ^9-THC and effects were monitored for over 5 hours. It were also undertaken in vitro binding studies on CD-1 murine and human CB_1/CB_2 receptors.

2. Material and Methods.

2.1. Animals

Male ICR (CD-1®) mice, 25-30 g (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8 to 10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm) on a 12:12-h light-dark cycle (light period from 6:30 AM to 6:30 PM), temperature of 20-22 °C, humidity of 45-55% and were provided with ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC. Moreover experimental protocols were approved by Italian Ministry of Health and by the Ethical Committee of the University of Ferrara. Adequate measures were taken to minimize the number of animals used, their pain and discomfort.

2.2. Identification of JWH-250 and JWH-073 in the herbal extract by HR-LC-MS analysis

JWH-250 and JWH-073 were isolated and purified by chromatography with a medium pressure system ISOLERA ONE (Biotage Sweden) and subsequently characterized by Agilent 6520 nano HPLC ESI-Q-TOF (Agilent Technologies) and Varian 400MHz NMR. Briefly, 200 mg of herbal sample were stirred at room temperature with 50 mL of dichloromethane (DCM) for 1 hour, the solid residue was filtered with a Gooch funnel and the organic portion was evaporate to dryness. The residual green oil obtained from the dichloromethane extract was dissolved in a solution of acetonitrile/water/trifluoroactic acid (60%/40%/0.1%), filtered over a regenerate cellulose 0.22 micron filter and injected directly to a ESI-Q-TOF-HPLC-MS instrument (Agilent 6520 equipped with a nano-HPLC-Chip Cube) using a Zorbax 300SB C18 5 micron column (separation column 43 mm x 75 micron, enrichment column 4 mm x 40 nL).

The HPLC-MS analysis was carried out by a linear gradient of solvent A (water 97%, CH_3CN 3%, formic acid 0.1%) and solvent B (CH_3CN 97%, water 3%, formic acid 0.1%); the optimal gradient for the separation was a linear gradient from 0% solvent B to 90% solvent B in 10 minutes, from 10 to 15 minutes the column was equilibrated to the starting conditions (0% solvent B).

Among different unknown peaks we clearly identify two different SCBs with 9.52 min retention time, that in these conditions the two different structures were inseparable (Fig 1S in Supplementary Materials). However, the MS analysis showed two $[M+H]^+$ ions at 328.17056u and 336.19654u that could correspond at the JWH-073 and JWH-250 chemicals structures with less than 3 ppm errors (Fig 2S in Supplementary Materials).

To confirm the chemical structures a LC-MS/MS analysis was performed; the MS/MS pattern for 328.17101u is in line with the fragmentation peak of (1-butyl-1*H*-indol-3-yl)(naphthalen-1-yl)-methanone (JWH-073). The same result was obtained from a MS/MS pattern of the 336.19561u, confirming the chemical structure of JWH-250 (2-(2-methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl)-ethanone) (Figure 3S in Supplementary Materials).

In conclusion, from the sample of herbaceous extract we were able to confirm the presence of the two different SCBs, JWH-073 and JWH-250. To perform in vivo studies the mixture of SCBs was separated by RP-HPLC using a Waters Delta Prep instruments (Waters, USA) equipped with a Phenomenex AXIA C_{18} column (10 μm x 300 Å, 100 x 30 mm) in a linear gradient from 10 % to 100 % of solvent B in 30 minutes.

2.3. Drug preparation and dose selection

JWH-250 and JWH-073 were obtained from a seizure of "herbal" material (as previously described). JWH-250 was also purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy) while AM 251 was purchased from Tocris (Tocris, Bristol, United Kingdom). Drugs were initially dissolved in absolute ethanol (final concentration was 2%) and Tween 80 (2%) and brought to the final volume with saline (0.9% NaCl). The solution made with ethanol, Tween 80 and saline was also used as the vehicle. The CB_1 receptor-preferring antagonist/inverse agonist AM 251 (6 mg/kg) was administered 20 minutes before JWH-250 and JWH-073 injections. Sodium Penthobarbital was obtained from Sigma-Aldrich, Italy. Drugs were administered by intraperitoneal injection at a volume of 4ul/g. The wide range of doses of JWH-250 and JWH-073 tested (0.01-15 mg/kg i.p.) was chosen basing on previous study (Vigolo et al., 2015, Ossato et al., 2015).

2.4. In vitro assays

2.4.1. *Mouse brain and spleen membrane preparation*

After mice were sacrificed by cervical dislocation, brain and spleen were removed and suspended in 50 mM Tris HCl buffer, pH 7.4 at 4°C. The mouse brain suspension was homogenized with a Polytron and centrifuged for 20 min at 40,000 x g. The mouse spleen was homogenized with a Polytron and centrifuged for 10 min at 2,000 x g. The supernatant was filtered and centrifuged for 20 min at 40,000 x g. The resulting pellets were used for competition binding experiments (Vincenzi et al., 2013).

2.4.2. *Cell culture and membrane preparation*

CHO cells transfected with human CB_1 or CB_2 receptors (Perkin Elmer Life and Analytical Sciences, USA) were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and Geneticin (G418, 0.4 mg/ml) at 37°C in 5% CO_2/95% air.

For membrane preparation the culture medium was removed and the cells were washed with PBS and scraped off plates in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and then centrifuged for 30 min at 40,000 x g. The membrane pellet was suspended in 50 mM Tris HCl buffer (pH 7.4) containing 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/ml BSA for CB_1 receptors or in 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 0.5% BSA for CB_2 adenosine receptors (Vincenzi et al., 2013).

2.4.3. *[^{3}H] CP-55,940 competition binding assays*

Competition binding experiments were performed using 0.5 nM [^{3}H]-CP-55,940 (Perkin Elmer Life and Analytical Sciences, USA) and different concentrations of the tested compounds with membranes obtained from CHO cells transfected with human CB_1 or CB_2 receptors (2 µg protein/100 µl). Competition binding experiments were also performed in mouse brain membranes (40 µg protein/100 µl) for CB_1 receptors and in mouse spleen membranes (80 µg protein/100 µl) for CB_2 receptors. The incubation time was 90 or 60 min at 30°C for CB_1 or CB_2 receptors, respectively. Non-specific binding was determined in the presence of 1 µM WIN 55,212-2 (Vincenzi et al., 2013). Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/C glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer Life and Analytical Sciences, USA).

2.4.4. *Cyclic AMP assays*

CHO cells transfected with human CB_1 or CB_2 receptors were washed with PBS, detached with trypsin and centrifuged for 10 min at 200 x g. The pellet containing $1x10^6$ cells/assay was suspended in 0.5 ml of incubation mixture: 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM Hepes, 10 mM $MgCl_2$, 5 mM glucose, pH 7.4 at 37°C. Then 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as a phosphodiesterase inhibitor was added and pre-incubated for 10 min in a shaking bath at 37°C. The potency of the examined compounds was studied in the presence of forskolin 1 µM. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA) and the final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay (Vincenzi et al., 2013).

2.5. Behavioural studies.

The effect of JWH-250, JWH-073 and their interactions was investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of new molecules in rodents (Irwin, 1968; Mattsson et al., 1996; Porsolt et al., 2002; Redfern et al., 2005; Hamdam et al., 2013; ICH S7A, 2001). These tests have been also validated to describe effects of cannabinoids on the “tetrad”, sensorimotor and neurological changes in mice (Compton et al., 1992; Marti et al., 2013; Vigolo et al., 2015; Ossato et al., 2015).

Behavioural tests were conducted into a thermostated (temperature 20-22 °C, humidity about 45-55 %) and light controlled (about 150 lux) room in which there is a background noise of about 40 ± 4 dB.

To reduce the number of animals used, mice were evaluated in functional observational behavioral tests carried out in a consecutive manner according to the following time scheme: observation of main neurological changes and aggressive responses, measures of visual object responses (frontal and lateral view), acoustic response, tactile response (pinna, vibrissae and corneal reflexes) and visual placing response, evaluation of catalepsy, measures of core (rectal measurement), body temperature, determination of the mechanical (tail pinch) and thermal (tail withdrawal) acute pain and stimulated motor activity (drag and accelerod test). All experiments were performed between 8:30 AM to 2:00 PM. Experiments were conducted in blind by trained observers working together in pairs (Redfern et al., 2005). The behavior of mice (neurologic and sensorimotor responses) was videotaped and analyzed off-line by a different trained operator that gives test scores.

2.5.1. Major neurological changes and aggressive response

A functional observational behaviour test (modified from Irwin, 1968) Vigolo et al., 2015; Ossato et al., 2015) was done immediately after synthetic cannabinoid administration to detect convulsions, hyperreflexia, myoclonus, and aggressive responses in mice. Neurological changes are expressed as frequency (percent of animals that develop symptoms), duration (total time in sec) and latency (time in sec of symptom onset). Aggressive response in mice is measured based on the number of bites that the mouse confers to an object of gray cloth that approaches the front of the snout of the animal. The object is placed in front of the nose of the mouse for 10 consecutive times (score 0/10 not aggressive, score 10/10 very aggressive). During the test, the mouse is free to move in its cage.

2.5.2. Sensorimotor studies

We studied the voluntary and involuntary animal sensorimotor responses resulting from different mouse reaction to visual, acoustic and tactile stimuli (Koch, 1999; Marti et al., 2013; Ossato et al., 2015). In particular, involuntary startle response in rodents consists of automatic eye-lid-closure with a fast twitch of facial and body muscles evoked by a sudden and intense visual, acoustic or tactile stimulus. Alternatively, the mouse can also react to external stimuli through a voluntary motor response by changing the ongoing behaviour. This voluntary response occurs when stimuli attract the attention of the mouse (i.e. visual placing response or mild acoustic stimulation) without inducing an automatic reflex of escape (i.e. sudden and intense acoustic or tactile stimuli). All of these responses are suggestive of a protective function of startle against injury from a predator or from a blow and are carried out for the preparation of a flight response (Koch, 1999).

In the visual object, acoustic and tactile sensorimotor tests, each mouse is housed in an experimental chamber (350 x 350 x 350 (h) mm) which is made with black methacrylate walls and a transparent front door. At the top and/or side of the box is placed a camera (B/W USB Camera day & night with varifocal lens; Ugo Basile, Italy). Before the experimental sessions each mouse is placed in the box and it is handled and trained in every other day (once a day) for a week (three days of training in total) in order to get used to the environment and to the experimenter. To avoid mice olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water.

2.5.2.1. Evaluation of the visual response

Mouse Visual response was verified by two behavioural tests, which evaluated the ability of the animal to capture visual information even when the animal is moving (the visual placing response) or when it is stationary (the visual object response).

Visual Placing response test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Ossato et al., 2015). Briefly, CD-1 mice were suspended 20 cm above the floor by an adhesive tape that it was placed approximately 1 cm from the tip of the tail (Steru et al., 1985). The downward movement of the mouse is videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) placed at the base of the tail suspension apparatus. Movies are analyzed off-line by a trained operator who does not know the drug treatments performed. The analysis frame by frame allows to evaluate the beginning of the reaction of the mouse while it is close to the floor. The first movement of the mouse when it perceives the floor is the extension of the front legs. When the mouse starts the reaction an electronic ruler evaluates the perpendicular distance in millimeters between the eyes of the mice to the floor. The mice untreated control perceives the floor and it prepares to contact at a

distance of about 27 ± 4.5 mm. Evaluation of the visual placing response was measured at 0, 15, 35, 70, 125, 185, 245 and 305 min post injection.

Visual object response test was used to evaluate the ability of the mouse to see an object approaching from the front (frontal view) or the side (lateral view), than inducing the animal to shift or turn the head, bring the forelimbs in the position of "defence" or retreat it. For the frontal visual response, a white horizontal bar was moved frontally to the mouse head and the manoeuvre was repeated 3 times. For the lateral visual response, a small dentist's mirror was moved into the mouse's field of view in an horizontal arc, until the stimulus was between the mouse's eyes. The procedure was conducted bilaterally (modified from Sooksawate et al., 2013) Ossato et al., 2015) and was repeated 3 times. The score assigned was a value of 1 if there was a reflection in the mouse movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score 9). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.5.2.2. Evaluation of acoustic response

Acoustic response measures the reflex of the mouse in replay to an acoustic stimulus produced behind the animal (Koch, 1999; Ossato et al., 2015). In particular, four acoustic stimuli of different intensity and frequency were tested. A snap of the fingers (four snaps repeated in 1.5 sec), a sharp click (produced by a metal instrument; four clicks repeated in 1.5 sec), an acute (produced by an audiometer that reproduces a high-pitched sound at a frequency of around 5.0-5.1 kHz) and a severe (produced by an audiometer that reproduces a sound at a frequency of around 125-150 Hz) sound. Each test was repeated 3 times, giving a value of 1 if there was a response, 0 if not present, for a total score of 3 for each sounds. The acoustic total score was calculated by adding scores obtained in the four tests (overall score 12). The background noise (about 40±4 dB) and the sound from the instruments are measured with a digital sound level meter. Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

The tactile response in the mouse was verified through vibrissae, pinna and corneal reflexes (Irwin, 1968; Ossato et al., 2015).

2.5.2.3. Evaluation of vibrissae reflex

Vibrissae reflex was evaluated by touching vibrissae (right and left) with a thin hypodermic needle once for side giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score 2). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.5.2.4. Evaluation of pinna reflex

Pinna reflex was assessed by touching pavilions (left and right) with a thin hypodermic needle. First the interior pavilions and then the external. This test was repeated twice for side giving a value of 1 if there was a reflex and 0 if not present (overall score 4). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.5.2.5. Evaluation of corneal reflex

Corneal reflex was assessed gently touching the cornea of the mouse with a thin hypodermic needle and evaluating the response, assigning a value of 1 if the mouse moved only the head, 2 if it only closed the eyelid, 3 if it closed the lid and moved the head. The procedure was conducted bilaterally (overall score 6). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 min post injection.

2.5.3. "Tetrad" paradigm for screening cannabinoid-like effect

2.5.3.1. Evaluation of core and surface body temperature

To better assess the effects of the ligands on thermoregulation, we measured both changes in the core (rectal) and surface (ventral fur) temperature. Rectal body temperature was used as an index of total body heat and ventral fur temperature was used as an index of blood flow to the skin (and therefore, of heat dissipation/conservation) at various times during the experiment. The core temperature was evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid vaseline, into the rectum of the mouse (to about 2 cm) and left in position until the stabilization of the temperature (about 10 sec; (Vigolo et al., 2015). The probe was connected to a Cole Parmer digital thermometer, model 8402. Stress was equalized to a normal routine clinical procedure. The surface temperature was measured by a Microlife FR 1DZ1 digital infrared thermometer (Microlife AG Swiss Corporation, Widnau/Switzerland), placed at 1 cm from the surface of the abdomen of the mouse (Vigolo et al., 2015). The measurement time was approximately 3-5 sec. Core (rectal) and surface (ventral fur) mouse body temperatures were measured at 0, 30, 50, 85, 140, 200, 260 and 320 min post injection.

2.5.3.2. Evaluation of pain induced by a mechanical stimulus

Acute mechanical nociception was evaluated using the tail pinch test (Vigolo et al., 2015). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed on the tail of the mouse (in the distal portion) and a progressive pressure was applied. When

the mouse flicked its tail, the pressure was stopped and the digital instrument saved the maximum peak of weight supported (g/force). A cut off (500 g/force) was set to avoid tissue damage. The test was repeated three times and the final value was calculated with the average of 3 obtained scores. Acute mechanical nociception was measured at 0, 35, 55, 90, 145, 205, 265 and 325 min post injection.

2.5.3.3. Evaluation of pain induced by a thermal stimulus.

Acute thermal nociception was evaluated using the tail withdrawal test (Vigolo et al., 2015). Mice were restrained in a dark plastic cylinder (3 cm long and 6.3 cm diameter) closed at the sides with plastic mesh which allowed the mice to breathe normally. Then half of the tail was dipped in water of 48 °C and the latency (in seconds) or time that the tail was left in water was recorded. A cut off (15 seconds) was set to avoid tissue damage. No signs of damage, burn or variation in mouse tail sensitivity were observed after the repetition of three consecutive tests at 48 °C. Acute thermal nociception was measured at 0, 35, 55, 90, 145, 205, 265 and 325 min post injection.

2.5.3.4. Motor activity assessment.

Alterations of motor activity induced by JWH-250, JWH-073 and their interaction were measured using a battery of behavioral tests validated to specifically assess different aspects of motor behavior (Marti et al., 2004; Marti et al., 2005; Vigolo et al., 2015) in static (bar test) and dynamic conditions (drag and accelerod test).

2.5.3.4.1. Bar test

The bar test measures the grade of akinesia/catalepsy, which is the time needed to initiate a movement. While on a table, each animal's forelimbs were placed on a bar made of plastic (block height 6 cm). The time spent on the bar was measured (immobility cut off: 20 sec) and the akinesia was calculated as total time spent on the bar after three consecutive trials (total maximal time of catalepsy: 60 sec). For each mouse the bar test was performed immediately before the drag test at 0, 20, 40, 75, 130, 190, 250 and 310 min post injection.

