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Pharmaco-toxicological effects of the novel third-generation fluorinate synthetic cannabinoids, 5F-ADBINACA, AB-FUBINACA, and STS-135 in mice. In vitro and in vivo studies

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Introduction: 5F-ADBINACA, AB-FUBINACA, and STS-135 are 3 novel third-generation fluorinate synthetic cannabinoids that are illegally marketed as incense, herbal preparations, or research chemicals for their psychoactive cannabis-like effects.

Methods: The present study aims at investigating the in vitro and in vivo pharmacological activity of 5F-ADBINACA, AB-FUBINACA, and STS-135 in male CD-1 mice, comparing their in vivo effects with those caused by the administration of Δ^9 -THC and JWH-018. In vitro competition binding experiments revealed a nanomolar affinity and potency of the 5F-ADBINACA, AB-FUBINACA, and STS-135 on mouse and human CB₁ and CB₂ receptors. Moreover, these synthetic cannabinoids induced neurotoxicity in murine neuro-2a cells.

Results: In vivo studies showed that 5F-ADBINACA, AB-FUBINACA, and STS-135 induced 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, and hyperreflexia; and promoted aggressiveness in mice. Behavioral and neurological effects were fully prevented by the selective CB₁ receptor antagonist/inverse agonist AM 251. Differently, the visual sensory response induced by STS-135 was only partly prevented by the AM 251, suggesting a CB₁-independent mechanism.

Conclusions: For the first time, the present study demonstrates the pharmaco-toxicological effects induced by the administration of 5F-ADBINACA, AB-FUBINACA, and STS-135 in mice and suggests their possible detrimental effects on human health.

KEYWORDS

5F-ADBINACA, AB-FUBINACA, human CB₁ receptor, neurotoxicity, STS-135, Δ^9 -THC

Isabella Canazza and Andrea Ossato contributed equally to this work.

Abbreviations: 5F-ADBINACA, (1-(5-fluoro-pentyl)1H-indole-3-carboxylic acid-(1-carbamoyl-2-methyl-propyl)-amide); AB-FUBINACA, N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide; AM 251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; JWH-018, Naphthalen-1-yl-(1-pentylindol-3-yl) methanone; STS-135, N-(Adamantan-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide; Δ^9 -THC, (-)- Δ^9 -THC or Dronabinol[®]

1 | INTRODUCTION

Since the appearance in 2009 of synthetic cannabinoids (SCBs) as psychotropic drugs (spice and herbal incense; EMCDDA, 2009), there has been a continuous stream of new SCBs; on the one side, they were aimed to mimic the psychotropic effects of Δ^9 -tetrahydrocannabinol

(Δ^9 -THC); on the other side, they circumvented the law through their novel chemical structure (EMCDDA, 2015).

Among the new SCBs, halogenated derivatives have become increasingly prominent in forensic drug and toxicology specimen analysis (Castaneto et al., 2014). Indeed, halogenation has become an important approach for drug development, both for the steric and lipophilic contributions of halogens and their ability to form stabilizing interactions, such as halogen bonding and multipolar interactions in biomolecular systems (Lu et al., 2012).

In particular, fluorination, which is a fairly old strategy, is still one of the most recently used approaches (together with other function constituents substitution, e.g., adding carboxamide moieties, as in N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide [AB-FUBINACA] and (1-(5-fluoro-pentyl)1H-indole-3-carboxylic acid-(1-carbamoyl-2-methyl-propyl)-amide) [5F-ADBINACA]) to synthesize new SCBs with greater biological activity (Banister, Stuart, et al., 2015b; Gurney, Scott, Kacinko, Presley, & Logan, 2014); it determines significant improvements in the affinity for CB₁ receptors (Banister, Stuart, et al., 2015b; Nikas et al., 2004; Wiley, Marusich, & Huffman, 2014) as well as high lipophilicity, which promotes blood-brain barrier penetration (Schifano, Orsolini, Duccio Papanti, & Corkery, 2015).

Halogenated compounds promoted toxicity in humans, as reported for nonhalogenated SCBs (e.g., for naphthalen-1-yl-(1-pentylindol-3-yl)methanone [JWH-018; Lapoint et al., 2011], JWH-073 [Schneir, Cullen, & Ly, 2011], JWH-210 [Hermanns-Clausen, Kneisel, Szabo, & Auwarter, 2013], and ADB-PINACA [Schwartz et al., 2015]). Indeed, several cases shown severe intoxications or deaths caused by fluorinated compounds such as AM-2201 (Corazza et al., 2014; Patton et al., 2013), MAM-2201 (Saito et al., 2013), XLR-11 (Shanks, Winston, Heidingsfelder, & Behonick, 2015), ADB-FUBINACA (Shanks, Clark, & Behonick, 2016), and 5F-PB-22 (Behonick et al., 2014).

In the first part of 2014, the novel fluorinated SCBs, 5F-ADBINACA, AB-FUBINACA, and N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide (STS-135) have been seized on the Italian territory by Law Enforcement (Carabinieri, Department of Scientific Investigation; RIS).

5F-ADBINACA, AB-FUBINACA, and STS-135 do not belong to any of the groups commonly used to classify SCBs: cyclohexylphenol (such as cannabicyclohexanol and CP-47497), classical cannabinoids (such as HU-210), naphthoylindoles (such as JWH-018 and JWH-073), phenylacetylindoles (such as JWH-250 and JWH-203), benzoylindoles (such as AM-694 and RCS-4), and naphthoynaphthalenes (such as CB-13), but they are carboxamide-indoles (5F-ADBINACA; Figure 1a), carboxamide-indazole

(AB-FUBINACA; Figure 1b), and adamantylindoles (STS-135; Figure 1c). In particular, these SCBs differ from earlier JWH-type SCBs, having an amide bridge connecting the indole/indazole structures to an adamantyl (STS-135) or carboxamide (AB-FUBINACA and 5F-ADBINACA) group. Furthermore, with the view to increase their biological activity, a fluorine atom was linked at the 5-pentyl position both in STS-135 and 5F-ADBINACA or at the para-benzyl position in AB-FUBINACA. This formulation strategy was previously carried out for AM-2201, XLR-11, 5F-PB-22, and 5F-AKB48, the fluorinated analogues of JWH-018, UR-144, PB-22, and AKB48, respectively (Banister, Stuart, et al., 2015b; Canazza et al., 2016; Gurney et al., 2014).

5F-ADBINACA is a synthetic cannabinoid showing an indole core with a 5-fluoropentyl moiety and a carboxamide-linked aminoxyobutane group (Figure 1b). Because of its recent identification, there are no pharmacological and toxicological information on the effects of this substance in animals and humans.

AB-FUBINACA was originally described in a patent filed by Pfizer Global Research and Development in 2009, as an alternative analog based on an indazole-carboxamide substructure (Buchler et al., 2009). As recreational drug, AB-FUBINACA was first detected in Japan in 2012 into herbal products (Uchiyama et al., 2014) and in United States in 2013 (DEA, 2013), where it was scheduled in 2014 (DEA, 2014). It was one of the top three synthetic cannabinoids identified in seizures and toxicological drug screening in Sweden during 2013 and 2014 (Vikingsson, Josefsson, & Green, 2015). Psychonauts reported that AB-FUBINACA assumption induces a euphoric state similar to AM2201, with a marked hallucinogenic and hypnotic action (<https://drugs-forum.com/forum/showthread.php?t=218821>). Hallucinations are more intense than those caused by cannabis but less than those caused by other synthetic cannabinoids. Mental effects are mild to intermediate, depending on different dosages; however, at very high doses, mental effects are like those caused by stronger cannabinoids (<https://www.erowid.org/experiences/exp.php?ID=105231>). Several hospitalizations following intake of a structurally similar indazole carboxamide, ADB-PINACA, have occurred. This combination produces nausea and vomiting, seizures, somnolence, hyperglycemia, hyperkalemia, tachycardia, myocardial infarction, pneumonia, rhabdomyolysis, anxiety, delirium, psychosis, and aggressive behaviors (CDCP, 2013; Martinotti et al., 2014; Schwartz et al., 2015). Fatalities and numerous hospitalizations were due to consumption of the methyl ester of AB-FUBINACA carboxylic acid, according to the Russian Federal Drug Control Service (RFDC, 2014). In vitro data show that this compound is a very potent ligand for CB₁ receptor, with a constant binding of 0.9nM and an EC₅₀ of 23.2nM for receptor activation as measured by GTPγS hydrolysis (Thomsen et al., 2015). Preclinical studies showed that AB-FUBINACA produces bradycardia and

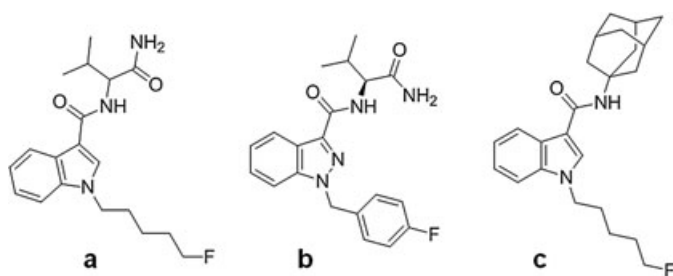


FIGURE 1 Chemical structures of (a) 5F-ADBINACA (1-(5-fluoro-pentyl)1H-indole-3-carboxylic acid-(1-carbamoyl-2-methyl-propyl)-amide), (b) AB-FUBINACA (N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide), and (c) STS-135 (N-(Adamantan-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide)

hypothermia in rats at doses of 0.3–3 mg/kg (Banister, Moir, et al., 2015a), depressed spontaneous locomotion in ND4 Swiss-Webster mice and positively substituted for the discriminative stimulus effects of Δ^9 -THC in rats (Gatch & Forster, 2015). Moreover, a recently published study (Kevin et al., 2017) reports several acute effects (decreased locomotor activity at high and low doses, increased anxiety-like behaviors and audible vocalizations, and reduced weight gain) and long-term effects (object recognition memory deficits) of AB-FUBINACA in rats.

STS-135, also known as 5F-APICA, is comparable to 5F-AKB48, where the core indazole structure is substituted with an indole base. Previous studies have demonstrated that STS-135 acts as potent cannabinoid receptor agonist in vitro with an EC_{50} of 13 nM for human CB_1 receptors and 51 nM for human CB_2 receptors, producing bradycardia and hypothermia in rats at doses of 1–10 mg/kg (Banister, Stuart, et al., 2015b). Recently, it has been shown that STS-135 facilitates dopamine release in the Shell Nucleus Accumbens of rats (De Luca, Castelli, et al., 2015b), introducing its potential positive role in reinforcing mechanisms, as already mentioned for other SCBs, as well as JWH-018 (De Luca, Bimpisidis, et al., 2015a), JWH-250 and JWH-073 (Ossato et al., 2016), AKB48, and 5F-AKB48 (Canazza et al., 2016).

The metabolism of AB-FUBINACA and STS-135 has been identified using a hepatocyte model, human liver microsomal incubation, and from human and rat urine samples (Castaneto et al., 2015; Ford & Berg, 2016; Gandhi et al., 2015; Hsin-Hung Chen et al., 2016; Sobolevsky, Prasolov, & Rodchenkov, 2015; Vikingsson et al., 2015). For both SCBs, several metabolites of phases I and II have been highlighted. In the case of AB-FUBINACA, the major liver metabolites were AB-FUBINACA carboxylic acid, hydroxy AB-FUBINACA carboxylic acid, dihydrodiol AB-FUBINACA, and dihydrodiol AB-FUBINACA carboxylic acid (Castaneto et al., 2015; Vikingsson et al., 2015). In the case of STS-135, the major liver metabolites were monohydroxy STS-135 and dihydroxy STS-135, both on the hydroxylatedadamantane system (Gandhi et al., 2015); on the contrary, the N-despentyl (desfluoropentyl) hydroxyadamantyl metabolite was the most present in the urine of STS-135 consumers (Sobolevsky et al., 2015). This evidence should be taken into account: as reported for others SCBs, a large number of metabolites could maintain agonistic activity at CB_1 receptors as demonstrated for JWH-018 and other SCBs (Brents et al., 2011; Brents et al., 2012).

Despite the presence of these studies, there is poor preclinical in vivo evidence on the overall pharmacotoxicological effects of AB-FUBINACA and STS-135, and there is no information for 5F-ADBINACA. Therefore, the current study aims at investigating the acute effect of 5F-ADBINACA, AB-FUBINACA, and STS-135 (0.01–6 mg/kg i.p.) 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), and aggressive response in CD-1 mice. To understand the behavioral effects of these drugs better, their actions were monitored for over 5 hr and compared with those of JWH-018 and Δ^9 -THC. In addition, in vitro binding studies on CD-1 murine and human CB_1 / CB_2 receptors and neurotoxic studies on neuro-2a cells have been performed for a full characterization of these three novel fluorinated SCBs.

2 | MATERIALS AND METHODS

2.1 | Animals

Male ICR (CD-1[®]) mice, 25–30 gr (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8–10 mice per cage; floor area per animal was 80 cm²; minimum enclosure height was 12 cm) in a colony room under constant temperature (20–22 °C) and humidity (45–55%). Food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and tap water were available ad libitum all the time the animals spent in their home cages. The daylight cycle was maintained artificially (dark between 6 pm–6 am). Experiments were performed during the light phase, and each mouse was used for only one experiment. Experimental protocols performed in the present study were in accordance with the new European Communities Council Directive of September 2010 (2010/63/EU), a revision of the Directive 86/609/EEC, and were approved by the Italian Ministry of Health (license n. 335/2016-PR) and by the Ethics Committee of the University of Ferrara. Moreover, adequate measures were taken to minimize the number of animals used and their pain and discomfort.

2.2 | Drug preparation and dose selection

5F-ADBINACA (Figure 1a), AB-FUBINACA (Figure 1b), and STS-135 (Figure 1c; LGC standards [LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy] and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide [AM 251; Tocris, Bristol, United Kingdom]) were used. Drugs were initially dissolved in absolute ethanol (final concentration 2%), Tween 80 (2%), and diluted in saline solution (0.9% NaCl) and administered intraperitoneally at a volume of 4 ul/gr. The solution made of 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 min before 5F-ADBINACA, AB-FUBINACA, and STS-135 injections. Doses of 5F-ADBINACA, AB-FUBINACA, and STS-135 (0.01–6 mg/kg i.p.) were chosen based on previous studies (Ossato et al., 2015; Ossato et al., 2016; Vigolo et al., 2015).

2.3 | Mouse tissues and cell culture membrane preparation

After mice were killed by cervical dislocation, brain and spleen were rapidly dissected. The mouse brain was suspended in 50 mM Tris HCl buffer, pH 7.4 at 4 °C, homogenized with a Polytron, and centrifuged for 20 min at 40,000 × g. The mouse spleen was suspended in 50 mM Tris HCl buffer, pH 7.4 at 4 °C, and, after homogenization by means of a Polytron, the suspension was centrifuged for 10 min at 2,000 × g. The supernatant was then filtered and centrifuged at 40,000 × g for 20 min. Mouse brain and spleen membranes were used for competition binding experiments (Vincenzi et al., 2013). CHO cells transfected with human CB_1 or CB_2 receptors (Perkin Elmer Life and Analytical Sciences, USA) were cultured 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% CO₂/95% air. To obtain membranes, cells were washed with PBS and

scraped into ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, and pH 7.4). After homogenization with a Polytron, the suspension was centrifuged for 30 min at 40,000 × g. For CB₁ receptors, the membranes were suspended in 50 mM Tris HCl buffer (pH 7.4) containing 2.5 mM EDTA, 5 mM MgCl₂, 0.5 mg/ml BSA; whereas for CB₂ receptors, membranes were suspended in 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, and 0.5 mg/ml BSA (Vincenzi et al., 2013).

2.4 | [³H] CP-55,940 competition binding assays and cyclic AMP assays

Competition binding experiments were carried out incubating various concentrations of the tested compounds in the presence of 0.5 nM [³H]-CP-55,940 (Vigolo et al., 2015; Vincenzi et al., 2013). Binding experiments were performed in membranes obtained from CHO cells transfected with human CB₁ or CB₂ receptors (2 μg protein/100 μl) as well as in mouse brain membranes (40 μg protein/100 μl) for CB₁ receptors and in mouse spleen membranes (80 μg protein/100 μl) for CB₂ receptors. The binding in the presence of 1 μM WIN 55,212-2 was defined as nonspecific binding. Bound and free radioligand was separated by filtration, and the radioactivity was measured in a Packard Tri Carb 2810 TR scintillation counter. Cyclic AMP experiments were performed in CHO cells transfected with human CB₁ or CB₂ receptors. After washing with PBS, cells were detached with trypsin and then centrifuged at 200 × g for 10 min (Vigolo et al., 2015; Vincenzi et al., 2013). Cell pellet was suspended in incubation buffer composed of 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5 mM HEPES, 10 mM MgCl₂, 5 mM glucose, and pH 7.4 at 37 °C. The phosphodiesterase inhibitor Ro 20-1724 (0.5 mM) was added to the cells during a preincubation of 10 min in a shaking bath at 37 °C. The effect of the tested compounds were evaluated on 1 μM forskolin-stimulated cAMP levels. An ice-cold 6% trichloroacetic acid solution was added to disrupt the cells and the final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay.

