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Abstract: JWH-018 is a synthetic CB1 and CB2 agonist illegally marketed as products named "Spice" or "herbal blend" for its psychoactive effects much higher than those produced by Cannabis. In the last year, the European Monitoring Centre for Drugs and Drug Addiction reported to the Italian National Early Warning System the seizure of plant material containing new halogenated derivatives of JWH-018 (JWH-018 Cl and JWH-018 Br). The present study was aimed at investigating the in vitro and in vivo activity of these two novel synthetic cannabinoids in mice. In vitro competition binding experiments performed on mouse and human CB1 receptors revealed a high affinity and potency of the halogenated compounds. Synthetic cannabinoids (0.01-6 mg/Kg i.p.) impaired motor activity and induced catalepsy in mice and their effects were more severe respect that evoked by Δ9-THC. Moreover, they increased mechanical and thermal pain threshold and induced a marked hypothermia. It is interesting to note that whereas high doses of JWH-018 causes seizures, myoclonia and hyperreflexia, the halogenated compounds were less effective. Behavioral and neurological changes were prevented by the selective CB1 receptor antagonist AM 251. These data for the first time demonstrated that JWH-018 Cl and JWH-018 Br act similarly to JWH-018 but they induce fewer neurological side effects, supporting the hypothesis that the halogenated compounds could have been introduced in the internet market in order to maintain an activity similar to that of JWH-018 but with a lower risk of side effects and therefore a lower detection by the Early Warning Systems

*Cover letter

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VNIVERSITÀ
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DI·FERRARA

Prof Thomas Gould
Neuropharmacology
Editor

Ferrara, September 25, 2014

Dear Prof Gould,

I take pleasure in submitting the enclosed paper entitled "**Novel halogenated derivates of JWH-018: behavioral and binding studies in mice"** by myself and colleagues for publication in **Neuropharmacology**.

The discovery of these new halogenated derivatives (N-(5-chloro-pentyl)- and N-(5-bromo-pentyl)) of JWH-018 in the illegal market and the lack of pharmacological and toxicological information suggested the need to study their in vitro and in vivo pharmacological profile to quickly understand their main adverse effects.

In recent preliminary studies (personal communication at national and international meetings) we provided evidence that the JWH-018Cl and JWH-018Br impaired sensory motor functions and object recognition memory in mice.

In the present study in vitro competition binding experiments performed on mouse and human CB_1 receptors revealed a high affinity and potency of the halogenated compounds. Synthetic cannabinoids impaired motor activity and induced catalepsy in mice and their effects were more severe respect that evoked by Δ^9-THC. Moreover, they increased mechanical and thermal pain threshold and induced a marked hypothermia. It is interesting to note that whereas high doses of JWH-018 causes seizures, myoclonia and hyperreflexia, the halogenated compounds were less effective.

In these study for the first time we demonstrated that JWH-018Cl and JWH-018Br act similarly to JWH-018 but they induce fewer neurological side effects, supporting the hypothesis that the halogenated compounds could have been introduced in the internet market in order to maintain an activity similar to that of JWH-018 but with a lower risk of side effects and therefore a lower detection by the Early Warning Systems.

We hope that our manuscript will be favorably considered by the Editors of **Neuropharmacology** and thank you for attention given to our work.

Sincerely

Matteo Marti

Highlights

- JWH-018Cl and JWH-018Br are new halogenated cannabinoids seized in Internet Market
- JWH-018-R compounds induced adverse effects through the stimulation of CB-1 receptor
- JWH-018Br act similarly to Δ^9-THC and could replace JWH-018 in the illegal market

*Title page

Novel halogenated derivates of JWH-018: behavioral and binding studies in mice.

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Abbreviations

AM 251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide
JWH-018	1-pentyl-3-(1-naphthoyl)indole
JWH-018Cl	(1-(5-chloro-pentyl)-3-(1-naphthoyl)indole)
JWH-018Br	(1-(5-bromo-pentyl)-3-(1-naphthoyl)indole)
JWH-018-R	JWH-018, JWH-018Cl and JWH-018Br
Δ^9-THC	(-)-Δ^9-THC or Dronabinol®

Abstract

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Keywords: Δ^9-THC; JWH-018; JWH-018 Br; JWH-018 Cl; CB_1 receptor; synthetic cannabinoids

Chemical compound studied in this article

AM 251 (PubChem CID: 2125); JWH-018 (PubChem CID: 10382701); Δ^9-THC (PubChem CID: 16078)

1.Introduction

JWH-018 (1-pentyl-3-(1-naphthoyl)indole) is a synthetic cannabinoid agonist developed in the early 1990's (Huffman et al., 1994) from a computational melding of the chemical structural features of Δ^9-tetrahydrocannabinol (Δ^9-THC) with the prototypic aminoalkylindole WIN 55,212-2 (D'Ambra et al., 1992). JWH-018 binds and activates in the low nanomolar range the CB_1 (Ki=9 nM) and CB_2 (Ki= 3nM) receptors (Huffman et al., 1994; Wiley et al., 1998), showing approximately four-fold increased activity to the CB_1 and about ten-fold affinity to the CB_2 receptor compared with Δ^9-THC (Auwarter et al., 2009). This aminoalkylindole is the first synthetic cannabinoid ever reported through the Early Warning System (EMCDDA 2009; Uchiyama et al., 2010) and marketed in "Spice" and "herbal blend" for its psychoactive effects similar to those produced by Cannabis. However, in addition to the "desired" psychoactive action, clinical data reported that JWH-018 induces significant psychiatric and physical adverse effects in consumers. The most common psychiatric effects reported were anxiety, psychosis, hallucination and alterations in cognitive abilities, while physical effects ranging in severity from nausea to more serious sympathomimetic-like symptoms such as psychomotor agitation, diaphoresis, palpitations, tachycardia, tachyarrhythmia, hyperreflexia and generalized convulsions (Auwarter et al., 2009; Hermanns-Clausen et al., 2013; Seely et al., 2012). In vivo animal studies revealed that JWH-018 reproduces the typical "tetrad" effects of Δ^9-THC as hypothermia, analgesia, hypolocomotion, akinesia (Brents et al., 2011; Macri et al., 2013; Wiebelhaus et al., 2012; Wiley et al., 2012) when delivered both via inhalation (Poklis et al., 2012; Wiebelhaus et al., 2012) or systemic injection (Fantegrossi et al., 2014; Wiley et al., 1998) with same differences in final "tetrad" symptoms between the two routes of administration (Marshell et al., 2014). Moreover, JWH-018 produces anxiolysis and depressive-related behavior in mice (Macri et al., 2013), sensory-motor and cardio-respiratory alterations (Marti et al., 2013b; Marti et al., 2014) and impaired more potently than Δ^9-THC working memory in adult mice (Marti et al., 2013a). In particular, the cannabinoid "tetrad" (Compton et al., 1992) has been extremely useful in the characterization of the biological activity of natural and synthetic agonist at CB_1 receptors. Ligands that fully activate cannabinoid receptors to produce maximal effects in a given system (i.e "tetrad") are referred to high efficacy agonists. In contrast, agonists that result in reduced maximal effects when compared to full agonists are designated as low efficacy agonists. Interestingly, Δ^9-THC that is a partial agonist both in vitro (Govaerts et al., 2004) and in vivo (Paronis et al., 2012) tends to elicit tetrad effects of similar magnitude to higher efficacy cannabinoids such as WIN-55,212-2 and CP-55,940 (Fan et al., 1994).

In the last year, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported to the Italian National Early Warning System (NEWS) the seizure of plant material

containing halogenated derivatives (N-(5-chloro-pentyl)- and N-(5-bromo-pentyl)) of JWH-018 (Fig 1). The discovery of these new halogenated derivatives of JWH-018 in the illegal market and the lack of pharmacological and toxicological information suggested the need to study their in vitro and in vivo pharmacological profile to quickly understand their main adverse effects. In fact, it is well known that halogenation of cannabinoid structure may lead to significant changes in the compound potency and affinities for the CB_1 receptors (Nikas et al., 2004; Wiley et al., 2014) as well as potential changes in the pharmacokinetic properties. Thus, the present study was aimed at investigating the effects of acute exposure to JWH-018, JWH-018 Cl and JWH-018 Br on main neurological changes, core and skin body temperature, modulation of acute thermal and mechanical pain stimuli and motor activity in CD-1 mice. Moreover, in vitro competition binding experiments were carried out to determine the selectivity and potency of action of the halogenated compounds for the CB_1 receptor. To better understand the behavioral profile of the JWH-018-R compounds the Δ^9-THC was used as a reference molecule and the effects were monitored for over 5 hours.

2. Material and Methods.

2.1. Animals

Male ICR (CD-1®) mice, 25-30 gr (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed on a reverse 12:12-h light-dark cycle, temperature of 20-22 °C, humidity of 45-55% and were provided 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 novel European Communities Council Directive of September 2010 (2010/63/EU) revising the Directive 86/609/EEC and were approved by Italian Ministry of Health (license n. 114/2013B) and by Ethical Committee of the University of Ferrara. Moreover, adequate measures were taken to minimize the number of animals used and animal pain and discomfort.

2.2. Drug Preparation

JWH-018 and (-)-Δ^9-THC (Dronabinol®) were purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy) while AM 251 from Tocris (Tocris, Bristol, United Kingdom). JWH-018 Cl and JWH-018 Br were purchased on Internet, isolated and purified by chromatography (in the laboratory of Dott. Claudio Trapella) with a medium pressure system ISOLERA ONE (Biotage Sweden) and subsequently characterized by Agilent 6520 nano HPLC ESI-Q-TOF (Agilent Technologies) and a Varian 400MHz NMR. 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-018-R compounds and Δ^9-THC injections. Drugs were administered by intraperitoneal injection in a volume of 4ul/gr.

2.3. In vitro assays

2.3.1. Mouse brain membrane preparation

After mice were sacrificed by cervical dislocation, brain was 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 pellet was then suspended in a buffer containing 50 mM Tris HCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.5% BSA, pH 7.4 at 30°C and used for competition binding experiments (Vincenzi et al., 2013).

2.3.2. 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, 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).

2.3.3. [^{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 a membrane suspension of mouse brain (40 µg protein/100 µl) for CB_1 binding experiments. Additional competition binding experiments were performed incubating [^{3}H]-CP-55,940 (0.5 nM) 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). 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 WIN 55,212-2 (1 µM). 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).

