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Behavioral and binding studies on the quinolinyl ester indoles 5F-PB22 (5F-QUPIC) and BB-22 (QUCHIC) in the mouse model

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ABSTRACT

Novel Psychoactive Substances newly introduced on the drug market are constantly changing patterns of drug consumption. Among these, Synthetic Cannabinoids (SCs) mainly dominated European seizures in the last years. Quinolin-8-yl 1-(5-fluoropentyl)-1H-indole-3-carboxylate (5F-PB22) and quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate (BB-22) are SCs whose abuse has been linked to a range of fatal intoxications and hospitalizations. Therefore, this study aims to investigate and compare in vitro and in vivo pharmacodynamics activity of these quinolinyl ester indoles. In vitro competition binding experiments performed on CD-1 murine and human cannabinoid CB_1 and CB_2 receptors revealed a sub-nanomolar affinity and potency of 5F-PB22 and BB-22. In vivo studies demonstrated that the acute systemic administration of 5F-PB22 and BB-22 (0.001-6 mg/kg) deeply impaired sensorimotor and motor responses, core temperature, breath rate and nociceptive threshold of CD-1 male mice. Pre-treatment with the selective cannabinoid CB_1 receptor antagonist/inverse agonist AM-251 (6 mg/kg) fully prevented the effects of both cannabinoids (at 1 mg/kg) suggesting a CB_1 receptor mediated action. Relying on these findings, this study showed for the first time the pharmaco-toxicological effects of these substances confirming their potential burden on human health. Furthermore, it revealed that the difference of the chemical structures of the two SCs results in their potency related disparities.

1. Introduction

Novel Psychoactive Substances (NPS), also known as 'legal highs', are synthetic or semi-synthetic compounds usually sold as legal alternatives of common drugs of abuse (Luethi and Liechtie, 2020). NPS newly introduced on the internet, social networks and smartphone apps are constantly changing patterns of drug consumption (Miliano et al., 2018). The health and social responses to NPS are prevented by the dynamic nature of this side of the market (United Nations, 2020). More than 50 new compounds are seized every year in European countries. Among these, synthetic cannabinoids represent one of the mainly detected classes (EMCDDA, 2020).

Synthetic cannabinoids (SCs) are a large family of compounds that has been popular as new psychoactive substances in the last decade (EMCDDA, 2020). 'Legal high' products containing SCs are mainly marketed on the internet as 'herbal smoking mixtures' under different brand names such as 'Spice' and 'K2' (DEA, 2014; EMCDDA, 2017). These compounds are available as powder or in liquid form spray dried on plant material or smoked through e-cigarettes (UNODC, 2011; EMCDDA, 2017). SCs are abused to mimic effects of Δ^9-THC, the main psychoactive component of *Cannabis sativa* plant (EMCDDA, 2017), and act as full agonist on CB_1 and CB_2 receptors inducing more severe ef-

Abbreviations: 5F-PB22, quinolin-8-yl 1-(5-fluoropentyl)-1H-indole-3-carboxylate; BB-22, quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate; AM-251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; Δ^9-THC, Δ9-tetrahydrocannabinol; SCs, Synthetic cannabinoids; NPS, Novel psychoactive substances.

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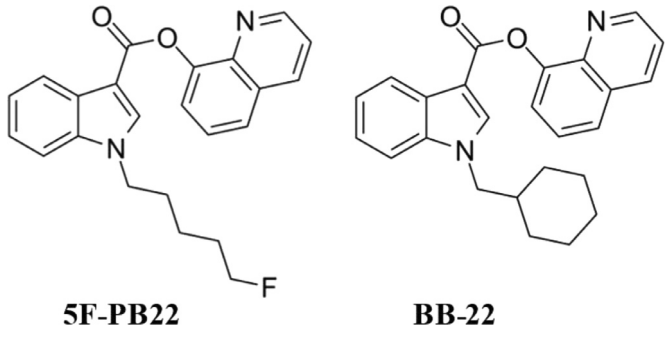

Fig. 1. Chemical structures of 5F-PB22 (quinolin-8-yl 1-(5-fluoropentyl)-1H-indole-3-carboxylate) and BB-22 (quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate; Cayman chemicals).

fects when compared to this (Tanama and Linch, 2020; Cohen and Weinstein, 2018; Fattore and Fratta, 2011).

Since the first appearance in the market, many new compounds have been synthetized in clandestine laboratories through chemical alterations of the main structures, leading to different generations of SCs. 5F-PB22 (quinolin-8-yl 1-(5-fluoropentyl)-1H-indole-3-carboxylate) and BB-22 (quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate), also known as 5F-QUPIC and QUCHIC, are quinolinyl ester indoles composed of an indole core substituted by a quinolinyl secondary structure through a carboxylate linker (Fig. 1; Carlier et al., 2018; WHO, 2017).

These substances have been first identified in 2013, in Japan and in various United Nations' member states respectively (Uchyiama et al., 2013; WHO, 2017). 5F-PB22 has been involved in seizures in Europe and in United States (DEA, 2014), while traces of BB-22 have been found in herbal material seized in Italy between 2013 and 2015 (Odoardi et al., 2016). In the last years, 5F-PB22 and BB-22 have been detected in biological samples from patients involved in a range of hospitalization and fatal intoxication cases (Abouchedid et al., 2016; Angerer et al., 2017; Behonick et al., 2014; EMCDDA, 2014; Hill et al., 2018; Schep et al., 2015) as well as medication-assisted treatment programs for drug dependence (Gundersen et al., 2019). Furthermore, a more recent D.E.A. report about 5F-PB22 (DEA, 2020) and its identification in recently seized herbal blends products (Ivanov et al., 2021). confirm that this substance has been currently marketed and abused. It is worth noting that this SC has been also found in e-cigarettes liquids that have become more and more popular over the last years (Angerer et al., 2015). These substances have been also linked to motor vehicle collisions. In 2013, 5F-PB22 has been detected in herbal mixtures smoked by a driver involved in a case of DUID (Driving Under the Influence of Drugs) in Japan (Kaneko et al., 2017). Indeed, abuse of these SCs has been frequently associated with adverse effects such as extreme agitation, unconsciousness, seizures, tachycardia, respiratory failures, nausea, vomiting and death (Abouchedid et al., 2016; Angerer et al., 2017; Behonick et al., 2014; Hill et al., 2018; Schep et al., 2015).