2.5 | Behavioral studies

The effect of 5F-ADBINA, AB-FUBINA, and STS-135 was investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" for preclinical characterization of new molecules in rodents (Hamdam et al., 2013; Irwin, 1968; Mattsson, Spencer, & Albee, 1996; Porsolt, Lemaire, Dürmüller, & Roux, 2002; Redfern et al., 2005; S7A, 2001). Those tests have been also validated to describe effects of cannabinoids on the "tetrad," sensorimotor, and neurological changes in mice (Compton, Johnson, Melvin, & Martin, 1992; Ossato et al., 2015; Ossato et al., 2016; Vigolo et al., 2015). To reduce the number of animals used, the behavior of mice was evaluated in five consecutive experimental sections (for detailed information, see Data S1). Moreover, in order to reduce stress in animals induced by manipulation, and to confirm the stability and reproducibility over time of the responses of our tests, they were trained 2 times per week for 2 weeks before pharmacological treatment. The behavior of mice (neurologic and sensorimotor responses) was videotaped and analyzed off-line by a different trained operator giving test scores.

2.5.1 | Major neurological changes and aggressive response

As previously described by other studies (Ossato et al., 2015; Ossato et al., 2016; Vigolo et al., 2015), convulsions, hyperreflexia, myoclonus, tail elevation, and aggressive responses in mice were observed immediately after SCBs administration. Neurological changes are expressed as frequency (percent of animals that develop symptoms), duration (total time in sec), latency (time in sec of symptom onset), and score (degree of tail elevation and number of bites connected to spontaneous and stimulated aggressiveness). The tail elevation was measured during the observation of freely moving mice in a square area. The elevation of the tail is described through four inclinations: absence elevation (score 0/4); inclination from 0 to 15 ° (score 1/4) from the ground surface; inclination from 15 to 70 ° (score 2/4); inclination from 70 to 90 ° (score 3/4); or inclination greater than 90 ° (score 4/4). The animal's spontaneous aggressiveness is measured through the number of bites that gives to an object, namely, a gray cloth, that approaches the front of the snout of the animal in an animal's mobility condition. Conversely, in stimulated aggressiveness, the animal is manually restrained and held in a supine position. For both aggressive behavior tests, a gray cloth was placed in front of the mouse nose for 10 consecutive times (score 0/10, *not aggressive*; score 10/10, *very aggressive*).

2.5.2 | Sensorimotor studies

We studied the voluntary and involuntary sensorimotor responses resulting from different mouse reaction to visual, acoustic, and tactile stimuli (Koch, 1999; Ossato et al., 2015).

Evaluation of the visual response

Mouse visual response was verified by two behavioral tests that evaluated the ability of the animals to capture visual information even when the animal is moving (the visual placing response) or when it is stationary (the visual object response). The first one 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). A camera videotaped the downward movement of the mouse. The analysis frame by frame allows to evaluate the beginning of the reaction of the mouse while it is close to the floor. When the mouse begins to react, an electronic ruler evaluates the perpendicular distance, in millimeters between the eyes of the mice and 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 postinjection. Visual object response test was used to evaluate the ability of the mouse to see an object approaching from the front or the side (Ossato et al., 2015). For the frontal visual response, a white horizontal bar was moved frontally to the mouse head for 3 times. For the lateral visual response, a dental mirror was moved in the field of animal, following a horizontal arc from behind to front the mouse's eyes. The procedure was conducted bilaterally and 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 visual object response with that obtained in the lateral one (overall score, 9). Evaluation of the visual

object response was measured at 0, 10, 30, 60, 120, 180, 240, and 300 min postinjection.

Evaluation of acoustic response

The perception of sounds by the animal was measured by the acoustic response test who values the reflex of the mouse in replay to an acoustic stimulus produced behind it (Koch, 1999). In particular, four acoustic stimuli of different intensity and frequency were tested (see Ossato et al., 2015). Each sound 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 sound. The acoustic total score was calculated by adding scores obtained in the four tests (overall score, 12). Evaluation of the acoustic response was measured at 0, 10, 30, 60, 120, 180, 240, and 300 min postinjection.

Evaluation of tactile response

The tactile response was verified through vibrissae, pinna, and corneal reflexes (for a more comprehensive description, see Ossato et al., 2015; Canazza et al., 2016). Each tactile response were measured at 0, 10, 30, 60, 120, 180, and 240 min postinjection.

2.5.3 | Tetrad paradigm for screening cannabinoid-like effect

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. As previously reported, the core temperature was assessed with a rectal probe connected to digital thermometer (Cole Parmer, model 8402) whereas the surface temperature was measured by a Microlife FR 1DZ1 digital infrared thermometer (Canazza et al., 2016; Vigolo et al., 2015). Core and surface mouse body temperatures were measured at 0, 30, 50, 85, 140, 200, 260, and 320 min postinjection.

Evaluation of pain induced by a mechanical and thermal stimuli

Acute mechanical nociception was evaluated using the tail pinch test (Vigolo et al., 2015). Pinch pressure was applied to the third of the tail extending from the root via a special rigid probe connected to a digital dynamometer (ZP-50 N, IMADA, Japan). Mice did not vocalize during the application of tail-pinch pressure, and when the mouse flicked its tail, the pressure was stopped and the digital instrument saved the maximum peak of weight supported (g/force). A cutoff (500 g/force) was set to avoid tissue damage. The test was repeated 3 times, and the final value was calculated with the average of 3 obtained scores. Acute thermal nociception was evaluated using the tail withdrawal test (Vigolo et al., 2015). The mouse was restrained in a dark plastic cylinder, and half of its tail was dipped in water of 48 °C and the withdrawal response to the hot stimulus was recorded as the latency (in seconds) of tail flicking in mechanical nociception tests lasting 15-s maximum each. The maximum heat exposure time was 15 s to prevent tissue damage. The test was repeated 3 times, and the final value was calculated with the average of 3 obtained scores. At the

end of each swimming session, the animal was removed from the cylinder and its tail was dried with paper towels. Acute mechanical and thermal nociception was measured at 0, 35, 55, 90, 145, 205, 265, and 325 min post injection.

Motor activity assessment

Motor activity alterations induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 were measured using the bar, drag, accelerod tests, and the analysis of spontaneous locomotor activity (Marti, Mela, Guerrini, Calò, & Bianchi, 2004; Marti et al., 2005; Ossato et al., 2015; Vigolo et al., 2015). In the bar test, each animal's forelimbs were placed on a bar made of plastic (height 6 cm). The time spent on the bar was measured (immobility cut off, 20 s) and the akinesia was calculated as total time spent on the bar after three consecutive trials (total maximal time of catalepsy, 60 s). The bar test was performed at 0, 20, 40, 75, 130, 190, 250, and 310 min postinjection. In the drag test, the mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20 cm/s for a fixed distance (100 cm). The number of steps made by each forepaw was counted by two separate observers. For each animal, five to seven measurements were collected. The drag test was performed at 0, 45, 70, 105, 160, 220, 280, and 340 min postinjection. In the accelerod test, animals were placed on a rotating cylinder that automatically and constantly (0–60 rotations/min in 5 min) increased velocity. The time spent on the cylinder was measured. The accelerod test was performed at 0, 40, 60, 95, 150, 210, 270, and 330 min postinjection. Spontaneous locomotor activity: in the open field test, the mice were placed in a square plastic cage (60 X 60 cm) located in a sound- and light-attenuated room. During the following 240 min, distance travelled (meter) and immobility time (second; the animal is considered immobile when 95% of his image remains in the same place for at least 2 s) were recorded and analyzed with a video-tracking system (Ugo Basile, application version 4.99 g Beta). Four mice were monitored at the same time in each experiment. The distance covered and the time of immobility were analyzed every 15 min for a maximum of 240 min. At the end of the experiment, fecal boli were removed and the floor was wiped clean with ethanol solution (5%) and washed with water.

2.6 | Neurotoxicity assay: mitochondrial membrane potential measurement

Neuro-2a cells, which endogenously express cannabinoid type 1 (CB₁) receptors (Graham et al., 2006; He et al., 2005), were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 10 µg/ml streptomycin, in a 5% CO₂ incubator at 37 °C. The cells were seeded in 6-well plates at a density of 150,000 cells. After 48 hr, the cells were loaded with 10 nM tetramethylrhodamine methyl ester (TMRM; Life Technologies), placed in a humidified chamber at 37 °C, and imaged every 1 min for 1 hr of treatment with a LiveScan Swept Field Confocal Microscope (Nikon Instruments Inc.) equipped with a 40× oil immersion lens. TMRM fluorescence was analyzed using the NIS Elements software package (Nikon Instruments Inc.), and depolarization rates were

defined as the slopes of the fluorescence trace over a poststimulation period, followed by 10 μ M carbonylcyanide-3-chlorophenylhydrazone (CCCP) treatment to collapse the $\Delta\Psi$.

2.7 | 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 IC₅₀ values according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + [C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^{*} its dissociation constant. Functional experiments were analyzed by nonlinear regression analysis using the equation for a sigmoid concentration-response curve using Prism (GraphPad Prism, USA). Effects of SCBs on mitochondrial membrane potential ($\Delta\Psi$) was expressed in histogram as Δ fluorescence intensity before and after the compound administration. All data are expressed as the mean \pm SEM of 3 independent experiments. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. 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)] \times 100}. Data are expressed in absolute values (seconds in neurological changes and immobility time, meter for distance travelled, and meter per second for calculation of maximum speed and number 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). All the numerical data are given as mean \pm SEM of four independent experimental replications. Data were analyzed by utilizing repeated measures ANOVA. Results from treatments showing significant overall changes were subjected to post hoc Tukey's for multiple comparison at $p < .05$. 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's test for multiple comparisons. The analysis of the total average effect induced by treatments (expressed in panel d) 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 ($p < .05$) 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 5F-ADBINACA, AB-FUBINACA, and STS-135 for CB₁ and CB₂ cannabinoid receptors

Competition binding experiments carried out in human CB₁ (Figure S1A) or CB₂ (Figure S1B) CHO cell membranes showed a good affinity of the examined compounds. AB-FUBINACA exhibited the highest affinity on human CB₁ receptors with a selectivity index (ratio between the Ki value to human CB₂ and the Ki value to human CB₁) of 1.3 (Table 1). The selectivity index for 5F-ADBINACA was 11.5, whereas STS-135 showed a similar affinity for CB₁ and CB₂ receptors (selectivity index = 1.3). The rank order of affinity for human CB₁ receptor was AB-FUBINACA > 5F-ADBINACA > STS-135. Similar results were observed in competition binding experiments performed in mouse brain membranes (for CB₁ receptors, Figure S1C) and in mouse spleen membranes (for CB₂ receptors, Figure S1D).

Cyclic AMP (cAMP) experiments were carried out to investigate the potency of the tested compounds in CHO cells transfected with human CB₁ (Figure S1E) or CB₂ (Figure S1F) receptors. The rank order of potency was the same obtained for the affinity either on human CB₁ or human CB₂ receptors. In particular, AB-FUBINACA was the most potent compound on human CB₁ and CB₂ receptors with potency values of 1.36 ± 0.09 nM and 1.95 ± 0.14 nM, respectively (Table 1). All the examined compounds were able to completely inhibit the forskolin-stimulated cAMP production, as full agonists (Figure S1E,F).

3.2 | Major neurological changes

Significant neurological alterations were observed in mice following systemic administration of 5F-ADBINACA, AB-FUBINACA, and STS-135 (0.01–6 mg/kg i.p.) but not in vehicle mice (Table 2). In particular, administration of high dose (6 mg/kg, i.p.) of AB-FUBINACA and STS-135 induced spontaneous convulsions, hyperreflexia, and myoclonias in mice: those effects were not observed after the administration of 5F-ADBINACA and Δ^9 -THC (Table 2). STS-135 and AB-FUBINACA induced convulsions in 75% and 60% of treated animals, respectively. STS-135 and AB-FUBINACA promoted seizures with similar latency

TABLE 1 Binding and functional parameters of 5F-ADBINACA, AB-FUBINACA, and STS-135 to human and mouse CB₁ and CB₂ receptors

Compound	hCB1 CHO membranes ^a Ki (nM)	hCB2 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 IC50 (nM)	hCB ₂ CHO cells ^b IC50 (nM)
5F-ADBINACA	2.37 \pm 0.18	27.2 \pm 2.4	5.17 \pm 0.43	23.6 \pm 1.9	6.26 \pm 0.56	42.4 \pm 3.8
AB-FUBINACA	0.734 \pm 0.071	0.933 \pm 0.082	1.26 \pm 0.08	1.12 \pm 0.08	1.36 \pm 0.09	1.95 \pm 0.14
STS-135	4.32 \pm 0.36	5.45 \pm 0.49	4.82 \pm 0.46	6.84 \pm 0.43	13.1 \pm 1.1	16.4 \pm 1.0

Note. Data are expressed as mean \pm SEM.

^a[³H]-CP-55,940 competition binding experiments.

^bCyclic AMP experiments.

TABLE 2 Effects of the systemic administration of Δ^9 -THC (0.01–100 mg/kg), JWH-018, 5F-ADBINAACA, AB-FUBINAACA, and STS-135 (0.01–6 mg/kg i.p.), on the neurological changes of the mouse

Compound	Vehicle	Δ^9 -THC ^a			JWH-018 ^b			5F-ADBINAACA			AB-FUBINAACA			STS-135			
		0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	6
Elevation tail																	
Frequency (%)	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	75
Score	-	-	0.6 ± 0.76	1.1 ± 0.8	-	0.5 ± 0.41	2.4 ± 0.33	-	-	0.4 ± 0.22**	-	-	0.4 ± 0.35	1.4 ± 0.3	-	-	2.6 ± 0.4**
Duration (sec)	-	-	654.7 ± 82.9	934.7 ± 88.2	-	412.6 ± 132.9	1766.6 ± 189.7	-	-	422 ± 101.5***	-	-	410 ± 102	542 ± 95**	-	-	1000 ± 208 *
Latency (sec)	-	-	112.5 ± 33.9	103.6 ± 17.4	-	92.5 ± 17.1	88.6 ± 13.4	-	-	100.7 ± 15	-	-	125 ± 20	185.5 ± 20**	-	-	110.6 ± 20
Hypertilexia																	
Frequency (%)	-	-	-	-	-	-	80	-	-	-	-	-	-	60	-	-	75
Duration (sec)	-	-	-	-	-	-	1439.8 ± 45.3	-	-	-	-	-	-	1010 ± 197.5	-	-	963 ± 108
Latency (sec)	-	-	-	-	-	-	93.5 ± 21.2	-	-	-	-	-	-	193 ± 41	-	-	209 ± 82
Myoclonie																	
Frequency (%)	-	-	-	-	-	-	80	-	-	-	-	-	-	60	-	-	80
Duration (sec)	-	-	-	-	-	-	669.7 ± 36.6	-	-	-	-	-	-	1552.3 ± 307*	-	-	1103 ± 218
Latency (sec)	-	-	-	-	-	-	109.7 ± 16.35	-	-	-	-	-	-	215 ± 48.2	-	-	254 ± 41
Convulsion																	
Frequency (%)	-	-	-	-	-	-	70	-	-	-	-	-	-	60	-	-	75
Duration (sec)	-	-	-	-	-	-	369.7 ± 32.2	-	-	-	-	-	-	821 ± 198	-	-	2234 ± 502***
Latency (sec)	-	-	-	-	-	-	109.7 ± 16.3	-	-	-	-	-	-	220 ± 71	-	-	211.2 ± 75
Spontaneous aggressiveness																	
Frequency (%)	-	-	-	-	-	-	90	-	-	-	-	-	-	-	-	-	-
Score (n° of bites)	-	-	-	-	-	-	8.2 ± 3.1	-	-	-	-	-	-	-	-	-	-
Duration (sec)	-	-	-	-	-	-	2750 ± 621	-	-	-	-	-	-	-	-	-	-
Stimulated aggressiveness																	
Frequency (%)	-	-	-	-	-	-	100	-	-	-	-	-	-	70	-	-	100
Score (n° of bites)	-	-	-	-	-	-	9 ± 1	-	-	-	-	-	-	2.5 ± 2	-	-	2.9 ± 0.5***
Duration (sec)	-	-	-	-	-	-	15832 ± 307	-	-	-	-	-	-	3587 ± 823	-	-	4294.7 ± 931***

Note. 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.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ versus JWH-018 at the same dosage.