2.3.4. Cyclic AMP assays

CHO cells transfected with human CB_1 (hCB_1) 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 compounds in comparison with a well known CB agonist, WIN 55,212-2 was studied in the presence of forskolin 1 µM. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay (Vincenzi

et al., 2013).

2.4. Behavioural studies

The compounds were studied using a battery of behavioural tests widely used for the preclinical characterization of cannabinoid ligands and validated to describe the typical “tetrad” effect and the major neurological changes in mice (Compton et al., 1992). To reduce the number of animals used, mice were evaluated in functional observational and behavioral tests carried out in a consecutive manner according to the following time scheme: observation of main neurological changes, measures of internal (rectal measurement) and external (skin measurement) body temperature, determination of the mechanical (tail pinch) and thermal (tail withdrawal) acute pain, evaluation of catalepsy and stimulated motor activity (drag and rotarod test).

2.4.1. Major neurological changes

Functional observational behaviour (FOB; modified from Irwin, 1968) was made immediately after drug administration to detect convulsions, hyperreflexia, myoclonus, tremors and tail elevation in mice treated with synthetic cannabinoids. 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).

2.4.2. Evaluation of the internal and external body temperature

To better assess the effects of the molecules on thermoregulation, we measured both changes in the internal (rectal) and external (skin) temperature. Rectal body temperature was used as an index for total body heat and skin temperature was used as an index for the blood flow to the skin (and therefore, of heat dissipation/conservation) at various times during the experiment. The internal 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; Ruzza et al., 2012). The probe was connected to a digital thermometer Cole Parmer model 8402. Stress was equalized to a normal routine clinical procedure. The skin temperature was measured by a digital infrared thermometer Microlife FR 1DZ1 (Microlife AG Swiss Corporation, Widnau/Switzerland) placed at 1 cm from the surface of the abdomen of the mouse. The measurement time was approximately 3-5 sec. Internal (rectal) and external (skin) mouse body temperatures were measured at 10, 30, 60, 120, 180, 240 and 300 min post injection.

2.4.3. Evaluation of pain induced by a mechanical stimulus

Acute mechanical nociception was evaluated using the tail pinch test (modified by Maeda et al., 2005). 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. As soon as the mouse felt the weight and wiggle 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 at tissue. The test was repeated three times and the final value was calculated as the average of 3 obtained scores. Acute mechanical nociception was measured at 20, 40, 75, 130, 190, 250, and 310 min post injection.

2.4.4. Evaluation of pain induced by a thermal stimulus.

Acute thermal nociception was evaluated using the tail withdrawal test (Calo et al., 1998). The animals were placed on a supportive cylinder and half of the tail was dived in water of 48 °C. The latency (in seconds) or time that the tail was left in water was counted. 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 30, 50, 85, 140, 200, 260 and 320 min post injection.

2.4.5. Motor activity assessment.

Alterations of motor activity induced by JWH-018, JWH-018 Cl, JWH-018 Br and Δ^9-THC were measured using a battery of behavioural tests validated to specifically assess different aspects of motor behaviour (Marti et al., 2005; Marti et al., 2004) in static (bar test) and dynamic conditions (drag and accelerod test).

2.4.5.1. Bar test

It measures the grade of akinesia/catalepsy, that 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 40, 60, 95, 150, 210, 270 and 330 min post injection.

2.4.5.2. Drag test

The 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., 2005; Marti et al., 2004). It provides

information about the time that the mouse takes to start and run a movement (bradykinesia). 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. Drag test was performed at 40, 60, 95, 150, 210, 270 and 330 min post injection.

2.4.5.3. Accelerod test

The test measures different motor parameters as the motor coordination, the locomotive ability (akinesia/bradykinesia), the balance ability, the muscular tone and the 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. Accelerod test was performed at 45, 70, 105, 150, 220, 280 and 340 min post injection.

2.5. Data and statistical analysis

The protein concentration was determined according to a Bio-Rad method with bovine serum albumin as reference standard. Inhibitory binding constants, Ki, was 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 (GraphPad Prism, USA). All the data are expressed as the mean ± SEM of 3 independent experiments. Core and skin temperature values were 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) were 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), Δ°C (core and skin temperature), Emax% (tail withdrawal, tail pinch and bar test), percentage of basal (drag test and accelerod test). The statistical analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies in histograms were performed by two-way ANOVA followed by Bonferroni 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, San Diego, CA).

3. Results

3.1. Affinity and potency of JWH-018 Cl and JWH-018 Br in comparison with JWH-018

In hCB_1 cell membranes, competition binding experiments resulted in Ki values in the nanomolar range for JWH-018 Cl and JWH-018 Br (Table 1). The affinity value of the reference compound JWH-018 (9.53 ± 0.88 nM; Table 1) was similar to those found for the novel halogenated derivatives with JWH-018 Cl showing a slightly higher affinity than the other examined compounds (3.92 ± 0.31 nM; Table 1). Tested compounds were also able to bind hCB_2 receptors with high affinity values suggesting their non-selectivity versus hCB_1 receptors (Table 1). The affinity values of JWH-018 and its halogenated derivatives obtained in mouse brain membranes were comparable to those found in human CB_1 receptors. Cyclic AMP assays performed in hCB_1 CHO cells revealed a good potency of the examined compounds with a lower IC_{50} value for JWH-018 Cl (Table 1). The complete inhibition of forskolin-stimulated cAMP production suggests that JWH-018 Cl and JWH-018 Br behave as full agonists showing a maximum effect comparable to that of JWH-018.

3.2. Behavioural studies

3.2.1. Major neurological changes

Administration of high dose (6 mg/Kg, i.p.) of JWH-018-R compounds induced spontaneous and handling-induced convulsions, hyperreflexia, myoclonias and tail elevation in mice that were not observed after the administration of Δ^9-THC (Table 2). In particular, convulsions were evoked in the 70% and 30% of animal treated respectively with JWH-018 and JWH-018 Cl, while the brominated compound was ineffective. JWH-018 induced seizures with shorter latency ($t=4.689$, $df=18$, $P=0.0002$) and longer duration ($t=2.793$, $df=18$, $P=0.012$) than those produced by JWH-018 Cl (Table 2). Hyperreflexia was observed in 80%, 100% and 50% of the animal treated with JWH-018, JWH-018 Cl and JWH-018 Br respectively. JWH-018 induced hyperreflexia with shorter latency ($F_{2,27}=5.417$, $P=0.01$) only with respect to JWH-018 Br (Table 2). Myoclonias were present in the 80% and 30% of animal treated respectively with JWH-018 and JWH-018 Cl, while JWH-018 Br was ineffective. JWH-018 induced myoclonias with shorter latency ($t=4.698$, $df=18$, $P=0.0002$) and longer duration ($t=4.827$, $df=18$, $P=0.0001$) than those caused by JWH-018 Cl (tab 2). Finally, the JWH-018-R compounds induced tail elevation in mice with comparable latency and duration. The rank order potency of the compounds studied in inducing neurological changes was JWH-018>JWH-018 Cl>JWH-018 Br=Δ^9-THC (Table 2). The neurological changes were

prevented by the pretreatment with the selective CB-1 receptor antagonist AM 251 (6 mg/Kg, i.p. injected 20 min before JWH-018-R administration; data not shown).

3.2.2. Evaluation of the internal and external body temperature

Systemic administration of JWH-018-R compounds (0.01-6 mg/kg ip) reduced both core (Fig 2) and skin (Fig 3) temperatures in mice, while the treatment with Δ^9-THC in the same range of doses (0.01-6 mg/kg ip) was ineffective (Fig 2-3). In particular, JWH-018 induced a transient reduction in core temperature at 1 mg/Kg (-5°C at 60 min time point) and a prolonged and significant hypothermia at 6 mg/Kg (-8.55°C at 60 min time point; Fig 2 A) that was maintained up to 300 minutes. JWH-018 Cl (Fig 2 C) and JWH-018 Br (Fig 2 D) also induced a prolonged and marked hypothermia at 6 mg/Kg (~-9.5°C and ~-6.1°C at 60 min time point respectively) in mice. The halogenated compound were ineffective in the range of doses of 0.01-1 mg/Kg. The resulted rank order potency of the compounds studied in inducing core hypothermia was JWH-018=JWH-018 Cl>JWH-018 Br>Δ^9-THC (Fig 2 E). Internal body hypothermia was matched by a reduction of the external body temperature which was observed only at the higher dose tested (6 mg/Kg; Fig 3). The maximal effects were observed at 120 min time point for the JWH-018 (~-8.5°C, Fig 3B), JWH-018 Cl (~-8.5°C, Fig 3C) and JWH-018 Br (~-8.5°C, Fig 3D). The resulted rank order potency of the compounds studied in inducing skin hypothermia was JWH-018=JWH-018 Cl>JWH-018 Br>Δ^9-THC (Fig 3E). Core and skin temperature changes were prevented by the pretreatment with AM 251 (Fig 3 F) which did not affect body temperature when administered alone.

3.2.3. Evaluation of pain induced by a mechanical stimulus

Systemic administration of JWH-018-R compounds and Δ^9-THC (0.01-6 mg/kg i.p.) increased the threshold to acute mechanical pain stimulus in mice in the tail pinch test (Fig 4). All the JWH-018-R compounds and Δ^9-THC were active in the dose range of 0.01-6 mg/Kg (Fig 4E) and the effects were prolonged up to 5 hours after injection of the compounds (Fig 4 B, C, D). It is interesting to note that the anti-nociceptive effect was already significant at the lower dose tested (0.01 mg/Kg). The maximal effects were observed at 75 min time point for Δ^9-THC (EMax%= 63.4 ±6.2; Fig 4 A), at 20 min for JWH-018 (EMax%= 84.1 ±4.6; Fig 4 B), at 130 min for JWH-018 Cl (EMax%= 83.6 ±3.5; Fig 4 C) and at 75 min for JWH-018 Br (EMax%= 76.5 ±3.9; Fig 4 D). At 6 mg/Kg the JWH-018-R compounds induced an increase in the pain threshold greater than that induced by the same dose of Δ^9-THC and the resulted rank order potency was JWH-018=JWH-018 Cl=JWH-018 Br>Δ^9-THC (Fig 4 E). The effects were prevented by the pretreatment with AM 251 (Fig 4 F) which alone did not alter the threshold to acute mechanical pain stimuli.