Effects and adverse effects can be influenced by the metabolic profile of these substances that has been recently characterized using human hepatocytes. The incubation with human hepatocytes leads to the identification of 22 5F-PB22 metabolites. Among these, ester hydrolysis products are the main represented and have been also identified through a study on human liver microsomes (Wohlfarth et al., 2014; Takayama et al., 2014). Furthermore, a recent study has shown that two of the main metabolites of 5F-PB22 retained activity (Cannaert et al., 2016). 10 different BB-22 metabolites have been identified instead. Those have been produced through the hydrolysis of the carboxylate linker that leads to the quinolinyl loss.

Both 5F-PB22 (Ki=0.13 nM) and BB-22 (Ki=0.11 nM) retain high affinity for CB_1 receptors in rat cortex homogenates and high potency and efficacy in a [35S]GTPγS binding essay (De Luca et al., 2016) and both substances were tested and fully substituted for the discriminative stimulus effect of Δ^9-THC in male rats (Gatch et al., 2018). Moreover, *in vitro* electrophysiological data has shown that 5F-PB-22 cause a significative reduction of fEPSP (Barbieri et al., 2019). 5F-PB22 has also been investigated for its capacity to reduce viability of cardiac and neuronal cells and a recent study has shown the mutagenic capability of BB-22, underling the high potential toxicity of these SCs (Santos-Carvalho et al., 2016; Lenzi et al., 2020).

According to *in vivo* results, these compounds can induce effects typical of the SCs. Indeed, 5F-PB22 and BB-22 dose-dependently induce hypothermic effect and reduced heart rate in male rats (Banister et al., 2015). Locomotor activity decrease and alterations of brain electrical EEG activity in both cortical and hippocampal areas have been also registered in male mice after the injection of 5F-PB22 (Barbieri et al., 2019; Gatch et al., 2015).

To deepen the insight of their pharmaco-toxicological profile, this study aims to characterize *in vivo* effects of 5F-PB22 and BB-22 acute administration on sensorimotor (visual, acoustic and tactile) and motor responses, breath rate, body temperature and nociceptive responses to mechanical and thermal stimuli in CD-1 male mice. The compounds were also tested through *in vitro* cyclic AMP and binding competition experiments, to evaluate their potency and their affinity for murine and human CB_1 and CB_2 receptors.

2. Materials and methods

2.1. Animals

Male ICR (CD-1®) mice weighing 30–35 g (Centralized Preclinical Research Laboratory, University of Ferrara, Italy) were group housed (5 mice per cage; floor area per animal was 80 cm2; minimum enclosure height was 12 cm), exposed to a 12:12-h light-dark cycle (light period from 6:30 AM to 6:30 PM) at a temperature of 20–22°C and humidity of 45–55% and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU). Experimental protocols were approved by the Italian Ministry of Health (license n. 335/2016-PR) and by the Animal Welfare Body of the University of Ferrara. According to the ARRIVE guidelines, all possible efforts were made to minimize the number of animals used, to minimize the animals' pain and discomfort. For the overall study 110 mice were used. In the analysis of the behavioral responses relative to each treatment (vehicle and each of 6 different 5F-PB22 and BB-22 doses (0.0001, 0.001, 0.01, 0.1, 1 and 6 mg/kg)) 6 mice were used (total used mice: 78). In the analysis of the interaction of the compounds with AM-251 (each of 2 different AM-251 doses (1 and 6 mg/kg); interaction of 5F-PB22 and BB-22 (0.1 mg/kg) with AM-251 (1 mg/kg); 2 different interactions of 5F-PB22 and BB-22 (1 mg/kg) with AM-251 (1 and 6 mg/kg)) 4 mice were used (total used mice: 32).

Data concerning the use of SCs among young adults in Europe state the higher lifetime prevalence of use for male (3.5%) than female (2.7%; ESPAD, 2019). This is in line with previous reports showing that emergency room assistance after SCs intoxication have been most frequently required for male (78%) than female (22%) patients (Bush and Woodwell, 2014). Taken together these data suggest that males are at greater risk of abuse and intoxication related to the use of these compounds. Thereby, male mice were used in this study. However, previous preclinical studies have also pointed out that gender can affect pharmacotoxicological effects induced by SCs (Fattore et al., 2020; Fattore et al., 2007, 2010; Wiley et al., 2017). Therefore, further research should be carried out to investigate this point.

Table 1
Correlation between mouse doses (mg/kg) and human equivalent doses (HED, mg/kg) of 5F-PB22. Effects in human were also presented, according to their increasing severity.

Mouse dose (mg/kg)	HED (mg/kg)	Human Dose (mg)	Human Dosage	Effects	References
0.0001	0.0000081	0.00046	*Threshold*	Spontaneous physical sensation ("body high"),	Schep et al., 2015; Abouchedid et al., 2017;
0.001	0.000081	0.00486		perception of bodily heaviness or lightness, emotion	https://psychonautwiki.org/wiki/5F-PB-22;
0.01	0.00081	0.046		enhancement, euphoria, sedation and drowsiness,	
0.1	0.0081	0.486		vertigo, appetite enhancement, dry mouth.	
				Changes in visual and auditory perceptions, anxiety,	Abouchedid et al., 2017; Hill et al., 2017;
1	0.081	4.86	*Common*	confusion, agitation or aggression, motor control loss,	Schep et al., 2015;
6	0.486	29.16	*Heavy*	tachycardia, hypertension, nausea and vomiting.	https://psychonautwiki.org/wiki/5F-PB-22
				Reduced consciousness, hallucination and paranoid	Abouchedid et al., 2017; Angerer et al., 2017; Hill
				features, psychosis, seizures, circulatory failure,	et al., 2017; Schep et al., 2015; Behonick et al.,
				respiratory failure, central nervous system failure,	2014; https://psychonautwiki.org/wiki/5F-PB-22
				renal failure, severe metabolic derangement and death.	