^aFrom Vigolo et al., 2015

^bFrom Ossato et al., 2016

($F_{2,29} = 1.031$, $p = 0.3702$) but longer duration ($F_{2,29} = 9.711$, $p = 0.0007$) to those produced by JWH-018 (Table 2).

STS-135 administered at 1 and 6 mg/kg induced hyperreflexia in 20% and 75% of treated animals, whereas AB-FUBINACA at 6 mg/kg caused hyperreflexia in 60% of mice (Table 2). STS-135 and AB-FUBINACA provoked hyperreflexia with latency ($F_{2,29} = 1.508$, $p = 0.2393$) and duration ($F_{2,29} = 3.340$, $p = 0.1255$) similar to those produced by JWH-018. STS-135 administered at 1 and 6 mg/kg caused myoclonias in 80% and 50% of treated mice, respectively, whereas AB-FUBINACA at 6 mg/kg induced myoclonias in 60% of treated animals (Table 2). STS-135 and AB-FUBINACA at 6 mg/kg caused myoclonias with same latency ($F_{2,29} = 3.052$, $p = 0.0639$) but longer duration ($F_{2,29} = 15.66$, $p < 0.0001$) than those produced by JWH-018. STS-135, AB-FUBINACA, and JWH-018 administered at 1 and 6 mg/kg induced tail elevation in mice, whereas 5F-ADBINACA was effective only at 6 mg/kg. STS-135 at 1 mg/kg promoted tail elevation with grater score ($F_{2,29} = 10.28$, $p = 0.0005$), duration ($F_{2,29} = 4.859$, $p = 0.0158$), but with comparable latency ($F_{2,29} = 0.7283$, $p = 0.4920$) than those produced by JWH-018 and AB-FUBINACA (Table 2). Finally, 5F-ADBINACA, AB-FUBINACA, and STS-135 promoted stimulated aggressiveness in mice, whereas spontaneous episodes did not appear. In particular, STS-135 and AB-FUBINACA caused stimulated aggressiveness at 1 and 6 mg/kg, whereas 5F-ADBINACA was effective only at 6 mg/kg. 5F-ADBINACA, AB-FUBINACA, and STS-135 at 6 mg/kg induced stimulated aggressiveness with less duration ($F_{3,39} = 103.5$, $p < .0001$) and score ($F_{3,39} = 13.02$, $p < .0001$) than JWH-018. All neurological changes were prevented by pretreatment with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p. injected 20 min before of the 5F-ADBINACA, AB-FUBINACA, and STS-135 administration; data not shown).

3.3 | Sensorimotor studies

3.3.1 | Evaluation of the visual object response

Visual object response tended to be reduced in vehicle-treated mice over 5 hr observation (~23% of reduction at 300 min; Figure 2a–c,f), and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-ADBINACA (0.01–6 mg/kg i.p.) dose dependently reduced the visual object response in mice and the effect persisted up to 5 hr (Figure 2a: significant effect of treatment, $F_{4,280} = 56.14$, $p < .0001$; time $F_{7,280} = 32.16$, $p < .0001$; and time x treatment interaction, $F_{28,280} = 3.306$, $p < .0001$). AB-FUBINACA (0.01–6 mg/kg i.p.) transiently reduced the visual object response in mice only at higher doses tested (1 and 6 mg/kg i.p.), and the effect persisted up to 180 min only for the dose of 6 mg/kg (Figure 2b: significant effect of treatment, $F_{4,280} = 286.3$, $p < .0001$; time, $F_{7,280} = 48.43$, $p < .0001$; and time x treatment interaction, $F_{28,280} = 37.74$, $p < .0001$). Similarly, STS-135 (0.01–6 mg/kg i.p.) long lasting inhibited the visual object response, and its effect was more deep than those caused by others SCBs considered (Figure 2c: significant effect of treatment, $F_{4,280} = 508.8$, $p < .0001$; time, $F_{7,280} = 105.2$, $p < .0001$; and time x treatment interaction, $F_{28,280} = 34.74$, $p < .0001$). The inhibition of visual object response induced by the highest dose of 5F-ADBINACA

(6 mg/kg), and AB-FUBINACA (6 mg/kg) was fully prevented by the pretreatment with AM 251 (6 mg/kg i.p.; Figure 2e: significant effect of treatment, $F_{3,56} = 13.60$, $p < .0001$; time, $F_{1,56} = 26.59$, $p < .0001$; and time x treatment interaction, $F_{3,56} = 5.586$, $p = 0.0020$). Surprisingly, AM 251 (6 mg/kg i.p.) partially prevented the effect induced by STS-135 at 6 mg/kg (Figure 2f: significant effect of treatment, $F_{3,224} = 240.6$, $p < .0001$; time, $F_{7,224} = 46.15$, $p < .0001$; and time x treatment interaction, $F_{21,224} = 16.70$, $p < .0001$). Conversely, AM 251 (1 mg/kg i.p.) totally prevented the inhibition of the visual object response induced by administration of STS-135 at 1 mg/kg (Figure S2A: significant effect of treatment, $F_{3,224} = 179.6$, $p < .0001$; time, $F_{7,224} = 17.61$, $p < .0001$; and time x treatment interaction, $F_{21,224} = 9.838$, $p < .0001$). The administration of AM 251 (1–6 mg/kg i.p.) did not alter the visual object response in mice. 5F-ADBINACA, AB-FUBINACA, and STS-135 inhibited the visual placing response in a prolonged manner although the effect appeared to be lower with respect to those induced by JWH-018 and Δ^9 -THC at the same doses. Conversely, in the case of STS-135 (1 and 6 mg/kg), the effects were similar to those induced by Δ^9 -THC (Figure 2d; $F_{23,191} = 35.81$, $p < .0001$).

3.3.2 | Evaluation of the acoustic response

Acoustic response tended to be reduced in vehicle-treated mice over 5 hr observation (~13% of reduction at 300 min; Figure 3a–c), and the effect was similar in naïve untreated animals (data not shown). Systemic administration of 5F-ADBINACA (6 mg/kg i.p.) transiently reduced the acoustic response in mice, and the effect was evident up to 180 min after drug injection. Interestingly, that 5F-ADBINACA inhibited acoustic response also at 0.1 mg/kg, although the effect appeared at the end of observation (Figure 3a: significant effect of treatment, $F_{4,280} = 22.70$, $p < .0001$; time, $F_{7,280} = 14.40$, $p < .0001$; and time x treatment interaction, $F_{28,280} = 1.502$, $p = 0.0541$). Also AB-FUBINACA (6 mg/kg i.p.) inhibited the acoustic response, and its effect was prompt but shorter (up to 60 min) compared to that caused by 5F-ADBINACA administration (Figure 3b: significant effect of treatment, $F_{4,280} = 39.88$, $p < .0001$; time, $F_{7,280} = 4.988$, $p < .0001$; and time x treatment interaction, $F_{28,280} = 4.205$, $p < .0001$). Systemic administration of STS-135 (0.01–6 mg/kg i.p.) reduced the acoustic response in mice in a dose dependent manner. The onset of the effect at 1–6 mg/kg was deeper compared to those induced by other SCBs (Figure 3c: significant effect of treatment, $F_{4,280} = 145.1$, $p < .0001$; time, $F_{7,280} = 14.63$, $p < .0001$; and time x treatment interaction, $F_{28,280} = 7.596$, $p < .0001$). The inhibition of acoustic response induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 (6 mg/kg) was prevented by the pretreatment with AM 251 (6 mg/kg i.p.; Figure 3e: significant effect of treatment, $F_{3,56} = 15.03$, $p < .0001$; time, $F_{1,56} = 56.88$, $p < .0001$; and time x treatment interaction, $F_{3,56} = 13.30$, $p < .0001$), which alone did not alter the acoustic response in mice (data not shown). The inhibitory effect caused by 5F-ADBINACA and AB-FUBINACA appeared to be less potent than those evoked by JWH-018 and Δ^9 -THC administration. Conversely, the administration of STS-135 at 6 mg/kg induced an effect similar to that caused by Δ^9 -THC administration at the same dose (Figure 3 d; $F_{23,191} = 24.97$, $p < .0001$).

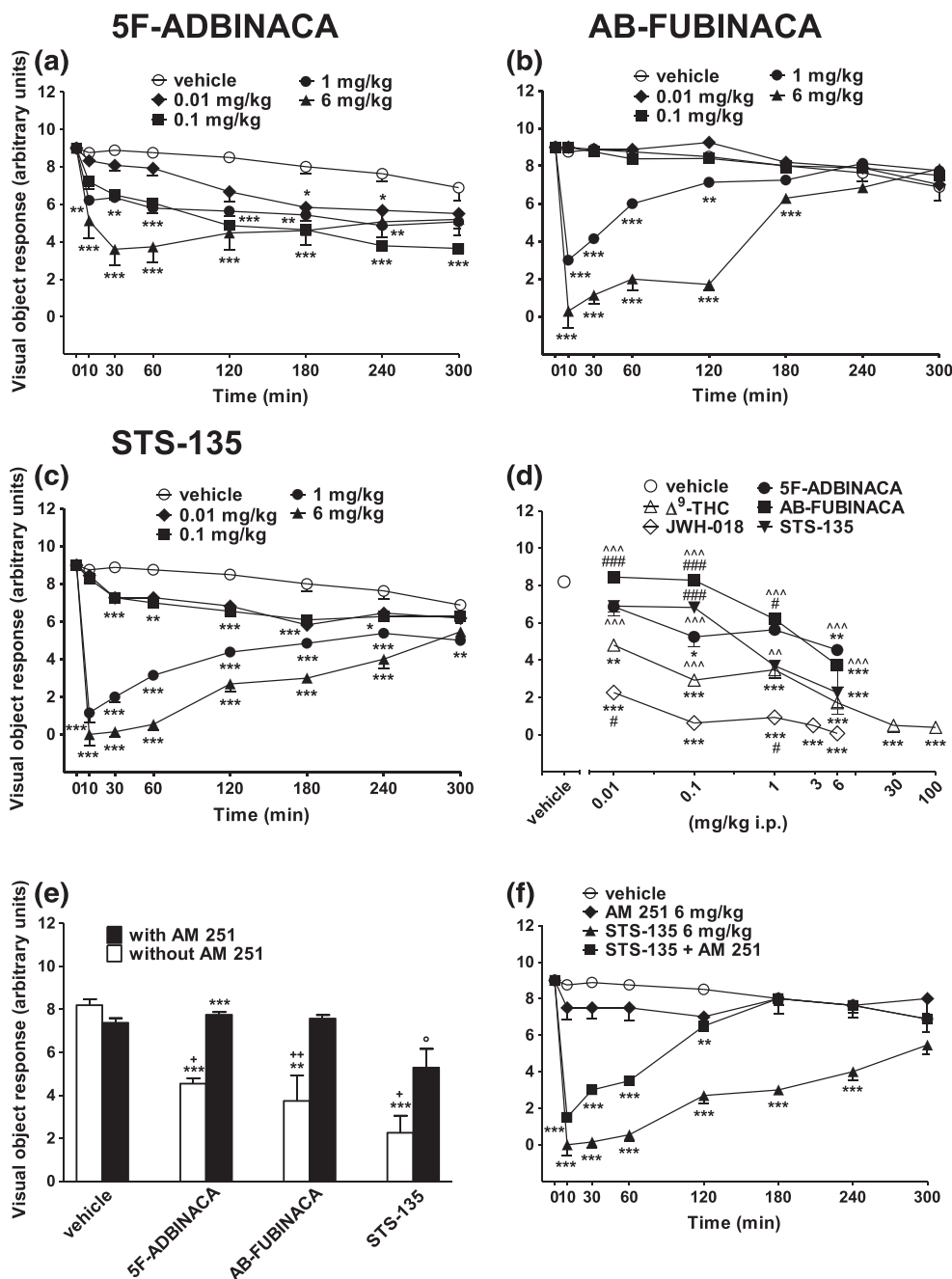


FIGURE 2 Intraperitoneal injection (0.01–6 mg/kg) of 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the visual object test in mice; (d) evaluation of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg) and JWH-018 (0.01–6 mg/kg i.p.); (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.) and (f) time course of evaluation of STS-135 with AM 251 at 6 mg/kg. Data are expressed as arbitrary units and represent the mean \pm 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 the dose response curve of each compound at different times (panels a, b, and c), and for the interaction with the AM 251 (panels e and f), whereas the statistical analysis of 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; + $p < 0.05$, ++ $p < 0.01$ versus AM 251 + agonist and ° $p < 0.05$, versus AM 251. ^cFrom Ossato et al., 2015

3.3.3 | Evaluation of tactile response

Vibrissae reflex did not change in vehicle-treated mice over 5 hr observation (Figure S3A–C), and the response was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-ADBINACA, AB-FUBINACA, and STS-135 did not alter the vibrissae reflex (Figure S3A: significant effect of treatment, $F_{4,280} = 2.423$, $p = 0.0485$; time, $F_{7,280} = 0.2142$, $p = 0.9820$; and time \times treatment

interaction, $F_{28,280} = 0.2142$, $p = 1$; Panel SB: significant effect of treatment, $F_{4,280} = 1.564$, $p = 0.1842$; time, $F_{7,280} = 0.3723$, $p = 0.9180$; and time \times treatment interaction, $F_{28,280} = 0.3723$, $p = 0.9987$; Panel SC: significant effect of treatment, $F_{4,280} = 0.9608$, $p = 0.4294$; time, $F_{7,280} = 0.1612$, $p = 0.9923$; and time \times treatment interaction, $F_{28,280} = 0.3495$, $p = 0.9993$). These data demonstrate that all three SCBs behave as Δ^9 -THC (Figure S3D; $F_{23,191} = 4.879$, $p < 0.0001$).

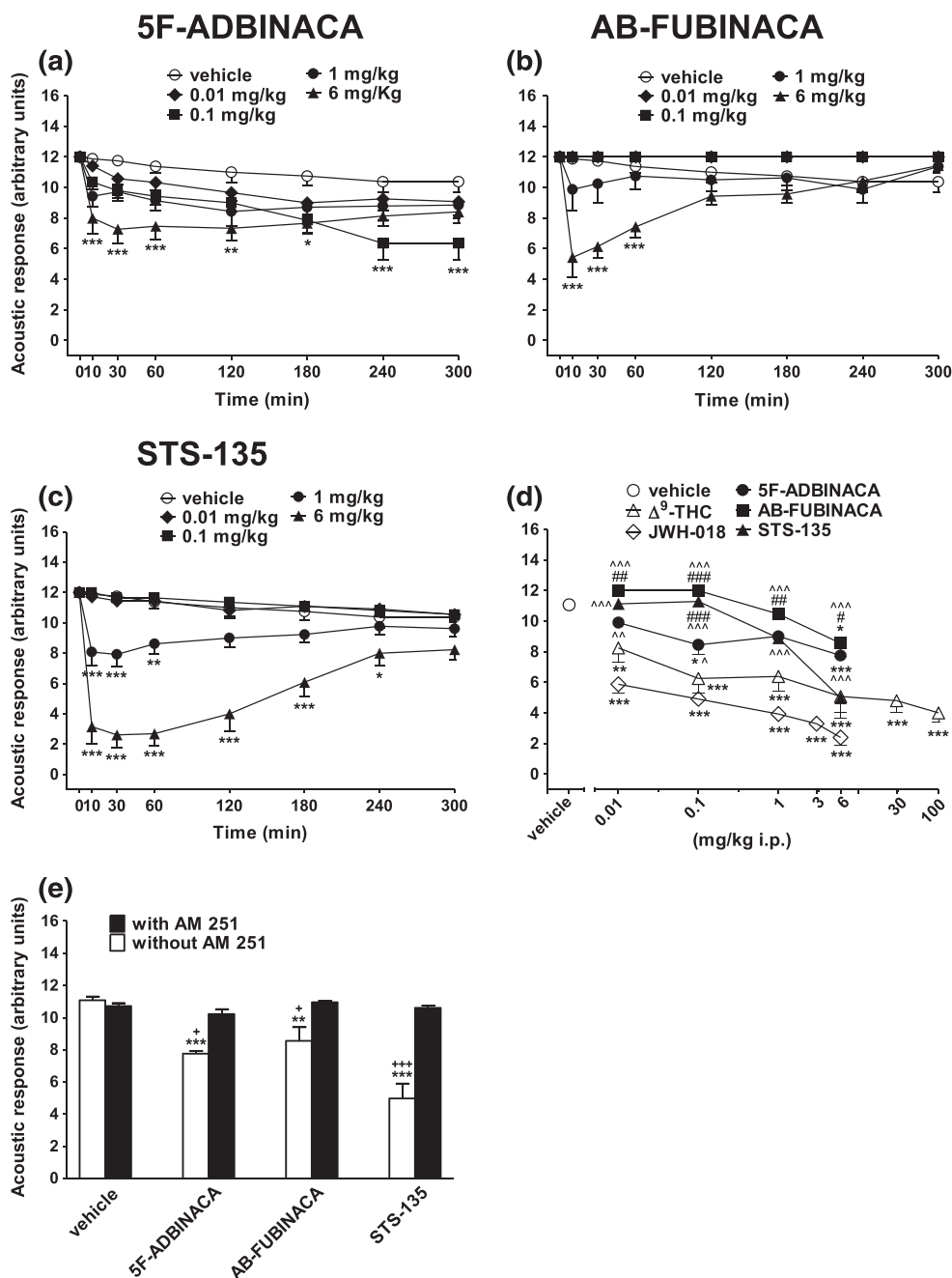


FIGURE 3 Intraperitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the acoustic response test in the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg i.p.)^c and JWH-018 (0.01–6 mg/kg i.p.)^c; (e) Interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compound at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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. ^cFrom Ossato et al., 2015