2.5.3.4.2. Drag test

The drag test measures the ability of the animal to balance the body posture with the front legs in response to a externally dynamic stimulus (Marti et al., 2004; Marti et al., 2005). The mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20cm/sec for a fixed distance (100 cm). The number of steps performed by each paw was

recorded by two different observers. For each animal from five to seven measurements were collected. The drag test was performed at 0, 45, 70, 105, 160, 220, 280 and 340 min post injection.

2.5.3.4.3. Accelerod test

The accelerod test measures different motor parameters, such as motor coordination, locomotive ability (akinesia/bradykinesia), balance ability, muscular tone and motivation to run. The animals were placed on a rotating cylinder that increases velocity automatically in a constant manner (0-60 rotations/min in 5 min). The time spent on the cylinder was measured. The accelerod test was performed at 0, 40, 60, 95, 150, 210, 270 and 330 min post injection.

2.5.3.5. *In vivo* brain microdialysis studies

Surgery. Male ICR (CD-1®) mice, 25-30 g (Harlan Italy; S. Pietro al Natisone, Italy) were anaesthetized with Sodium Penthobarbital (50 mg/kg ip; Sigma-Aldrich, Italy) and implanted with vertical dialysis probe (1 mm dialyzing portion) prepared with AN69 fibers (Hospal Dasco, Bologna, Italy) in the Nucleus Accumbens shell (NAc shell; A+1.4, L 0.4 from bregma, V-4.8 from dura) according to the mouse brain atlas by Paxinos and Franklin (Second Edition, 2001).

Analytical Procedure. On the day following surgery, probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. Dialysate samples (15 µl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The first electrode of the detector was set at +130 mV (oxidation) and the second at -175 mV (reduction). The composition of the mobile phase was: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5. The sensitivity of the assay for dopamine (DA) was 5 fmol/sample.

Histology. At the end of the each experiment, animals were sacrificed and their brains removed and stored in formalin (8%) for histological examination to verify the correct placement of the microdialysis probe.

2.6. Data and statistical analysis

Protein concentrations were determined according to a Bio-Rad method with bovine serum albumin as reference standard. Inhibitory binding constants (Ki) were calculated from the IC_{50} values according to the Cheng and Prusoff equation: $Ki = IC_{50}/(1 + [C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant. Functional experiments were

analyzed by non-linear regression analysis using the equation for a sigmoid concentration-response curve using Prism (GraphPad Prism, USA). All data are expressed as the mean ± SEM of 3 independent experiments. Core and surface temperature values are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C). Antinociception (tail withdrawal and tail pinch tests) and catalepsy (bar test) are calculated as percent of maximal possible effect {EMax%=[(test - control latency)/(cut off time - control)] X 100}. Data are expressed in absolute values (sec in neurological changes, n° of bites in the aggressive response test), Δ°C (core and surface temperature), Emax% (tail withdrawal, tail pinch and bar test) and percentage of basal (drag test and accelerod test). In sensorimotor response experiments data are expressed in arbitrary units (visual objects response, acoustic response, vibrissae, corneal and pinna reflex) and percentage of baseline (visual placing response). In microdialysis experiments data are expressed as percentage of DA basal values. All the numerical data are given as mean ± SEM. Data were analyzed by utilizing repeated measures ANOVA. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests with significance for $p < 0.05$.

The statistical analysis of the effects of the individual substances in different concentrations over time, effects of interaction between JWH-250 and JWH-073 and that of antagonism studies in histograms were performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. The analysis of the total average effect induced by treatments (expressed in the panels E) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. The Student's t-test was used to determine statistical significance ($P<0.05$) between two groups (see neurological changes). The statistical analysis was performed with the program Prism software (GraphPad Prism, USA).

3. Results

3.1. Affinity and potency of JWH-250 and JWH-073 for CB_1 and CB_2 cannabinoid receptors

Competition binding experiments performed in CHO cell membranes transfected with human CB_1 (Fig 1 A) or CB_2 (Fig 1 B) receptors revealed a good affinity of the examined compounds. JWH-250 displayed a slight lower affinity than JWH-073 for both human CB_1 and CB_2 receptors but a similar ratio between Ki values (CB_2/CB_1) of 2.10. JWH-073 showed the highest affinity on human CB_1 receptors and a ratio between the Ki value to human CB_2 and the Ki value to human CB_1 of 2.05 (Table 1). Similar data were obtained in competition binding experiments performed in mouse brain membranes (for CB_1 receptors, Fig 1 C) and in mouse spleen membranes (for CB_2 receptors, Fig 1 D). In particular, JWH-250 and JWH-073 showed a higher affinity for CB_1 than CB_2 receptors despite a lower ratio between Ki values (CB_2/CB_1) than in human receptors (Table 1).

Cyclic AMP experiments were performed to evaluate the potency of the two compounds in CHO cells transfected with human CB_1 (Fig 1 E) or CB_2 (Fig 1 F) receptors. As expected, JWH-073 resulted the most potent with a greater potency for CB_1 than CB_2 receptors (Table 1). JWH-073 and JWH-250 behaved as full agonists as demonstrated by the capability to completely inhibit the forskolin-stimulated cAMP production (Fig 1 E-F).

3.2. Behavioural studies

3.2.1. Major neurological changes and aggressive behaviour

Systemic administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) caused important neurological changes in mice (Table 2). In particular, injection of high doses (6 and 15 mg/kg, i.p.) of JWH-like compounds induced spontaneous and handling-induced convulsions, hyperreflexia, myoclonias and aggressive responses in mice that were not observed after the administration of Δ^9-THC (Table 2) or vehicle (data not shown). JWH-250 administered at 6 and 15 mg/kg induced convulsions in 25% and 80% of treated animals respectively, while JWH-073 induced convulsions in 80% of mice only at 15 mg/kg. JWH-250 at 6 mg/kg induced seizures with same latency ($t=1.276$, $df=18$, $P=0.2182$) and same duration ($t=17.82$, $df=18$, $P=0.8605$) than those produced by JWH-018, while JWH-250 at 15 mg/kg induced seizures with same latency ($t=0.1368$, $df=18$, $P=0.8927$) but shorter duration ($t=2.888$, $df=18$, $P=0.0098$) than those produced by JWH-073 (Table 2).

JWH-250 administered at 3, 6 and 15 mg/kg induced hyperreflexia in 18%, 87% and 100% of treated animals, while JWH-073 at 3, 6 and 15 mg/kg induced hyperreflexia in 6%, 75% and

100% (Table 2). JWH-250 at 6 mg/kg induced hyperreflexia with same latency ($F_{2,29}$=1.287, p=0.2924) and longer duration ($F_{2,29}$=4.535, p=0.02) than those produced by JWH-073, and hyperreflexia was similar to those induced by JWH-018. While, JWH-250 at 15 mg/kg induced hyperreflexia with same latency (t=0.3057, df=18, P=0.7634) and same duration (t=1.076, df=18, P=0.2963) than those produced by JWH-073.

JWH-250 administered at 6 and 15 mg/kg induced myoclonias in 87.5% and 100% of treated animals, while JWH-073 at 6 and 15 mg/kg induced myoclonias in 75% and 100% (Table 2). JWH-250 at 6 mg/kg induced myoclonias with same latency respect to those produced by both JWH-073 and JWH-018 ($F_{2,29}$=1.070, p=0.3570) but with shorter and longer duration respect to those produced by both JWH-073 and JWH-018 respectively ($F_{2,29}$=18.15, p<0.0001). While JWH-250 at 15 mg/kg induced myoclonias with same latency (t=0.1368, df=18, P=0.8927) and shorter duration (t=8.653, df=18, P=0.0001) than those produced by JWH-073.

JWH-250 and JWH-073 administered at 15 mg/kg induced aggressive responses in 80% and 22% of treated animals respectively. Mice showed the same type of aggressiveness for the number of bites (t=0.1368, df=18, P=0.8927), latency to first attack (t=0.4451, df=18, P=0.6615) and duration (t=0.1751, df=18, P=0.8629) of aggressive responses (Table 2). JWH-018 administered at 6 mg/kg induced aggressiveness in 90% of treated mice, while JWH-250 and JWH-073 were ineffective at this dose (Table 2).

Neurological changes and aggressive responses were prevented by the pre-treatment with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p. injected 20 min before JWH-250 and JWH-073 administration; data not shown).

3.2.2. Sensorimotor studies

3.2.2.1. Evaluation of the visual object response

Visual object response tended to be reduced in vehicle-treated mice over the 5 hours observation (~26% of reduction at 300 min; Fig 2 A-B-C-E) and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of JWH-250 (0.01-15 mg/kg i.p.) reduced in a dose dependent manner the visual object response in mice and the effect persisted up to 5 hours at higher doses (Fig 2 A; effect of treatment ($F_{6,392}$=74.49, p<0.0001), time ($F_{7,392}$=15.27, p<0.0001) and time x treatment interaction ($F_{42,392}$=2.692, p<0.0001)). Also JWH-073 (0.01-15 mg/kg i.p.) inhibited the visual object response in a prolonged manner (Fig 2 B; effect of treatment ($F_{6,392}$=179.8, p<0.0001), time ($F_{7,392}$=46.44, p<0.0001) and time x treatment interaction ($F_{42,392}$=8.951, p<0.0001)). The visual impairment induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 2

C; effect of treatment ($F_{4,280}$=109.5, p<0.0001), time ($F_{7,280}$=14.86, p<0.0001) and time x treatment interaction ($F_{28,280}$=5.349, p<0.0001)) which alone did not alter the visual response in mice. The inhibitory effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and Δ^9-THC (Fig 2 D; ($F_{23,191}$=60.39, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) caused a marked inhibition of the visual object response in mice (of about 75 % at 10 min after drug administration) and the effect persisted up to 2 hours (Fig 2 E; effect of treatment ($F_{3,224}$=61.50, p<0.0001), time ($F_{7,224}$=7.777, p<0.0001) and time x treatment interaction ($F_{21,224}$=5.462, p<0.0001)).

3.2.2.2. Evaluation of the acoustic response

Acoustic response did not change in vehicle-treated mice over the 5 hours observation (Fig 3 A-B-C). Systemic administration of JWH-250 (0.01-15 mg/kg i.p.) reduced the acoustic response in mice and the effect persisted up to 2 hours at higher doses (Fig 3 A; effect of treatment ($F_{6,392}$=17.57, p<0.0001), time ($F_{7,392}$=1.525, p=0.1571) and time x treatment interaction ($F_{42,392}$=0.5595, p=0.9885)). Also JWH-073 (0.01-15 mg/kg i.p.) inhibited the acoustic response and the onset of the effect was more rapid compared to that induced by the administration of JWH-250. The inhibition of acoustic response persisted up to 5 hours at the highest dose (Fig 3 B; effect of treatment ($F_{6,392}$=80.34, p<0.0001), time ($F_{7,392}$=14.93, p<0.0001) and time x treatment interaction ($F_{42,392}$=4.097, p<0.0001)). The acoustic impairment induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 3 C; effect of treatment ($F_{4,280}$=16.44, p<0.0001), time ($F_{7,280}$=1.787, p=0.0898) and time x treatment interaction ($F_{28,280}$=1.077, p=0.3655)) which alone did not alter the acoustic response in mice. The inhibitory effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and Δ^9-THC (Fig 3 D; ($F_{23,191}$=29.24, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown)

3.2.2.3. Evaluation of the vibrissae reflex

Vibrissae reflex did not change in vehicle-treated mice over the 5 hours observation (Fig 4 A-B). Systemic administration of JWH-250 (0.01-15 mg/kg i.p.) did not affect acoustic response in mice (Fig 4 A; effect of treatment ($F_{6,392}$=17.57, p<0.0001), time ($F_{7,392}$=1.525, p=0.1571) and time x treatment interaction ($F_{42,392}$=0.5595, p=0.9885)). JWH-073 (0.01-15 mg/kg i.p.) slightly inhibited the acoustic response at the highest dose tested (15 mg/kg i.p.) and the effect was transient and persisted up to 1 hour (Fig 4 B; effect of treatment ($F_{6,368}$=9.515, p<0.0001), time ($F_{7,368}$=0.07338,

p=0.04994) and time x treatment interaction ($F_{42,368}$=1.909, p=0.0009)). The acoustic impairment induced by JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., data not shown) which alone did not alter the acoustic response in mice. The inhibitory effect caused by JWH-073 appeared to be less potent than that induced by JWH-018 (Fig 4 C; ($F_{23,191}$=7.133, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.2.4. Evaluation of the pinnae reflex

Pinnae reflex did not change in vehicle-treated mice over the 5 hours observation (Fig 5 A-B-C). Systemic administration of JWH-250 (0.01-15 mg/kg i.p.) did not significantly affect the pinnae reflex in mice (Fig 5 A; effect of treatment ($F_{6,368}$=4.685, p<0.0001), time ($F_{7,368}$=2.455, p=0.85120) and time x treatment interaction ($F_{42,368}$=0.4419, p=0.98871)). Otherwise, JWH-073 (0.01-15 mg/kg i.p.) slightly and transiently inhibited the acoustic response at the higher dose tested (6 and 15 mg/kg i.p.). The effect was transient and persisted up to 1 hour (Fig 5 B; effect of treatment ($F_{6,368}$=12.42, p<0.0001), time ($F_{7,368}$=0.5997, p=0.7562) and time x treatment interaction ($F_{42,368}$=1.789, p=0.0027)). The effects induced JWH-073 (6 mg/kg i.p.) were prevented by the pretreatment with AM 251 (6 mg/Kg i.p., data not shown; effect of treatment ($F_{4,280}$=19.45, p<0.0001), time ($F_{7,280}$=0.2124, p=0.9824) and time x treatment interaction ($F_{28,280}$=1.787, p=0.0104)) which alone did not alter the pinnae response in mice. The inhibitory effect caused by JWH-073 appeared to be less potent than that induced by JWH-018 but not Δ^9-THC (Fig 5 D; ($F_{23,191}$=9.178, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.2.5. Evaluation of the corneal reflex

Corneal reflex did not change in vehicle-treated mice over the 5 hours observation (Fig 6 A-B-C). Systemic administration of JWH-250 (0.01-15 mg/kg i.p.) transiently reduced corneal reflex in mice at the highest dose tested (15 mg/kg i.p.) and the effect persisted up to 2 hours (Fig 6 A; effect of treatment ($F_{6,392}$=11.00, p<0.0001), time ($F_{7,392}$=0.4007, p=0.9018) and time x treatment interaction ($F_{42,392}$=0.8375, p=0.7551)). Likewise, JWH-073 (0.01-15 mg/kg i.p.) transiently inhibited the corneal reflex at the higher dose tested (6 and 15 mg/kg i.p.). The effect persisted up to 4 hours (Fig 6 B; effect of treatment ($F_{6,392}$=11.38, p<0.0001), time ($F_{7,392}$=1.760, p=0.0939) and time x treatment interaction ($F_{42,392}$=0.7161, p=0.09076)). The effect induced by JWH-250 (15 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 6 C; effect of treatment ($F_{4,280}$=7.722, p<0.0001), time ($F_{7,280}$=0.9558, p=0.4639) and time x

treatment interaction ($F_{28,280}$=1.199, p=0.2305)) which alone did not alter the pinnae response in mice. The inhibitory effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 (Fig 6 D; ($F_{23,191}$=18.71, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.2.6. Evaluation of the visual placing response

Visual placing response tended to be reduced in vehicle-treated mice over the 5 hours observation (~20% of reduction at 305 min; Fig 7 A-B-C) and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of JWH-250 (0.01-15 mg/kg i.p.) reduced in a dose dependent manner the visual placing response in mice and the effect persisted up to 5 hours at higher doses (Fig 7 A; effect of treatment ($F_{6,392}$=84.37, p<0.0001), time ($F_{7,392}$=32.65, p<0.0001) and time x treatment interaction ($F_{42,392}$=4.720, p<0.0001)). Also, JWH-073 (0.01-15 mg/kg i.p.) inhibited the visual placing response but the effect was transient and persisted up to 2 hours (Fig 7 B; effect of treatment ($F_{6,392}$=29.53, p<0.0001), time ($F_{7,392}$=22.33, p<0.0001) and time x treatment interaction ($F_{42,392}$=3.933, p<0.0001)). The effect induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 7 C; effect of treatment ($F_{4,280}$=85.94, p<0.0001), time ($F_{7,280}$=11.11 p<0.0001) and time x treatment interaction ($F_{28,280}$=3.770, p<0.0001)) which alone did not alter the pinnae response in mice. The inhibitory effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and Δ^9-THC (Fig 7 D; ($F_{23,191}$=46.28, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.3. "Tetrad" paradigm for screening cannabinoid-like effect