2.2. Drug preparation and dose selection

5F-PB22 and BB-22 were purchased from LGC Standards (LGC Standards, Milan, Italy). AM-251 was purchased from Tocris (Tocris, Bristol, United Kingdom). All compounds were initially dissolved in absolute ethanol (final concentration: 2%) and Tween 80 (2%) and brought to the final volume with saline (0.9% p/v NaCl). The solution made with ethanol, Tween 80 and saline was also used as the vehicle. The CB_1 receptor-preferring antagonist/inverse agonist AM-251 (1-6 mg/kg) were administered 20 minutes before 5F-PB22 and BB-22 injections. Drugs were administered by intraperitoneal route at a volume of 4ul/gr. Range of drugs doses (0.0001-6 mg/kg i.p.) were chosen basing on interspecies dose scaling (Nair and Jacob, 2016) and our previous studies (Table 1; Vigolo et al., 2015; Ossato et al., 2015; Ossato et al., 2016), in order to test mice doses that correspond to threshold, common and heavy doses in humans (Table 1; https://psychonautwiki.org/wiki/5F-PB-22). Both 5F-PB22 and BB-22 were ineffective at the lowest dose tested (0.0001 mg/kg i.p.; data not shown).

2.3. In vitro studies

2.3.1. Chemicals

WIN 55,212-2 was purchased from Tocris Biosciences (Bristol, UK) while JWH-018 was purchased from LGC Standards (LGC Standards, Milan, Italy). Drugs were dissolved in dimethyl sulfoxide and further diluted in water containing 5% Tween 20. The vehicle is composed of water containing 5% Tween 20 and 5% dimethyl sulfoxide (Vincenzi et al., 2013). All other reagents were of analytical grade and obtained from commercial sources.

2.3.2. Mouse brain and spleen membrane preparation

To evaluate the affinity of synthetic cannabinoids for murine CB_1 and CB_2 receptors, membranes from mouse brain and spleen were used, respectively. Following excision from mice, tissues were suspended in 50 mM Tris HCl, pH 7.4 at 4°C. The mouse brain and spleen tissues were homogenized with a Polytron and subsequently centrifuged for 10 min at 2,000 x g. The resulting supernatants were filtered, centrifuged for 20 min at 40,000 x g and the pellets were used for competition binding experiments (Vincenzi et al., 2013).

2.3.3. Cell culture and membrane preparation

CHO cells transfected with human CB_1 or CB_2 receptors (Perkin Elmer Life and Analytical Sciences, USA) were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and Geneticin (G418, 0.4 mg/ml) at 37°C in 5% CO_2/95% air. To obtain membranes, cells were washed with PBS (Phosphate Buffer Saline) and scraped off with ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and then centrifuged for 30 min at 40,000 x g. The membrane pellet was suspended in 50 mM Tris HCl buffer (pH 7.4) containing 2.5 mM EDTA (Ethylenediaminetetraacetic acid), 5 mM $MgCl_2$, 0.5 mg/ml BSA (Bovine Serum Albumine) for CB_1 receptors or in 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 0.5% BSA for CB_2 receptors (Vincenzi et al., 2013).

2.3.4. [^{3}H] CP-55,940 competition binding assays

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

2.3.5. Cyclic AMP assays

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

2.3.6. Data analysis

The protein concentration was determined according to a Bio-Rad method with bovine serum albumin as a reference standard. Inhibitory binding constants, Ki, were calculated from the IC_{50} values according to the Cheng and Prusoff equation: $Ki = IC_{50}/(1 + [C^*]/K_D^*)$, where $[C^*]$ is the concentration of the radioligand and K_D^* its dissociation constant. Functional experiments were analyzed by non-linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism, USA). All the data are expressed as the mean ± SEM of three independent experiments.

2.4. Behavioral tests

In the present study, effect induced by 5F-PB22 and BB-22 on sensorimotor responses was investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" and routinely adopted in our laboratory for the preclinical characterization of new molecules in rodents (Irwin, 1968; Ossato et al., 2015, 2018; Vigolo et al., 2015; Bilel et al., 2019; Arfè et al., 2021). Voluntary and involuntary motor responses of the animal to different visual, acoustic, and tactile stimuli were evaluated according to the procedure previously described by Ossato et al., 2015. To reduce the number of animals used, mice were evaluated in functional observational tests carried out in a consecutive manner according to the following time scheme: observation of visual object responses (frontal and lateral view), acoustic response, tactile response (vibrissae, corneal, and pinnae reflexes) and visual placing response. Behavioral tests were conducted in a thermostatic (temperature: 20-22°C, humidity: 45-55 %) and light (150 lux) controlled room with a background noise of 40 ± 4 dB. The apparatus for the visual object, acoustic and tactile sensorimotor tests consisted of an experimental chamber (350×350×350 mm) with black methacrylate walls and a transparent front door. During the week before the experiment, each mouse was placed in the box and handled (once a day) every other day, i.e., 3 times, to get used to both the environment and the experimenter. To avoid mice olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and rinsed with water. All experiments were performed between 8:30 AM to 2:00 PM and conducted in blind by trained observers working in pairs (Ossato et al., 2015). The behavior of mice was videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) placed at the top or on one side of the box and analyzed off-line by a different trained operator.