Pinnae reflex did not change in vehicle-treated mice over 5 hr observation (Figure S4A–C), and the response was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-ADBINACA transiently and slightly impaired pinnae reflex in mice only at the highest dose tested (6 mg/kg; Figure S4A: significant effect of treatment, $F_{4,280} = 12.36$, $p < 0.0001$; time, $F_{7,280} = 3.507$, $p = 0.0013$; and time \times treatment interaction, $F_{28,280} = 1.095$, $p = 0.3428$). Similarly, AB-FUBINACA at 6 mg/kg

transiently inhibited pinnae reflex (Panel 4SB: significant effect of treatment, $F_{4,280} = 14.95$, $p < 0.0001$; time, $F_{7,280} = 1.5$, $p = 0.1669$; and time \times treatment interaction, $F_{28,280} = 1.155$, $p = 0.2740$). STS-135 was effective both at 1 and 6 mg/kg and the effect was prolonged at the highest dose tested (6 mg/kg; Panel 4SC: significant effect of treatment, $F_{4,280} = 34.24$, $p < 0.0001$; time, $F_{7,280} = 4.405$, $p = 0.0001$; and time \times treatment interaction, $F_{28,280} = 2.694$, $p < 0.0001$). The inhibition of pinnae reflex induced by the highest

dose of 5F-ADBINACA, AB-FUBINACA, and STS-135 (6 mg/kg) was fully prevented by pretreatment with AM 251 (6 mg/kg i.p.; Figure S4 E: significant effect of treatment, $F_{3,56} = 10.05$, $p < 0.0001$; time, $F_{1,56} = 14.76$, $p = 0.0003$; and time \times treatment interaction, $F_{3,56} = 4.710$, $p = 0.0053$), which alone did not alter the pinnae reflex in mice (data not shown). Inhibitory effects caused by 5F-ADBINACA, AB-FUBINACA, and STS-135 appeared similar to those induced by Δ^9 -THC and less potent than those induced by JWH-018 (Figure S4D; $F_{23,191} = 5.568$, $p < 0.0001$).

Corneal reflex did not change in vehicle-treated mice over 5 hr observation (Figure 4a–c), and the response was similar in naïve untreated animals (data not shown). Systemic administration of 5F-ADBINACA did not alter the corneal reflex in mice (Figure 4a: significant effect of treatment, $F_{4,280} = 4.810$, $p = 0.0009$; time, $F_{7,280} = 0.8266$, $p = 0.5659$; and time \times treatment interaction, $F_{28,280} = 1.083$, $p = 0.3585$). Conversely, the administration of AB-FUBINACA transiently inhibited at 6 mg/kg the corneal reflex in mice (Figure 4b: significant effect of treatment, $F_{4,280} = 20.21$, $p < 0.0001$;

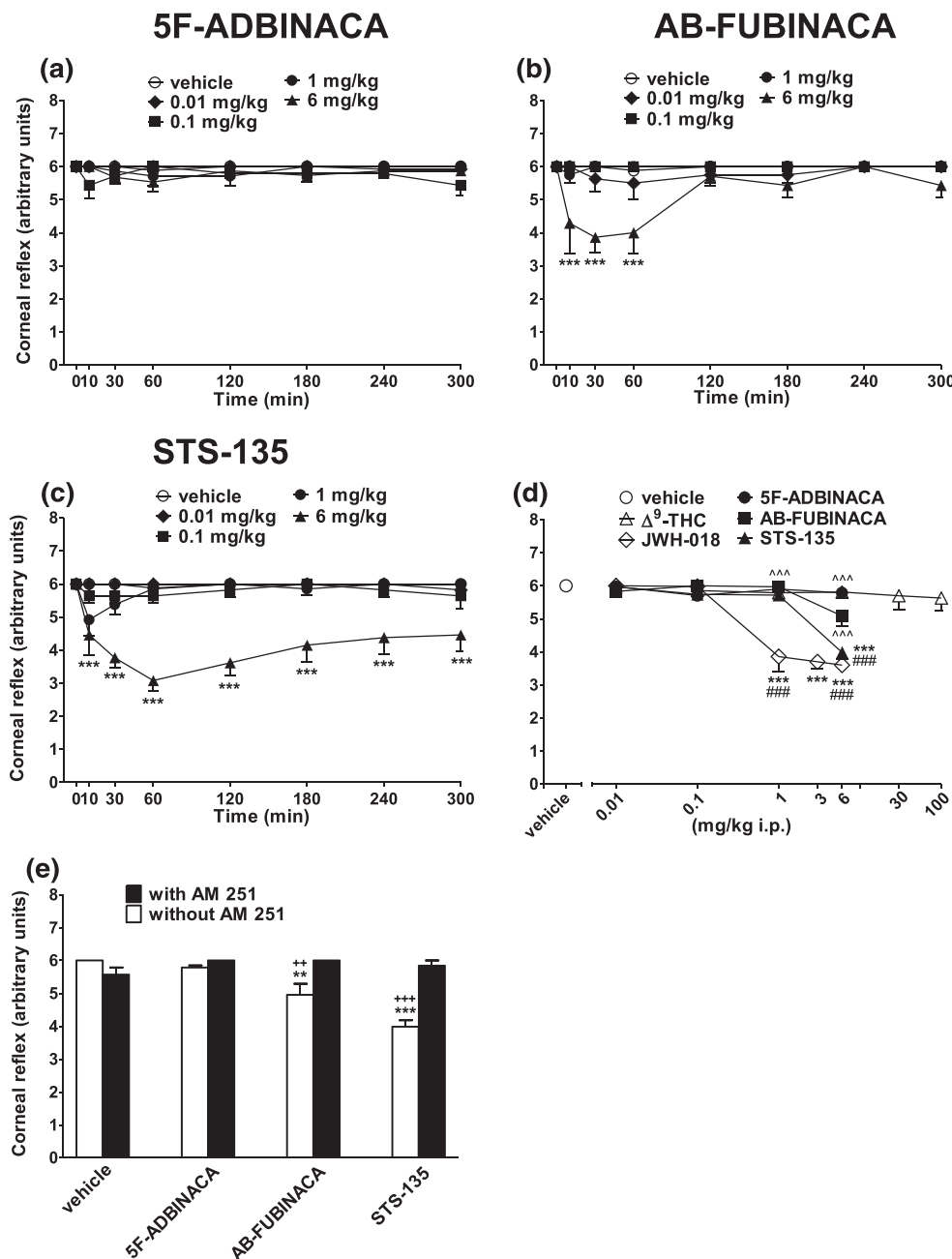


FIGURE 4 Intraperitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the corneal reflex in the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)^f and JWH-018 (0.01–6 mg/kg i.p.)^f. (e) Interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compound at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of panel d was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. ** $p < 0.01$, *** $p < 0.001$ versus vehicle; ^^^ $p < 0.001$ versus JWH-018 and ++ $p < 0.01$, +++ $p < 0.001$ versus AM 251 + agonist. ^fFrom Ossato et al., 2015

time, $F_{7,280} = 3.882$, $p = 0.0005$; and time x treatment interaction, $F_{28,280} = 2.587$, $p < 0.0001$). STS-135 (6 mg/kg) deeply inhibited the corneal reflex in mice and the effect persisted up to 300 min (Figure 4c: significant effect of treatment, $F_{4,280} = 76.44$, $p < 0.0001$; time, $F_{7,280} = 4.307$, $p = 0.0002$; and time x treatment interaction, $F_{28,280} = 2.988$, $p < 0.0001$). The inhibition of corneal reflex induced by the highest dose of AB-FUBINACA and STS-135 (6 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p.; Figure 4e: significant effect of treatment, $F_{3,56} = 13.74$, $p < 0.0001$; time, $F_{1,56} = 31.88$, $p < 0.0001$; and time x treatment interaction,

$F_{3,56} = 17.59$, $p < 0.0001$), which alone did not alter the corneal reflex in mice (data not shown). The effects of STS-135 at 6 mg/kg were higher than those induced by 5F-ADBINACA and AB-FUBINACA and similar to those caused by JWH-018 at the same doses (Figure 4d; $F_{23,191} = 18.55$, $p < 0.0001$).

3.3.4 | Evaluation of the visual placing response

Visual placing response tended to be reduced in vehicle-treated mice over 5 hr observation (~20% of reduction at 300 min; Figure 5a,b),

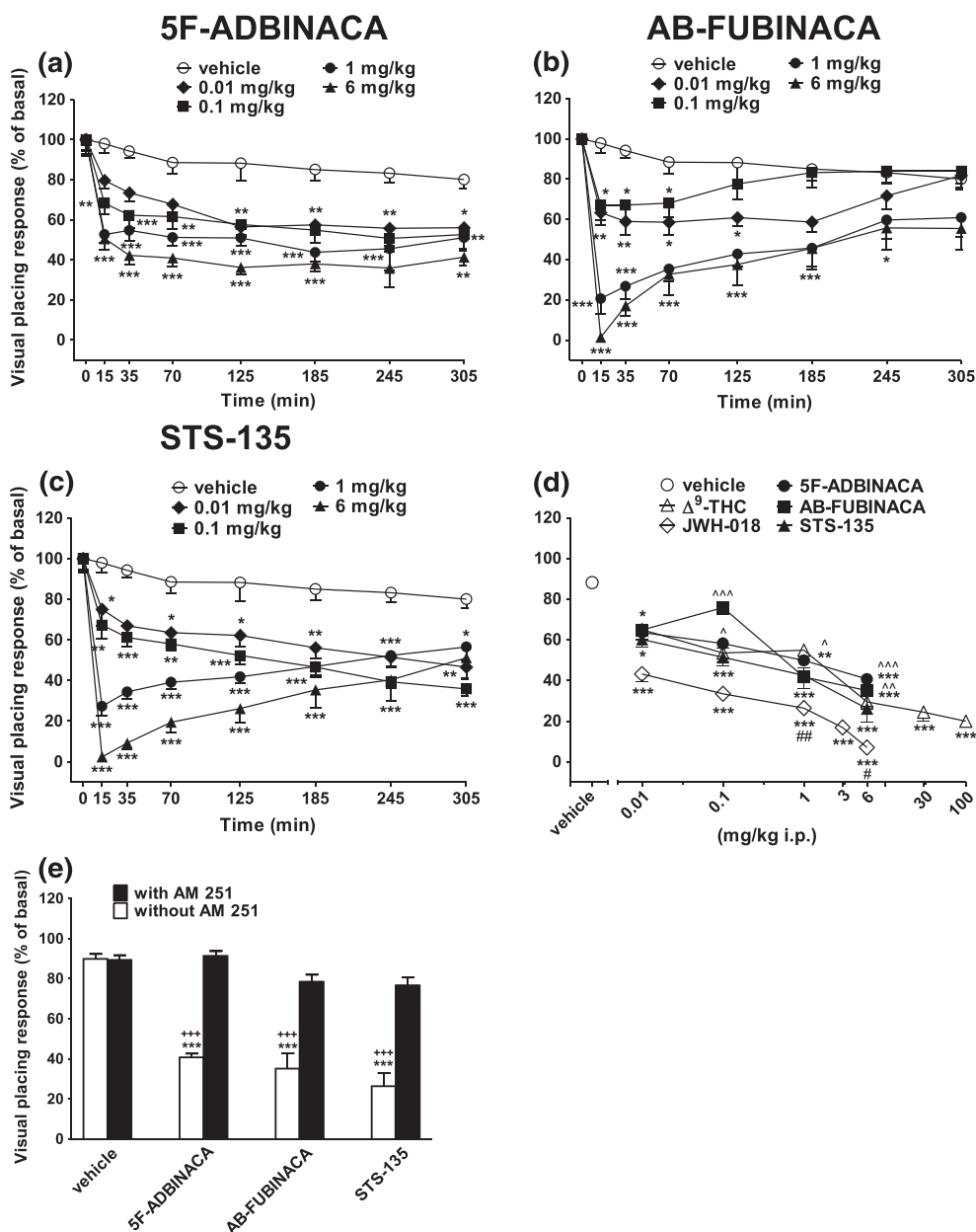


FIGURE 5 Intrapertoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the visual placing test in the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)^c and JWH-018 (0.01–6 mg/kg i.p.)^c; (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compound at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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; ## $p < 0.01$ versus Δ^9 -THC; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ versus JWH-018; +++ $p < 0.001$ versus AM 251 + agonist. ^cFrom Ossato et al., 2015

and the effect was similar in naïve untreated animals (data not shown). Systemic administration of 5F-ADBINACA (0.01–6 mg/kg i.p.) dose dependently reduced the visual placing response in mice and the effect persisted up to 5 hr (Figure 5a: significant effect of treatment, $F_{4,280} = 58.69$, $p < 0.0001$; time, $F_{7,280} = 33.89$, $p < 0.0001$; and time \times treatment interaction, $F_{28,280} = 1.507$, $p = 0.0528$). Furthermore, AB-FUBINACA (0.01–6 mg/kg i.p.) dose dependently reduced the visual placing response in mice, and the effect was prompt but tended to reverse after 185 min of drug administration (Figure 5b: significant effect of treatment, $F_{4,280} = 66.05$, $p < 0.0001$; time, $F_{7,280} = 26.92$, $p < 0.0001$; and time \times treatment interaction, $F_{28,280} = 3.443$, $p < 0.0001$). STS-135 (0.01–6 mg/kg i.p.) dose dependently impaired visual placing response (Figure 5c: significant effect of treatment, $F_{4,280} = 98.70$, $p < 0.0001$; time, $F_{7,280} = 40.90$, $p < 0.0001$; and time \times treatment interaction, $F_{28,280} = 6.406$, $p < 0.0001$). The visual impairment induced by the highest dose of 5F-ADBINACA, AB-FUBINACA, and STS-135 (6 mg/kg i.p.) was prevented by pretreatment with AM 251 (6 mg/kg i.p., Figure 5e: significant effect of treatment, $F_{3,56} = 20.84$, $p < 0.0001$; time, $F_{1,56} = 95.93$, $p < 0.0001$; and time \times treatment interaction, $F_{3,56} = 11.10$, $p < 0.0001$), which alone did not alter the parameter. The inhibition of the visual response induced by STS-135 was higher than those induced by 5F-ADBINACA and AB-FUBINACA administration and was similar to those of Δ^9 -THC (Figure 5d; $F_{23,191} = 22.00$, $p < 0.0001$).

3.4 | Tetrad paradigm for screening cannabinoid-like effect

3.4.1 | Bar test

Systemic administration of 5F-ADBINACA did not induce catalepsy in the bar test (Figure 6a: significant effect of treatment, $F_{4,245} = 0.6317$, $p = 0.6404$; time, $F_{6,245} = 0.2635$, $p = 0.9534$; and time \times treatment interaction, $F_{24,245} = 0.7410$, $p = 0.8065$). Conversely, AB-FUBINACA increased the time spent on bar at 6 mg/kg (Figure 6b: significant effect of treatment, $F_{4,245} = 19.22$, $p < 0.0001$; time, $F_{6,245} = 2.677$, $p = 0.0155$; and time \times treatment interaction, $F_{24,245} = 2.220$, $p = 0.0013$). STS-135 caused a transient increase (up to 75 min) in the time spent on bar at 1 mg/kg and a marked catalepsy at 6 mg/kg, which persisted up to 5 hr (Figure 6c: significant effect of treatment, $F_{4,245} = 329.7$, $p < 0.0001$; time, $F_{6,245} = 21.76$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 14.44$, $p < 0.0001$). The effects were prevented by pretreatment with AM 251, which alone did not induce akinesia and catalepsy (Figure 6e: significant effect of treatment, $F_{3,56} = 15.09$, $p < 0.0001$; time, $F_{1,56} = 29.13$, $p < 0.0001$; and time \times treatment interaction, $F_{3,56} = 12.89$, $p < 0.0001$). STS-135 was more potent in inducing catalepsy than AB-FUBINACA, and its effect was similar to that induced by JWH-018 at 6 mg/kg (Figure 6d; $F_{23,191} = 25.93$, $p < 0.0001$).