3.2.4. Evaluation of pain induced by a thermal stimulus

Systemic administration of JWH-018-R compounds and Δ^9-THC (0.01-6 mg/kg i.p.) increased the threshold to acute thermal pain stimulus in mice in the tail withdrawal test (Fig 5). In particular, JWH-018 induced a mild and transient increase in the thermal pain threshold at 1 mg/Kg (EMax%= 18.1 ±3.9 at 30 min after JWH-018 injection) and robust elevation of the pain threshold at 6 mg/Kg which ended after 250 min after administration of the compound (Fig 5 B). The Δ^9-THC, JWH-018 Cl and JWH-018 Br were effective only at the higher dose tested (6 mg/Kg; Fig 5 A, C, D). The maximal effects were observed at 85 min for Δ^9-THC (EMax%= 40.7 ± 10; Fig 5 A), at 30 min for JWH-018 (EMax%= 76.8 ± 13.7; Fig 5 B), at 30 min for JWH-018 Cl (EMax%= 56.0 ±11; Fig 5 C) and at 30 min for JWH-018 Br (EMax%= 41.7 ± 11; Fig 5 D). At 6 mg/Kg JWH-018 induced an increase in the pain threshold greater than that induced by the same dose of Δ^9-THC. Otherwise, JWH-018 Br induced an effect similar to that of THC and significantly lower than that induced by the JWH-018 (Fig 5 E). The resulted rank order potency was JWH-018=JWH-018 Cl=JWH-018 Br>Δ^9-THC (Fig 5 E). The effects were prevented by the pretreatment with AM 251 (Fig 5 F) which alone did not alter the threshold to acute thermal pain stimuli.

3.2.5. Motor activity assessment

3.2.5.1. Bar test

JWH-018-R compounds induced catalepsy in the bar test (Fig 5 B, C, D), while the treatment with Δ^9-THC was ineffective (Fig 6 A). In particular, JWH-018 induced a transient increase in the time spent on bar at 1 mg/Kg (EMax%= 30.7 ±5.1 at 60 min after JWH-018 injection) and marked catalepsy at 6 mg/Kg (EMax%= 82 ±13.6 at 95 min) which gradually decreases to return to baseline levels after 270 min from administration of JWH-018 (Fig 6 B). The JWH-018 Cl and JWH-018 Br were effective only at the higher dose tested (6 mg/Kg; Fig 6 C, D). The maximal effects were observed at 40 min for the JWH-018 Cl (EMax%= 22.6 ± 11.7; Fig 6 B) and for the JWH-018 Br (EMax%= 18.9 ±9.2; Fig 6 C). At 6 mg/Kg the JWH-018 induced a cataleptic state greater than that induced by the same dose of JWH-018 Cl and JWH-018 Br, that induced a similar effect (Fig 6 E). The resulted rank order potency was JWH-018>JWH-018 Cl=JWH-018 Br>Δ^9-THC (Fig 6 E). The effects were prevented by the pretreatment with AM 251 (Fig 6 F) which alone did not induce akinesia and catalepsy.

3.2.5.2. Drag test

JWH-018-R compounds induced at the higher dose tested (6 mg/kg i.p.) a prolonged and significant reduction of the number of steps performed with the front legs of the mice (Fig 7 B, C, D), while the treatment with Δ^9-THC was ineffective (Fig 7, A). The brominate compound caused an effect lower than that induced by JWH-018 and JWH-018 Cl (Fig 7 E). The resulted rank order potency was JWH-018=JWH-018 Cl>JWH-018 Br>Δ^9-THC (Fig 7 E). The inhibitory effects were prevented by the pretreatment with the AM 251 (Fig 7 F) which alone did not affect mice performance.

3.2.5.3. Accelerod test

Also in the accelerod test the JWH-018-R compounds induced at the higher dose tested (6 mg/kg i.p.) a prolonged and significant impairment of locomotion (Fig 8 B, C, D), while the treatment with Δ^9-THC was ineffective (Fig 8, A). The brominate compound caused an effect lower than that induced by JWH-018 and JWH-018 Cl (Fig 8 E). The resulted rank order potency was JWH-018=JWH-018 Cl>JWH-018 Br>Δ^9-THC (Fig 8 E). The inhibitory effects were prevented by the pretreatment with the AM 251 (Fig 8 F) which alone did not affect mice performance.

4. Discussion

The present study investigates for the first time the in vitro and in vivo activity of two novel synthetic halogenated cannabinoids, JWH-018 Cl and JWH-018 Br, molecules available in the European Internet market (EMCDDA–Europol 2012). The discovery of these compounds in products seized by the law enforcement has placed the urgency for their rapid study since the halogenation in the terminal portion of the pentylic side chain of the molecule could bring to the potentiation of the effects of JWH-018, that is known as one of the most potent naphthoyl-indole derivatives (Wiley et al., 1998; Wiley et al., 2012) identified within the "Spice" and K2 products (Seely et al., 2012; Uchiyama et al., 2010). This hypothesis is supported by the evidence that halogen substitution at the terminal carbon of the side chain in cannabinoids leads to an enhancement in affinity and producing the largest effects (Nikas et al., 2004; Marti et al., 2014). Moreover, the spread of new synthetic cannabinoids specifically halogenated in the pentylic side chain is increasing in the illegal market with important consequences for public health (Gugelmann et al., 2014; McQuade et al., 2013; Wohlfarth et al., 2014).

Our in vitro binding studies show that the insertion of a Cl or Br atom on the N-1 pentyl side chain of the JWH-018 core structure did not change significantly the binding properties of the compounds since they retain a nanomolar affinity for both murine and human CB_1 receptors and human CB_2 receptors similar to that of JWH-018 (Table 2). In particular, in murine preparation JWH-018 Cl displays an affinity on CB_1 receptors (Ki = 4.21 nM) analogous to that of JWH-018 (Ki = 5.82 nM) and slightly higher than that of JWH-018 Br (Ki = 7.13 nM). Whereas, on human CB_1 receptors, the JWH-018 Cl shows an higher affinity (Ki = 3.92 nM) compared to JWH-018 (Ki = 9.53 nM) and to JWH-018 Br (Ki = 6.24 nM). This small enhanced CB_1 receptor affinity of the chlorinated compound could justify its major efficacy in inhibiting the cyclic AMP formation as suggested by its potency value (IC_{50} = 8.53 nM).

In the behavioural tests halogenated compounds produced the same profile of effects (i.e. hypothermia, analgesia and motor inhibition) as JWH-018 (Brents et al., 2011; Wiebelhaus et al., 2012; Wiley et al., 1998; Wiley et al., 2012) and Δ^9-THC (Compton et al., 1992). However, JWH-018 Br appears to be less potent respect to JWH-018 Cl and JWH-018 in changing some physiological and behavioral parameters. Consistently with previous study JWH-018 induced a marked hypothermia (Brents et al., 2011; Marshell et al., 2014; Poklis et al., 2012; Wiebelhaus et al., 2012; Wiley et al., 1998) reaching the maximum effect (-8.5±1°C) at 60 min after injection. Hypothermia was completely prevented by the administration of AM 251 confirming that this effect is mediated by the stimulation of CB_1 receptors (Brents et al., 2011). Also JWH-018 Cl and JWH-018 Br induced a robust and prolonged inhibition of core and skin temperatures that was fully

dependent on CB_1 receptor activation, although the brominated compound was less effective than JWH-018 and JWH-018 Cl. As reported previously (Paronis et al., 2012), Δ^9-THC in the range of doses tested (0.01-6 mg/kg) did not induce a significant reduction in core and skin temperature confirming its lower efficacy compared to the effect of synthetic cannabinoids on body thermoregulation (Brents et al., 2012; Fantegrossi et al., 2014; Marshell et al., 2014; McMahon and Koek, 2007; Paronis et al., 2012). Cannabinoid-induced hypothermia has been amply demonstrated after systemic (Rawls et al., 2002), intracerebroventricular administration (Schmeling and Hosko, 1980) and direct microinjection in the preoptic area of the hypothalamus (Fitton and Pertwee, 1982; Rawls et al., 2002) of structurally different cannabinoids such as Δ^9-THC, WIN 55212-2, CP55,940, AM 4054 and JWH-like compounds (Fan et al., 1994; Fitton and Pertwee, 1982; McLaughlin et al., 2013; Rawls et al., 2002; Wiley et al., 2014; Wiley et al., 2012). So it is possible to assume that JWH-018 and its halogenated derivatives, JWH-018 Cl and JWH-018 Br, induces hypothermia by the stimulation of the same neural circuits. Our results show that JWH-018-R compounds induce a marked and prolong core hypothermia accompanied by a synchronous time-correlated decrease of the temperature of the skin. Since the body temperature balance is determined by two variables, heat production and heat dissipation, and cannabinoids decrease oxygen consumption (Athanasiou et al., 2007; Fitton and Pertwee, 1982) without evident direct effects on vascular tone (O'Sullivan et al., 2007), metabolic inhibition with diminished heat production, but not increased heat loss, appears to be the primary mechanism underlying body hypothermia induced by cannabinoids (Fitton and Pertwee, 1982). Therefore, prolonged decrease in skin temperature could be the result of a reduction in body core temperature and a decrease in the temperature of arterial blood supply. On the other hand, lower body temperature could cause a compensatory increase in sympathetic output and an enhanced vasoconstrictor activity of cutaneous vessels in order to diminish heat dissipation to the external environment (Honda et al., 2007). Both these effects could contribute to decrease skin temperature after the administration of JWH-018-R compounds and Δ^9-THC.

The analysis of the responses obtained in the tests of acute mechanical (tail pinch) and thermal analgesia (tail withdrawal) confirm that cannabinoids exert an important control of nociceptive signals which is known to be comparable with opiates in potency and efficacy in a variety of animal models (Walker and Huang, 2002). Indeed, JWH-018-R compounds and Δ^9-THC that are ineffective in modulating body temperature and inhibiting stimulated locomotion up to a dose of 6 mg/kg, induce analgesia in the tail pinch test at low doses (0.01-0.1 mg/kg) and increase the threshold to noxious thermal stimulus at the higher dose (6 mg/Kg).

At low doses (0.01-1 mg/kg) JWH-018-R compounds and Δ^9-THC induce a similar analgesic response to mechanical noxious stimuli while at higher dose (6 mg/kg) the synthetic cannabinoids cause a rapid and greater response respect to that induced by Δ^9-THC.