2.4.1. Evaluation of the visual response

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

Visual object response test was performed to evaluate the ability of the mouse to see an object approaching from the front (frontal view) or the side (lateral view) that typically induces the animal to shift or turn the head or retreat from it. For the frontal visual response, a white horizontal bar was moved frontally to the mouse head and the maneuver was repeated 3 times. For the lateral visual response, a small dentist's mirror was moved into the mouse's field of view in a horizontal arc, until the stimulus was between the mouse's eyes. The procedure was conducted bilaterally and was repeated 3 times (Ossato et al., 2015). The score assigned was 1 if there was a reflection in the mouse movement or 0 if it was not present. The total value was calculated by adding the scores obtained in the frontal with those obtained in the lateral visual object response test (overall score: 9). Tests were measured at 10, 30, 60, 120, 180, 240 and 300 min after the injection for the evaluation of the visual object response.

Visual Placing response test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Irwin, 1968; Ossato et al., 2015). The downward movement of the mouse was videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) placed at the base of the tail suspension apparatus. Movies were analyzed off-line by a trained operator who was unaware of the drug treatments performed. The analysis frame by frame allows evaluating the beginning of the reaction of the mouse while it was approaching the floor. The first movement of the mouse when it perceives the floor is the extension of the front legs. When the mouse started the reaction, an electronic ruler evaluated the perpendicular distance in millimeters between the eyes of the mice to the floor. Untreated control mice typically perceive the floor and prepare to contact at a distance of about 23.6 ± 4.8 mm. Tests were measured at 15, 35, 70, 125, 185, 245 and 305 min after the injection for the evaluation of the visual placing response.

2.4.2. Evaluation of acoustic response

Acoustic response measures the reflex of the mouse in response to an acoustic stimulus produced behind the animal (Ossato et al., 2015) that typically induces mice to shift or turn the head, stop and stay in defense position moving pinnae or vibrissae or promptly increasing breath rate. In particular, four acoustic stimuli of different intensity and frequency were tested. A snap of the fingers (four snaps repeated in 1.5 sec), a sharp click (produced by a metal instrument; four clicks repeated in 1.5 sec), an acute sound (produced by an audiometer; frequency: 5.0-5.1 kHz) and a severe sound (produced by an audiometer; frequency: 125-150 Hz). Each test was repeated 3 times. The score assigned was 1 if there was a response or 0 if it was not present, for a total score of 3 for each sound. The acoustic total score was calculated by adding the scores obtained in the four tests (overall score: 12). The background noise (about 40 ± 4 dB) and the sound from the instruments were measured with a digital sound level meter. Sensorimotor tests were measured at 10, 30, 60, 120, 180, 240 and 300 min after the injection for the evaluation of the acoustic response.

2.4.3. Evaluation of tactile response

Tactile response in the mouse was verified through vibrissae, corneal and pinnae reflexes (Irwin, 1968; Ossato et al., 2015). Data are expressed as the sum of the three above-mentioned parameters (overall score: 12). *Vibrissae reflex* was evaluated by touching vibrissae (right and left) with a thin hypodermic needle once for side giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score: 2). *Corneal reflex* was assessed by gently touching the cornea of the mouse with a thin hypodermic needle and evaluating the response: the score assigned was 1 if the mouse moved only the head, 2 if it only closed the eyelid, 3 if it closed the lid and moved the head. The procedure was conducted bilaterally (overall score: 6). *Pinna reflex* was assessed by touching pinnae (left and right) with a thin hypodermic needle: first the interior pinna and then the external. This test was repeated twice for side giving a score of 1 if a reflex was present and 0 if it was not present (overall score: 4). Sensorimotor tests were measured at 10, 30, 60, 120, 180, 240 and 300 min after the injection for the evaluation of the tactile response.

2.4.4. Motor activity assessment

Motor activity alterations were measured performing the *Drag test* and the *Accelerod test* (Vigolo et al., 2015; Ossato et al., 2015). The *Drag test* measures the ability of the animal to balance the body posture with the front legs in response to an externally dynamic stimulus (Marti et al., 2005). The mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20 cm/s for a fixed distance (100 cm). The number of steps performed by each paw (24.3 ± 3.5 steps as basal stepping activity) was recorded by two different observers. For each animal from five to seven measurements were collected (Vigolo et al. 2015). The drag test was performed at 45, 70, 105, 160, 220, 280 and 340 min after injections. The *Accelerod test* measures different motor parameters, such as motor coordination, locomotor ability (akinesia/bradykinesia), balance ability, muscular tone and motivation to run. The animals were placed on a rotating cylinder that increases speed automatically in a constant manner (0-60 rotations/min in 5 min). The time spent on the cylinder (264.7 ± 24.6 sec as basal motor activity) was measured (Vigolo et al. 2015). The accelerod test was performed at 40, 65, 95, 150, 210, 270 and 330 min after injections.

2.4.5. Evaluation of core temperature

To better assess the effects of the ligands on thermoregulation, we measured changes in the core (rectal) temperature. Rectal body temperature was used as an index of total body heat. The core temperature was

evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid Vaseline, into the rectum of the mice (to about 2 cm) and left in position until the stabilization of the temperature (about 10 sec; Vigolo et al., 2015). The probe was connected to a Cole Parmer digital thermometer, model 8402. A basal value of 38.5 ± 2°C was considered and a cut-off for core body temperature was set at 22°C (room temperature) as the lowest value reached by animals (Arfè et al., 2021). Core temperature was measured at 30, 50, 85, 140, 200, 260 and 320 min after injections.

2.4.6. Evaluation of breath rate

The experimental protocol for the detection of respiratory parameter in this study provides for monitoring of the animal awake, freely moving, with a non-invasive and minimal handling. The animal was left moving free in a cage and the respiration patterns of the mice were videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) placed above observation's cage. A trained operator unaware of the drug treatments performed analyses movies offline. The analysis frame by frame allows to better estimate the number of breath rates (brpm) of the mouse evaluated considering the count of about 257±11 ribcage expansions per minutes as basal value. Breath rate was measured at 40, 65, 95, 150, 210, 270 and 330 min after injections (Arfè et al., 2021).