3.4.2 | Evaluation of the core and surface body temperature

Systemic administration of 5F-ADBINACA and STS-135 (0.01–6 mg/kg i.p.) reduced both core (Figure 7) and surface (Figure S5) body temperature in mice. AB-FUBINACA slightly reduced only core temperature at 6 mg/kg (Figure 7b) but did not change surface temperature

(Figure S5). In particular, 5F-ADBINACA and AB-FUBINACA provoked a transient reduction in core temperature at 6 mg/kg (-2.5 °C at 50 min and -3 °C at 85 min time point, respectively; Figure 7a: significant effect of treatment, $F_{4,245} = 15.24$, $p < 0.0001$; time, $F_{6,245} = 11.02$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 1.207$, $p = 0.2365$; Panel b: significant effect of treatment, $F_{4,245} = 10.16$, $p < 0.0001$; time, $F_{6,245} = 6.546$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 4.638$, $p < 0.0001$). On the other hand, STS-135 at 1 mg/kg evoked a transient hypothermia in mice (-2 °C at 50 min time point); whereas at 6 mg/kg, the effect (-4.8 °C at 85 min time point) persisted up to 260 min (Figure 7c: significant effect of treatment, $F_{4,245} = 93.68$, $p < 0.0001$; time, $F_{6,245} = 3.972$, $p = 0.0008$; and time \times treatment interaction, $F_{24,245} = 4.562$, $p < 0.0001$). 5F-ADBINACA, AB-FUBINACA, and STS-135 were ineffective in the range of doses of 0.01–0.1 mg/kg. In studies with 5F-ADBINACA and STS-135 (at 1 and 6 mg/kg) core body hypothermia was accompanied by a reduction of the surface body temperature (Figure S5A: significant effect of treatment, $F_{4,245} = 14.86$, $p < 0.0001$; time, $F_{6,245} = 4.314$, $p = 0.0004$; and time \times treatment interaction, $F_{24,245} = 0.5821$, $p = 0.9423$; Figure S5 C: significant effect of treatment, $F_{4,245} = 50.58$, $p < 0.0001$; time, $F_{6,245} = 6.842$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 3.074$, $p < 0.0001$). Core and surface body temperature changes were prevented by pretreatment with AM 251, which did not affect body temperature when administered alone (Figure 7 E: significant effect of treatment, $F_{3,56} = 17.61$, $p < 0.0001$; time, $F_{1,56} = 55.21$, $p < 0.0001$; and time \times treatment interaction, $F_{3,56} = 8.385$, $p = 0.0001$; Figure S5 E: significant effect of treatment, $F_{3,56} = 12.24$, $p < 0.0001$; time, $F_{1,56} = 59.09$, $p < 0.0001$; and time \times treatment interaction, $F_{3,56} = 11.34$, $p < 0.0001$). Furthermore, overall changes on core and surface temperature induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 administration were similar to those induced by Δ^9 -THC but less potent compared to those caused by JWH-018 (Figure 7d; $F_{23,191} = 19.10$, $p < 0.0001$; Figure S5d: $F_{23,191} = 11.70$, $p < 0.0001$).

3.4.3 | Evaluation of pain induced by mechanical and thermal stimuli

Systemic administration of 5F-ADBINACA (0.01–6 mg/kg i.p.) increased in a dose dependent manner the threshold to acute mechanical pain stimulus in mice, and the analgesic effect was prolonged up to 5 hr (Figure 8a: significant effect of treatment, $F_{4,245} = 50.53$, $p < 0.0001$, time, $F_{6,245} = 7.263$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 2.037$, $p = 0.0038$). On the other hand, systemic administration of AB-FUBINACA and STS-135 transiently induced an analgesic effect at 1 and 6 mg/kg (Figure 8 b: significant effect of treatment, $F_{4,245} = 16.02$, $p < 0.0001$; time, $F_{6,245} = 12.17$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 2.271$, $p = 0.0009$; Figure 8c: significant effect of treatment, $F_{4,245} = 134.9$, $p < 0.0001$; time, $F_{6,245} = 42.93$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 16.51$, $p < 0.0001$). Effects were prevented by pretreatment with AM 251, which alone did not alter the threshold to acute mechanical pain stimuli (Figure 8e: significant effect of treatment, $F_{3,56} = 5.422$, $p = 0.0024$; time, $F_{1,56} = 35.50$, $p < 0.0001$; and time \times treatment interaction, $F_{3,56} = 4.442$, $p = 0.0072$). The analgesic effect induced by each one SCBs was similar to those induced by Δ^9 -THC and lower to that caused by JWH-018 (Figure 8d: $F_{23,191} = 25.13$, $p < 0.0001$).

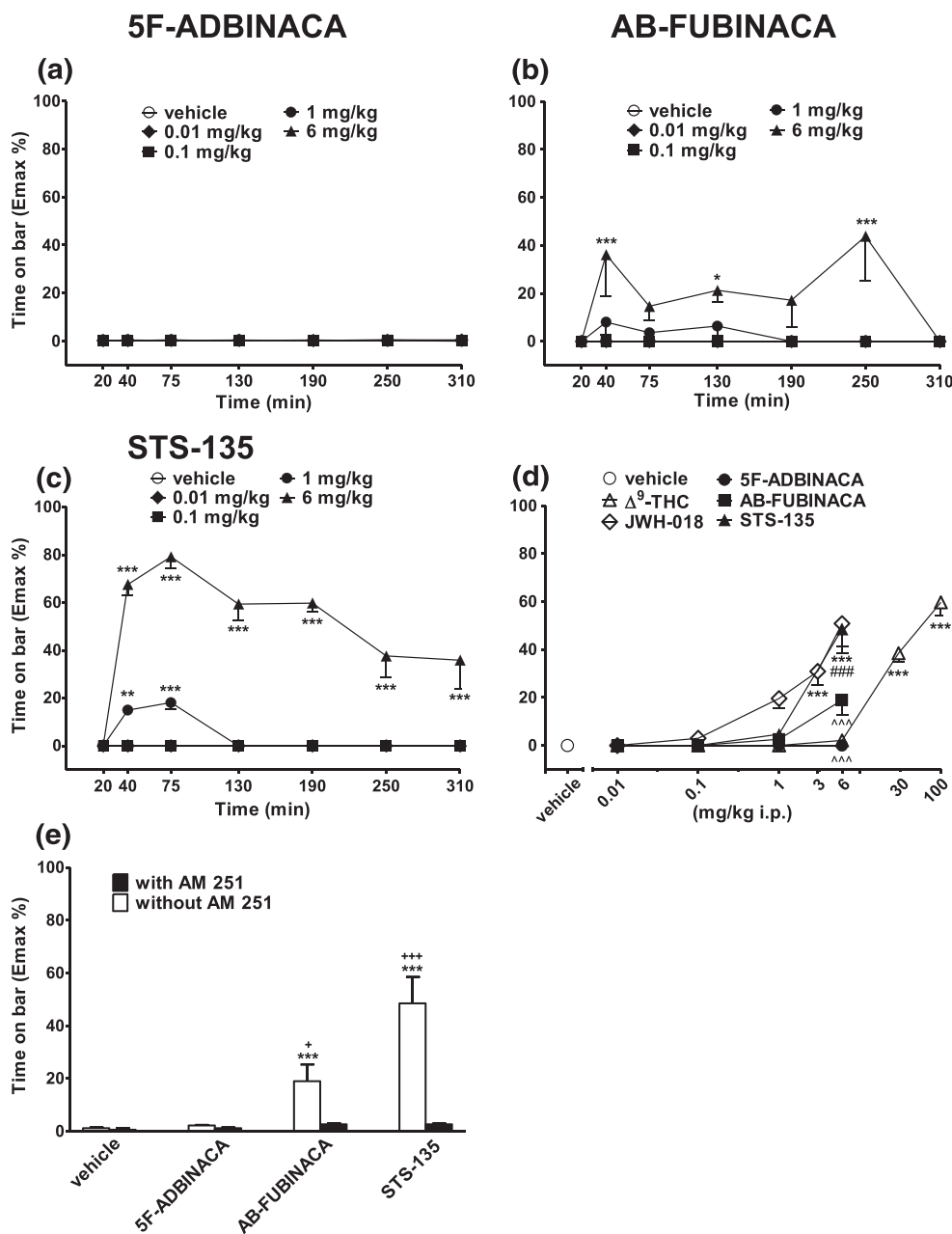


FIGURE 6 Intrapерitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINA, (b) AB-FUBINA, and (c) STS-135 on the bar test of the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)^a and JWH-018 (0.01–6 mg/kg i.p.)³; (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compounds at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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; ^^ $p < 0.001$ versus JWH-018; + $p < 0.05$, +++ $p < 0.001$ versus AM 251 + agonist. ^afrom Vigolo et al., 2015

Systemic administration of 5F-ADBINA, AB-FUBINA, and STS-135 (0.01–6 mg/kg i.p.) transiently increased the threshold to acute thermal pain stimulus in mice only at the highest dose tested (Figure 9a: significant effect of treatment, $F_{4,245} = 7.550$, $p < 0.0001$; time, $F_{6,245} = 2.761$, $p = 0.0129$; and time \times treatment interaction, $F_{24,245} = 1.920$, $p = 0.0075$; Figure 9b: significant effect of treatment, $F_{4,245} = 11.42$, $p < 0.0001$; time, $F_{6,245} = 2.537$, $p = 0.0212$; and time \times treatment interaction, $F_{24,245} = 1.152$, $p = 0.2883$). In particular, STS-135 quickly induced a robust elevation of the pain threshold, which persisted up to 145 min after administration (Figure 9c: significant

effect of treatment, $F_{4,245} = 52.10$, $p < 0.0001$; time, $F_{6,245} = 10.79$, $p < 0.0001$; and time \times treatment interaction, $F_{24,245} = 4.903$, $p < 0.0001$). SCB effects were prevented by pretreatment with AM 251, which alone did not alter the threshold to acute thermal pain stimuli (Figure 9e: significant effect of treatment, $F_{3,56} = 9.829$, $p < 0.0001$; time, $F_{1,56} = 12.85$, $p = 0.0007$; and time \times treatment interaction, $F_{3,56} = 4.258$, $p = 0.0089$).

5F-ADBINA and AB-FUBINA were less effective than STS-135, Δ^9 -THC, and JWH-018 administration, whereas STS-135 was equally effective to Δ^9 -THC (Figure 9d: $F_{23,191} = 15.73$, $p < 0.0001$).

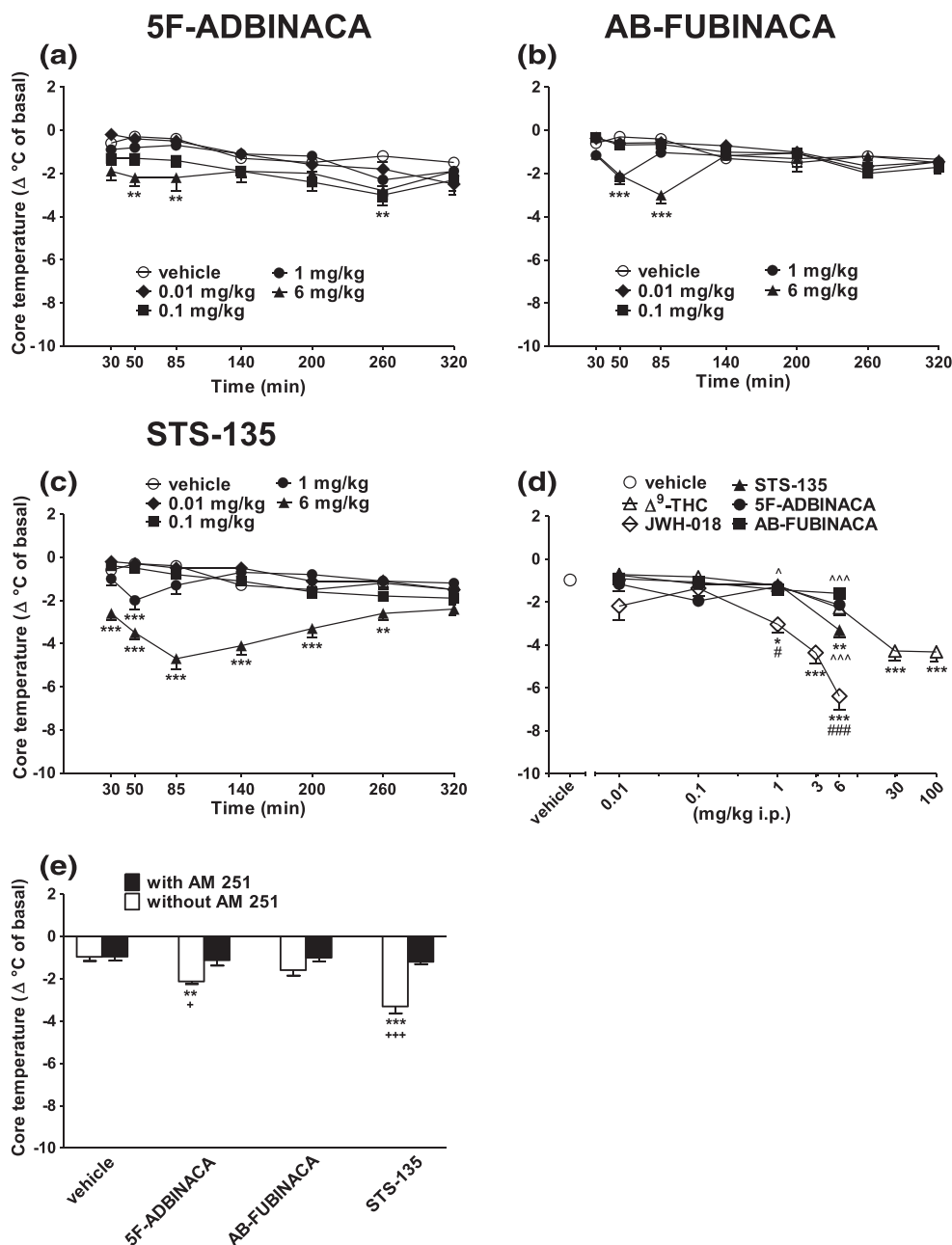


FIGURE 7 Intrapertoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on mouse core temperature; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)^a and JWH-018 (0.01–6 mg/kg i.p.)³; (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compound at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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; ^ $p < 0.05$, ^^ $p < 0.001$ versus JWH-018 and * $p < 0.05$, + $p < 0.01$, +++ $p < 0.001$ versus AM 251 + agonist. ^aFrom Vigolo et al., 2015

3.4.4 | Accelerod test

In the accelerod test, AB-FUBINACA and STS-135 induced only a transient impairment of stimulated locomotion (inhibition of about 55% and 70%, respectively; Figure 10b: significant effect of treatment, $F_{4,280} = 13.75$, $p < 0.0001$; time, $F_{7,280} = 2.602$, $p = 0.0129$; and time \times treatment interaction, $F_{28,280} = 0.6474$, $p = 0.9165$; Figure 10c: significant effect of treatment, $F_{4,280} = 16$, $p < 0.0001$; time, $F_{7,280} = 0.7354$, $p = 0.6421$; and time \times treatment interaction,

$F_{28,280} = 0.7652$, $p = 0.7999$). On the contrary, 5F-ADBINACA was ineffective (Figure 10a: significant effect of treatment, $F_{4,280} = 4.893$, $p = 0.0008$; time, $F_{7,280} = 0.1784$, $p = 0.9896$; and time \times treatment interaction, $F_{28,280} = 0.501$, $p = 0.9993$). The inhibitory effects were prevented by pretreatment with AM 251, which alone did not affect mice performance (Figure 10e: significant effect of treatment, $F_{3,56} = 25.08$, $p < 0.0001$; time, $F_{1,56} = 29.36$, $p < 0.0001$; and time \times treatment interaction, $F_{3,56} = 21.22$, $p < 0.0001$). AB-FUBINACA and