This response could be due to the fact that JWH-018 and its halogenated derivatives have a higher affinity for the CB_1 receptor compare to that of Δ^9-THC ((Wiley et al., 1998); present data) and they behave as full agonists at the CB_1 receptor (present date) while Δ^9-THC is reported to act as a partial agonist both in vitro (Govaerts et al., 2004) and in vivo (Paronis et al., 2012) models. It was also demonstrated that the synthetic cannabinoids are biotransformed into active metabolites that retain a high affinity and agonist activity on the CB_1 receptors (Brents et al., 2012; Brents et al., 2011). This could support the evidence that JWH-018-R compounds are more potent than Δ^9-THC in some in vivo assays (Fantegrossi et al., 2014; Marshell et al., 2014).

In our experimental conditions the possibility that the greater analgesic effect induced by JWH-018-R compounds 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 CB1 selective antagonist AM 251.

The greater efficacy of the JWH-018-R compounds and Δ^9-THC in reducing nociception to mechanical stimulation compared to thermal stimulus highlights that cannabinoid agonists exert their analgesic effect acting on different sensory components of pain generated by a mechanical (Martin et al., 1996) or thermal (Hohmann et al., 1999) stimuli. In fact, in our study at the highest dose tested (6 mg/kg) the increase of the pain threshold to mechanical stimulation is rapid in onset and prolonged in time up to 310 min (Emax ~ 60% at 310 min for the JWH-018 R compounds), while the increase in thermal pain threshold, although it is rapid in onset, it is transient and it decreases over time.

It is known that axonal mechanical and thermal sensitivity of cutaneous afferent neurones (McGlone and Reilly, 2010; Teliban et al., 2011) is perceived through the activation of different receptor-mediated mechanisms (Basbaum et al., 2009) expressed on different sensory nerve fibers of type C or Aδ (McGlone and Reilly, 2010). Moreover, it was reported that the duration of mechanical hyperalgesia (lasting up to 4 hours) long outlasted that of heat hyperalgesia (lasting up to 45 min) in rat treated with capsaicin (Gilchrist et al., 1996). So it is possible that cannabinoid agonists could modulate the perception of mechanical and thermal pain acting on the different mechanisms controlling the nociceptive signals at peripheral and spinal level (Walker and Huang, 2002). In particular, a possible interaction could occur with the transient receptor potential (TRP) channels, which are important transducers of noxious stimuli in nociceptors (Basbaum et al., 2009).

In fact it is known that cannabinoid-induced peripheral antihyperalgesic and antinociceptive effects by inhibiting the TRPV1-mediated currents in sensory neurons (Akopian et al., 2009).

It is interesting to note that the analgesic effects induced by Δ^9-THC are completely independent from the motor alterations, since the Δ^9-THC did not change the locomotion in mice in the range of doses tested. Similarly, the JWH-018-R compounds induced analgesia in the tail pinch test at doses (ie 0.01-0.1 mg/kg,) that did not reduce the motor performance of the mice. Moreover, the duration of the analgesic effect on the tail-pinch reflex induced by JWH-018 Br at higher dose (6 mg/Kg) was significantly greater than the duration of the motor effects on the rotarod and bar test. If the analgesic effects were the result of motor impairment, one would expect catalepsy, rotarod performance and tail pinch to be similar.

These findings point out that the actions of JWH-018-R compounds and Δ^9-THC on nociception and movement are mediated by separate processes and provide further evidence that the decreased behavioral responsiveness to noxious stimuli induced by cannabinoid agonists does not result merely from a disruption of motor function (Martin et al., 1996).

Our data reinforce previous studies that have shown that the JWH-018 proves to be the most potent compound among the napthoyl-indoles in reducing the motor activity in the rodent (Wiley et al., 2012). In fact, it induces a marked catalepsy and deeply impairment of motor performance in the drag and rotarod test. Otherwise, halogenated compounds are less active than the JWH-018 in inducing catalepsy and as evidenced by JWH-018 Br, in reducing the motor performance in the rotarod.

This reduced activity of the halogenated compounds and in particular of the JWH-018 Br, in reducing motor performance but also body temperature and thermal nociception could be due to a different pharmacokinetic of halogenated compounds compared to that of JWH-018 (i.e. greater lipophilicity and reduced tissue distribution) rather than to differences in pharmacodynamic, since the JWH-018-R compounds show a similar affinity for both native murine and transfected human CB_1 receptor and possess a similar potency in the activation of the CB_1 receptors (present date).

This aspect could also justify the different pro-convulsant activity induced by the administration of the JWH-018-R compounds at higher dose (6 mg/Kg i.p.). In fact, the JWH-018 causes convulsions, hyperreflexia and myoclonia in the majority of animals (Marshell et al., 2014), while the JWH-018 Cl is less effective than JWH-018 in inducing convulsions and the JWH-018-Br does not induce seizures and myoclonia but causes hyperreflexia only in 50% of treated mice.

Although the neurobiological mechanisms underlying pro-convulsant effect of JWH-018-R compounds has not yet been investigated, it has been hypothesized that excessive stimulation of CB_1 receptors leads to an imbalance between inhibitory (GABAergic) and excitatory

(glutamatergic) signals in epileptogenic brain areas (i.e. hippocampus, amygdala and cortex) thus favoring the appearance of convulsions (Vilela et al., 2013). These preclinical data are in agreement with the increasing clinical reports showing the occurrence of seizures and hyperreflexia in young people who have smoked spice containing different types of synthetic cannabinoids (Gugelmann et al., 2014; Lapoint et al., 2011; McQuade et al., 2013; Schneir and Baumbacher, 2012; Simmons et al., 2011) and clearly outline that these compounds are extremely dangerous for human health.

5 Conclusion

The present data, together preliminary evidence (Marti et al., 2013b), shows that JWH-018 Cl and JWH-018 Br alter the sensory-motor response in mice similarly to JWH-018 but with fewer adverse effects on motor skills and neurologic functions. These observations allow us to hypothesize that the halogenated derivatives (in particular JWH-018 Br) may have been placed on the illegal market to replace JWH-018 because of its severe side effects (convulsions, hyperreflexia) that have limited its use by consumer and have alerted the health care facilities, prevention centers and law enforcement agencies that registered intoxication cases related to its consumption.

In conclusion, our results showed for the first time that the halogenated compounds, JWH-018 Cl and JWH-018 Br, maintain a pharmaco-toxicological profile similar to that of JWH-018 (rank order of potency: JWH-018≥JWH-018 Cl≥JWH-018 Br>Δ^9-THC) and as reported for the JWH-018 they may affect negatively human health as well as greatly increase the risk factors for road traffic accidents (Musshoff et al., 2014; Tuv et al., 2014).

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Table 1

Binding and functional parameters of halogenated JWH-018 compounds in comparison with JWH-018 to CB_1 and CB_2 receptors.

Compound	*Mouse brain membranes*[a] *Ki (nM)*	*hCB1 CHO membranes*[a] *Ki (nM)*	*hCB1 CHO cells*[b] *IC50 (nM)*	*hCB2 CHO membranes*[a] *Ki (nM)*
JWH-018	5.82 ± 0.61	9.53 ± 0.88	14.12 ± 1.23	8.62 ± 0.71
JWH-018 Cl	4.21 ± 0.49	3.92 ± 0.31	8.53 ± 0.82	6.13 ± 0.64
JWH-018 Br	7.13 ± 0.62	6.24 ± 0.53	16.24 ± 1.41	7.38 ± 0.79

Data are expressed as mean ± SEM.
[a] [^{3}H]-CP-55,940 competition binding experiments
[b] cyclic AMP experiments

Table 2

Neurological changes induced by the administration of JWH-018–R compounds at 6 mg/Kg (i.p.).

Convulsions

Compound	*Frequency (%)*	*Duration (sec)*	*Latency (sec)*
Δ^9-THC	**–**	**–**	**–**
JWH-018	70%	369.7±32.2	109.7±16.3
JWH-018 Cl	30%	238.9±34*	270.5±30.1 ***
JWH-018 Br	**–**	**–**	**–**

Hyperreflexia

Compound	*Frequency (%)*	*Duration (sec)*	*Latency (sec)*
Δ^9-THC	**–**	**–**	**–**
JWH-018	80%	1439.8±45.3	93.5±21.2
JWH-018 Cl	100%	1362.6±39.1	139.3±19.1
JWH-018 Br	50%	1405.6±41	179.4±11.7*

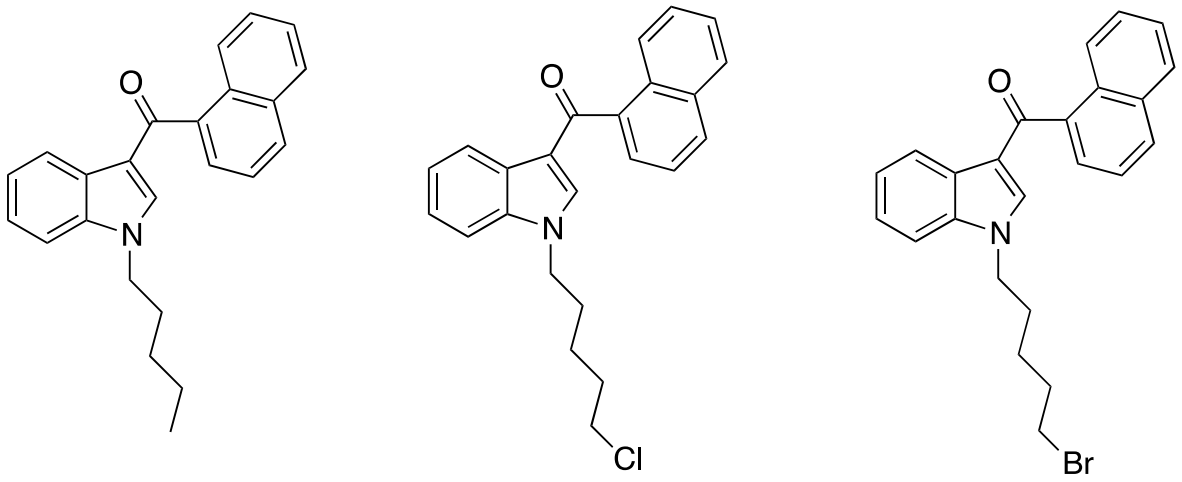
Myoclonias

Compound	*Frequency (%)*	*Duration (sec)*	*Latency (sec)*
Δ^9-THC	**–**	**–**	**–**
JWH-018	80%	669.7±36.6	109.7±16.3
JWH-018 Cl	30%	452.9±26***	270.5±30.1***
JWH-018 Br	**–**	**–**	**–**

Tail elevation

Compound	*Frequency (%)*	*Duration (sec)*	*Latency (sec)*
Δ^9-THC	**–**	**–**	**–**
JWH-018	80%	1766.6±189.7	88.6±13.4
JWH-018 Cl	100%	2167.8±321.8	122.3±27.3
JWH-018 Br	50%	1549.9±275.8	119.9±34.2

Data are expressed as percentage (frequency of animal with neurological signs) or seconds (duration and latency of neurological signs) and represent the mean ± SEM of 10 animals for each treatment. *$p<0.05$ and ***$p<0.0001$ different from JWH-018.