2.4.7. Evaluation of pain induced by mechanical and thermal stimulation of the tail

Tail pinch test was performed to evaluate acute mechanical nociception (Vigolo et al., 2015). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed in the distal portion of the tail, and progressive pressure was applied. When the mouse flicked its tail, the pressure was stopped, and the digital instrument saved the maximum peak of weight supported (g/force). A peak of 246 ± 10.2 g/force was considered as basal value and a cut off (500 g/force) was set to avoid tissue damage. The test was repeated three times and the final value was calculated with the average of three obtained scores. *Tail withdrawal test* was performed to evaluate acute thermal nociception (Vigolo et al., 2015). Mice were restrained in a dark plastic cylinder, which allowed the animals to breathe normally. Then half of the tail was dipped in water of 48°C and the latency (in seconds) until the tail was left in the water was recorded. 2.3 ± 0.6 sec was considered as the mean response time in abovementioned experimental condition and a cut off of 15 seconds was set to avoid tissue damage. Acute mechanical and thermal nociception were measured at 35, 55, 90, 145, 205, 265 and 325 min after injections.

2.5. Statistical analysis

In sensorimotor response experiments, data were expressed in arbitrary units (visual objects response, acoustic response, vibrissae, corneal and pinnae reflex) and percentage of baseline (visual placing response, drag and rotarod tests, reaction time, and breath rate). Core temperature values were expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C) for each substance and presented in maximal possible effect {EMax% = [(test T° – control T°) / (cut off T° – control T°)] x 100} for dose-response curves (ED_{50}). Antinociception (tail withdrawal and tail pinch tests) were calculated as percentage of maximal possible effect {EMax% = [(test – control latency) / (cut off time – control)] x 100}. All data are shown as mean ± SEM of 6 or 4 independent experimental replications. Statistical analysis of the effects of each compound at different concentrations over time was performed by two-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons. Analysis of the total average effect induced by treatments and the interaction with AM-251 was performed with one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. ED_{50} (dose of agonist to obtain 50% of the maximal effect) values were calculated by non-linear regression analysis of dose-response data performed using Prism software (GraphPad Prism, San Diego CA). Curves have been compared, when possible, performing the F test (curves comparison). The calculation of an inter-rater reliability score for the two blinded observer have revealed the highest discrepancy in visual placing test results. Mean basal values in this specific experimental session are 21.4 ± 2.6 and 22.2 ± 1.3 mm (variation percentage: 3.7%) for the two blinded observer respectively. The Student-T test did not reveal any statistical difference between these values. Statistical analyses were performed using the program Prism software (GraphPad Prism, San Diego CA).

3. Results

3.1. Affinity and potency of the examined compounds for CB_1 and CB_2 receptors

Competition binding experiments performed in CHO cell membranes transfected with human CB_1 receptors revealed affinity values in the picomolar range for 5F-PB22 and BB-22 in comparison with the reference compound JWH-018 that showed an affinity value for human CB_1 receptors in the nanomolar range (Fig. 2; Table 2). 5F-PB22 showed a greater affinity for human CB_1 receptors than for human CB_2 receptors with a selectivity index (ratio between the Ki value to human CB_2 and the Ki value to human CB_1) of 47. The selectivity index for BB-22 and JWH-018 were 1.8 and 0.9, respectively, denoting a lower selectivity for the two receptor subtypes. Similar results were obtained evaluating affinity values of the synthetic cannabinoids in mouse tissues suggesting no species selectivity between murine and human CB receptors. As with the human CB_1 receptors, 5F-PB22 showed a very high affinity for mouse CB_1 receptors (Table 2).

Cyclic AMP experiments were performed to evaluate the potency of the examined compounds in CHO cells transfected with human CB_1 or CB_2 receptors. Both 5F-PB22 and BB-22 showed a picomolar potency at the human CB_1 receptors while a nanomolar potency at the human CB_1 receptors. Interestingly, 5F-PB22 was 302 times more potent at the human CB_1 receptors than the reference compound JWH-018 (Table 2). All the tested compounds were able to completely inhibit the forskolin-stimulated cAMP production, thus behaving as full agonists.

3.2. Behavioral studies

3.2.1. Evaluation of the visual object response

Visual object response did not change in vehicle-treated mice (Fig. 3A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-PB22 and BB-22 (0.001-6 mg/kg i.p.) significantly ($p<0.0001$) and dose-dependently reduced the visual object response in mice (Fig. 3A and B). 5F-PB22 produced an immediate impairment, especially at the highest doses (1-6 mg/kg i.p.), that persisted up to 5 h (Fig. 3A; significant effect of treatment ($F_{5,224}=334.8$, $p<0.0001$), time ($F_{7,224}=129.2$, $p<0.0001$) and time x treatment interaction ($F_{35,224}=7.744$, $p<0.0001$)). Similarly, BB-22 inhibited in a transient (0.001 mg/kg) or prolonged manner (0.01-6 mg/kg) the visual object response in mice (Fig. 3B; significant effect of treatment ($F_{5,224}=274.0$, $p<0.0001$), time ($F_{7,224}=87.29$, $p<0.0001$) and time x treatment interaction ($F_{35,224}=6.497$, $p<0.0001$)). Pretreatment with AM-251 (1 mg/kg i.p.), which alone did not alter the response in mice (Fig. 3C e D), prevented the inhibition of visual object response induced by 0.1 mg/kg of 5F-PB22 (Fig. 3C; significant effect of treatment ($F_{4,19}=59.65$, $p<0.0001$)) and BB-22 (Fig. 3D; significant effect of treatment ($F_{4,19}=20.87$, $p<0.0001$)). Otherwise, pretreatment with the same dose of AM-251 partially prevented the impairment provoked by 1 mg/kg of 5F-PB22 and BB-22 (Fig. 3C and D). Administration of higher doses of AM-251 is required to observe the full prevention of the effect induced by 1 mg/kg of this SCs. Indeed, pretreatment