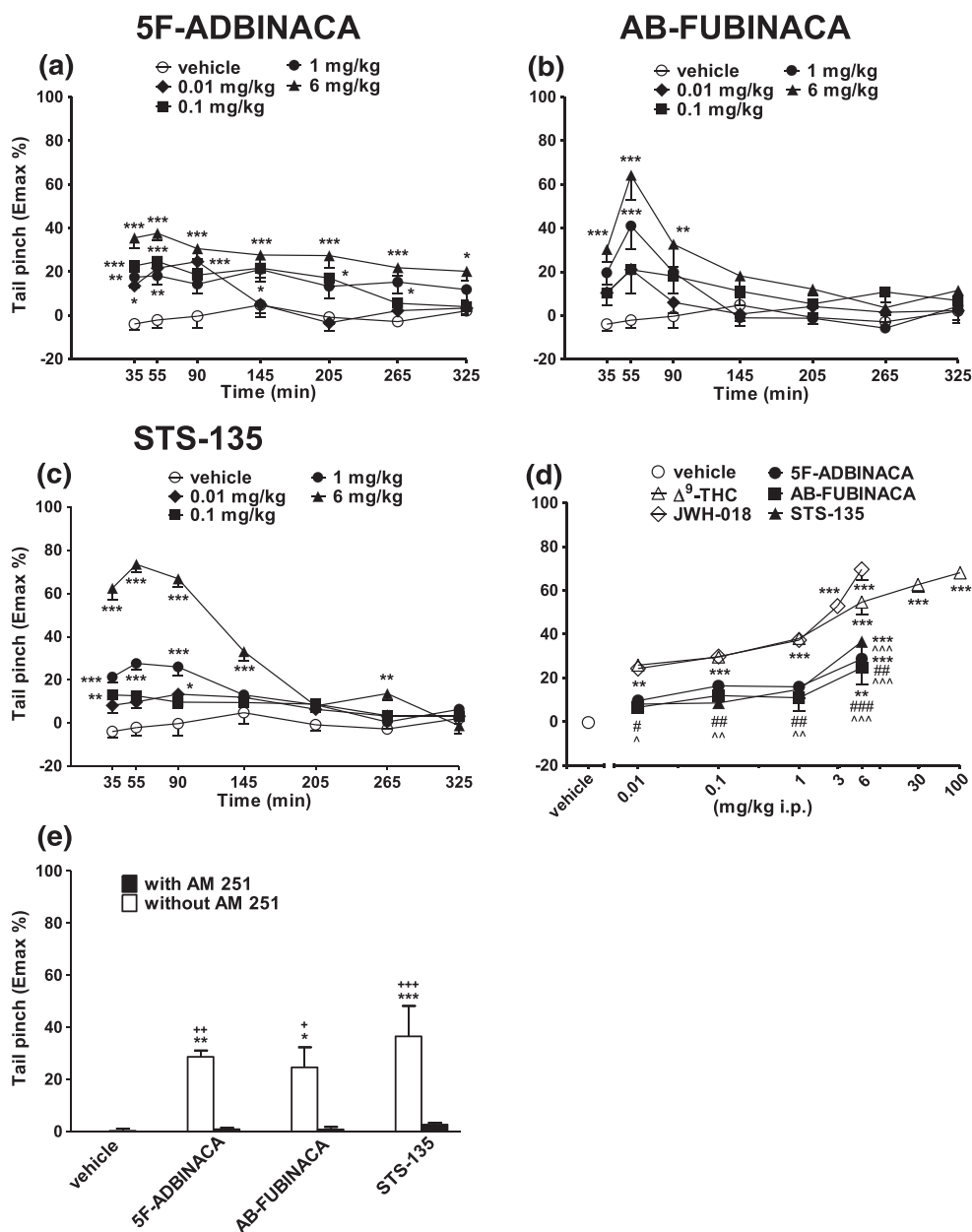


FIGURE 8 Intrapерitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINAICA, (b) AB-FUBINACA, and (c) STS-135 on the tail pinch test of the mouse; (d) comparison of the total average effect observed in 5 hours with Δ^9 -THC (0.01–100 mg/kg)^a and JWH-018 (0.01–6 mg/kg i.p.); (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compounds at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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 + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ versus AM 251 + agonist. ^aFrom Vigolo et al., 2015

STS-135 were less effective in comparison to JWH-018 but more potent than Δ^9 -THC (Figure 10d: $F_{23,191} = 63.79$, $p < 0.0001$).

3.4.5 | Drag test

Systemic administration of AB-FUBINACA and STS-135 (1 and 6 mg/kg) induced a long lasting reduction of number of steps performed with the front paws of the mice (Figure 11b: significant effect of treatment, $F_{4,280} = 11.82$, $p < 0.0001$; time, $F_{7,280} = 2.032$, $p = 0.0512$; and time x treatment interaction, $F_{28,280} = 0.9066$, $p = 0.6055$; Figure 11c:

significant effect of treatment, $F_{4,280} = 37.56$, $p < 0.0001$; time, $F_{7,280} = 1.713$, $p = 0.1058$; and time x treatment interaction, $F_{28,280} = 1.384$, $p = 0.0994$). On the contrary, 5F-ADBINAICA was ineffective (Figure 11 A: significant effect of treatment, $F_{4,280} = 2.108$, $p = 0.0800$; time, $F_{7,280} = 0.5695$, $p = 0.7806$; and time x treatment interaction, $F_{28,280} = 0.2714$, $p = 0.9999$). Inhibitory effects were prevented by pretreatment with AM 251 (Figure 11e: significant effect of treatment, $F_{3,56} = 48.65$, $p < 0.0001$; time, $F_{1,56} = 109.3$, $p < 0.0001$; and time x treatment interaction, $F_{3,56} = 37.73$, $p < 0.0001$). 5F-ADBINAICA, AB-FUBINACA, and STS-135 at the highest dose

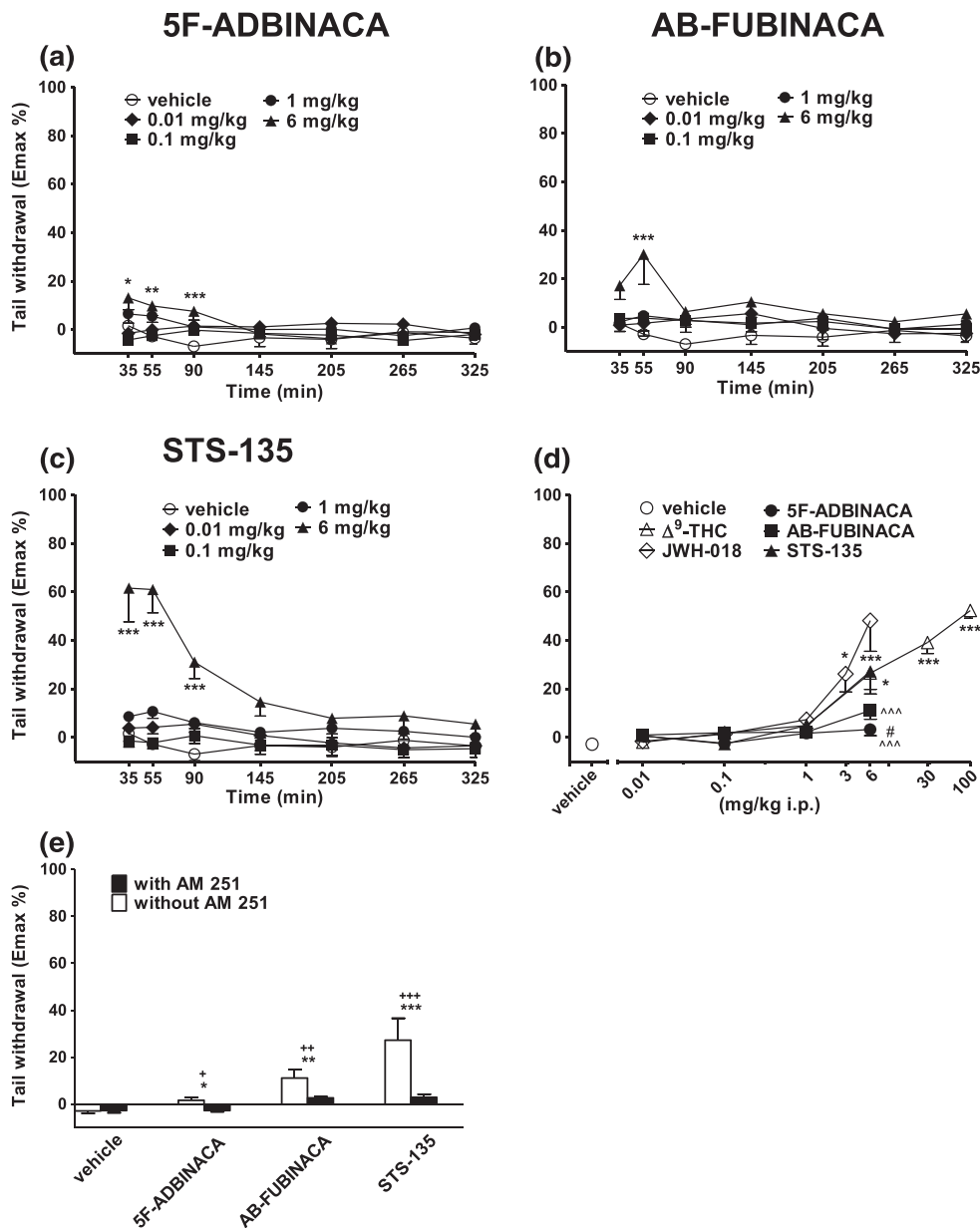


FIGURE 9 Intraperitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the tail withdrawal test of the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)⁹ and JWH-018 (0.01–6 mg/kg i.p.)²; (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compounds at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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 Δ^9 -THC; ^^ $p < 0.001$ versus JWH-018 and + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ versus AM 251 + agonist. ^AFrom Vigolo et al., 2015

tested (6 mg/kg) exerted a more severe effect respect to those of Δ^9 -THC but less than those of JWH-018 (Figure 11d: $F_{23,191} = 27.84$, $p < 0.0001$).

3.4.6 | Studies on spontaneous locomotor activity in mice

To exclude the possibility that reduction of sensorimotor responses could be due to the inhibition of motor activity, we investigated the effect of 5F-ADBINACA, AB-FUBINACA, and STS-135 administration (0.01–6 mg/kg i.p.) on spontaneous locomotor activity in mice. All three SCBs at low doses, facilitated, whereas they transiently inhibited

the spontaneous locomotor activity in mice at higher doses. 5F-ADBINACA increased at 0.1 mg/kg while reduced at 6 mg/kg the total distance travelled (Figure 12a: significant effect of treatment, $F_{4,720} = 12.62$, $p < 0.0001$; time, $F_{15,720} = 52.82$, $p < 0.0001$; and time \times treatment interaction, $F_{60,720} = 1.073$, $p = 0.3337$). Moreover, it reduced the immobility time at 0.1 mg/kg and increased it at 6 mg/kg (Figure 13a: significant effect of treatment, $F_{4,720} = 13.95$, $p < 0.0001$; time, $F_{15,720} = 24.04$, $p < 0.0001$; and time \times treatment interaction, $F_{60,720} = 0.9117$, $p = 0.6650$).

AB-FUBINACA reduced at 6 mg/kg the total distance travelled (Figure 12b: significant effect of treatment, $F_{4,720} = 3.850$,

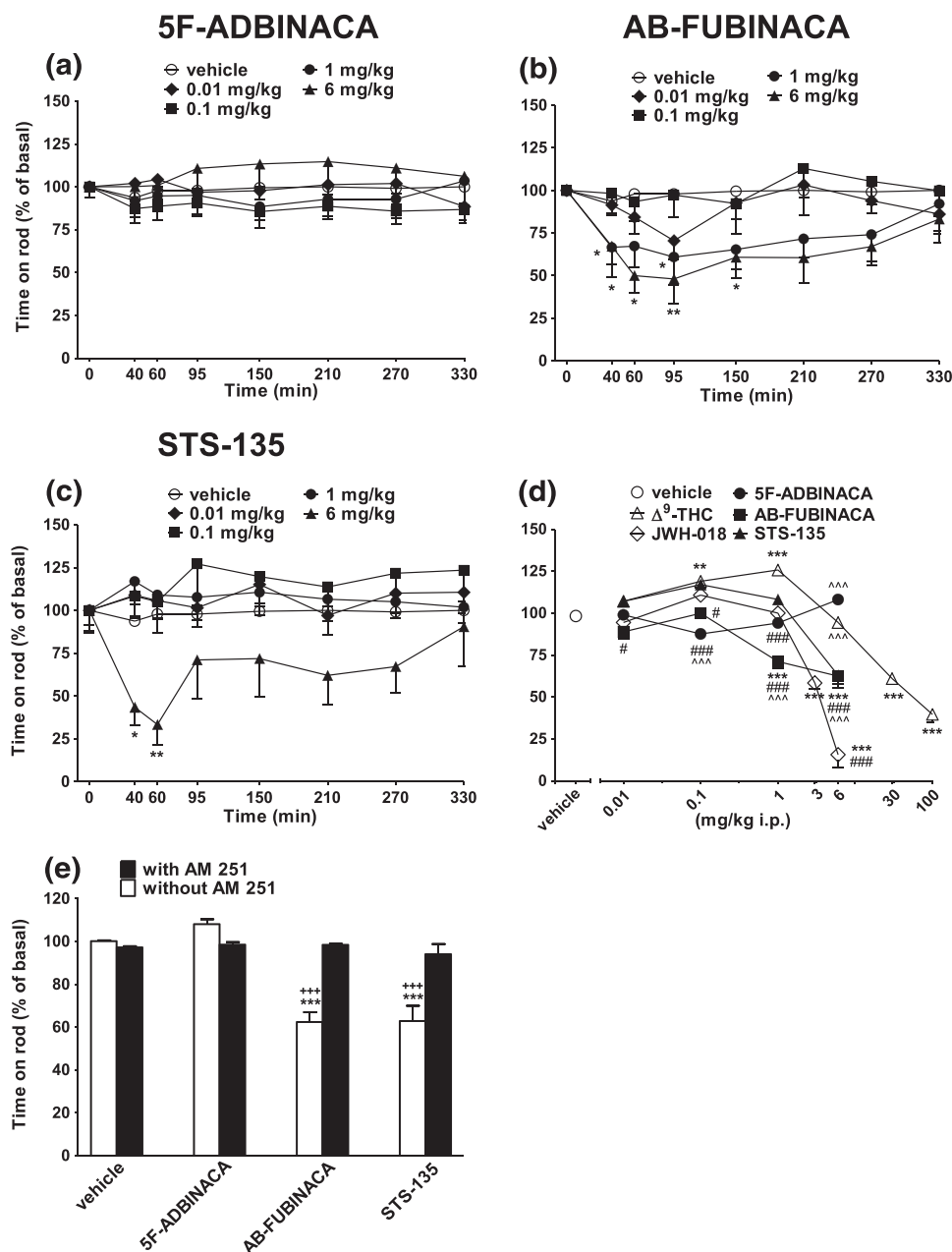


FIGURE 10 Intraperitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINAACA, (b) AB-FUBINAACA, and (c) STS-135 on the accelerated test of the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)^a and JWH-018 (0.01–6 mg/kg i.p.)^a; (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compounds at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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 +++ $p < 0.001$ versus AM 251 + agonist. ^aFrom Vigolo et al., 2015

$p < 0.0042$; time, $F_{15,720} = 24.47$, $p < 0.0001$; and time \times treatment interaction, $F_{60,720} = 1.070$, $p = 0.3407$), and it increased the immobility time at 1 and 6 mg/kg (Figure 13b: significant effect of treatment, $F_{4,720} = 6.382$, $p < 0.0001$; time, $F_{15,720} = 4.765$, $p < 0.0001$; and time \times treatment interaction, $F_{60,720} = 0.8971$, $p = 0.6944$).

STS-135 increased at 1 mg/kg while biphasically affected at 6 mg/kg the total distance travelled (Figure 12c: significant effect of treatment, $F_{4,560} = 11.87$, $p < 0.0001$; time, $F_{15,560} = 47.75$, $p < 0.0001$; and time \times treatment interaction, $F_{60,560} = 2.874$,

$p < 0.0001$). Moreover, STS-135 increased the immobility time at 1 and 6 mg/kg (Figure 13d: significant effect of treatment, $F_{4,560} = 32.94$, $p < 0.0001$; time, $F_{15,560} = 15.44$, $p < 0.0001$; and time \times treatment interaction, $F_{60,560} = 1.914$, $p < 0.0001$). The overall motor analysis showed that 5F-ADBINAACA, AB-FUBINAACA, and STS-135 facilitated spontaneous locomotion at low doses and inhibited it at higher ones (Figure 12d: significant effect of agonists, $F_{14,139} = 362.3$, $p < 0.0001$; Figure 13d, significant effect of agonists, $F_{14,139} = 1111$, $p < 0.0001$).