JWH-018 **JWH-018 Cl** **JWH-018 Br**

Figure 1.

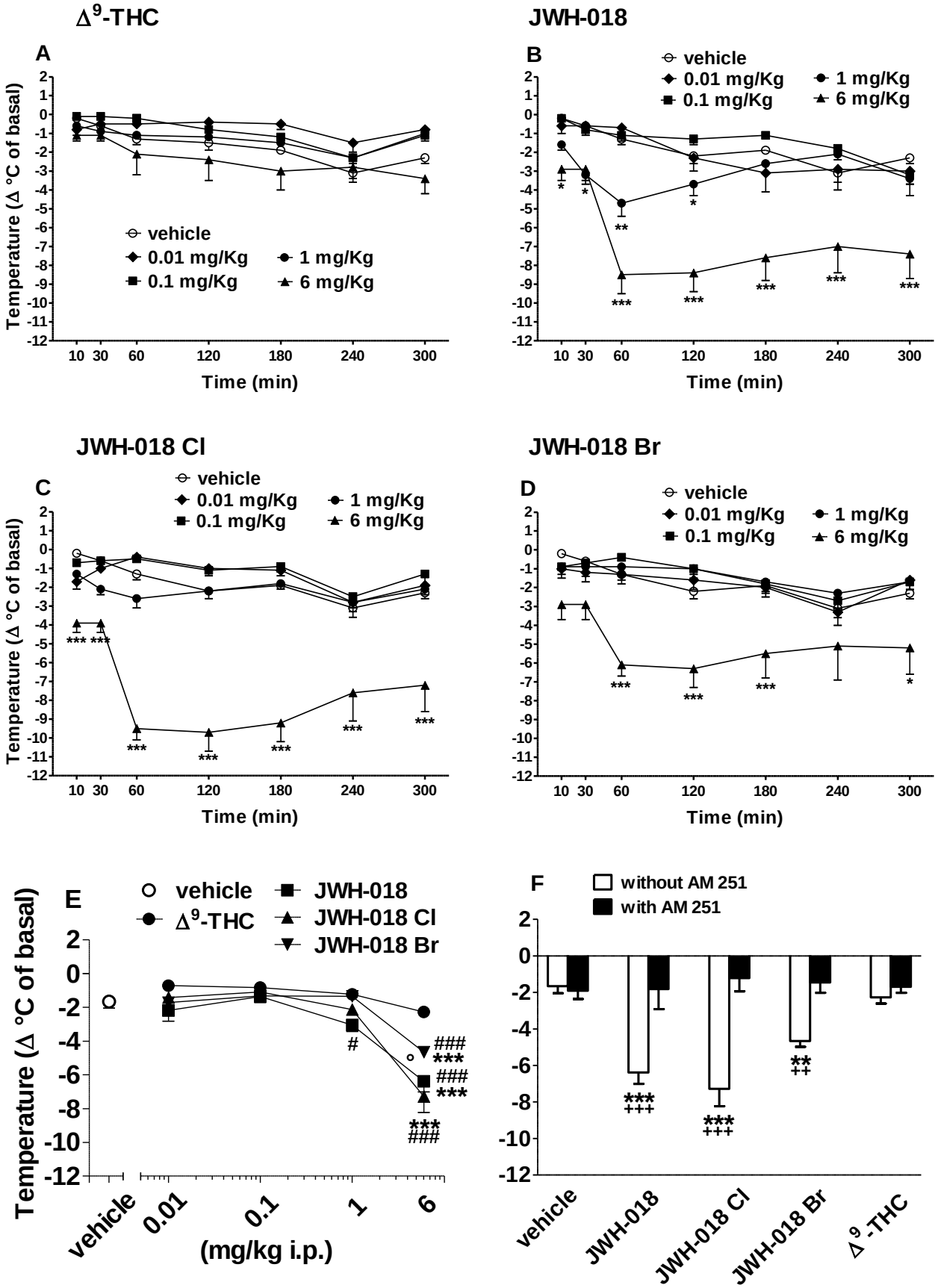

Δ9-THC
A
Temperature (Δ °C of basal)
vehicle
0.01 mg/Kg
1 mg/Kg
0.1 mg/Kg
6 mg/Kg
Time (min)
JWH-018
B
JWH-018 Cl
C
JWH-018 Br
D
E
Δ9-THC
JWH-018
JWH-018 Cl
JWH-018 Br
(mg/kg i.p.)
F
without AM 251
with AM 251

Figure 2.

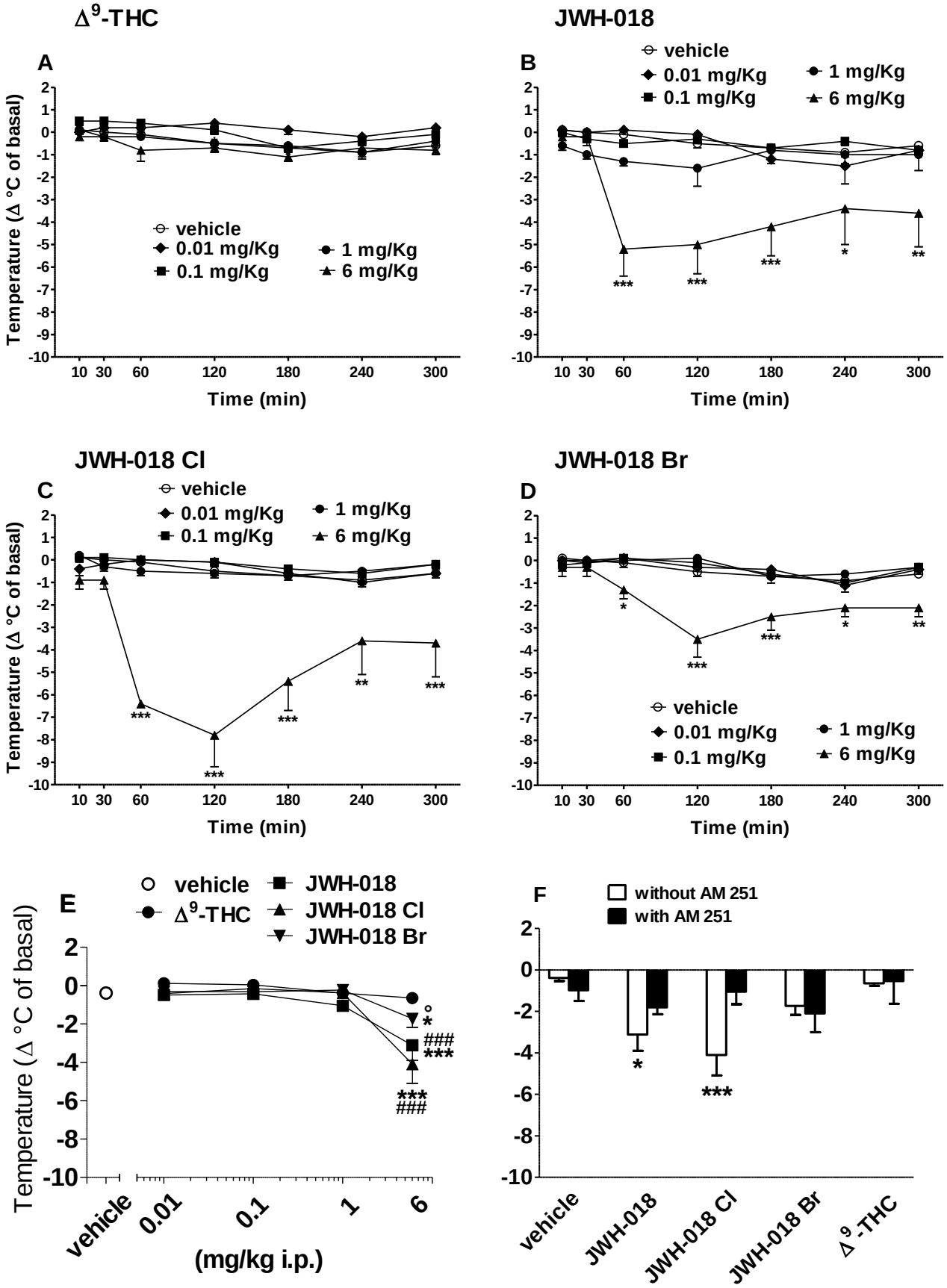

Δ9-THC
A
Temperature (Δ °C of basal)
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
6 mg/Kg
Time (min)
JWH-018
B
JWH-018 Cl
C
JWH-018 Br
D
E
vehicle
Δ9-THC
JWH-018
JWH-018 Cl
JWH-018 Br
(mg/kg i.p.)
F
without AM 251
with AM 251

Figure 3.