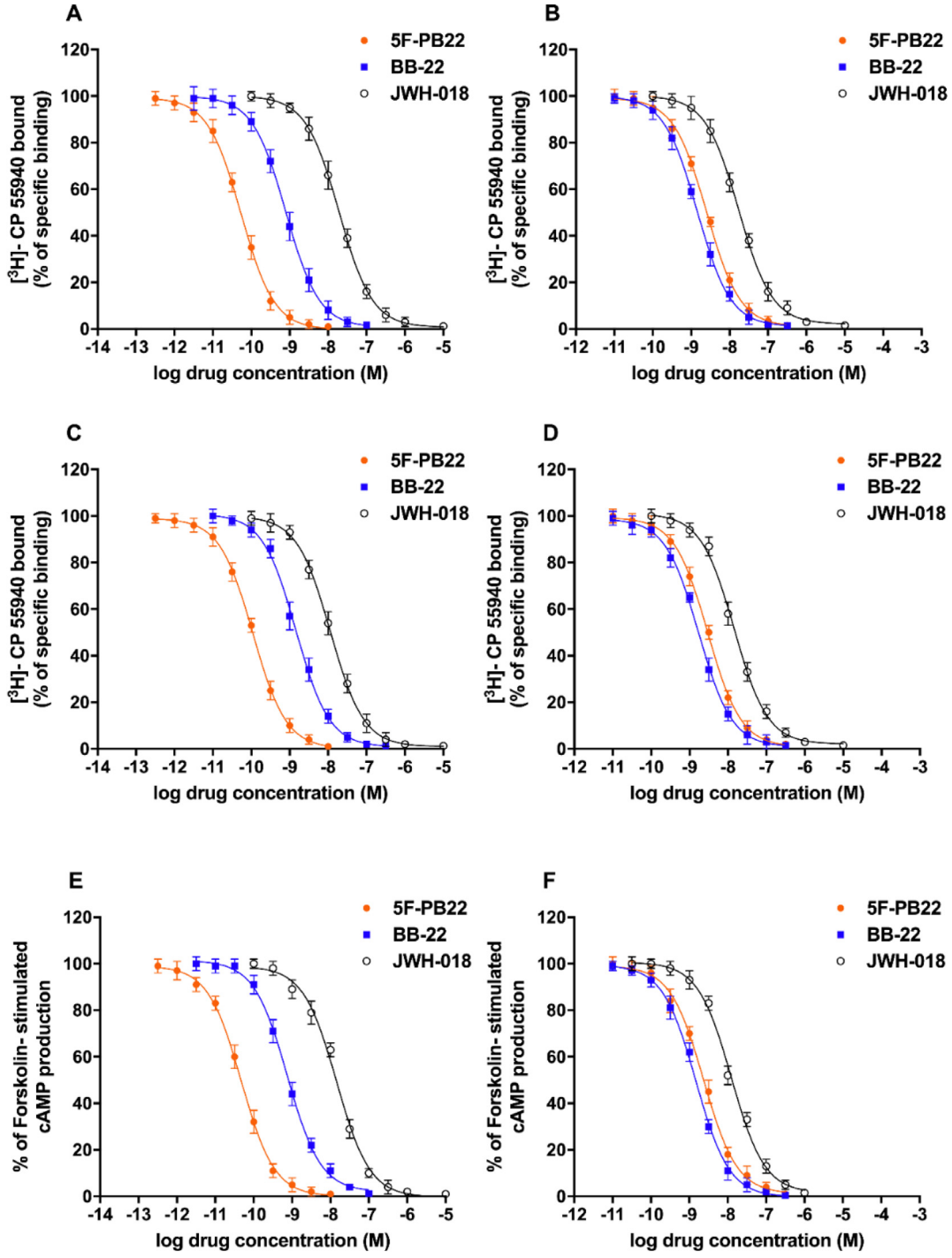

Fig. 2. Competition curves of specific [^{3}H]-CP 55940 binding by synthetic cannabinoids 5F-PB22, BB-22 and JWH-018 in CHO cell membranes transfected with human CB_1 receptors (A) or human CB_2 receptors (B) and to CB_1 receptors expressed in mouse brain membranes (C) or CB_2 receptors expressed in mouse spleen membranes (D). Inhibition curves of forskolin-stimulated cAMP accumulation by synthetic cannabinoids in CHO cells transfected with human CB_1 receptors (E) or human CB_2 receptors (F). Results are given as the mean ± SEM of three independent experiments performed in duplicate.

with AM-251 (6 mg/kg i.p.) totally prevented the inhibitory effect induced by 1 mg/kg of 5F-PB22 (Fig. 3C; significant effect of treatment ($F_{5,22}$=72.73, p<0.0001)) and BB-22 (Fig. 3D; significant effect of treatment ($F_{5,22}$=38.50, p<0.0001)). 5F-PB22 appeared to be more potent in reducing visual object response in mice, when compared to BB-22 (Fig. 3E; curves comparison ($F_{(1,64)}$=117.5, p < 0.0001)). ED_{50} values are presented in Table 3.

3.2.2. Evaluation of the visual placing response

Visual placing response did not change in vehicle-treated mice (Fig. 4A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-PB22 and BB-22 (0.001-6 mg/kg; i.p.) dose-dependently reduced the visual placing response in mice (Fig. 4A and B). 5F-PB22 induced an inhibitory effect, especially at the highest doses (1-6 mg/kg;

Table 2
Binding and functional parameters of synthetic cannabinoids to human and mouse CB_1 and CB_2 receptors.