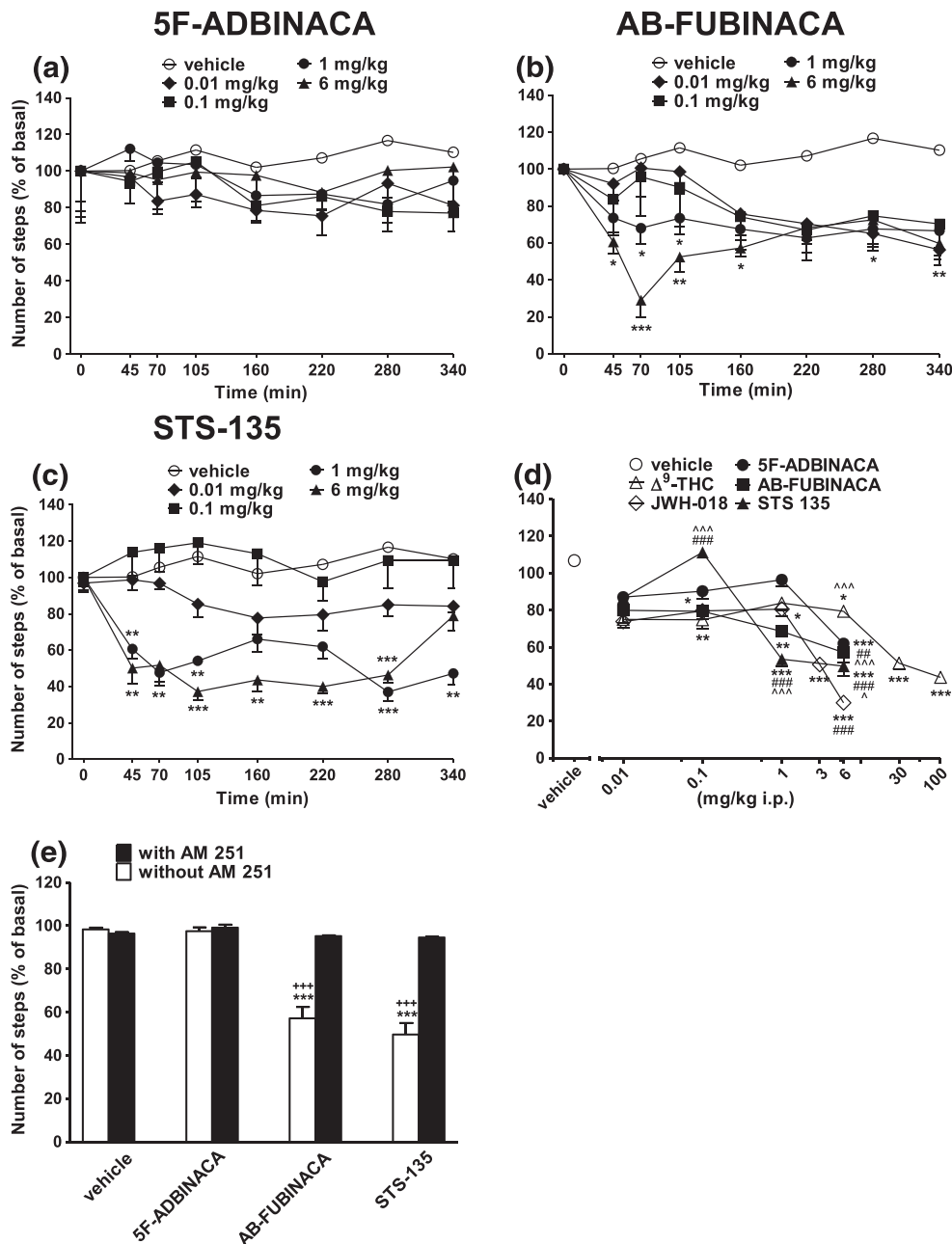


FIGURE 11 Intraperitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the drag test of the mouse; (d) comparison of the total average effect observed in 5 hr with Δ^9 -THC (0.01–100 mg/kg)^a and JWH-018 (0.01–6 mg/kg i.p.)^a; (e) interaction of different SCBs (6 mg/kg) with the selective CB₁ receptor antagonist AM 251 (6 mg/kg, i.p.). Data are expressed as arbitrary units and represent the mean \pm SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compound at different times (panels a, b, and c), and for the interaction with the AM 251 (panel e), whereas the statistical analysis of 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; ^ $p < 0.05$, ^^ $p < 0.001$ versus JWH-018 and +++ $p < 0.001$ versus AM 251 + agonist. ^aFrom Vigolo et al., 2015

3.4.7 | Neurotoxicity in vitro

The mitochondria are essential organelles in cell life, responsible for many biological processes including energy production, lipid metabolism, intracellular Ca²⁺ signaling, reactive oxygen species (ROS) production, autophagy, inflammation, and apoptosis (Rimessi, Giorgi, Pinton, & Rizzuto, 2008; Rimessi et al., 2013; Rimessi et al., 2015). A distinctive feature of the early stages of apoptotic cell death is the alteration of this organelle, inducing the mitochondrial permeability transition pore opening that permits the proton flow across the inner

mitochondrial membrane, which causes a reduction in mitochondrial membrane potential ($\Delta\Psi$). In order to verify the levels of SCBs-induced toxicity on living neuro-2a cells that endogenously express CB₁ receptors (Graham et al., 2006; He et al., 2005), we investigated whether the administration of these compounds influenced mitochondrial functionality in intact viable cells. SCBs effects on mitochondrial function were studied by measuring changes in $\Delta\Psi$, as typical marker of cellular viability, mediating the fluorescent dye TMRM. The mitochondrial confocal imaging approach was used to monitor the fast changes in $\Delta\Psi$ in

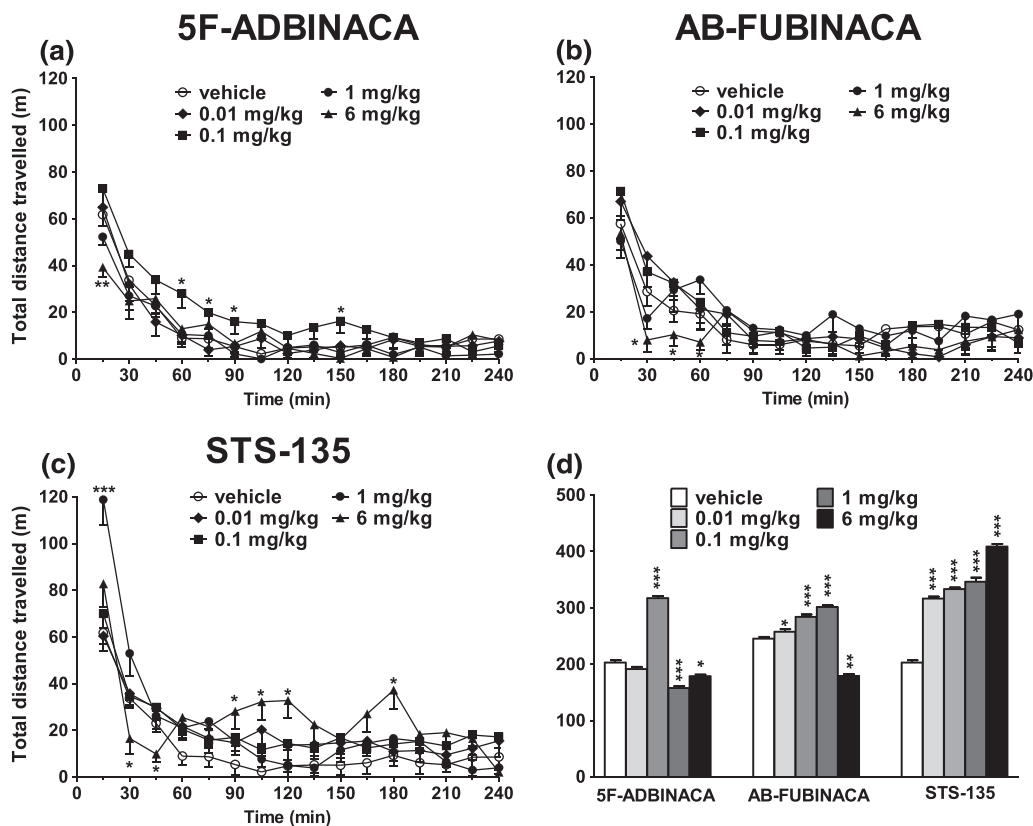


FIGURE 12 Intrapерitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the total distance travelled test of mice; (d) the overall effect observed in the 5-hr period was also reported. Data are expressed as meters (total distance travelled; panels a, b, c, and d) and represent mean \pm SEM of 10 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose response curve of total distance travelled (panels a, b, and c); (d) the analysis of the overall effect in the 5-hr period was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle

cells for 1hr after addition of SCBs at the doses of 3 ($F_{3,128} = 3.185$, $p < 0.0261$), 30 ($F_{3,490} = 7.782$, $p < 0.0001$), and 60 μM ($F_{3,210} = 13.12$, $p < 0.0001$; Figure 14). The administration of STS-135, at all the doses tested, resulted toxic for the cell as shown by the drop in membrane potential. The fluorescence slope was analyzed and expressed in histogram as Δ fluorescence intensity before and after the compound administration (Figure 14). The higher neurotoxic effect was induced by STS-135 administration that at lower dose presented the same dramatic effect of AB-FUBINACA at 60 μM . However, a weak neurotoxic effect was provoked by 5F-ADBINACA administration at the dose of 60 μM .

4 | DISCUSSION

This is the first study that has carried out a comparative analysis of the in vitro and in vivo effects caused by new third-generation fluorinate SCBs, 5F-ADBINACA, AB-FUBINACA, and STS-135. In vitro studies show that these SCBs retain nanomolar affinity for both CD-1 murine and human CB₁ and CB₂ receptors, promoting dramatic effect on cellular viability as reported by neurotoxicity assay. In vivo studies demonstrate that 5F-ADBINACA, AB-FUBINACA, and STS-135 systemic administration induce the typical "tetrad effect" in mice as reported for other JWH-type SCBs (Macri et al., 2013; Ossato et al., 2016; Vigolo

et al., 2015; Wiebelhaus et al., 2012; Wiley et al., 1998) and Δ^9 -THC (Compton et al., 1992; Vigolo et al., 2015). In particular, effects induced by 5F-ADBINACA on tetrad appears to be less potent than those induced by AB-FUBINACA, STS-135, and JWH-018 but more comparable with those of Δ^9 -THC. Conversely, STS-135 is the most effective of the compounds studied, and it displays an overall activity on tetrad similar to that caused by JWH-018 (Ossato et al., 2015; Vigolo et al., 2015). Moreover, all three SCBs caused important alteration of sensorimotor reflexes, and they promoted aggressive response in mice. As previously reported for JWH-018, JWH-250, and JWH-073, also AB-FUBINACA and STS-135 induced neurological alterations such as convulsions, hyperreflexia, and myoclonias. Those effects were not observed after administration of Δ^9 -THC (Marshall et al., 2014; Ossato et al., 2016; Vigolo et al., 2015). Physiological, behavioral, and neurological effects induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 were fully dependent on CB₁ receptor stimulation because they were completely prevented by the administration of the selective CB₁ receptor antagonist/inverse agonist AM 251. Surprisingly, the impairment of the visual sensorimotor response induced by high dose of STS-135 (6 mg/kg i.p.) was only partially blocked by AM 251, whereas visual impairment induced by lower dose of STS-135 (1 mg/kg i.p.) was completely dependent on CB₁ receptor stimulation.

The protocol used in this research is widely utilized in studies of "safety pharmacology" for preclinical characterization of new

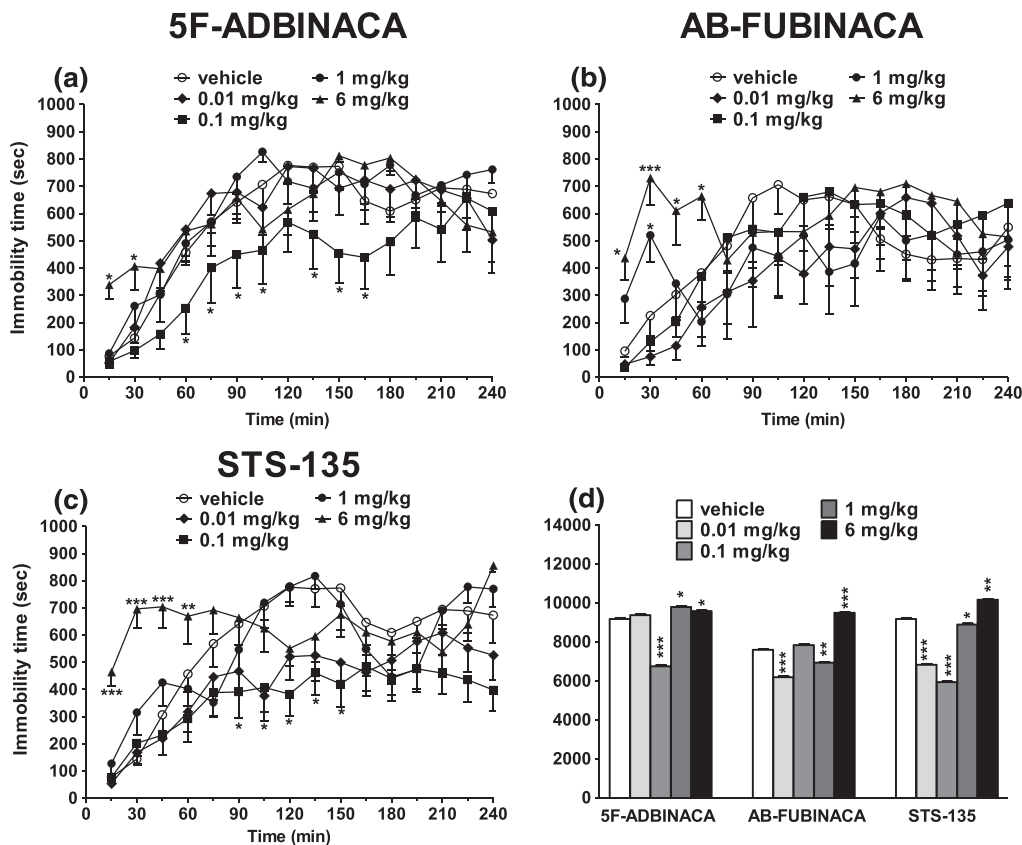


FIGURE 13 Intraperitoneal injection (0.01–6 mg/kg) of (a) 5F-ADBINACA, (b) AB-FUBINACA, and (c) STS-135 on the total time immobile of mice; (d) the overall effect observed in the 5-hr period was also reported. Data are expressed as second of immobility (total time immobile; panels a, b, c, and d) and represent mean \pm SEM of 10 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose response curve of total time immobile (panels a, b, and c). The analysis of the overall effect in the 5-hr period (panel d) was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle

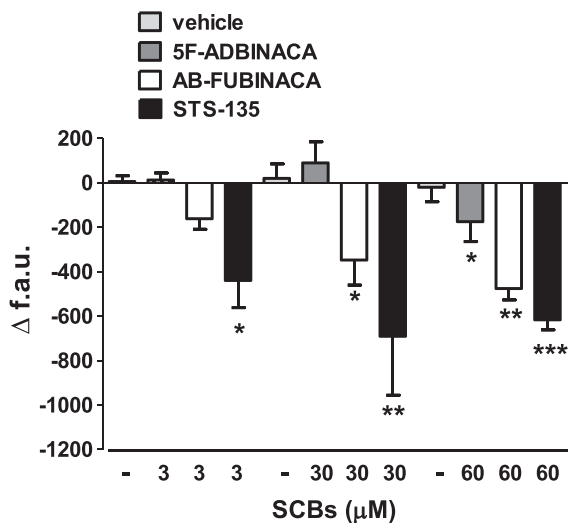


FIGURE 14 Effects of SCBs on mitochondrial membrane potential. Measurements of the $\Delta\psi$ in neuro-2a cells during SCBs administration. Neuro-2a cells were treated with 5F-ADBINACA, AB-FUBINACA, and STS-135 for 1 hr. The bars show the change in the TMRM fluorescence level, expressed as the Δ change with respect to vehicle treating cells, before and after the treatment. The depolarization rates were defined as the slopes of the fluorescence trace over a poststimulation period. F.a.u., fluorescent arbitrary units, $n = 3$ independent experiments

molecules in rodents (Hamdam et al., 2013; Irwin, 1968; Mattsson et al., 1996; Porsolt et al., 2002; Redfern et al., 2005; S7A, 2001). Moreover, we previously validated this protocol to describe the effects of cannabinoids on the tetrad, sensorimotor, and neurological changes in mice (Canazza et al., 2016; Ossato et al., 2015; Ossato et al., 2016; Vigolo et al., 2015). Additionally, to show that our protocol causes a mild or no stress in animals, we compared and analyzed the behavioral motor, sensorimotor responses, nociceptive, and body temperature in both naïve (untreated) and vehicle or saline (treated) animals (Ossato et al., 2016) and present data). Despite the repetition of tests over time, all animals showed no changes in the parameters above described due to stress or discomfort. In particular, changes in body temperature (core temperatures) and responses to noxious stimuli, parameters sensitive to stressful situations (Bouwknicht et al., 2007; Kozlov, Abramova, Chekhlov, Grigorochuk, & Pertsov, 2015), were not significantly different in naïve animals and in saline or vehicle animals.