3.2.3.1. Bar test

Administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) induced catalepsy in mice in the bar test only at the higher dose tested (6 and 15 mg/kg i.p.; Fig 8 A-B). JWH-250 at 15 mg/kg induced a prolonged catalepsy that was maximal at 75 minutes (EMax%= 90.05 ± 2.49; Fig 8 A) and persisted up to 5 hours (Fig 8 A; effect of treatment ($F_{6,343}$=152.4, p<0.0001), time ($F_{6,343}$=1.871, p=0.0850) and time x treatment interaction ($F_{36,343}$=0.7105, p=0.08939)). JWH-073 readily induced catalepsy which is already maximal after 20 minutes (EMax%= 90.05 ± 2.49; Fig 8 B) and lasted up to 5 hours (Fig 8 B; effect of treatment ($F_{6,343}$=152.7, p<0.0001), time ($F_{6,343}$=1.325, p=0.2451) and time x treatment interaction ($F_{36,343}$=0.4650, p=0.9957)). Catalepsy

induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 8 C; effect of agonists ($F_{4,245}$=112.3, p<0.0001), time ($F_{6,245}$=1.682, p=0.1259) and time x treatment interaction ($F_{24,245}$=0.7891, p=0.7493)) which alone did not alter the catalepsy in mice. The inhibitory effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 but more potent respect that of Δ^9-THC (Fig 8 D; $F_{23,191}$=51.90, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.3.2. Evaluation of the core and surface body temperature

Systemic administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) reduced both core (Fig 9 A-B) and surface (Fig 10 A-B) temperatures in mice. In particular, JWH-250 induced a transient reduction in core temperature at 6 and 15 mg/Kg (-4.8°C and -6.2°C at 50 and 85 min time point, respectively; Fig 9 A: effect of treatment ($F_{6,343}$=38.84, p<0.0001), time ($F_{6,343}$=3.004, p=0.0071) and time x treatment interaction ($F_{36,343}$=2.573, p<0.0001)). While, JWH-073 induced a transient reduction in core temperature at 3, 6 and 15 mg/Kg (-3.2°C, -4.7°C and -6°C at 50 min time point, respectively; Fig 9 B: effect of treatment ($F_{6,343}$=47.45, p<0.0001), time ($F_{6,343}$=6.002, p<0.0001) and time x treatment interaction ($F_{36,343}$=6.614, p<0.0001)). JWH-250 did not affect surface body temperature (Fig 10 A) while JWH-073 transient and slight reduced surface body temperature only at highest dose tested (Fig 10 B; effect of treatment ($F_{6,343}$=10.06, p<0.0001), time ($F_{6,343}$=1.175, p=0.3190) and time x treatment interaction ($F_{36,343}$=1.260, p=0.1519)). Core and surface hypothermia induced by JWH-250 and JWH-073 were prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 10 C; data not shown for surface hypothermia) Core and surface hypothermia caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and similar to that induced by Δ^9-THC (Fig 9 D; ($F_{23,191}$=16.99, p<0.0001) and Fig 10 D; ($F_{23,191}$=11.63, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect on both core and surface temperature (data not shown).

3.2.3.3. Evaluation of pain induced by a mechanical stimulus

Systemic administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) increased the threshold to acute mechanical pain stimulus in mice in the tail pinch test (Fig. 11 A-B). In particular, JWH-250 at 6 and 15 mg/kg induced a transient analgesic effect that reached the maximum at 55 min (EMax%= 39.9 ±7.84 and EMax%= 62.7 ±7, respectively; Fig 11 A: effect of treatment ($F_{6,343}$=20.96, p<0.0001), time ($F_{6,343}$=14.13, p<0.0001) and time x treatment interaction

($F_{36,343}$=3.506, p<0.0001)) and which tended to decrease within 5 hours of observation. Similarly to JWH-250, JWH-073 at 6 and 15 mg/kg induced a transient analgesic effect that reached the maximum at 55 min (EMax%= 43.9 ±7.6 and EMax%= 81.5 ±7.5; Fig 11 B: effect of treatment ($F_{6,343}$=54.11, p<0.0001), time ($F_{6,343}$=17.52, p<0.0001) and time x treatment interaction ($F_{36,343}$=6.717, p<0.0001)). Analgesic effect induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 11 C; effect of treatment ($F_{4,245}$=27.79, p<0.0001), time ($F_{6,245}$=11.93, p<0.0001) and time x treatment interaction ($F_{24,245}$=5.458, p<0.0001)) which alone did not alter the pain threshold in mice. The analgesic effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and Δ^9-THC (Fig 11 D; ($F_{23,191}$=33.03, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) increased the threshold to acute mechanical pain stimulus in mice at 55 min (EMax%= 23.5 ±5.7; Fig 11 E: effect of treatment ($F_{4,245}$=27.79, p<0.0001), time ($F_{6,245}$=11.93, p<0.0001) and time x treatment interaction ($F_{24,245}$=5.458, p<0.0001)).

3.2.3.4. Evaluation of pain induced by a thermal stimulus

Systemic administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) increased the threshold to acute thermal pain stimulus in mice in the tail withdrawal test (Fig 12 A-B). In particular, JWH-250 at 6 and 15 mg/kg induced a transient analgesic effect that reached the maximum at 55 min (EMax%= 41.3 ±9.3, and EMax%= 36.0 ±7.9, respectively; Fig 12 A: effect of treatment ($F_{6,343}$=17.04, p<0.0001), time ($F_{6,343}$=3.196, p=0.0046) and time x treatment interaction ($F_{36,343}$=0.4811, p=0.9954)) and which tended to decrease within 5 hours of observation. Also JWH-073 at 3, 6 and 15 mg/kg induced a transient analgesic effect that reached the maximum at 55 min (EMax%= 31.0 ±5.0, EMax%= 44.0 ±8.1 and EMax%= 44.2 ±3.6; Fig 12 B: effect of treatment ($F_{6,343}$=60.79, p<0.0001), time ($F_{6,343}$=12.94, p<0.0001) and time x treatment interaction ($F_{36,343}$=2.789, p<0.0001)). Analgesic effect induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/Kg i.p., Fig 12 C; effect of treatment ($F_{4,245}$=23.57, p<0.0001), time ($F_{6,245}$=2.533, p=0.0213) and time x treatment interaction ($F_{24,245}$=0.8663, p=0.6482)) which alone did not alter the pain threshold in mice. The analgesic effect caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 but similar to that induced by Δ^9-THC (Fig 12 D; ($F_{23,191}$=15.33, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.3.5. Accelerod test

Systemic administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) induced a significant impairment of locomotion in the accelerod test in mice (Fig 13 A-B). In particular, JWH-250 at 6 and 15 mg/kg induced a transient inhibition of motor performance that reached the maximum effect at 60 min (inhibition of about 27% and 58%, respectively; Fig 13 A: effect of treatment ($F_{6,392}$=15.92, p<0.0001), time ($F_{7,392}$=2.787, p=0.0077) and time x treatment interaction ($F_{42,392}$=0.9688, p=0.5300)) and which tended to revert within 5 hours of observation. Also JWH-073 at 6 and 15 mg/kg induced a transient analgesic effect that reached the maximum at 60 min (inhibition of about 32% and 64%, respectively; Fig 13 B: effect of treatment ($F_{6,392}$=11.63, p<0.0001), time ($F_{7,392}$=1.425, p=0.01937) and time x treatment interaction ($F_{42,392}$=0.9143, p=0.06266)). Inhibitory effect induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 13 C; effect of treatment ($F_{4,280}$=14.32, p<0.0001), time ($F_{7,280}$=2.289, p=0.0278) and time x treatment interaction ($F_{28,280}$=0.6559, p=0.91100)) which alone did not alter the motor performance in mice. The motor impairment caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and similar to that induced by Δ^9-THC (Fig 13 D; ($F_{23,191}$=50.63, p<0.0001)). The co-administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.2.3.6. Drag test

Systemic administration of JWH-250 and JWH-073 (0.01-15 mg/kg i.p.) caused a prolonged and significant reduction of the number of steps performed with the front legs of in mice (Fig 14 A-B). In particular, JWH-250 at 6 and 15 mg/kg induced a transient inhibition of motor performance that reached the maximum effect at 70 min (inhibition of about 42% and 67%, respectively; Fig 14 A: effect of treatment ($F_{6,392}$=15.32, p<0.0001), time ($F_{7,392}$=9.173, p<0.0001) and time x treatment interaction ($F_{42,392}$=0.9648, p=0.5370)) and which tended to revert within 5 hours of observation. Also JWH-073 at 3, 6 and 15 mg/kg induced a transient analgesic effect that reached the maximum at 60 min (inhibition of about 54%, 45% and 64%, respectively; Fig 14 B: effect of treatment ($F_{6,392}$=26.19, p<0.0001), time ($F_{7,392}$=5.567, p<0.0001) and time x treatment interaction ($F_{42,392}$=1.233, p=0.1590)). Inhibitory effect induced by JWH-250 (6 mg/kg i.p.) and JWH-073 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/Kg i.p., Fig 14 C; effect of treatment ($F_{4,245}$=20.15, p<0.0001), time ($F_{6,245}$=0.4430, p=0.8495) and time x treatment interaction ($F_{23,191}$=28.03, p<0.0001)) which alone did not alter the motor performance in mice. The motor impairment caused by JWH-250 and JWH-073 appeared to be less potent than that induced by JWH-018 and similar to that induced by Δ^9-THC (Fig 14 D; ($F_{23,191}$=50.63, p<0.0001)). The co-

administration of ineffective doses of JWH-250 (1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) did not caused additive or synergic effect (data not shown).

3.3. *In vivo* brain microdialysis

Effect of JWH-250 and JWH-073 administration on DA transmission in the NAc shell

Basal values of extracellular DA in NAc shell were 38±14 fmoles/15ul sample. Systemic administration of JWH-250 and JWH-073 (0.1-3 mg/kg i.p.) increased extracellular DA release in NAc shell of awake and freely moving mice (Fig. 15 A-B) in a dose-dependent manner. In particular, JWH-250 facilitated extracellular DA release at 1 mg/kg and 3 mg/kg (effect of treatment ($F_{3,280}$=30.16, p<0.0001), time ($F_{13,280}$=3.52, p<0.0001) and time x treatment interaction ($F_{39,280}$=1.14, p=0.2653)). JWH-250 at 1 mg/kg induced a prolonged release of DA (up to 75 minutes) that reached the maximum at 15 min after drug administration (max increase of about +50%) while at the highest dose the effect was transient and disappeared after 15 min (Fig. 15 A). Similarly, JWH-073 at 1 and 3 mg/kg facilitated DA release (effect of treatment ($F_{3,280}$=17.99, p<0.0001), time ($F_{13,280}$=4.21, p<0.0001) and time x treatment interaction ($F_{39,280}$=1.04, p=0.4179)). JWH-073 at 1 mg/kg induced a prolonged release of DA (up to 60 minutes) that reached the maximum at 30 min after drug administration (max increase of about +50%) while at the highest dose the effect was transient and disappeared after 15 min (Fig. 15 B). The facilitatory effect induced by JWH-250 (1 mg/kg i.p.) and JWH-073 (1 mg/kg i.p.) was prevented by AM 251 (1 mg/kg i.p. injected 30 minutes before cannabinoid agonists (Fig. 15 C; effect of treatment ($F_{4,280}$=34.27, p<0.0001), time ($F_{13,280}$=4.02, p<0.0001) and time x treatment interaction ($F_{52,280}$=1.06, p=0.3663)) which alone did not alter the motor performance in mice.

The co-administration of ineffective doses of JWH-250 (0.1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) caused a marked facilitation of DA release in NAc shell of mice (of about 40 % at 15 min after drug administration) and the effect persisted up to 45 minutes (Fig. 15 D; effect of treatment ($F_{3,280}$=8.86, p<0.0001), time ($F_{13,280}$=1.92, p=0.0282) and time x treatment interaction ($F_{39,280}$=0.77, p=0.8384)).

4. Discussion

This study demonstrates that the systemic administration of JWH-250 (Huffman et al., 2005) and JWH-073 (Huffman et al., 1994) induces the typical tetrad effect characterized by thermal and mechanical analgesia, core and surface hypothermia, motor impairment in the drag and accelerod tests and catalepsy. Moreover, for the first time we demonstrated that JWH-250 and JWH-073 cause important alteration of visual, acoustic and tactile sensorimotor reflexes and they promote aggressive response in CD-1 mice. Furthermore, as previously reported for the synthetic cannabinoid JWH-018 (Marshell et al., 2014; Vigolo et al., 2015; Ossato et al., 2015), JWH-250 and JWH-073 induce neurological alterations such as convulsions, hyperreflexia and myoclonias that are not observed after the administration of Δ^9-THC (Vigolo et al., 2015; Ossato et al., 2015). Finally, by the microdialysis technique in awake and freely moving mice we demonstrated that systemic administration of JWH-250 and JWH-073 transiently facilitates extracellular DA release in the NAc shell. All these behavioural and neurochemical effects were fully dependent on CB_1 receptor stimulation since they are completely prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM 251. In addition, this study demonstrates that the co-administration of ineffective doses of JWH-250 and JWH-073 synergistically improves mechanical analgesia, impairs visual response and facilitates mesolimbic DA transmission in mice, suggesting that the simultaneous presence of synthetic cannabinoids in the same package (Uchiyama et la., 2011) may potentiate the detrimental effects of individual compounds (Brents et al., 2013) increasing their dangerousness and abuse potential.

In vitro binding studies show that JWH-250 and JWH-073 retain nanomolar affinity for both CD-1 murine and human CB_1 and CB_2 receptors (Huffman et al., 2005; Wiley et al., 1998) with a slightly greater preference for CB_1 receptor. In particular, in CD-1 murine preparation JWH-250 displays an affinity for CB_1 receptors (Ki = 25.7 nM) similar to that of JWH-073 (Ki = 17.9 nM) but lower respect to that of JWH-018 (Ki = 5.82 nM; (Vigolo et al., 2015). Whereas, on human CB_1 receptors, JWH-250 shows a lower affinity (Ki = 22.5 nM) compared to that of JWH-073 (Ki = 12.3 nM) and JWH-018 (Ki = 9.53 nM; (Vigolo et al., 2015). The reduced CB_1 receptor affinity of JWH-250 and JWH-073 could justify their lower potency value (JWH-250, IC_{50} = 33.7 nM and JWH-073, IC_{50} = 22.5 nM) in inhibiting cyclic AMP formation respect to that of JWH-018 (IC_{50} = 14.1 nM; (Vigolo et al., 2015). Although this evidence was obtained in CHO cells transfected with human CB_1 receptors, however, it could justify the lower efficacy and potency of JWH-250 and JWH-073 compared to those induced by JWH-018 in behavioural studies.

Indeed, JWH-250 and JWH-073 reproduce the typical tetrad effect (i.e. hypothermia, analgesia and motor inhibition) as reported for JWH-018 (Wiebelhaus et al., 2012; Wiley et al.,

1998; Macri et al., 2013; Vigolo et al., 2015) and Δ^9-THC (Compton et al., 1992; Vigolo et al., 2015) but their activity appear to be less potent than those induced by JWH-018 and more comparable with that of Δ^9-THC.

In this regard administration of JWH-250 and JWH-073 in the dose-range up to 15 mg/kg induces a core and surface hypothermia which is significantly lower respect to that induced by JWH-018 but that it was similar to that induced by administration of high doses of Δ^9-THC (Vigolo et al., 2015). In particular, JWH-073 induces an hypothermia in mice which reaches the maximum effect between 60 and 90 minutes (Brents et al., 2012) and that it was lower respect to that induced by JWH-018 but comparable to that induced by Δ^9-THC (Marshell et al., 2014). However, in the present study we cannot exclude that administration of JWH-250 and JWH-073 at higher doses might induce a greater body and surface hypothermia. Nevertheless, the occurrence of major neurological changes avoid us to increase doses of JWH-250 or JWH-073. As reported for others cannabinoid agonists, hypotermia induced by JWH-250 or JWH-073 is completely prevented by pretreatment with AM 251 confirming that this effect is clearly mediated by the stimulation of CB_1 receptors (Marshell et al., 2014; Vigolo et al., 2015), possibly expressed in the preoptic area of the hypothalamus (Fitton and Pertwee, 1982; Rawls et al., 2002).

Systemic administration of JWH-250 and JWH-073 increases the threshold to acute mechanical pain stimulus in mice, although the analgesic effect is less intense respect to that induced by JWH-018 and Δ^9-THC administration (Vigolo et al., 2015). This lower response, in particular to mechanical stimuli, could be due to the fact that JWH-250 and JWH-073 have a lower affinity for the CB_1 receptor in CD-1 mice preparation, compare to that of JWH-018 (Vigolo et al., 2015). Moreover, it has been reported that SCBs are biotransformed into glucuronitated or monohydroxylated metabolites that are inactive or that even can act as neutral antagonists at CB_1 receptors dampening the overall activity of the parent compound (Seely et al., 2012; Brents et al., 2012). The latter hypothesis would justify the fact that Δ^9-THC, which has a reduced affinity compared to that of JWH-018 (Wiley et al., 1998) and acts as a partial agonist at CB_1 receptors both in vitro (Govaerts et al., 2004) and in vivo (Paronis et al., 2012), it induces a mechanical analgesia higher than that of JWH-250 and JWH-073.