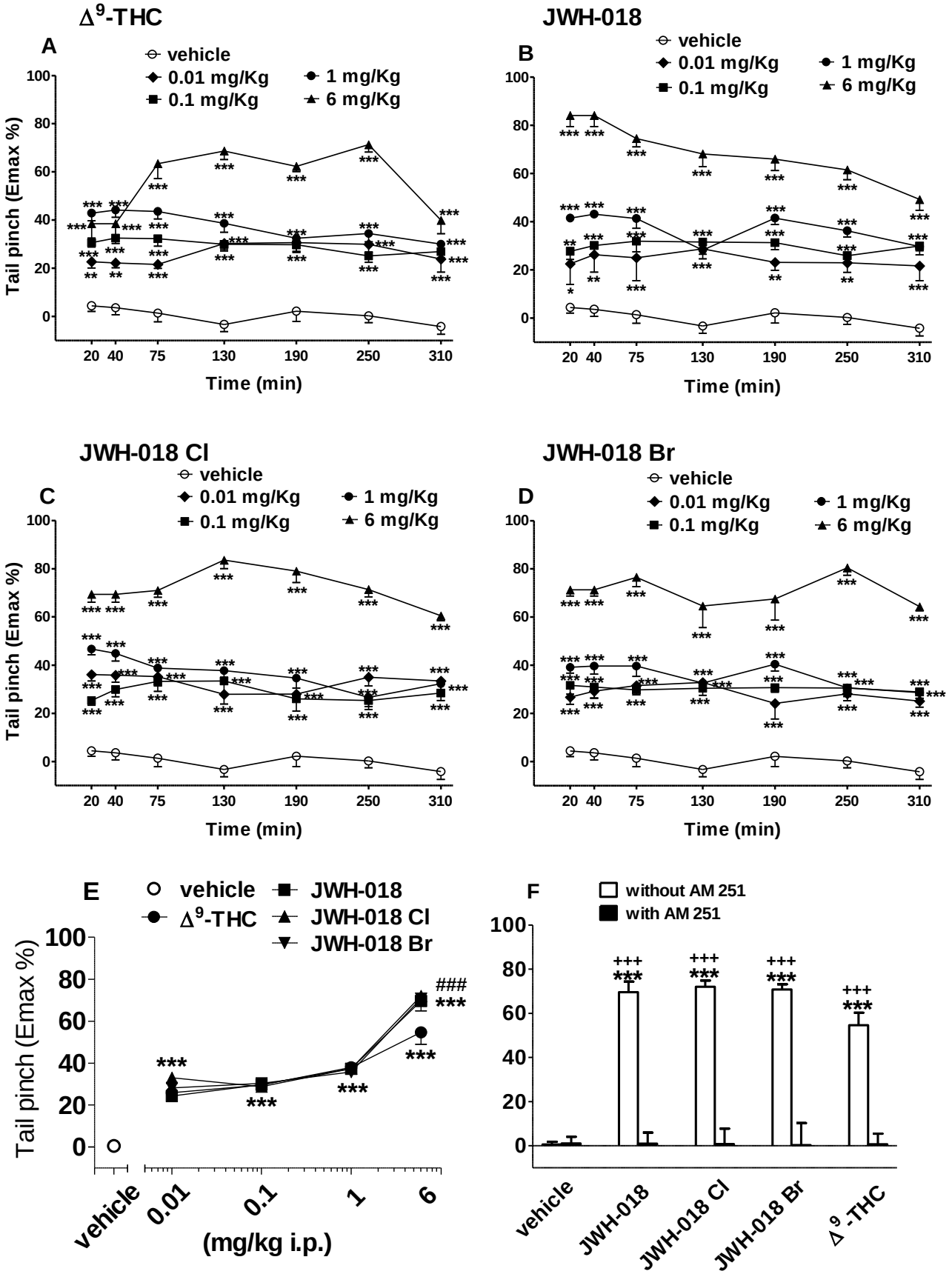

Δ9-THC
A
vehicle
0.01 mg/Kg
1 mg/Kg
0.1 mg/Kg
6 mg/Kg
Tail pinch (Emax %)
Time (min)
JWH-018
B
JWH-018 Cl
C
JWH-018 Br
D
E
JWH-018
Δ9-THC
JWH-018 Cl
JWH-018 Br
(mg/kg i.p.)
F
without AM 251
with AM 251

Figure 4.

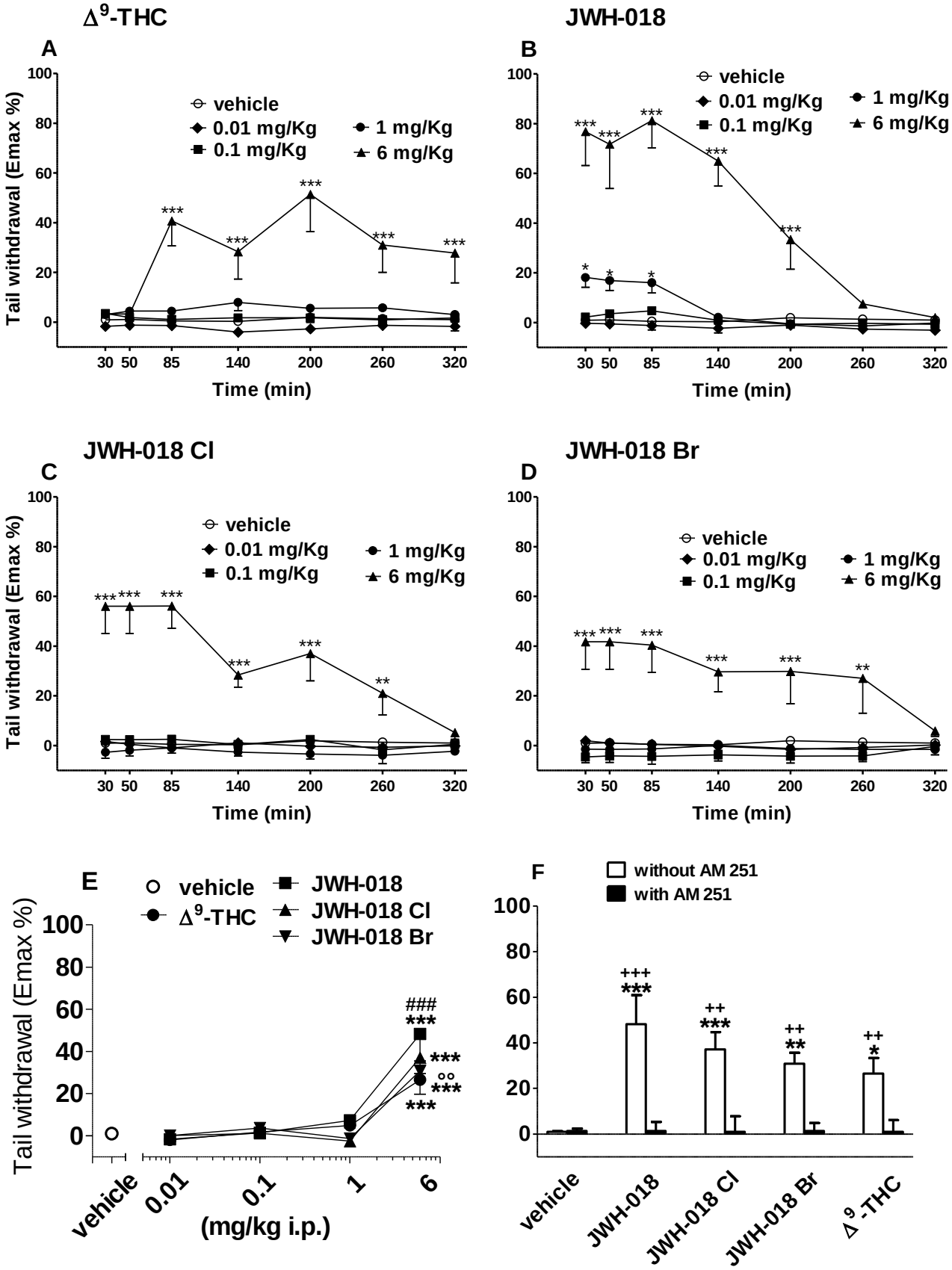

Δ9-THC
A
Tail withdrawal (Emax %)
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
6 mg/Kg
Time (min)
JWH-018
B
JWH-018 Cl
C
JWH-018 Br
D
E
vehicle
Δ9-THC
JWH-018
JWH-018 Cl
JWH-018 Br
(mg/kg i.p.)
F
without AM 251
with AM 251
JWH-018
JWH-018 Cl
JWH-018 Br
Δ9-THC

Figure 5.

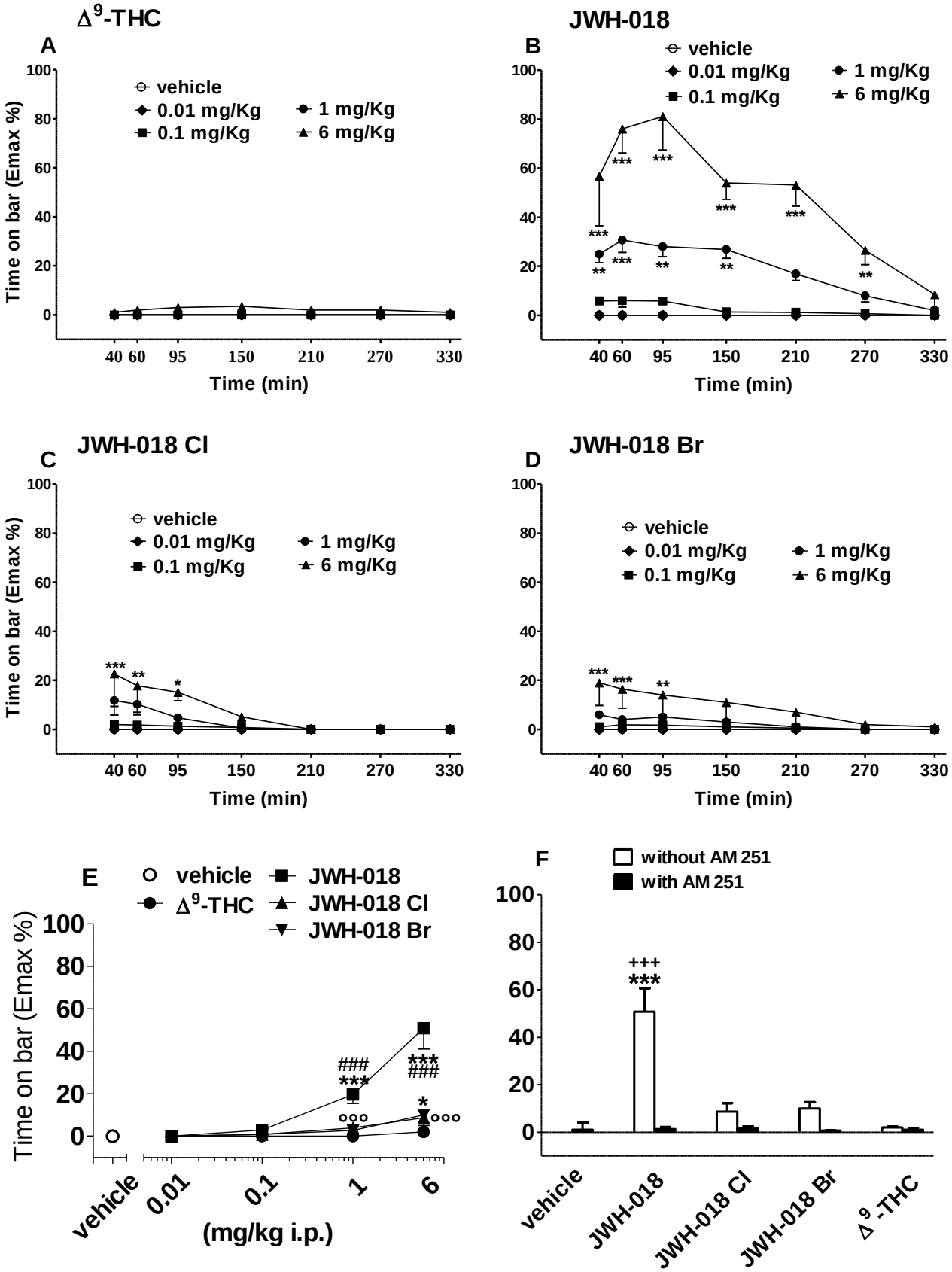

A
Δ9-THC
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
6 mg/Kg
Time on bar (Emax %)
Time (min)
B
JWH-018
C
JWH-018 Cl
D
JWH-018 Br
E
vehicle
Δ9-THC
JWH-018
JWH-018 Cl
JWH-018 Br
(mg/kg i.p.)
F
without AM 251
with AM 251

Figure 6.