In vitro binding studies show that 5F-ADBINACA, AB-FUBINACA, and STS-135 retain a nanomolar affinity for both CD-1 murine and human CB₁ and CB₂ receptors and the rank order was AB-FUBINACA > 5F-ADBINACA > STS-135. In particular, it has been observed a preference of 5F-ADBINACA for CB₁ receptor, whereas AB-FUBINACA and STS-135 had a similar affinity for both CB₁ and CB₂ receptors (Table 1). In CD-1 murine preparation, 5F-ADBINACA displayed an affinity for CB₁ receptors ($K_i = 5.17$ nM) similar to that

of STS-135 ($K_i = 4.82\text{nM}$) and JWH-018 ($K_i = 5.82\text{nM}$; Vigolo et al., 2015) but slightly lower respect to AB-FUBINACA ($K_i = 1.26\text{nM}$). On human CB_1 receptors, 5F-ADBINACA showed an affinity ($K_i = 2.37\text{nM}$) compared to STS-135 ($K_i = 4.32\text{nM}$) but slightly higher than JWH-018 ($K_i = 9.53\text{nM}$; Vigolo et al., 2015), whereas AB-FUBINACA showed a subnanomolar affinity for human CB_1 receptors ($K_i = 0.73\text{nM}$). The increased CB_1 receptor affinity of AB-FUBINACA could justify its potency ($IC_{50} = 1.36\text{nM}$) in inhibiting cyclic AMP formation respect to 5F-ADBINACA ($IC_{50} = 6.26\text{nM}$), STS-135 ($IC_{50} = 13.1\text{nM}$) and to JWH-018 ($IC_{50} = 14.1\text{nM}$; Vigolo et al., 2015). Despite the *in vitro* evidence showing that AB-FUBINACA and 5F-ADBINACA have an affinity for the CB_1 receptors greater or slightly greater than for JWH-018, *in vivo* data show a reduced efficacy and potency among AB-FUBINACA, 5F-ADBINACA, and JWH-018. These data suggest that the efficacy of these compounds *in vivo* does not depend exclusively on pharmacodynamic (i.e., receptor affinity) but possibly by pharmacokinetic (i.e., absorption and metabolism) parameters (Schifano et al., 2015). Recent studies support this hypothesis showing that the halogenation in the pentilic side chain of JWH-018 (i.e., JWH-018Cl and JWH-018Br) does not significantly change the binding affinity of the compounds at the cannabinoid CB_1 and CB_2 receptors, but it influences their biological activity *in vivo* (Barbieri et al., 2016; Vigolo et al., 2015). *In vitro* neurotoxicity studies in murine neuro-2a cells also suggest that different mechanisms, not directly related to receptor affinity, are involved in biological effects of novel SCBs.

The administration *in vitro* of AB-FUBINACA and in particular of STS-135 provoked significant perturbations on mitochondrial functionality, compromising radical changes in $\Delta\Psi$ after few minutes. The maintenance of $\Delta\Psi$ is essential for the cells and for ATP synthesis. On physiological conditions, $\Delta\Psi$ is highly negative due to the chemiosmotic gradient of protons across the inner mitochondrial membrane, the energy of which is used to synthesize ATP by the mitochondrial respiratory chain (Bonora et al., 2012). The loss of $\Delta\Psi$ alters the energetic status of the cells, compromising the mitochondrial ATP machinery and favoring the production of detrimental reactive oxygen species. Those events have a considerable impact on cell viability. The present data are in agreement with recent studies showing the neurotoxicity of SCBs in mice (Cha et al., 2015; Tomiyama & Funada, 2014). Although the three SCBs tested presented similar affinity for both murine $CD-1$ and human CB_1 and CB_2 receptors, they have different neurotoxic properties. This aspect opens new experimental perspectives and is currently under deeper investigations.

In vivo studies show that administration of AB-FUBINACA and 5F-ADBINACA in the dose-range up to 6 mg/kg induced a core and surface hypothermia significantly lower respect to that induced by STS-135 and JWH-018, but similar to that induced by Δ^9 -THC (Vigolo et al., 2015). Nevertheless, we cannot exclude that administration of AB-FUBINACA and 5F-ADBINACA at higher doses than those tested might induce a greater hypothermia. However, the occurrence of major neurological changes (i.e., AB-FUBINACA) prevents us to increase doses. As reported for other cannabinoid agonists, hypothermia induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 was completely prevented by pretreatment with AM 251, confirming that this effect is clearly mediated by CB_1 receptors stimulation (Marshall et al., 2014; Ossato et al., 2016; Vigolo et al., 2015).

Systemic administration of 5F-ADBINACA, AB-FUBINACA, and STS-135 increased the threshold to acute mechanical and thermal pain stimulus in mice and the rank order was STS-135 > AB-FUBINACA > 5F-ADBINACA. In particular, the analgesic effect induced by 5F-ADBINACA was less intense than AB-FUBINACA, STS-135, JWH-018, and Δ^9 -THC administration (Vigolo et al., 2015), but it was similar to the analgesic profile of other SCBs as JWH-250 and JWH-073 (Ossato et al., 2016). This lower response could be due to the fact that 5F-ADBINACA and AB-FUBINACA, as well as others SCBs, may be biotransformed into glucuronitated or monohydroxylated metabolites (Castaneto et al., 2015; Vikingsson et al., 2015) that may act as neutral antagonists at CB_1 receptors dampening the overall activity of the parent compound (Seely, Lapoint, Moran, & Fattore, 2012; Brents et al., 2012).

As previously reported for other JWH-type SCBs (Vigolo et al., 2015), 5F-ADBINACA shows a greater efficacy in reducing nociception to mechanical stimulation compared to thermal stimulus with a different profile of action (i.e., long lasting) respect to AB-FUBINACA and STS-135 (i.e., transient). In particular, 5F-ADBINACA increases the threshold to mechanical pain for prolonged periods over time, similarly to those induced by treatment with JWH-018, JWH-018Cl, and JWH-018Br compounds and Δ^9 -THC (Vigolo et al., 2015). Whereas, AB-FUBINACA and STS-135 have a transitory analgesic profile over time, similar to JWH-073 and JWH-250 (Ossato et al., 2016). This evidence strengthens the hypothesis that SCBs exert their analgesic effect not only by acting on different sensory components of pain generated by a mechanical (Martin, Hohmann, & Walker, 1996) or thermal (Hohmann, Tsou, & Walker, 1999) stimuli but also with a different kinetics.

Unlike previous studies showing that the analgesic effect caused by JWH-018, JWH-018Cl, and JWH-018Br compounds precedes the motor impairment (Vigolo et al., 2015), analgesia induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 overlap almost completely to the motor alterations. This responsiveness is in line with previous studies reporting that changes in the molecular structure of SCBs induce consistent disparities among potencies and efficacies of their *in vivo* effects (Ossato et al., 2016; Wiley et al., 1998; Wiley et al., 2014).

In our experimental conditions, the possibility that the acute analgesic effect induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 is due to the activation of peripheral CB_2 receptors (Guindon & Hohmann, 2008) should be ruled out because their analgesic effects are fully prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM 251.

Administration of 5F-ADBINACA, AB-FUBINACA, and STS-135 affects the startle response to visual, acoustic, and tactile stimuli in mice (Ossato et al., 2015), less effectively than JWH-018 and Δ^9 -THC. A recent study has shown that visual information in mice is elaborated in a subpopulation of neurons selectively localized in the dorsomedial striatum (Reig & Silberberg, 2014), in which CB_1 receptors are expressed (Marsicano & Lutz, 1999; Tsou, Brown, Sanudo-Pena, Mackie, & Walker, 1998). 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 5F-ADBINACA, AB-FUBINACA, and STS-135 could inhibit visual function through the stimulation of CB_1 receptors

expressed in thalamocortical-striatal visual circuitry (Dasilva, Grieve, Cudeiro, & Rivadulla, 2012; Marsicano & Lutz, 1999; Tsou et al., 1998; Yoneda et al., 2013).

It is interesting to note that STS-135 inhibits visual sensorimotor responses with a CB₁ receptor dependent mechanism up to a dose of 1 mg/kg; whereas at the higher dose (6 mg/kg), the effect was only partially prevented by AM 251. The evidence that the effects induced by STS-135 at 6 mg/kg in all the other behavioral parameters tested are blocked by AM 251 excludes that the synthetic cannabinoid loses its selectivity towards the CB₁ receptors. Otherwise, it is possible to hypothesize that STS-135 can be metabolized into compounds with indole structure or adamantyl derivatives (Gandhi et al., 2015; Sobolevsky et al., 2015) that may act directly on receptor systems involved in the control of visual sensory responses. In fact, metabolites with indole structure may interact with serotonin receptors (i.e., 5HT_{2A}); whereas those with adamantyl group with the NMDA glutamate receptors, both of which receptor types are involved in visual dysperceptive serotonergic effects of hallucinogens and dissociative anesthetics (Fantegrossi, Murnane, & Reissig, 2008; Hanks & Gonzalez-Maeso, 2013; van Loon et al., 2015). However, further studies will be needed to understand which neural mechanisms are involved in visual alterations caused by STS-135.

Our study also demonstrates that all the three SCBs impair the acoustic startle response in mice by the selective stimulation of CB₁ receptors. This is in agreement with previous findings that demonstrated the effectiveness of acute administration of Δ^9 -THC (Malone & Taylor, 2006; Nagai et al., 2006; Ossato et al., 2015), CP 55940 (Mansbach, Rovetti, Winston, & Lowe, 1996; Martin et al., 2003), WIN 55,212-2 (Bortolato et al., 2005), JWH-018 (Ossato et al., 2015), JWH-250, and JWH-073 (Ossato et al., 2016) 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). Indeed, it has been reported that administration of WIN-55,212-2 (Tzounopoulos, Rubio, Keen, & Trussell, 2007) or the activation of the endogenous cannabinoid system affected the short-term synaptic plasticity (Sedlacek, Tipton, & Brenowitz, 2011; Tzounopoulos et al., 2007; Zhao, Rubio, & Tzounopoulos, 2011). Therefore, 5F-ADBINACA, AB-FUBINACA, and STS-135 could impair the acoustic startle reflex in mice by stimulating CB₁ receptors expressed on the presynaptic terminals of parallel fibers in the dorsal cochlear nucleus (Tzounopoulos et al., 2007).

Based on the present study, it is not possible to define whether visual and acoustic alterations induced by 5F-ADBINACA, AB-FUBINACA, and STS-135 in mice are an expression of hallucinatory states, as suggested for Δ^9 -THC in human studies (Winton-Brown et al., 2011). However, our data support the hypothesis that SCBs by stimulating CB₁ receptors could impair the sensorimotor gating in mice similarly to Δ^9 -THC (Malone & Taylor, 2006; Nagai et al., 2006), CP 55940 (Mansbach et al., 1996; Martin et al., 2003), and WIN 55,212-2 (Schneider & Koch, 2002; Wegener, Kuhnert, Thuns, Roese, & Koch, 2008).

We also underline that STS-135 is more effective than 5F-ADBINACA and AB-FUBINACA in inhibiting the sensorimotor responses in mice in reply to tactile stimuli and that the three SCBs

inhibit pinnae reflex but are ineffective on vibrissae sensorimotor response. This inefficacy was unexpected because Δ^9 -THC, JWH-018, and JWH-073 are effective in inhibiting vibrissae responses in mice (Ossato et al., 2015; Ossato et al., 2016) possibly by activating CB₁ receptors (Cristino et al., 2006; Tsou et al., 1998) expressed in the inferior olive, somatosensory cortex, and superior colliculus (Hemelt & Keller, 2008). However, this lack of response may be due to the peculiar chemical structure of these SCBs (carboxamide-indole, Figure 1a; carboxamide-indazole, Figure 1b; adamantylindoles, Figure 1c), which may strongly influence their biological response in vivo as previously shown for indole- and pyrrole-derived cannabinoids (Wiley et al., 1998; Wiley et al., 2014). On the contrary, 5F-ADBINACA, AB-FUBINACA, and STS-135 might inhibit sensorimotor responses of pinna and cornea through the stimulation of CB₁ receptors directly expressed in trigeminal structures (Herkenham et al., 1991; Price, Helesic, Parghi, Hargreaves, & Flores, 2003; Tsou et al., 1998) as hypothesized for JWH-018, JWH-073, and JWH-250 (Ossato et al., 2015; Ossato et al., 2016). These results are consistent with previous studies showing that the administration of HU 210 and WIN-55,212-2 suppressed central trigeminal transmission (Jenkins, Worthington, Harris, & Clarke, 2004; Papanastassiou, Fields, & Meng, 2004) and that topical application of WIN-55,212-2 reduced cornea-evoked trigeminal brainstem activity (Bereiter, Bereiter, & Hirata, 2002).

Noteworthy, 5F-ADBINACA, AB-FUBINACA, and STS-135 impair visual sensorimotor responses at a low dose (0.1 mg/kg) that does not cause catalepsy or reduce spontaneous (open field studies) and stimulated motor activity (drag test and accelerod) in mice. This finding points out that effects induced by SCBs 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). This is further supported by evidence that the administration of low doses of 5F-ADBINACA, AB-FUBINACA, and STS-135 facilitates spontaneous locomotion and at the same time impairs visual and acoustic sensorimotor responses, as previously reported for Δ^9 -THC and JWH-018 (Ossato et al., 2015). The biphasic profile induced by the 5F-ADBINACA, AB-FUBINACA, and STS-135 on motor activity fits well with the time- and dose-dependent biphasic effect caused in rodents by anandamide (Sulcova, Mechoulam, & Fride, 1998), Δ^9 -THC (Ossato et al., 2015) and WIN 55,212-2 (Drews, Schneider, & Koch, 2005), and it suggests that this modulation is typical of the cannabinoid system and not of a single molecule class (Rodriguez de Fonseca, Del Arco, Martin-Calderon, Gorriti, & Navarro, 1998).

The present study increases preclinical evidence showing that SCBs caused convulsions, hyperreflexia, and myoclonia in mice (Marshall et al., 2014; Ossato et al., 2016; Vigolo et al., 2015). These data confirm the proconvulsant effect of SCBs, and they are in agreement with the increasing clinical reports showing the occurrence of seizures and hyperreflexia in young people smoking "spice" products containing different SCBs (Gugelmann et al., 2014; Lapoint et al., 2011; McQuade, Hudson, Dargan, & Wood, 2013; Schneur & Baumbacher, 2012; Simmons et al., 2011).

As previously reported, SCBs promote aggressive response in mice (Ossato et al., 2016). Pharmacological modulation of cannabinoid signal alters spontaneous aggressive behavior in mice, rats, and squirrel

monkeys (Ham & De Jong, 1975; Miczek, 1978; van Ree, Niesink, & Nir, 1984); this behavior was exacerbated in stressful situations in rodents (Carder & Olson, 1972; Carlini & Gonzales, 1972; Carlini, Lindsey, & Tufik, 1976). Therefore, even though in our experimental conditions this behavior was observed in a simple test that is not fully representative for an overall and accurate assessment of aggressive behavior in mice (Miczek et al., 2007; Takahashi & Miczek, 2014), it is possible that the aggressive response caused by the administration of 5F-ADBINACA, AB-FUBINACA, and STS-135 in mice is mainly due to sensorimotor alterations and neurological symptoms rather than a direct effect on neural circuits that control aggressive behavior.

5 | CONCLUSION

For the first time, the present study demonstrates the pharmacotoxicological effects induced by the acute administration of novel third-generation fluorinated SCBs 5F-ADBINACA, AB-FUBINACA, and STS-135 in mice. In particular, *in vivo* studies show that SCBs impair sensorimotor responses (0.1 mg/kg) first, and motor activity (1–6 mg/kg) then. At higher doses (6 mg/kg), they induce severe neurological effects (seizures, myoclonia, and hyperreflexia) and promote aggressiveness in mice. *In vitro* studies highlight the neurotoxic potential of these drugs on murine cells. Although obtained in animal model, these data reinforce the hypothesis that SCBs may have a detrimental effect for human health.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

The authors declare that the manuscript contains original unpublished work and is not being submitted for publication elsewhere.

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SUPPORTING INFORMATION

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