As previously reported for JWH-018-R compounds and Δ^9-THC (Vigolo et al., 2015), also JWH-250 and JWH-073 show a greater efficacy in reducing nociception to mechanical stimulation (Emax ~60% for JWH-250 and Emax ~80% for JWH-073) compared to thermal stimulus (Emax ~40% for JWH-250 and Emax ~45% for JWH-073). Moreover, the evidence that the co-administration of ineffective doses of JWH-250 and JWH-073 synergistically provokes an analgesic effect in the tail pinch (Emax ~24%) but not in the tail withdrawal test strengthens the hypothesis

that cannabinoid agonists exert their analgesic effect by acting on different sensory components of pain generated by a mechanical (Martin et al., 1996) or thermal (Hohmann et al., 1999) stimuli.

In our experimental conditions the possibility that the analgesic effect induced by JWH-250, JWH-073 and/or their metabolites (Rajasekaran et al., 2013) is due to the activation of peripheral CB_2 receptors (Guindon and Hohmann, 2008) should be ruled out since their analgesic effects are fully prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM 251.

Unlike previous studies (Vigolo et al., 2015), the analgesic effects induced by JWH-250 and JWH-073 overlap almost completely to theirs motor impairment. This profile of action may be due to the fact that JWH-250 and JWH-073 act with greater effectiveness and potency in inhibiting motor activity in bar (Fig 8 D) and drag (Fig 14 D) tests compared to the modulation of analgesic effect to mechanical (Fig 11 D) and thermal (Fig 12 D) stimulation.

This responsiveness biased towards the motor inhibition is in line with previous studies that have reported that small changes in the molecular structure of indole- and pyrrole-derived cannabinoids induce consistent disparities among potencies and efficacies of in vivo effects (Wiley et al., 1998; Wiley et al., 2014). In particular, the small difference in length of the side chain between the JWH-073 (butyl chain) and JWH-018 (pentyl chain) is sufficient for determining the different responsiveness of the two compounds in tests of locomotion (ED_{50} ~ 0.34 µM/kg for JWH-073 and ED_{50} ~ 0.44 µM/kg for JWH-018), analgesia (ED_{50} ~ 1.3 µM/kg for JWH-073 and ED_{50} ~ 0.09 µM/kg for JWH-018) and hypothermia (ED_{50} ~ 3.3 µM/kg for JWH-073 and ED_{50} ~ 1.7 µM/kg for JWH-018; (Wiley et al., 1998).

Administration of JWH-250 and JWH-073 affects the startle response to visual, acoustic and tactile stimuli in mice through the stimulation of CB_1 receptors, although effects are less potent than those induced by JWH-018 and Δ^9-THC (Ossato et al., 2015). In particular, JWH-250, as well as JWH-073, causes a marked inhibition of visual response in mice. Some studies have shown that CB_1 receptors are critically involved in the modulation of visual cortical plasticity in mice (Liu et al., 2008; Garkun and Maffei, 2014) and that Δ^9-THC inhibits the visual processes in rat by impairing the thalamocortical transmission (Dasilva et al., 2012). Moreover, a recent study has shown that visual information in mice is elaborated in a subpopulation of neurons selectively localized in the dorsomedial striatum (Reig and Silberberg, 2014), a brain area of the basal ganglia in which CB_1 receptors are expressed (Tsou et al., 1998; Marsicano and Lutz, 1999). Even though in our study we are not able to understand which brain areas and neural mechanisms are responsible for the reduced visual response of the mouse, it is possible to hypothesize that JWH-250 and JWH-073 could inhibit visual function through the stimulation of CB_1 receptors expressed in

thalamocortical-striatal visual circuitry (Tsou et al., 1998; Marsicano and Lutz, 1999; Yoneda et al., 2013).

For the first time we demonstrate that the co-administration of ineffective doses of JWH-250 and JWH-073 synergistically impairs visual responses in mice without affecting other motor and sensorimotor parameters. This selectivity might be due to the high sensitivity of the visual system to CB_1 receptor stimulation. Our data are in agreement with previous study that has been showed that co-administration of JWH-018 and JWH-073 in mice produces additive, synergistic or antagonistic interactions. In particular, synergistic interactions between JWH-018 and JWH-073 were observed for Δ^9-THC drug discrimination, analgesia and displacement of radioligand from CB_1 receptors (Brents et al., 2013). Further studies will be undertaken to understand the cellular mechanisms which underline these synergetic actions, since these interactions can have a primary role in the genesis of adverse effects in humans.

Our study also demonstrates that JWH-250 and JWH-073 impair the acoustic startle response in mice by the selective stimulation of CB_1 receptors. This finding is in agreement with previous studies that have demonstrated the effectiveness of acute administration of Δ^9-THC (Malone and Taylor, 2006; Nagai et al., 2006; Ossato et al., 2015), CP 55940 (Mansbach et al., 1996; Martin et al., 2003), WIN 55,212-2 (Bortolato et al., 2005) and JWH-018 (Ossato et al., 2015) in reducing the acoustic startle reflex in rodents. Acoustic startle reflex is induced by the activation of three serially connected structures that involve the activation of the dorsal cochlear nucleus (Gomez-Nieto et al., 2014). Therefore, JWH-250 and JWH-073 could impair the acoustic startle reflex in mice by stimulating CB_1 receptors expressed on the presynaptic terminals of parallel fibers in the dorsal cochlear nucleus (Tzounopoulos et al., 2007). In support of this hypothesis, it has been reported that administration of the synthetic cannabinoid agonist WIN-55,212-2 (Tzounopoulos et al., 2007) or the activation of the endogenous cannabinoid system affected the short-term synaptic plasticity right in the dorsal cochlear nucleus of mice (Tzounopoulos et al., 2007; Zhao et al., 2011; Sedlacek et al., 2011).

Albeit, it is not possible to define whether visual and acoustic alterations induced by JWH-250, JWH-073 and JWH-018 (Ossato et al., 2015) in mice are an expression of hallucinatory states, as suggested for the Δ^9-THC in human studies (Winton-Brown et al., 2011), our data support the hypothesis that SCBs by stimulating CB_1 receptors could impair the sensorimotor gating in mice similarly to what demonstrated for other cannabinoid agonists such as Δ^9-THC (Malone and Taylor, 2006; Nagai et al., 2006), CP 55940 (Mansbach et al., 1996; Martin et al., 2003) and WIN 55,212-2 (Schneider and Koch, 2002; Wegener et al., 2008). Further studies will be conducted using the pre-pulse inhibition test to investigate the potential psychogenic effect of JWH-250 and JWH-073.

We also underline that JWH-073 is more effective than JWH-250 in inhibiting the sensorimotor responses in mice in reply to tactile stimuli of vibrissae, pinna and cornea. The inhibitory effect induced by JWH-073 administration on vibrissae responses is consistent with previous studies showing that endocannabinoid system and exogenous Δ^9-THC or WIN 55,212-2 administration directly modulated whisking activity in rodent (Patel et al., 2002; Pietr et al., 2010; Ho et al., 2010). Neuronal circuits that are associated with whisking control include brain areas, such as the inferior olive, somatosensory cortex and superior colliculus (Hemelt and Keller, 2008), which expressed CB_1 receptors (Tsou et al., 1998; Cristino et al., 2006). Therefore, it is possible to hypothesize that JWH-073 could inhibit responses of the vibrissae through stimulation of CB_1 receptors expressed in those neuronal circuitry.

Whereas, in agreement with what previously hypothesized for JWH-018 (Ossato et al., 2015), JWH-073 may inhibit sensorimotor responses of pinna and cornea through the stimulation of CB_1 receptors directly expressed in trigeminal structures (Herkenham et al., 1991; Tsou et al., 1998; Price et al., 2003). These results are consistent with previous studies showing that the administration of HU 210 and WIN55,212-2 suppressed central trigeminal transmission (Jenkins et al., 2004; Papanastassiou et al., 2004) and that topical application of WIN55,212-2 reduced cornea-evoked trigeminal brainstem activity (Bereiter et al., 2002).

It is interesting to note that both JWH-250 and JWH-073 impair visual sensorimotor responses in mice at doses (1 and 3 mg/kg) that do not cause catalepsy (bar test) or reduce stimulated motor activity (drag and accelerod test). These findings point out that effects induced by JWH-250 and JWH-073 on sensorimotor responses and motor activity are mediated by separate processes and suggest that the decreased sensory responsiveness does not result merely from a disruption of motor function (Ossato et al., 2015). Recent evidence show that the administration of low doses of Δ^9-THC at the same time facilitates spontaneous locomotion and inhibits visual and acoustic sensorimotor responses (Ossato et al., 2015).

The present study increases preclinical evidence showing that SCBs caused convulsions, hyperreflexia and myoclonia (Marshell et al., 2014; Vigolo et al., 2015; Ossato et al., 2015). However, JWH-250 and JWH-073 are less potent in inducing convulsions respect to JWH-018 (Vigolo et al., 2015) and it is possible connected to their lower affinity and potency on CB_1 receptors. These data are in agreement with the increasing clinical reports showing the occurrence of seizures and hyperreflexia in young people who have smoked “Spice” products containing different SCBs (Gugelmann et al., 2014; Lapoint et al., 2011; McQuade et al., 2013; Schneir and Baumbacher, 2012; Simmons et al., 2011).

A further observation is that high doses of JWH-250, JWH-073 and JWH-018 promote aggressive response in mice. However, this behavior was observed in a simple test that is not fully representative for an overall and accurate assessment of aggressive behavior in mice (Takahashi and Miczek, 2014; Miczek et al., 2007). Nevertheless, our observation is consistent with previous studies that have shown that pharmacological modulation of cannabinoid signal alter aggressive behavior. In fact, Δ^9-THC induces a dose-dependent decrease in attack behavior in mice, rats, and squirrel monkeys (Ham and De Jong, 1975; Miczek, 1978; van Ree et al., 1984). However, other studies have highlighted how Cannabis Sativa extract or Δ^9-THC administration in stressful situations can cause or exacerbate aggression in rodents (Carder and Olson, 1972; Carlini and Gonzales, 1972; Carlini et al., 1976). Therefore, it is possible that the aggressive response caused by the administration of JWH-250, JWH-073 and JWH-018 in mice is mainly due to the stressful situation of the animal (sensorimotor alterations and neurological symptoms) rather than a direct effect on neural circuits that control aggressive behaviour. However, further studies will be undertaken in the model of the resident-intruder to better understand the effects of JWH-250, JWH-073 and JWH-018 on aggressive behavior in mice since irritability and aggressive response have been evidenced in consumers of SCBs admitted to the emergency room (McGuinness and Newell, 2012; Zawilska and Wojcieszak, 2014; Castaneto et al., 2014).

It is well established that Δ^9-THC and the synthetic cannabinoid agonist WIN 55,212-2 shares with drugs of abuse the property of increase DA transmission preferentially in the NAc shell (Tanda et al., 1997; Di Chiara et al., 2004; Lecca et al., 2006). Similarly, recent studies have shown that JWH-018 stimulates DA transmission preferentially in the NAc shell as compared to the NAc core and medial prefrontal cortex of rats and that this effect was observed at lower doses compared to those that produced tetrad-like effects (De Luca et al., 2015). In order to evaluate whether JWH-250 and JWH-073 are able to increase DA transmission in the NAc shell, the effect of both drugs (0.1-3 mg/kg ip) were evaluated by means of *in vivo* brain microdialysis in CD-1 mice. JWH-250 and JWH-073 induced a prolonged increase of DA release at 1 mg/kg while the lower dose (0.1 mg/kg) was ineffective. The highest dose tested (3 mg/kg) produced a transient effect that disappeared after 15 min. These data show that JWH-250 and JWH-073 on dialysate DA had an inverted U-shape, as observed for JWH-018 (De Luca et al., 2015). This unusual dose-response curve might be due to the synthesis of hydroxylated metabolites of JWH-250 and JWH-073 that can act as partial agonists or antagonists, thus inhibiting the effect of the parent drug (Dhawan et al., 2006; Wiebelhaus et al., 2012). Otherwise, the inhibition of DA release could be due to a retrograde signaling through presynaptic CB_2 receptors located on DArgic terminals of the NAc (Xi et al., 2011; Morales and Bonci, 2012). However, the facilitatory effect induced by the SCBs is fully

prevented by AM 251 confirming the involvement of CB_1 receptors. This observation is in agreement with the notion that genetic deletion of CB_1 receptors also prevents the effect of JWH-018 (De Luca et al., 2015). Importantly, the co-administration of ineffective doses of JWH-250 (0.1 mg/kg ip) and JWH-073 (0.1 mg/kg ip) caused a marked and persistent facilitation of DA release in NAc shell of mice. Thus demonstrating that the concurrent administration increases the rewarding properties of each one of the Spice cannabinoid component studied. Similarly, the use of different SCBs in humans can increase their abuse liability.

The present data show that JWH-250 and JWH-073 reproduce the typical cannabinoid tetrad effect, impaired sensorimotor responses (visual, acoustic and tactile), caused neurological alterations, promote aggressiveness and stimulate dopamine release in the NAc shell of mice. Of noteworthy relevance, the co-administration of ineffective doses of JWH-250 and JWH-073 synergistically impaired visual sensorimotor responses, improved mechanical pain threshold and stimulated mesolimbic DA transmission in mice, living unchanged all others behavioral and physiological parameters. For the first time the present study demonstrates the overall pharmacological effects induced by the administration of JWH-250 and JWH-073 in mice and it reveals their synergistic action suggesting that co-administration of different SCBs may potentiate the detrimental effects of individual compounds increasing their dangerousness and abuse potential.

Table 1

Binding and functional parameters of JWH-250 and JWH-073 at CB_1 and CB_2 receptors.

Compound	hCB_1 CHO membranes[a] Ki (nM)	hCB_2 CHO membranes[a] Ki (nM)	Mouse cortex membranes CB_1[a] Ki (nM)	Mouse spleen membranes CB_2[a] Ki (nM)	hCB_1 CHO cells[b] IC_{50} (nM)	hCB_2 CHO cells[b] IC_{50} (nM)
JWH-250	22.5 ± 1.7	47.3 ± 4.3	25.7 ± 2.2	42.9 ± 4.2	33.7 ± 2.7	75.6 ± 6.4
JWH-073	12.3 ± 0.9	25.2 ± 2.1	17.9 ± 1.3	21.3 ± 1.9	22.5 ± 1.6	48.7 ± 3.9

Data are expressed as mean ± SEM.
[a] [^{3}H]-CP-55,940 competition binding experiments.
[b] Cyclic AMP experiments.
Human CB_1 receptor (hCB_1) and human CB_2 receptor (hCB_2)

Table 2

Neurological changes and aggressive response induced by the administration of JWH-250, JWH-073 (0.01-15 mg/kg i.p.), Δ^9-THC (0.01-100 mg/kg i.p.) and JWH-018 (0.01-6 mg/kg i.p.).