Compound	hCB_1 CHO membranes[a] Ki (nM)	hCB_2 CHO membranes[a] Ki (nM)	Mouse cortex membranes CB_1 [a] Ki (nM)	Mouse spleen membranes CB_2 [a] Ki (nM)	hCB_1 CHO cells[b] IC_{50} (nM)	hCB_2 CHO cells[b] IC_{50} (nM)
5F-PB22	0.027 ± 0.002	1.27 ± 0.12	0.055 ±0.004	1.48 ± 0.08	0.048 ± 0.003	2.38 ± 0.12
BB22	0.394 ± 0.029	0.724 ± 0.059	0.736 ± 0.062	0.857 ± 0.063	0.762 ± 0.063	1.52 ± 0.11
JWH-018	9.64 ± 0.81	8.66 ± 0.68	5.73 ± 0.41	7.25 ± 0.63	14.49 ± 1.23	12.48 ± 1.13

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

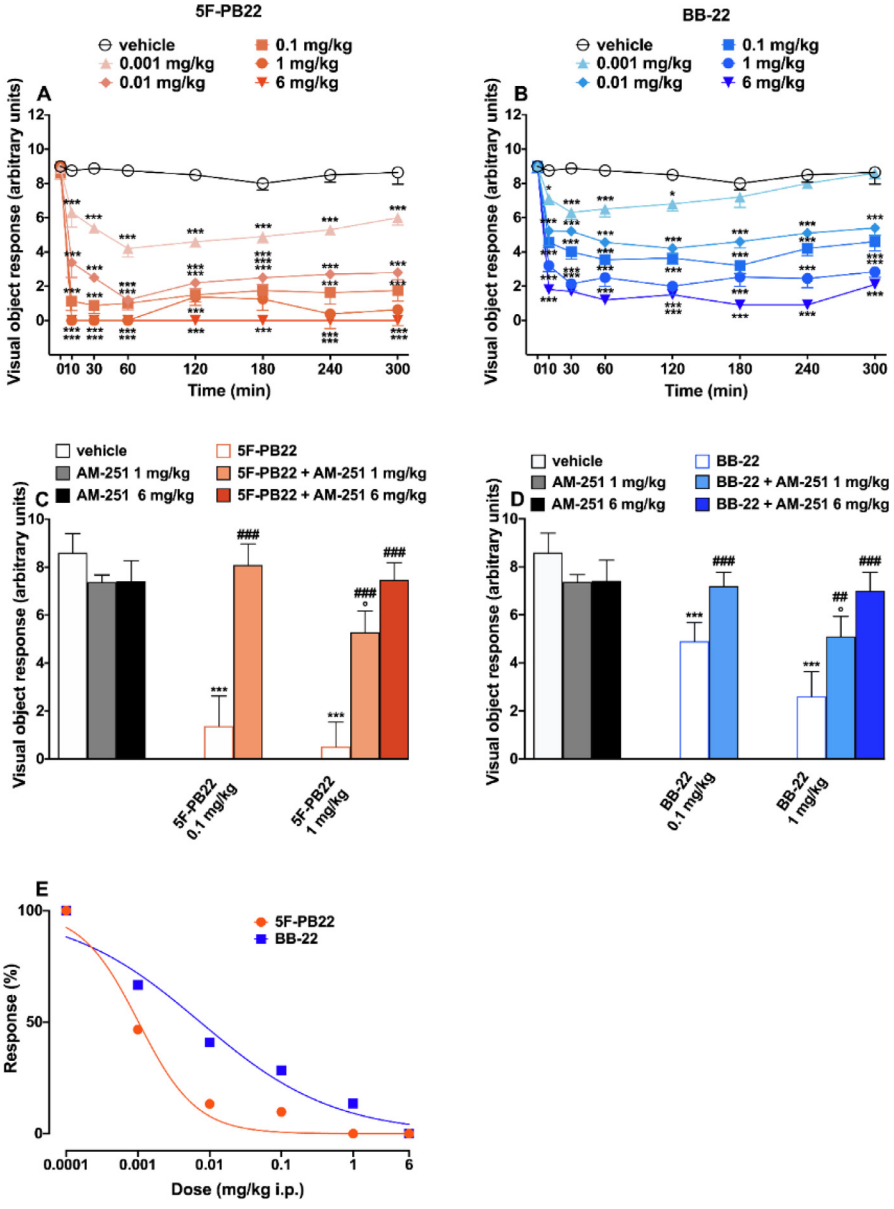

Fig. 3. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the visual object response of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as arbitrary units and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{***}p < 0.001$ versus vehicle; $^{\circ}p < 0.05$ versus AM-251 (1 mg/kg); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus agonist.

i.p.), that persisted up to 5 h (Fig. 4A; significant effect of treatment ($F_{5,224}$=316.7, p<0.0001), time ($F_{7,224}$=95.97, p<0.0001) and time x treatment interaction ($F_{35,224}$=8.008, p<0.0001)). Similarly, BB-22 (0.01-6 mg/kg; i.p.) provoked a significant impairment that persisted up to 5 h (Fig. 4B; significant effect of treatment ($F_{5,208}$=241.0, p<0.0001), time ($F_{7,208}$=51.52, p<0.0001) and time x treatment interaction ($F_{35,208}$=5.623, p<0.0001)). Otherwise, the lowest dose of BB-22 (0.001 mg/kg i.p.) did not inhibit the visual placing response in mice. Pretreatment with AM-251 (1 mg/kg; i.p.), which alone did not alter the response in mice (Fig. 4C and D), did not prevent the inhibition of visual placing response induced by 0.1 mg/kg of 5F-PB22 (Fig. 4C; significant effect of treatment ($F_{4,19}$=26.64, p<0.0001)). Otherwise, the impairment provoked by 0.1 mg/kg of BB-22 is prevented by pretreatment with the same dose of AM-251 (Fig. 4D; significant effect of treatment ($F_{4,25}$=22.71, p<0.0001)). Moreover, pretreatment with AM-251 (1 mg/kg i.p.) only partially prevented the inhibitory ef-