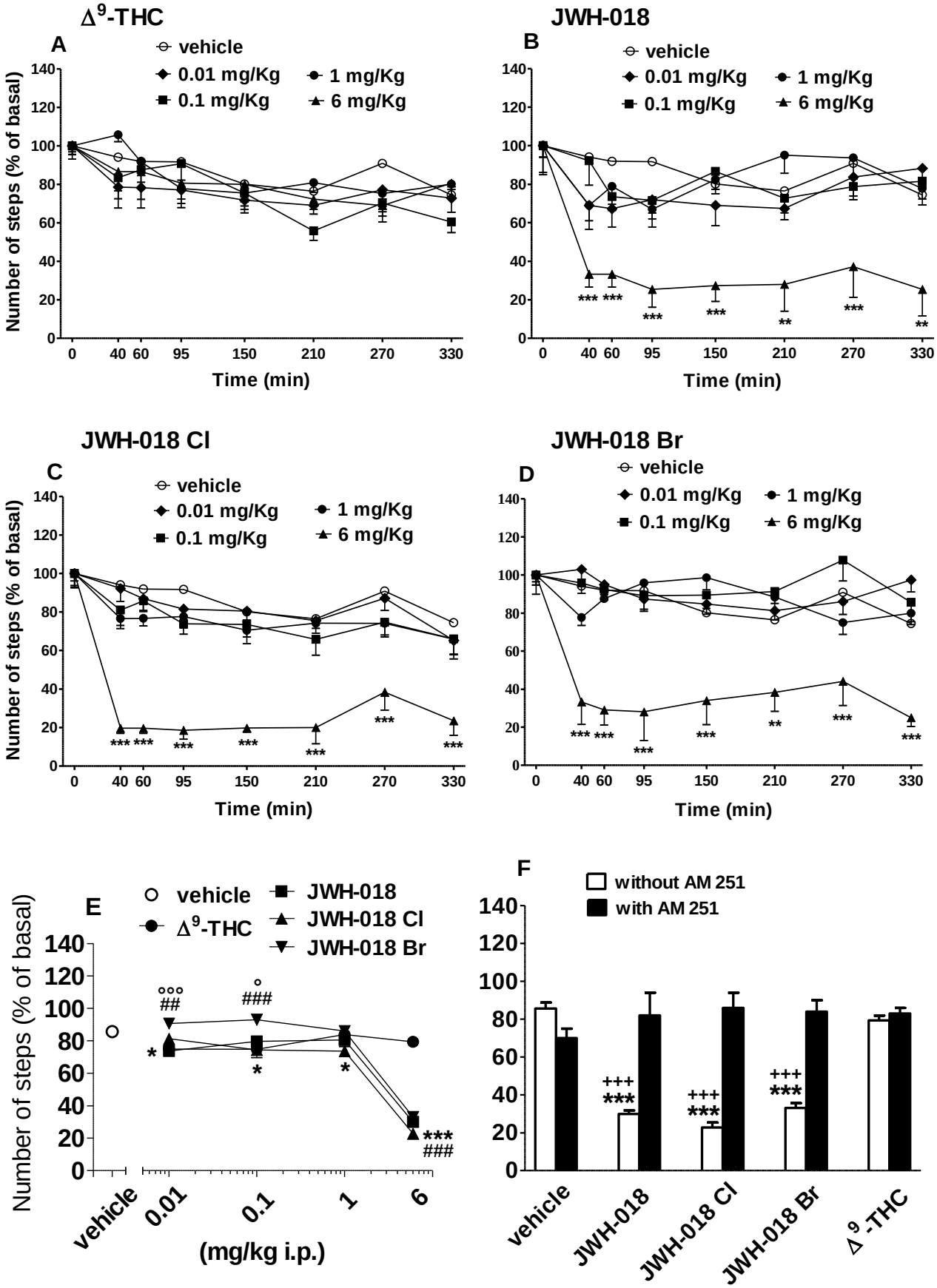

Δ9-THC
A
vehicle
0.01 mg/Kg
1 mg/Kg
0.1 mg/Kg
6 mg/Kg
Number of steps (% of basal)
Time (min)
JWH-018
B
JWH-018 Cl
C
JWH-018 Br
D
E
Δ9-THC
JWH-018
JWH-018 Cl
JWH-018 Br
(mg/kg i.p.)
F
without AM 251
with AM 251

Figure 7.

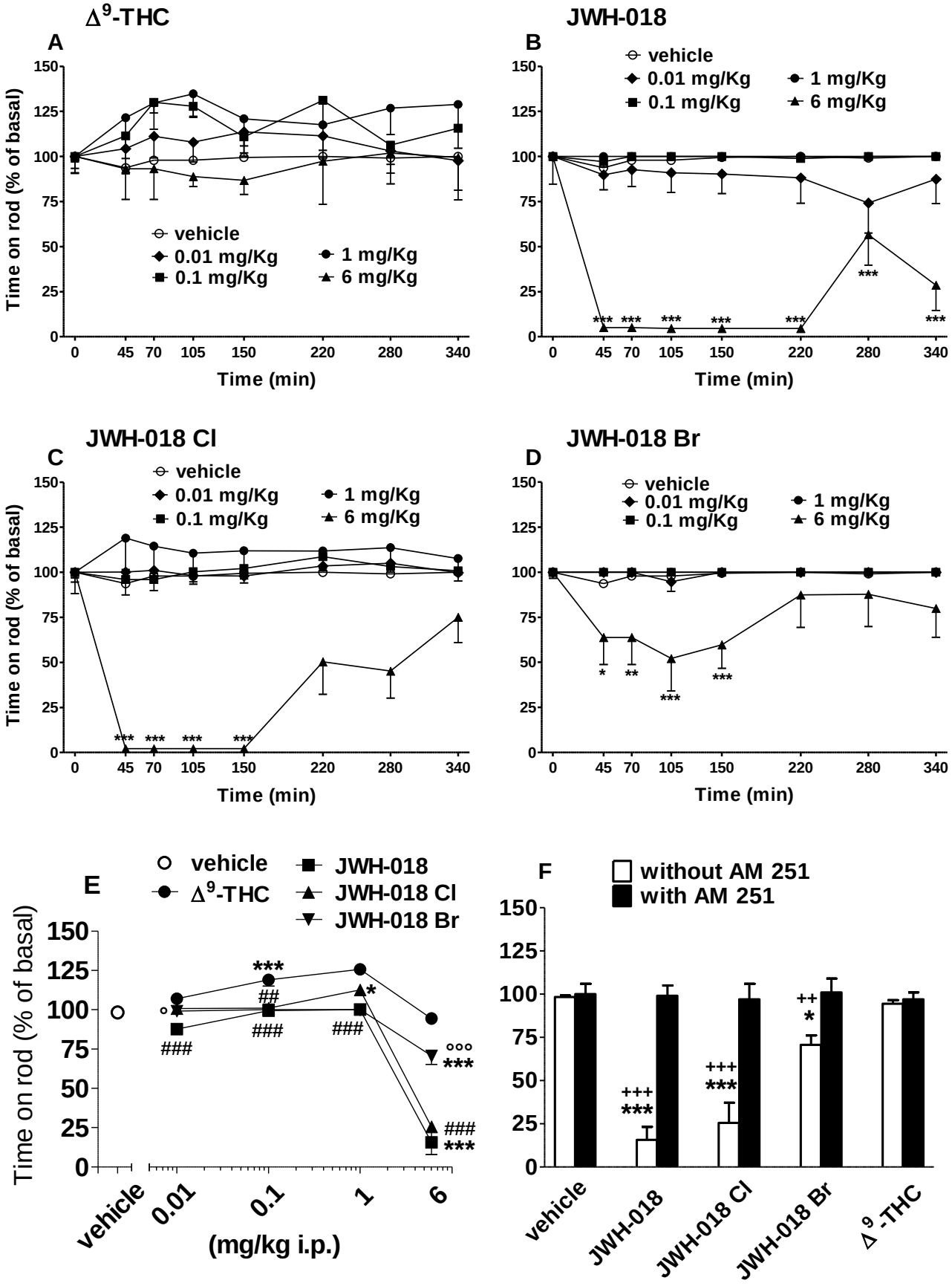

Δ9-THC
A
Time on rod (% of basal)
Time (min)
vehicle
0.01 mg/Kg
0.1 mg/Kg
1 mg/Kg
6 mg/Kg
JWH-018
B
JWH-018 Cl
C
JWH-018 Br
D
E
F
without AM 251
with AM 251
(mg/kg i.p.)
vehicle
JWH-018
JWH-018 Cl
JWH-018 Br
Δ9-THC

Figure 8.

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Figure captions

Figure 1. Chemical structures of JWH-018 (1-pentyl-3-(1-naphthoyl)indole), JWH-018 Cl (1-(5-chloro-pentyl)-3-(1-naphthoyl)indole) and JWH-018 Br (1-(5-bromo-pentyl)-3-(1-naphthoyl)indole).