Convulsions

Compound	*Vehicle*	**Δ^9-THC[a]**							**JWH-018[a]**					**JWH-250**						**JWH-073**					
Doses (mg/Kg)		*0.01*	*0.1*	*1*	*3*	*6*	*30*	*100*	*0.01*	*0.1*	*1*	*3*	*6*	*0.01*	*0.1*	*1*	*3*	*6*	*15*	*0.01*	*0.1*	*1*	*3*	*6*	*15*
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	70	–	–	–	–	25	80	–	–	–	–	–	80
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	369.7±32.2	–	–	–	–	357.2±62.3	921.8±67.2 ##	–	–	–	–	–	1928.4±342
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	109.7±16.3	–	–	–	–	268±123	220.5±54.1	–	–	–	–	–	231.9±63.4

Hyperriflexia

Compound	*Vehicle*	**Δ^9-THC[a]**							**JWH-018[a]**					**JWH-250**						**JWH-073**					
Doses (mg/Kg)		*0.01*	*0.1*	*1*	*3*	*6*	*30*	*100*	*0.01*	*0.1*	*1*	*3*	*6*	*0.01*	*0.1*	*1*	*3*	*6*	*15*	*0.01*	*0.1*	*1*	*3*	*6*	*15*
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	15	80	–	–	–	18	87	100	–	–	–	6	75	100
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	1354.8±67.2	1439.8±45.3	–	–	–	956.7±122	1665.2±157 #	1939.8±145	–	–	–	1120.8±151	1120.8±151	2198.6±192
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	124.9±31.7	93.5±21.2	–	–	–	193.4±42.3	132.7±36.1	98.6±37.5	–	–	–	219.8±67	184.8±56	110.9±14.6

Myoclonias

Compound	*Vehicle*	**Δ^9-THC[a]**							**JWH-018[a]**					**JWH-250**						**JWH-073**					
Doses (mg/Kg)		*0.01*	*0.1*	*1*	*3*	*6*	*30*	*100*	*0.01*	*0.1*	*1*	*3*	*6*	*0.01*	*0.1*	*1*	*3*	*6*	*15*	*0.01*	*0.1*	*1*	*3*	*6*	*15*
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	80	–	–	–	–	87.5	100	–	–	–	–	75	100
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	669.7±36.6	–	–	–	–	1587.6±233 ** #	1998.2±126 ###	–	–	–	–	2281.6±229 ***	3621.6±139
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	109.7±16.35	–	–	–	–	268±123	220.5±54.1	–	–	–	–	282.4±101.7	231.9±63.4

Aggressive response

Compound	*Vehicle*	**Δ^9-THC**							**JWH-018**					**JWH-250**						**JWH-073**					
Doses (mg/Kg)		*0.01*	*0.1*	*1*	*3*	*6*	*30*	*100*	*0.01*	*0.1*	*1*	*3*	*6*	*0.01*	*0.1*	*1*	*3*	*6*	*15*	*0.01*	*0.1*	*1*	*3*	*6*	*15*
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	90	–	–	–	–	–	80	–	–	–	–	–	22
Score (n° of bites)	–	–	–	–	–	–	–	–	–	–	–	–	8.2±3.1	–	–	–	–	–	7.5±1.6	–	–	–	–	–	6.45±1.7
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	2750±621	–	–	–	–	–	2481.6±668	–	–	–	–	–	2327.6±572
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	324.5±76	–	–	–	–	–	347±70	–	–	–	–	–	291.2±104

Table 2

Effect of the systemic administration of Δ^9-THC (0.01-100 mg/kg i.p. *from (Vigolo et al., 2015)* JWH-018 (0.01-6 mg/kg i.p. *from (Vigolo et al., 2015),* JWH-250 (0.01-15 mg/kg i.p.) and JWH-073 (0.01-15 mg/kg i.p.) on neurological changes and aggressive behavior in mice.

Data relating to the neurological changes (convulsions, hyperriflexia, mioclonias) induced by JWH-018 and Δ^9-THC are taken from *(Vigolo et al., 2015).* Data are expressed as percentage (frequency of animal with neurological signs), seconds (duration and latency of neurological signs) and score (number of bites), represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons and Student's t-test was used to determine statistical significance ($P<0.05$) between two groups.

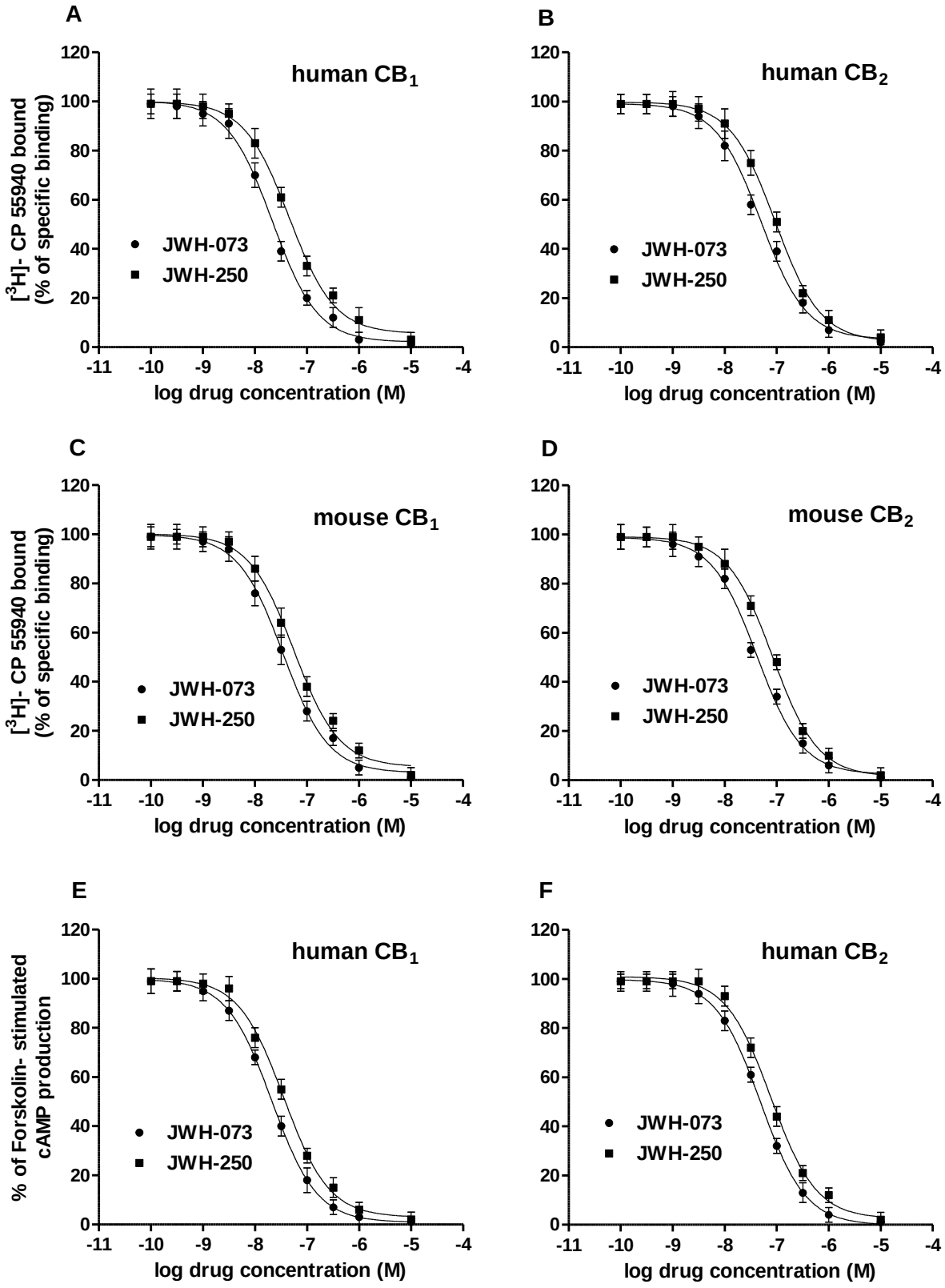
A
human CB1
[3H]- CP 55940 bound
(% of specific binding)
JWH-073
JWH-250
log drug concentration (M)
B
human CB2
JWH-073
JWH-250
log drug concentration (M)
C
mouse CB1
[3H]- CP 55940 bound
(% of specific binding)
JWH-073
JWH-250
log drug concentration (M)
D
mouse CB2
JWH-073
JWH-250
log drug concentration (M)
E
human CB1
% of Forskolin- stimulated
cAMP production
JWH-073
JWH-250
log drug concentration (M)
F
human CB2
JWH-073
JWH-250
log drug concentration (M)

Figure 1

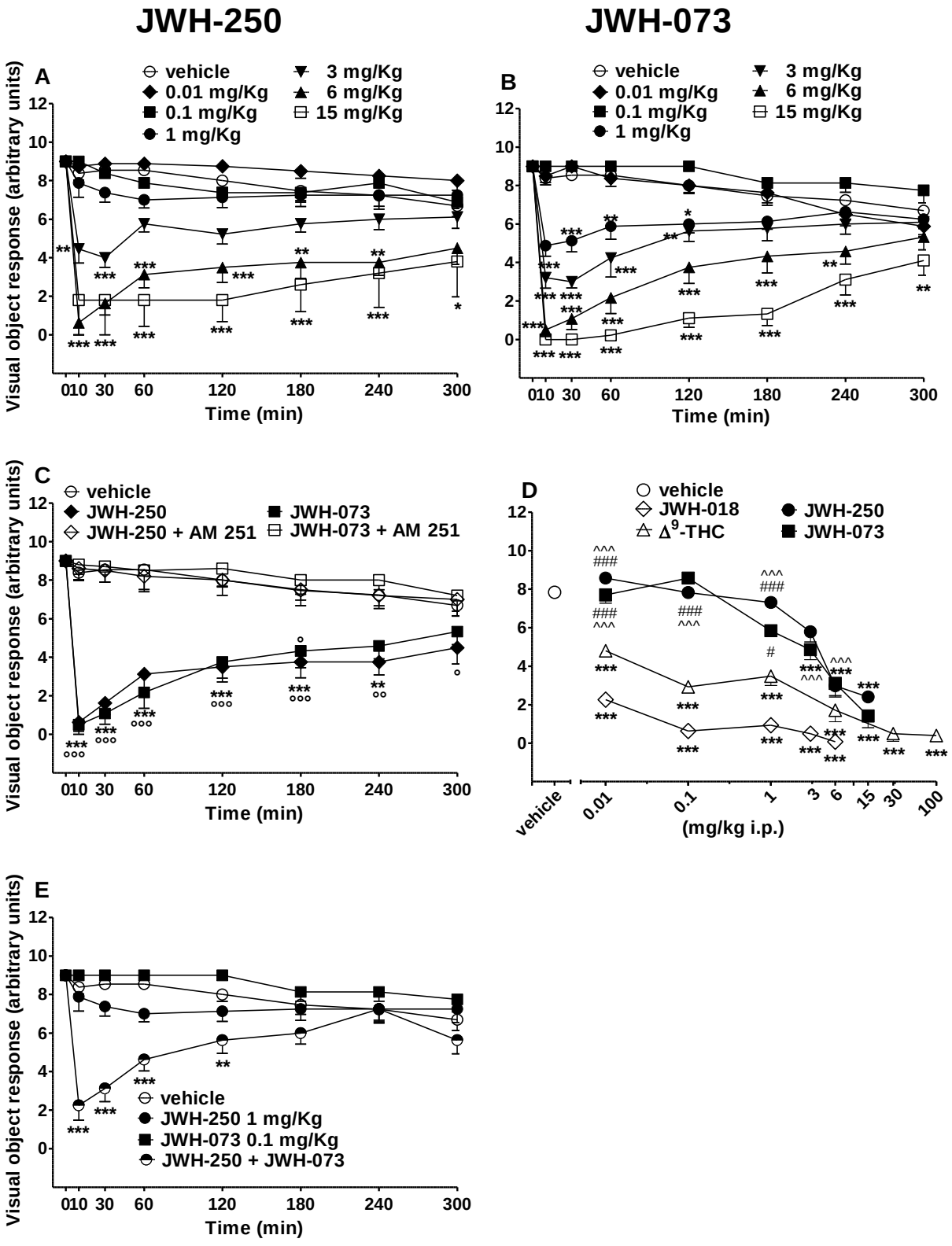
JWH-250
JWH-073
A
B
C
D
E
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Visual object response (arbitrary units)
Time (min)
JWH-250
JWH-250 + AM 251
JWH-073
JWH-073 + AM 251
JWH-018
Δ9-THC
(mg/kg i.p.)
JWH-250 1 mg/Kg
JWH-073 0.1 mg/Kg
JWH-250 + JWH-073

Figure 2

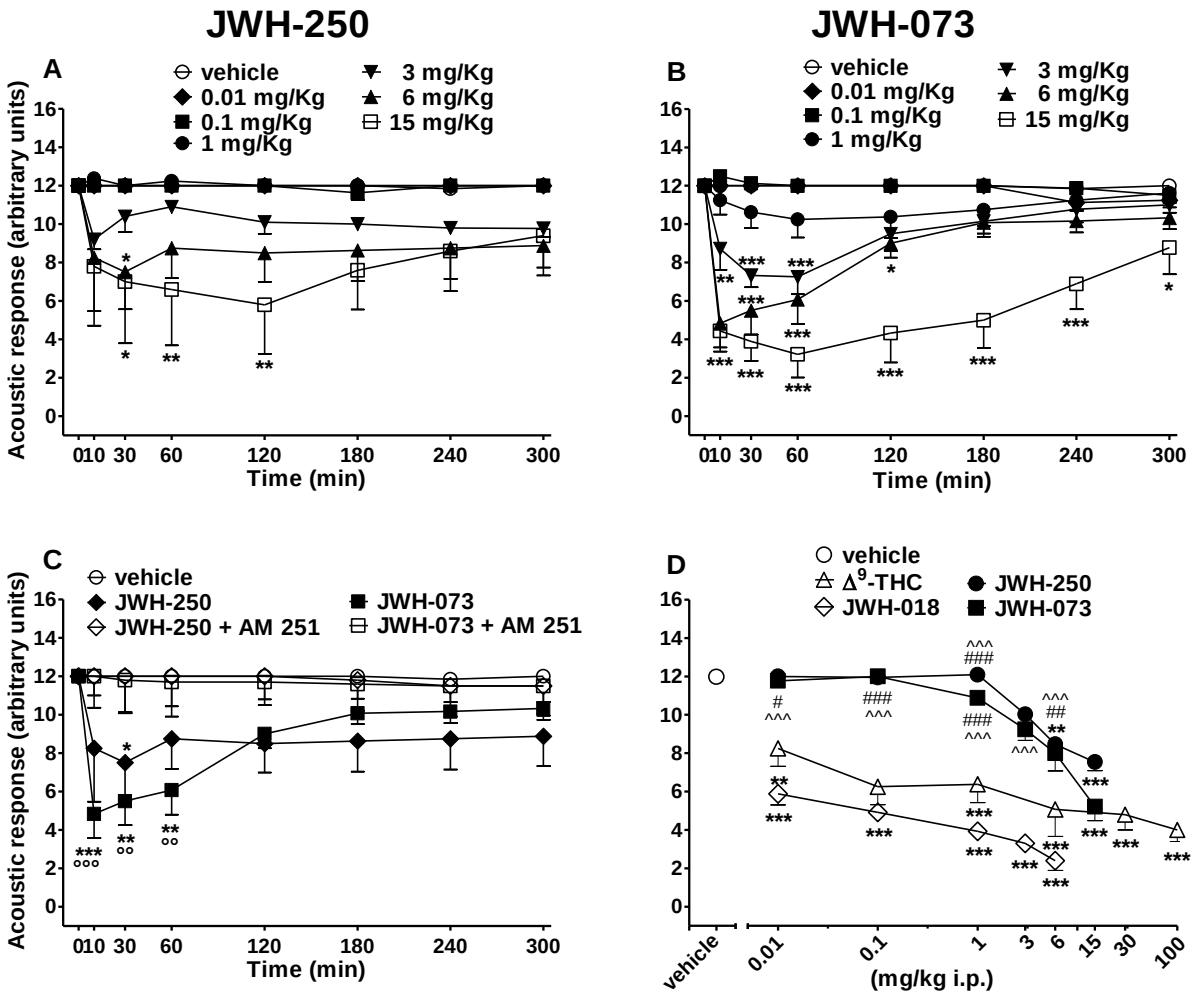
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
JWH-250
JWH-073
JWH-250 + AM 251
JWH-073 + AM 251
Δ9-THC
JWH-018
Acoustic response (arbitrary units)
Time (min)
(mg/kg i.p.)

Figure 3

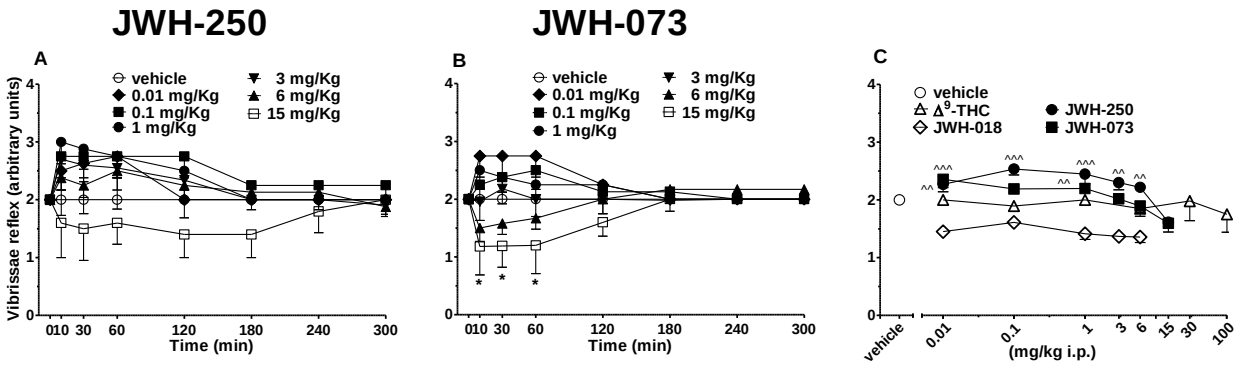
JWH-250
JWH-073
A
B
C
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Vibrissae reflex (arbitrary units)
Time (min)
Δ9-THC
JWH-018
JWH-250
JWH-073
(mg/kg i.p.)

Figure 4

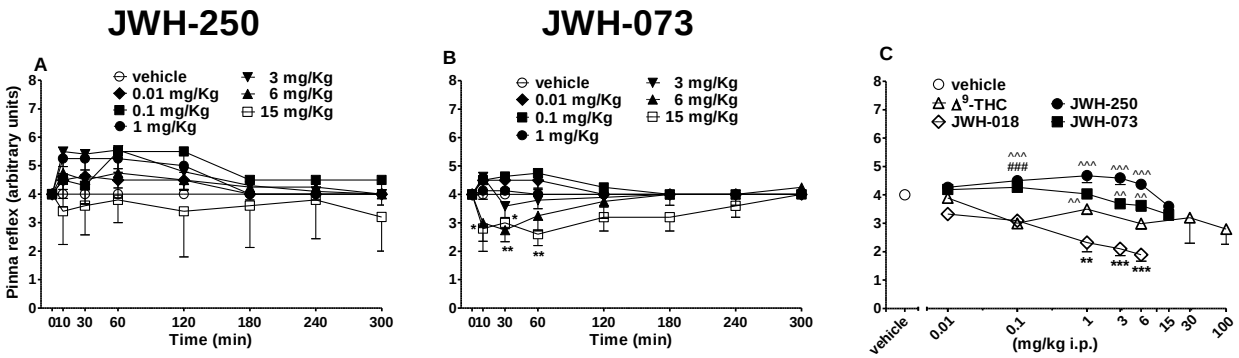
JWH-250
JWH-073
A
B
C
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Pinna reflex (arbitrary units)
Time (min)
Δ9-THC
JWH-018
JWH-250
JWH-073
(mg/kg i.p.)

Figure 5

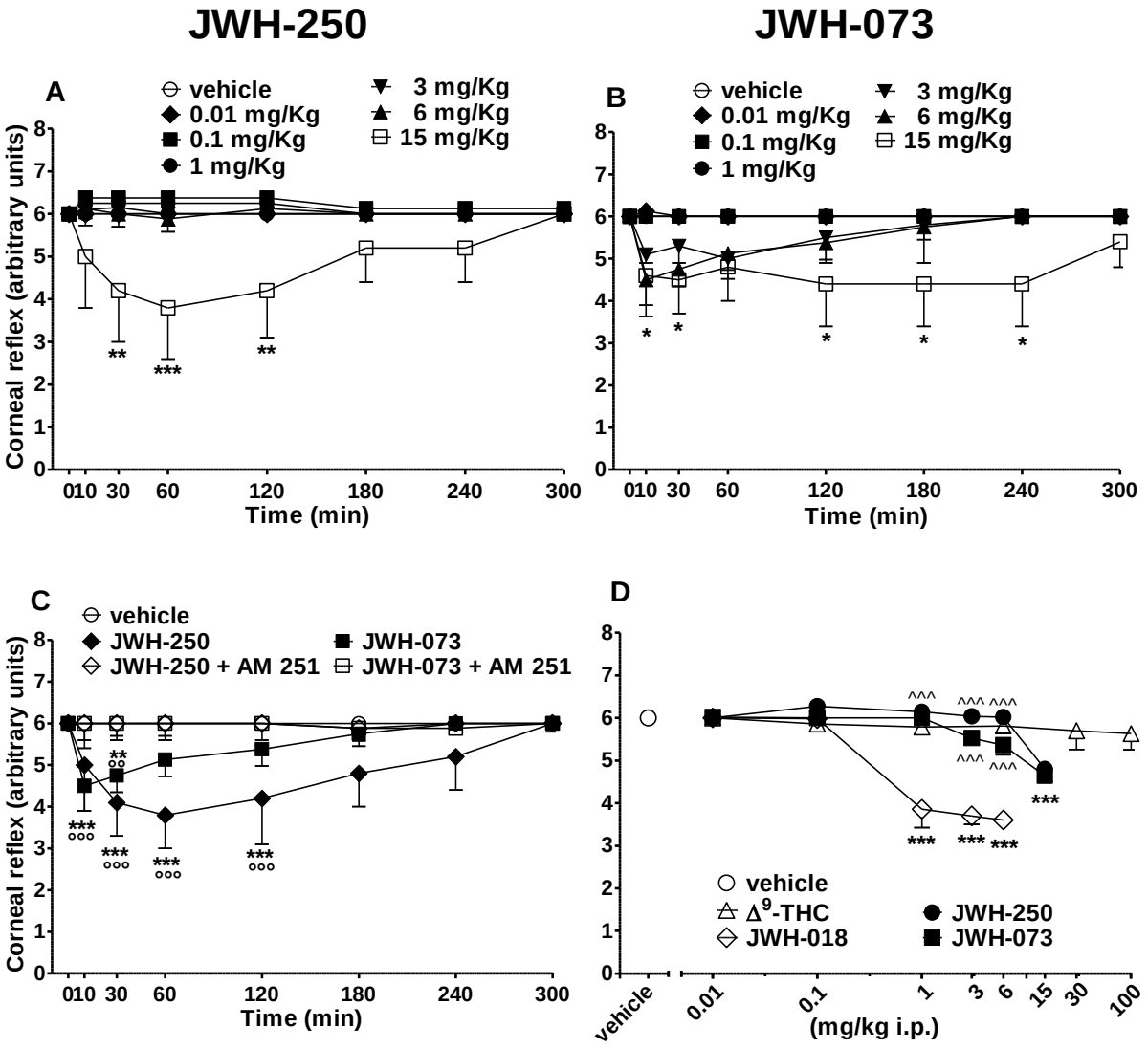
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Corneal reflex (arbitrary units)
Time (min)
JWH-250
JWH-073
JWH-250 + AM 251
JWH-073 + AM 251
Δ9-THC
JWH-018
(mg/kg i.p.)

Figure 6

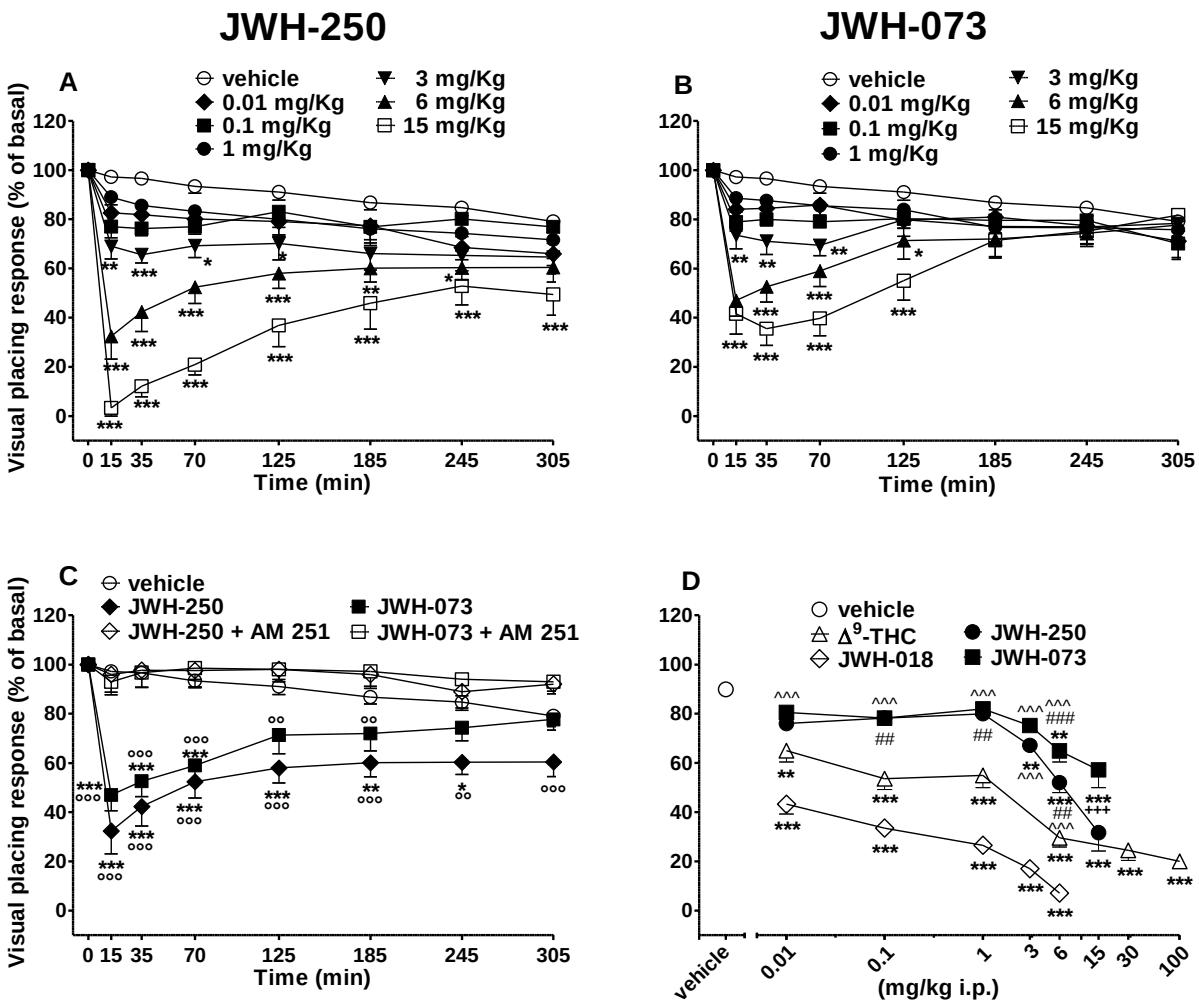
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
JWH-250 + AM 251
JWH-073 + AM 251
Δ9-THC
JWH-018
Visual placing response (% of basal)
Time (min)
(mg/kg i.p.)

Figure 7

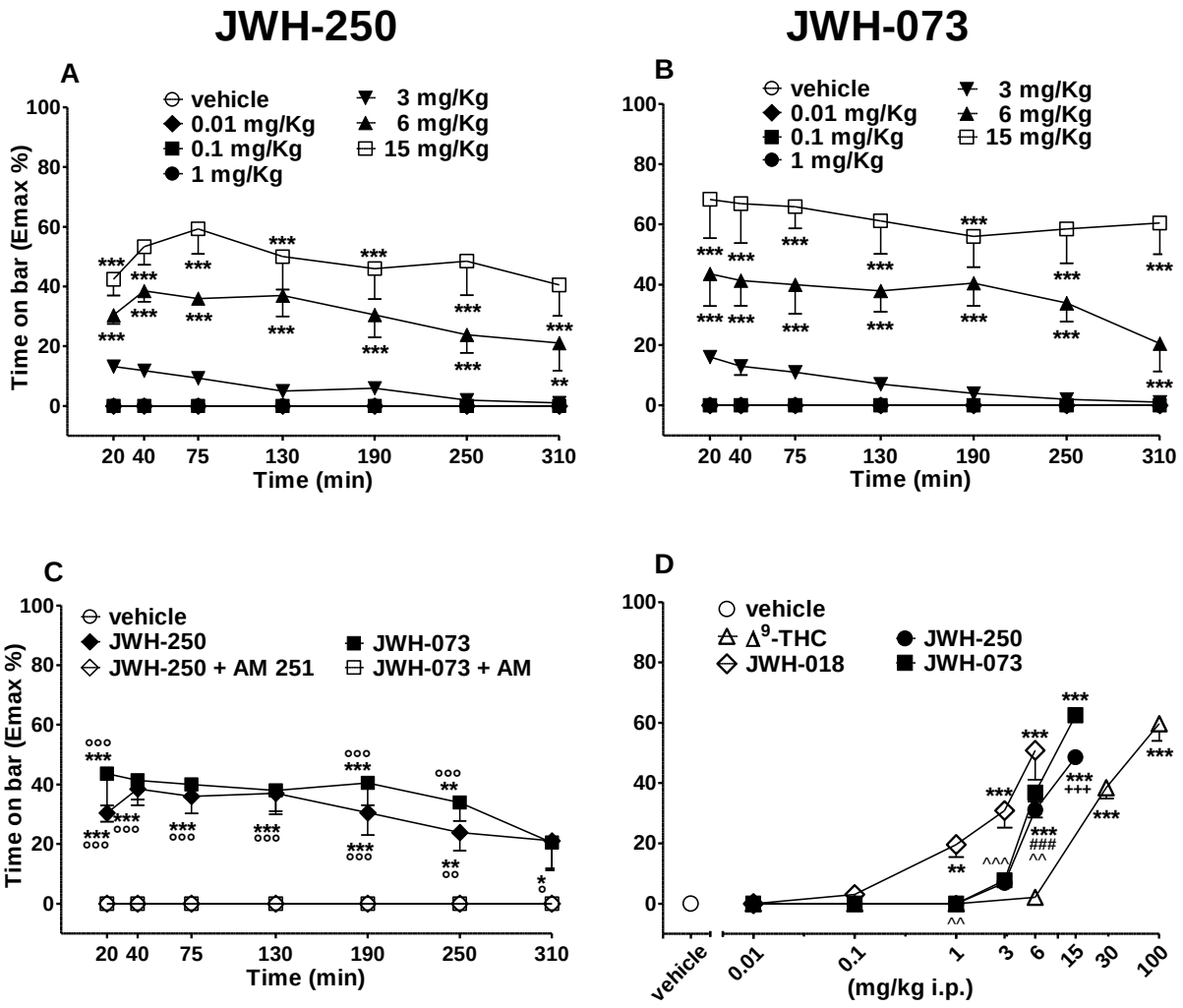
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Time on bar (Emax %)
Time (min)
20 40 75 130 190 250 310
JWH-250 + AM 251
JWH-073 + AM
Δ9-THC
JWH-018
(mg/kg i.p.)
vehicle 0.01 0.1 1 3 6 15 30 100

Figure 8

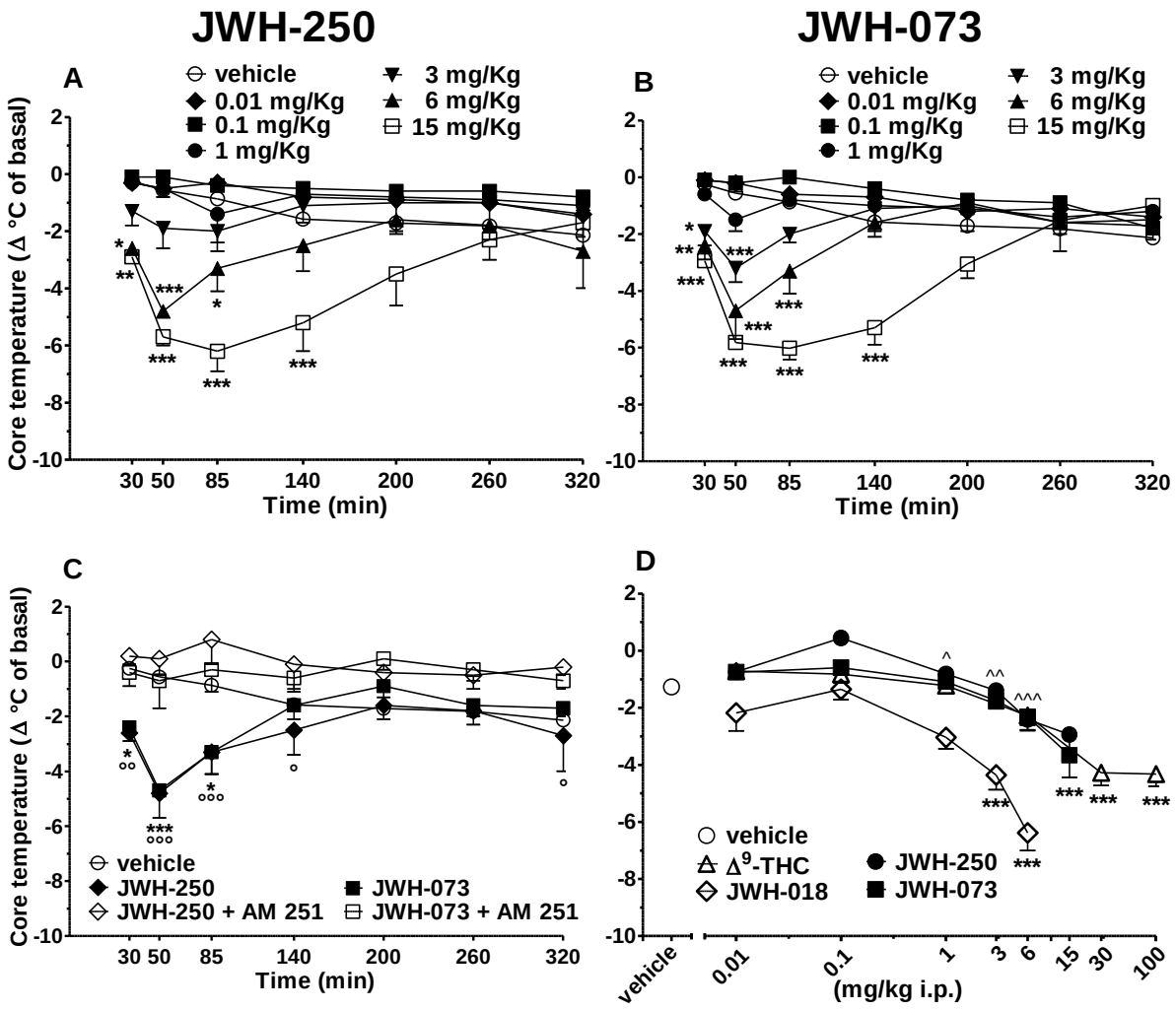
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Core temperature (Δ °C of basal)
Time (min)
30 50 85 140 200 260 320
JWH-250 + AM 251
JWH-073 + AM 251
Δ9-THC
JWH-018
(mg/kg i.p.)
0.01 0.1 1 3 6 15 30 100

Figure 9

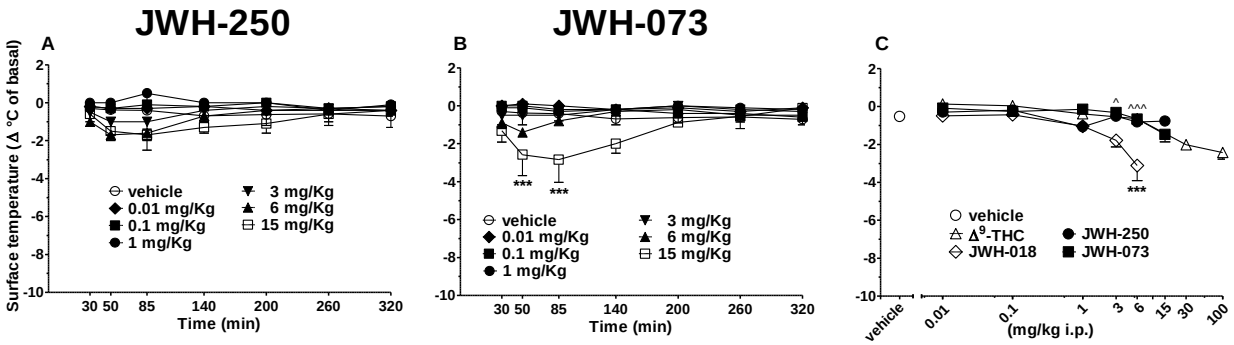
JWH-250
JWH-073
A
B
C
Surface temperature (Δ °C of basal)
Time (min)
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Δ9-THC
JWH-018
JWH-250
JWH-073
(mg/kg i.p.)