Figure 2. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the core temperature of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.
Panel A: significant effect of treatment ($F_{4,245}$=14.71, p<0.0001), time ($F_{6,245}$=10.89, p<0.0001) and time x treatment interaction ($F_{24,245}$=1.050, p=0.4031). Panel B: significant effect of treatment ($F_{4,245}$=68.00, p<0.0001), time ($F_{6,245}$=12.76, p<0.0001) and time x treatment interaction ($F_{24,245}$=2.715, p<0.0001). Panel C: significant effect of treatment ($F_{4,245}$=166.4, p<0.0001), time ($F_{6,245}$=11.89, p<0.0001) and time x treatment interaction ($F_{24,245}$=4.687, p<0.0001). Panel D: significant effect of treatment ($F_{4,245}$=39.17, p<0.0001), time ($F_{6,245}$=7.073, p<0.0001) and time x treatment interaction ($F_{24,245}$=1.031, p=0.4276). Panel E: significant effect of agonists ($F_{4,140}$=17.02, p<0.0001), doses ($F_{3,140}$=64.76, p<0.0001) and agonist x doses interaction ($F_{12,140}$=8.347, p<0.0001), Panel F: significant effect of agonists ($F_{4,70}$=6.562, p=0.0002), AM 251 ($F_{1,70}$=49.81, p<0.0001) and agonist x AM 251 interaction ($F_{4,70}$=8.722, p<0.0001). *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ^9-THC; °p<0.05 versus JWH-018; ++p<0.01 and +++p<0.001 versus AM 251.

Figure 3. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the skin temperature of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.

Panel A: significant effect of treatment ($F_{4,245}$=18.39, p<0.0001), time ($F_{6,245}$=10.60, p<0.0001) and time x treatment interaction ($F_{24,245}$=1.211, p=0.2328). Panel B: significant effect of treatment ($F_{4,245}$=25.83, p<0.0001), time ($F_{6,245}$=5.035, p<0.0001) and time x treatment interaction ($F_{24,245}$=2.280, p=0.0009). Panel C: significant effect of treatment ($F_{4,245}$=74.28, p<0.0001), time ($F_{6,245}$=7.511, p<0.0001) and time x treatment interaction ($F_{24,245}$=4.972, p<0.0001). Panel D: significant effect of treatment ($F_{4,245}$=33.59, p<0.0001), time ($F_{6,245}$=11.52, p<0.0001) and time x treatment interaction ($F_{24,245}$=2.810, p<0.0001). Panel E: significant effect of agonists ($F_{4,140}$=8.911, p<0.0001), doses ($F_{3,140}$=32.20, p<0.0001) and agonist x doses interaction ($F_{12,140}$=5.514, p<0.0001), Panel F: significant effect of agonists ($F_{4,70}$=3.965, p=0.0059), AM 251 ($F_{1,70}$=2.663, p=0.1072) and agonist x AM 251 interaction ($F_{4,70}$=2.458, p=0.0534). *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC and °p<0.05 versus JWH-018.

Figure 4. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the tail pinch test of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.

Panel A: significant effect of treatment ($F_{4,245}$=213.1, p<0.0001), time ($F_{6,245}$=4.810, p=0.0001) and time x treatment interaction ($F_{24,245}$=4.564, p<0.0001). Panel B: significant effect of treatment ($F_{4,245}$=239.5, p<0.0001), time ($F_{6,245}$=4.968, p<0.0001) and time x treatment interaction ($F_{24,245}$=1.734, p=0.0207). Panel C: significant effect of treatment ($F_{4,245}$=373.7, p<0.0001), time ($F_{6,245}$=2.734, p=0.0137) and time x treatment interaction ($F_{24,245}$=2.203, p=0.0014). Panel D: significant effect of treatment ($F_{4,245}$=316.7, p<0.0001), time ($F_{6,245}$=2.212, p=0.0426) and time x treatment interaction ($F_{24,245}$=0.9386, p=0.5493). Panel E: significant effect of agonists

($F_{4,140}$=244.2, p<0.0001), doses ($F_{3,140}$=199.3, p<0.0001) and agonist x doses interaction ($F_{12,140}$=15.23, p<0.0001), Panel F: significant effect of agonists ($F_{4,70}$=16.63, p<0.0001), AM 251 ($F_{1,70}$=253.1, p<0.0001) and agonist x AM 251 interaction ($F_{4,70}$=17.02, p<0.0001). *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC and +++p<0.001 versus AM 251.

Figure 5. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the tail withdrawal test of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.
Panel A: significant effect of treatment ($F_{4,245}$=41.34, p<0.0001), time ($F_{6,245}$=2.861, p=0.0103) and time x treatment interaction ($F_{24,245}$=2.916, p<0.0001). Panel B: significant effect of treatment ($F_{4,245}$=110.8, p<0.0001), time ($F_{6,245}$=14.07, p<0.0001) and time x treatment interaction ($F_{24,245}$=7.479, p<0.0001). Panel C: significant effect of treatment ($F_{4,245}$=107.4, p<0.0001), time ($F_{6,245}$=5.323, p<0.0001) and time x treatment interaction ($F_{24,245}$=4.316, p<0.0001). Panel D: significant effect of treatment ($F_{4,245}$=57.47, p<0.0001), time ($F_{6,245}$=1.117, p=0.3531) and time x treatment interaction ($F_{24,245}$=1.369, p=0.1221). Panel E: significant effect of agonists ($F_{4,140}$=5.561, p=0.0003), doses ($F_{3,140}$=63.71, p<0.0001) and agonist x doses interaction ($F_{12,140}$=5.356, p<0.0001), Panel F: significant effect of agonists ($F_{4,70}$=3.920, p=0.0063), AM 251 ($F_{1,70}$=48.73, p<0.0001) and agonist x AM 251 interaction ($F_{4,70}$=3.977, p=0.0058). *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; °°p<0.01 versus JWH-018; ++p<0.01 and +++p<0.001 versus AM 251.

Figure 6. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the bar test of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.

Panel A: significant effect of treatment ($F_{4,245}$=110.1, p<0.0001), time ($F_{6,245}$=3.185, p=0.0050) and time x treatment interaction ($F_{24,245}$=3.185, p<0.0001). Panel B: significant effect of treatment ($F_{4,245}$=120.4, p<0.0001), time ($F_{6,245}$=10.82, p<0.0001) and time x treatment interaction ($F_{24,245}$=4.468, p<0.0001). Panel C: significant effect of treatment ($F_{4,245}$=7.305, p<0.0001), time ($F_{6,245}$=3.645, p=0.0017) and time x treatment interaction ($F_{24,245}$=1.368, p=0.1229). Panel D: significant effect of treatment ($F_{4,245}$=14.50, p<0.0001), time ($F_{6,245}$=2.297, p=0.0355) and time x treatment interaction ($F_{24,245}$=1.011, p=0.4526). Panel E: significant effect of agonists ($F_{4,140}$=33.10, p<0.0001), doses ($F_{3,140}$=30.95, p<0.0001) and agonist x doses interaction ($F_{12,140}$=13.26, p<0.0001), Panel F: significant effect of agonists ($F_{4,70}$=17.35, p<0.0001), AM 251 ($F_{1,70}$=34.10, p<0.0001) and agonist x AM 251 interaction ($F_{4,70}$=17.16, p<0.0001). *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; °°°p<0.001 versus JWH-018 and +++p<0.001 versus AM 251.

Figure 7. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the drag test of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.

Panel A: significant effect of treatment ($F_{4,280}$=2.515, p=0.0418), time ($F_{7,280}$=7.860, p<0.0001) and time x treatment interaction ($F_{28,280}$=0.6818, p=0.8882). Panel B: significant effect of treatment ($F_{4,280}$=35.31, p<0.0001), time ($F_{7,280}$=6.914, p<0.0001) and time x treatment interaction ($F_{28,280}$=1.39, p=0.0963). Panel C: significant effect of treatment ($F_{4,280}$=94.96, p<0.0001), time ($F_{7,280}$=18.80, p<0.0001) and time x treatment interaction ($F_{28,280}$=2.518, p<0.0001). Panel D: significant effect of treatment ($F_{4,280}$=68.26, p<0.0001), time ($F_{7,280}$=6.159, p<0.0001) and time x treatment interaction ($F_{28,280}$=2.382, p=0.0002). Panel E: significant effect of agonists ($F_{4,140}$=35.40,

$p<0.0001$), doses ($F_{3,140}=127.8$, $p<0.0001$) and agonist x doses interaction ($F_{12,140}=23.81$, $p<0.0001$), Panel F: significant effect of agonists ($F_{4,70}=10.65$, $p<0.0001$), AM 251 ($F_{1,70}=76.46$, $p<0.0001$) and agonist x AM 251 interaction ($F_{4,70}=19.35$, $p<0.0001$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus vehicle; ##$p<0.01$, ###$p<0.001$ versus Δ^9-THC; °$p<0.05$, °°°$p<0.001$ versus JWH-018 and +++$p<0.001$ versus AM 251.

Figure 8. Effect of the systemic administration (0.01-6 mg/Kg i.p.) of Δ^9-THC (panel A), JWH-018 (panel B), JWH-018 Cl (panel C) and JWH-018 Br (panel D) on the accelerod test of the mouse. Comparison of the total average effect observed in 5 hours (panel E) and interaction with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel F). 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, C, D) and for the comparison of the total average effect of the compounds (panel E), while the statistical analysis of the interaction with the AM 251 (panel F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons.

Panel A: significant effect of treatment ($F_{4,280}=7.570$, $p<0.0001$), time ($F_{7,280}=0.5972$, $p=0.7580$) and time x treatment interaction ($F_{28,280}=0.4034$, $p=0.9973$). Panel B: significant effect of treatment ($F_{4,280}=178.5$, $p<0.0001$), time ($F_{7,280}=6.324$, $p<0.0001$) and time x treatment interaction ($F_{28,280}=5.525$, $p<0.0001$). Panel C: significant effect of treatment ($F_{4,280}=115.9$, $p<0.0001$), time ($F_{7,280}=4.516$, $p<0.0001$) and time x treatment interaction ($F_{28,280}=4.823$, $p<0.0001$). Panel D: significant effect of treatment ($F_{4,280}=21.15$, $p<0.0001$), time ($F_{7,280}=1.507$, $p=0.1647$) and time x treatment interaction ($F_{28,280}=1.133$, $p=0.2986$). Panel E: significant effect of agonists ($F_{4,140}=55.47$, $p<0.0001$), doses ($F_{3,140}=173.1$, $p<0.0001$) and agonist x doses interaction ($F_{12,140}=25.31$, $p<0.0001$), Panel F: significant effect of agonists ($F_{4,70}=16.48$, $p<0.0001$), AM 251 ($F_{1,70}=78.05$, $p<0.0001$) and agonist x AM 251 interaction ($F_{4,70}=15.78$, $p<0.0001$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus vehicle; ##$p<0.01$, ###$p<0.001$ versus Δ^9-THC; °$p<0.05$, °°°$p<0.001$ versus JWH-018; ++$p<0.01$ and +++$p<0.001$ versus AM 251.