Figure 10

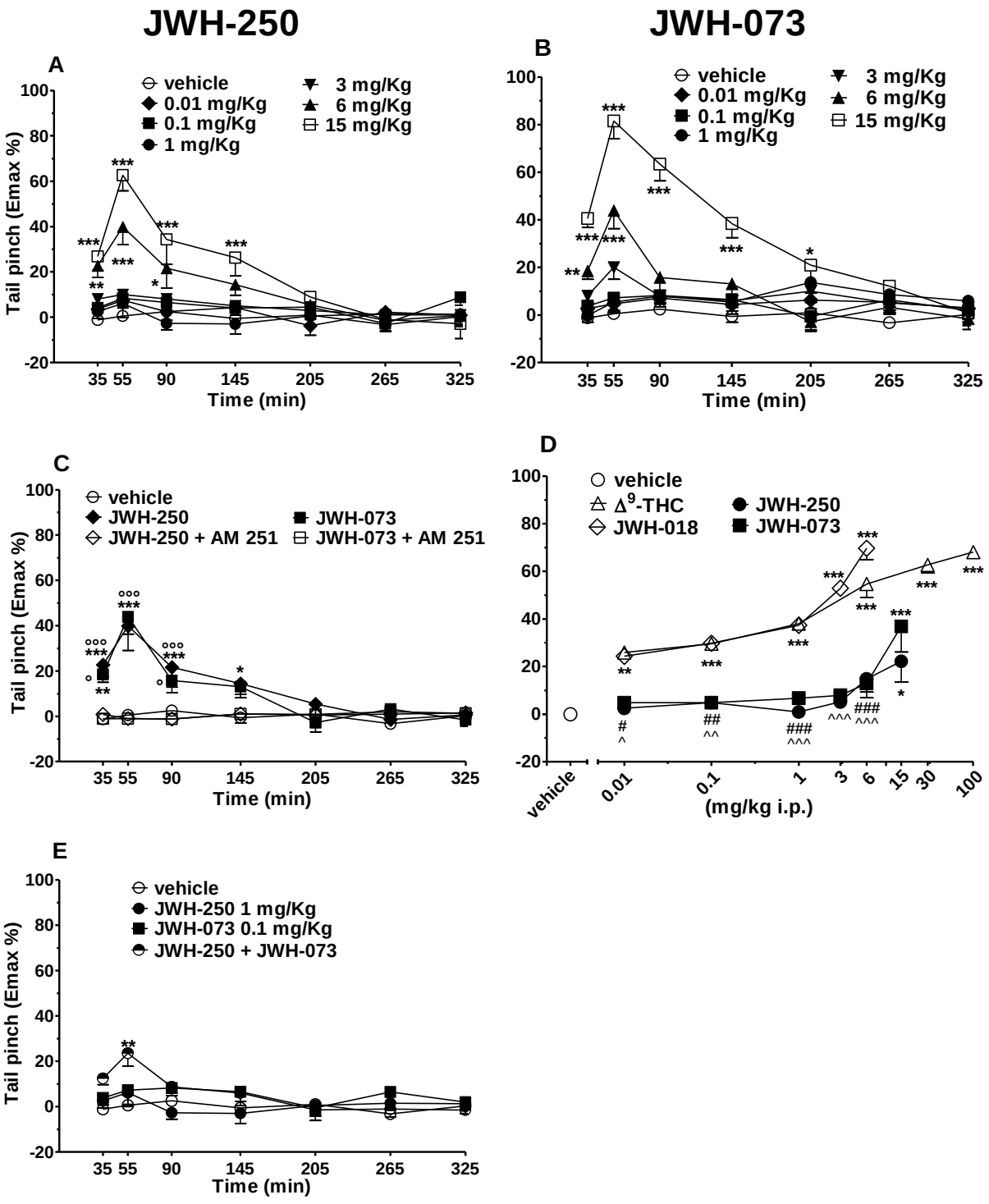
JWH-250
JWH-073
A
B
C
D
E
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Tail pinch (Emax %)
Time (min)
JWH-250 + AM 251
JWH-073 + AM 251
Δ9-THC
JWH-018
(mg/kg i.p.)
JWH-250 1 mg/Kg
JWH-073 0.1 mg/Kg
JWH-250 + JWH-073

Figure 11

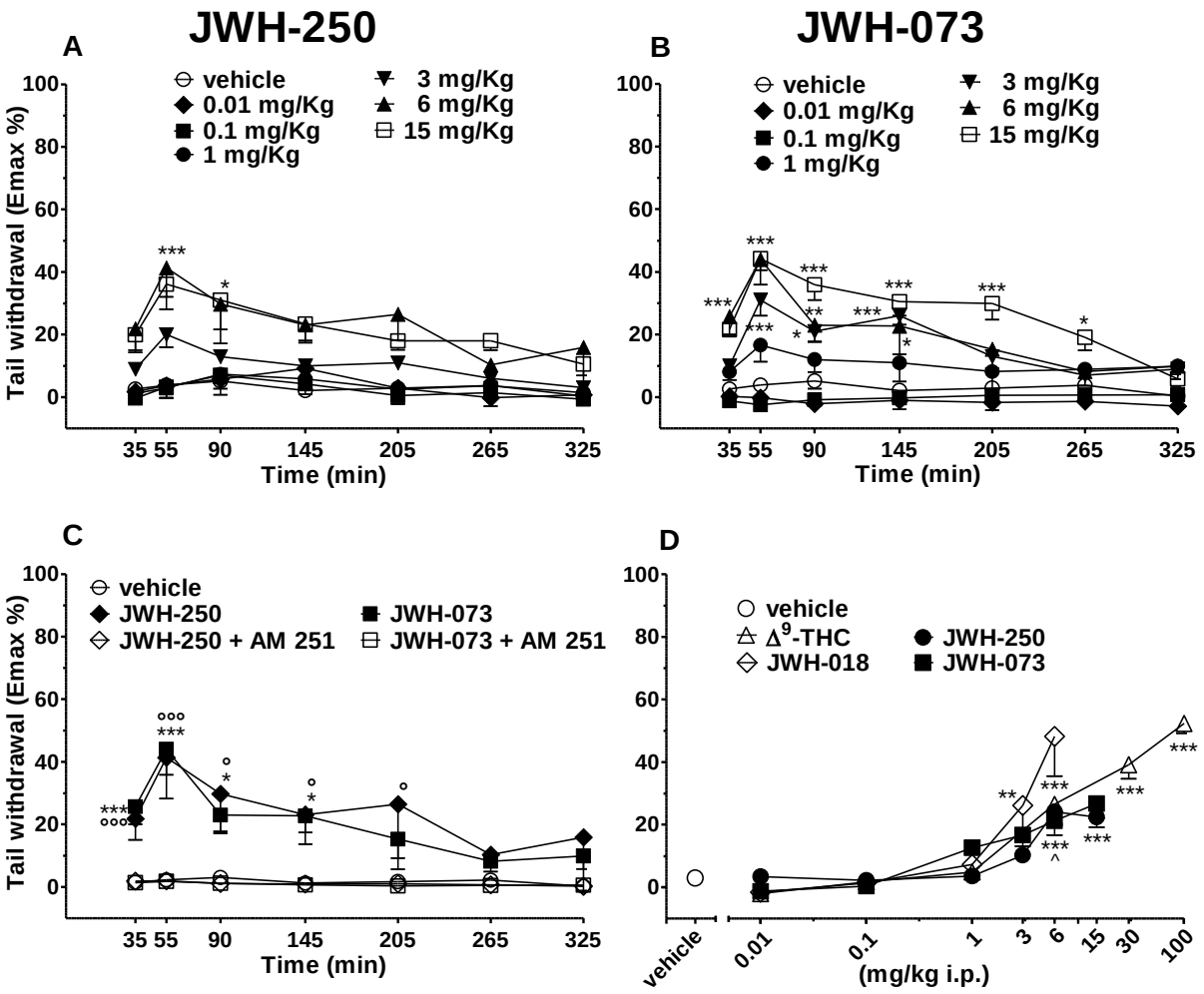
A
JWH-250
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Tail withdrawal (Emax %)
Time (min)
B
JWH-073
C
JWH-250 + AM 251
JWH-073 + AM 251
D
Δ9-THC
JWH-018
(mg/kg i.p.)

Figure 12

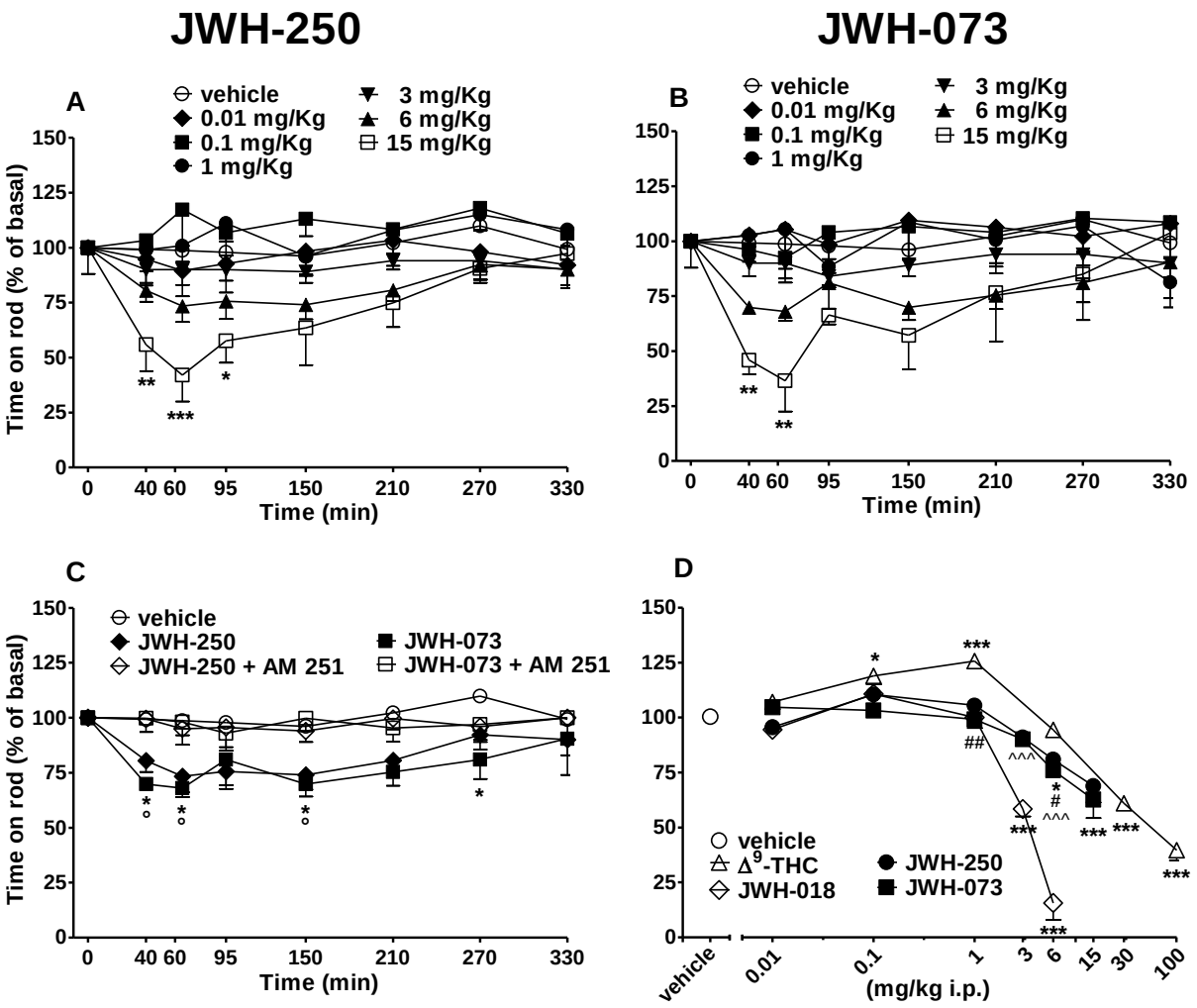
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
Time on rod (% of basal)
Time (min)
JWH-250
JWH-250 + AM 251
JWH-073
JWH-073 + AM 251
Δ9-THC
JWH-018
(mg/kg i.p.)

Figure 13

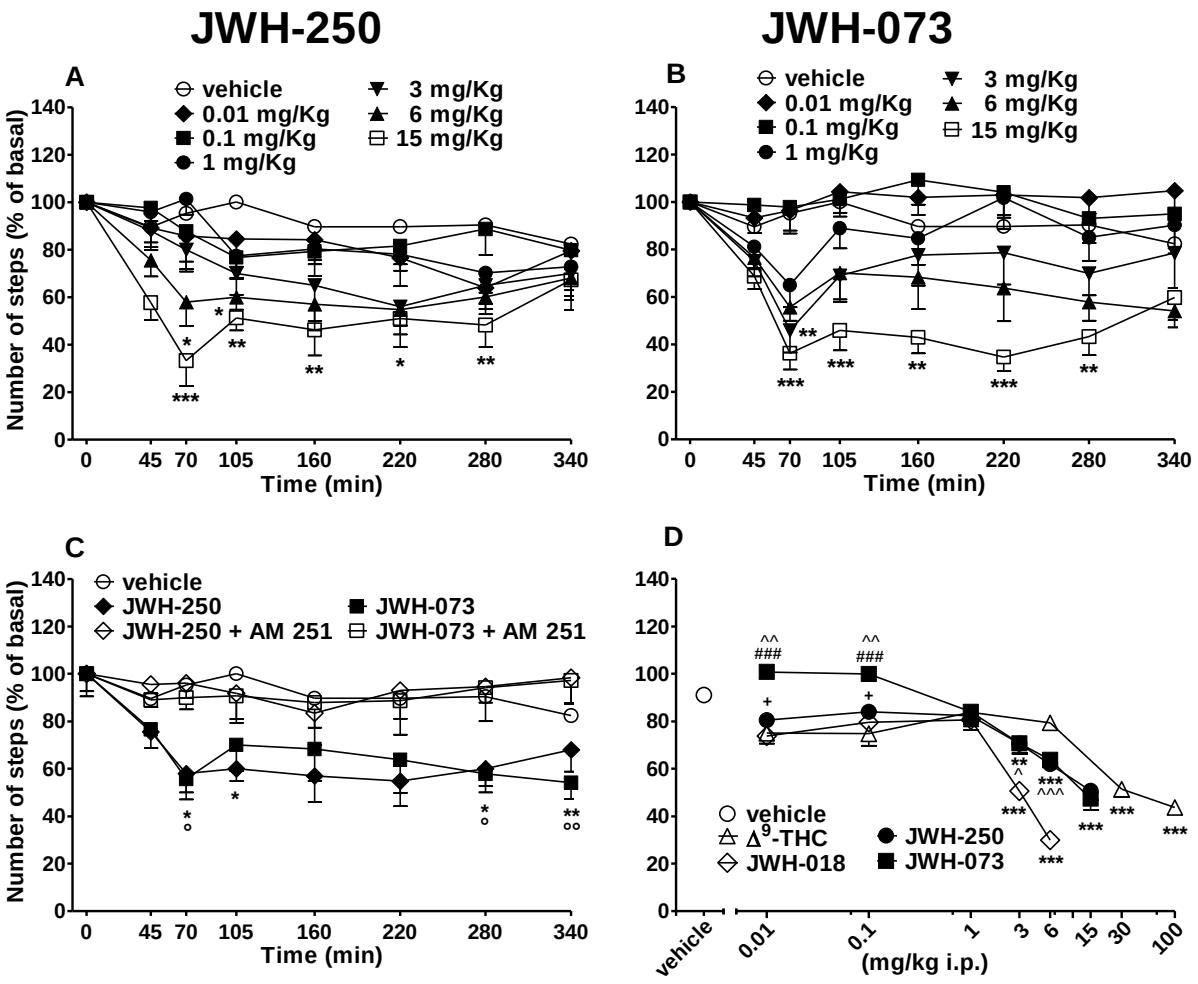
JWH-250
JWH-073
A
B
C
D
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
3 mg/Kg
6 mg/Kg
15 mg/Kg
JWH-250
JWH-073
JWH-250 + AM 251
JWH-073 + AM 251
Δ9-THC
JWH-018
Number of steps (% of basal)
Time (min)
(mg/kg i.p.)

Figure 14

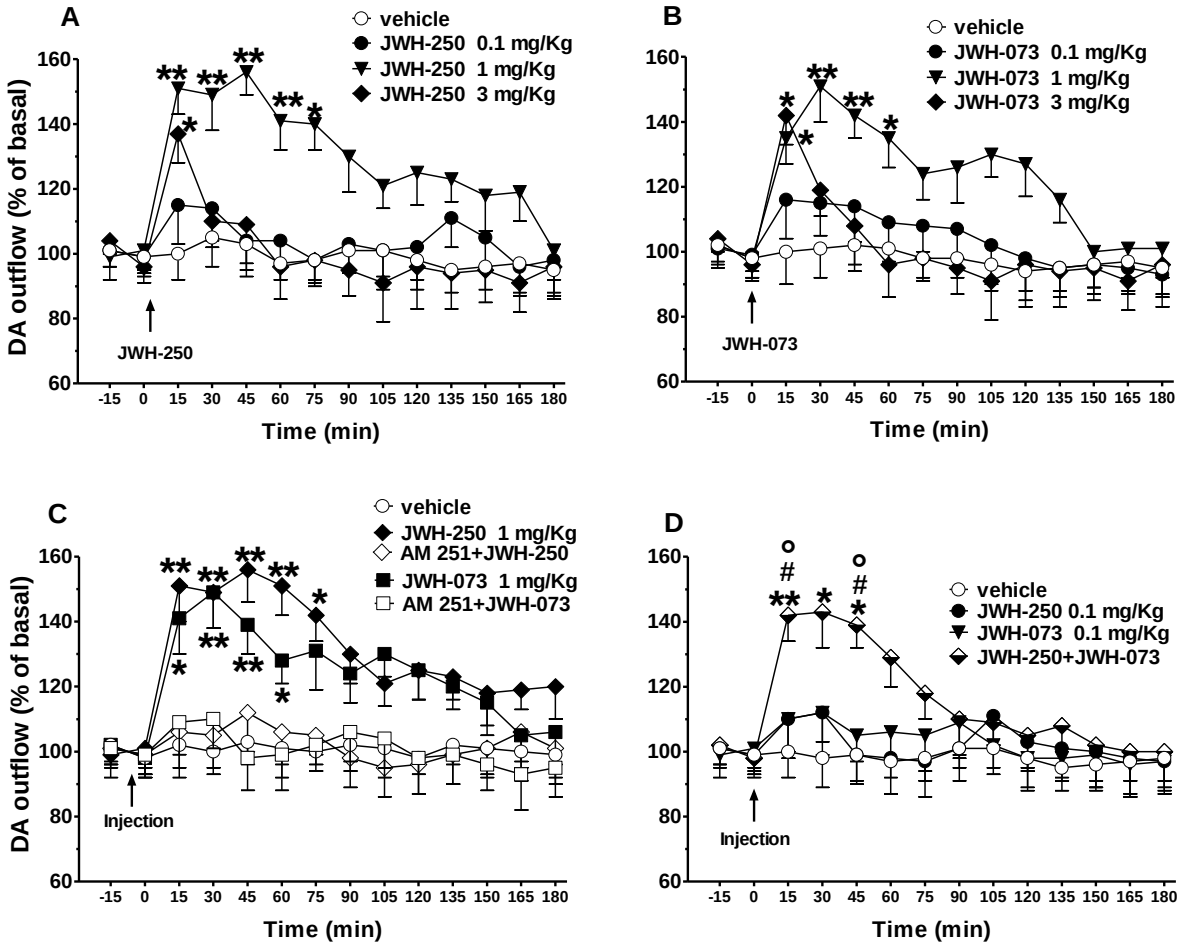
A
vehicle
JWH-250 0.1 mg/Kg
JWH-250 1 mg/Kg
JWH-250 3 mg/Kg
DA outflow (% of basal)
JWH-250
Time (min)
B
vehicle
JWH-073 0.1 mg/Kg
JWH-073 1 mg/Kg
JWH-073 3 mg/Kg
JWH-073
Time (min)
C
vehicle
JWH-250 1 mg/Kg
AM 251+JWH-250
JWH-073 1 mg/Kg
AM 251+JWH-073
DA outflow (% of basal)
Injection
Time (min)
D
vehicle
JWH-250 0.1 mg/Kg
JWH-073 0.1 mg/Kg
JWH-250+JWH-073
Injection
Time (min)

Figure 15

Figure legends

Figure 1. Competition curves of specific [^{3}H]-CP 55940 binding by JWH-073 and JWH-250 in CHO cell membranes transfected with human CB_1 receptors (panel A) or human CB_2 receptors (panel B) and to CB_1 receptors expressed in mouse brain membranes (panel C) or CB_2 receptors expressed in mouse spleen membranes (panel D). Inhibition curves of forskolin-stimulated cAMP accumulation by JWH-073 and JWH-250 in CHO cells transfected with human CB_1 receptors (panel E) or human CB_2 receptors (panel F). Results are given as the mean ± SEM of three independent experiments performed in duplicate.

Figure 2. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the visual object test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[c] and JWH-018 (0.01-6 mg/kg)[c]. Co-administration of ineffective doses of JWH-250 (1 mg/kg i.p.) and JWH-073 (0.1 mg/kg i.p., panel E). Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), for the interaction with the AM 251 (panel C) and for co-administration of JWH-250 and JWH-073 (panel E), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251 + agonist. [c]*Data from Ossato et al., 2015.*

Figure 3. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the acoustic response test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[c] and JWH-018 (0.01-6 mg/kg)[c]. Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B),

for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ##p<0.01, ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018 and °°p<0.01, °°°p<0.001 versus AM 251 + agonist. [c]*Data from Ossato et al., 2015.*

Figure 4. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the vibrissae reflex test in the mouse. Comparison of the total average effect observed in 5 hours (panel C) with Δ^9-THC (0.01-100 mg/kg)[c] and JWH-018 (0.01-6 mg/kg)[c]. Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), while the statistical analysis of the comparison of the total average effect of the compounds (panel C) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05 versus vehicle and ^^p<0.01, ^^^p<0.001 versus JWH-018. [c]*Data from Ossato et al., 2015.*

Figure 5. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the pinna reflex test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[c] and JWH-018 (0.01-6 mg/kg)[c]. Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01 versus vehicle; ###p<0.001 versus Δ^9-THC. ^^p<0.01, ^^^p<0.001 versus JWH-018 and °p<0.05 versus AM 251 + agonist. [c]*Data from Ossato et al., 2015.*

Figure 6. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the corneal reflex test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C).

Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[c] and JWH-018 (0.01-6 mg/kg)[c]. Data are expressed (see material and methods) as arbitrary units and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ^^^p<0.001 versus JWH-018 and °°p<0.01, °°°p<0.001 versus AM 251 + agonist. [c]*Data from Ossato et al., 2015.*

Figure 7. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the visual placing response test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[c] and JWH-018 (0.01-6 mg/kg)[c]. Data are expressed (see material and methods) as percentage of baseline and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ##p<0.01, ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018; +++p<0.001 versus JWH-073 and °°p<0.01, °°°p<0.001 versus AM 251 + agonist. [c]*Data from Ossato et al., 2015.*

Figure 8. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the bar test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Data are expressed as percentage of maximum effect (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for

both the dose response curve of each compounds at different times (panel A, B), and for the interaction with the AM 251 (panel C). while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; ^^p<0.01, ^^^p<0.001 versus JWH-018 ; +++p<0.001 versus JWH-073 °p<0.05, °°p<0.01 and °°°p<0.001 versus AM 251 + agonist. [d]*Data from Vigolo et al., 2015.*

Figure 9. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the core temperature test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Data are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C; see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018 and °p<0.05, °°°p<0.001 versus AM 251 + agonist. [d]*Data from Vigolo et al., 2015.*

Figure 10. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the surface temperature test in the mouse. Comparison of the total average effect observed in 5 hours (panel C) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Data are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C; see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), while the statistical analysis of the comparison of the total average effect of the compounds (panel C) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. ***p<0.001 versus vehicle; ^p<0.05, ^^^p<0.001 versus JWH-018. [d]*Data from Vigolo et al., 2015.*

Figure 11. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the tail pinch test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Co-administration of ineffective doses of JWH-250 (1 mg/kg i.p.) and JWH-073 (0.1 mg/kg i.p., panel E). Data are expressed as percentage of maximum effect (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), for the interaction with the AM 251 (panel C) and for co-administration of JWH-250 and JWH-073 (panel E) while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ##p<0.01, ###p<0.001 versus Δ^9-THC; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018 and °p<0.05, °°°p<0.001 versus AM 251 + agonist. [d]*Data from Vigolo et al., 2015.*

Figure 12. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the tail withdrawal test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Data are expressed as percentage of maximum effect (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B), and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ^p<0.05 versus JWH-018 and °p<0.05, °°°p<0.001 versus AM 251 + agonist. [d]*Data from Vigolo et al., 2015.*

Figure 13. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the accelerod test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C).

Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Data are expressed as percentage of baseline (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, Panel D: significant effect of JWH-250 and JWH-073 versus Δ^9-THC and JWH-018 ($F_{23,191}$=50.63, p<0.0001); ***p<0.001 versus vehicle; #p<0.05, ##p<0.01 versus Δ^9-THC ; ^^^p<0.001 versus JWH-018 and °p<0.05 versus AM 251 + agonist. [d]*Data from Vigolo et al., 2015.*

Figure 14. Effect of the systemic administration (0.01-15 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on the drag test in the mouse. Interaction of effective dose of JWH-R compounds (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg)[d] and JWH-018 (0.01-6 mg/kg)[d]. Data are expressed as percentage of baseline (see material and methods) and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018; +p<0.05 versus JWH-073 and °p<0.05, °°p<0.01 versus AM 251 + agonist. [d]*Data from Vigolo et al., 2015.*

Figure 15. Effect of the systemic administration (0.1-3 mg/kg i.p.) of JWH-250 (panel A) and JWH-073 (panel B) on DA transmission in the NAc shell of mice. Interaction of effective dose of JWH-R compounds (1 mg/kg, i.p.) with the selective CB_1 receptor antagonist AM 251 (1 mg/kg, i.p.; panel C). Co-administration of ineffective doses of JWH-250 (0.1 mg/kg i.p.) and JWH-073 (0.1 mg/kg i.p., panel D). Data are expressed as percentage of basal values (see material and methods) and represent the mean ± SEM of 5-8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B),

for the interaction with the AM 251 (panel C) and for the co-administration studies (panel D). *p<0.05, **p<0.01, versus vehicle; #p<0.05 versus JWH-250; and °p<0.05, versus JWH-073

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Supplementary Material

Identification of JWH-250 and JWH-073 in the herbal extract by ESI-Q-TOF-HPLC-MS analysis.

Fig 1S

Mass chromatograms of ESI-Q-TOF-HPLC-MS analysis. HPLC-MS analysis of herbal extract (Panel A). MS analysis of compounds at 9.5 min time retention (Panel B).

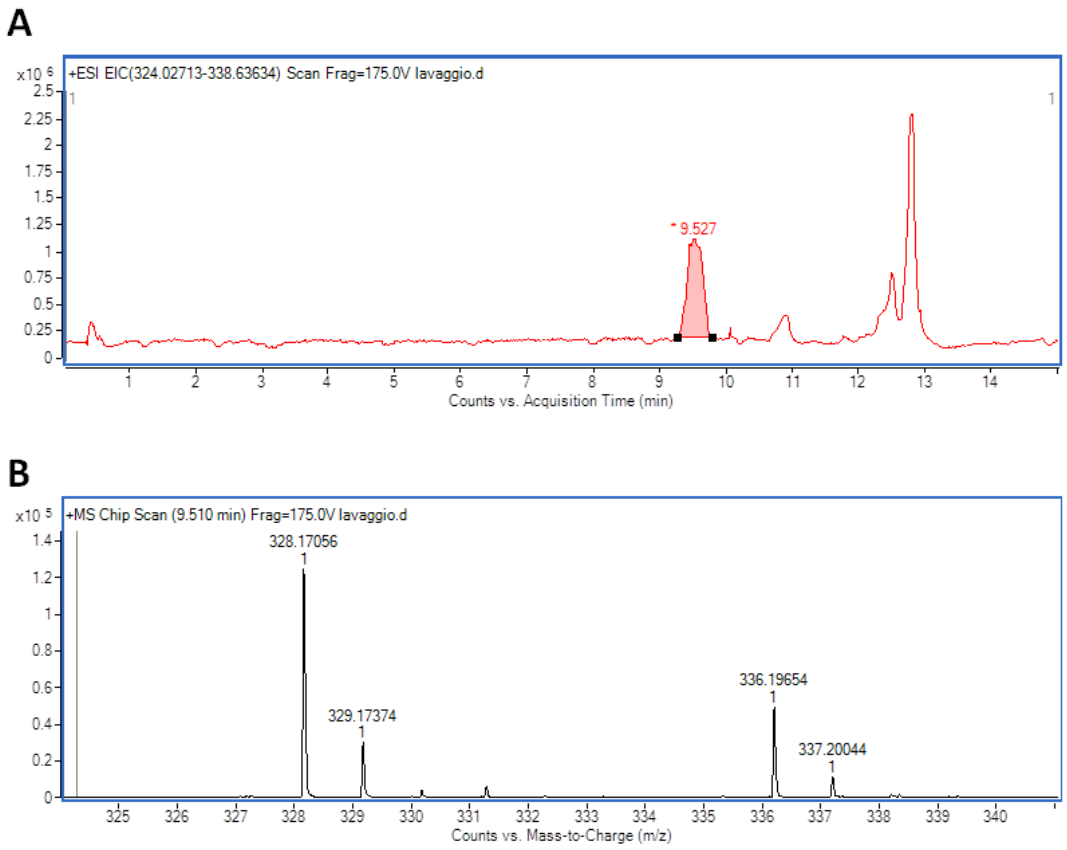

Fig 2S

MS analysis showed two $[M+H]^+$ ions at 328.17056u and 336.19654u that could correspond at the JWH-073 and JWH-250 chemicals structures with less than 3 ppm errors.

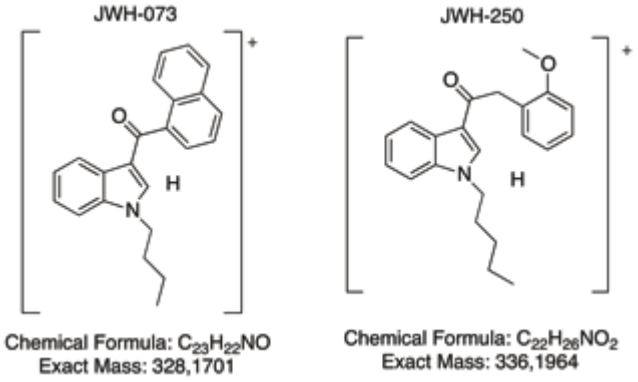

Fig 3S

Mass spectra of JWH-073 (Panel A) and JWH-250 (Panel B) with reported their fragmentation products. Mass spectra analysis of the fragment of JWH-250 with MW of 214.12370 (Panel C).