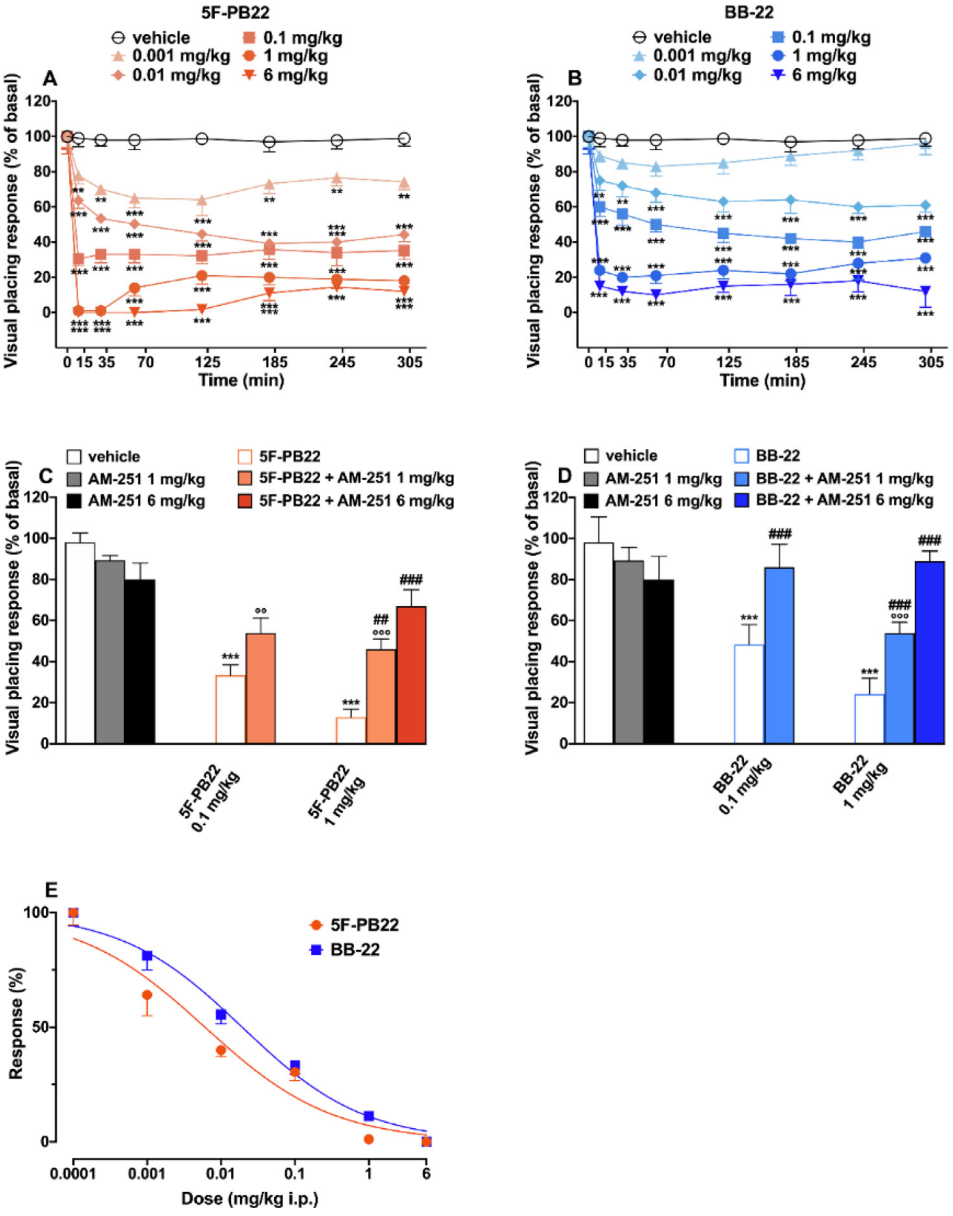

Fig. 4. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the visual placing response of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as percentage of baseline and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 were performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{°°}p < 0.01$, $^{°°°}p < 0.001$ versus AM-251 (1 mg/kg); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus agonist.

Table 3
ED_{50} values of 5F-PB22 and BB-22 based on *in vivo* performed behavioral tests. Data are expressed as value ± SEM. ED_{50} (dose of agonist to obtain 50% of the maximal effect) has been calculated by non-linear regression curve fitting of the dose-response curves determined using Prism 8.0 software (GraphPad Prism, San Diego CA). ED_{50} curves relative to each test were compared performing the F test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus BB-22.

Test	5F-PB-22ED_{50} (mg/kg)	BB-22ED_{50} (mg/kg)
Visual object	0.001±0.036***	0.007±0.068
Visual Placing	0.006±0.129**	0.028±0.057
Startle reflex	0.004±0.066***	0.018±0.095
Overall tactile	0.081±0.032***	0.187±0.054
Rotarod	0.394±0.218	0.481±0.213
Drag	0.008±0.194**	0.042±0.128
Core temperature	0.159±0.086	0.199±0.069
Breath rate	0.069±0.115	0.118±0.107
Tail pinch	0.032±0.086***	0.181±0.092
Tail withdrawal	0.892±0.016	1.134±0.065

fect induced by 1 mg/kg of 5F-PB22 and BB-22. Instead, the higher dose of AM-251 (6 mg/kg i.p.) prevented the inhibition of the response induced by 1 mg/kg of 5F-PB22 (Fig. 4C; significant effect of treatment ($F_{5,22}$=41.44, p<0.001)) and BB-22 (Fig. 4D; significant effect of treatment ($F_{5,28}$=58.35, p<0.0001)). 5F-PB22 appeared to be more potent in reducing visual placing response in mice, when compared to BB-22 (Fig. 4E; curves comparison ($F_{(1,64)}$=9.745, p = 0.0027)). ED_{50} values are presented in Table 3.

3.2.3. *Evaluation of the acoustic response*

Acoustic response did not change in vehicle-treated mice over the 5-hour observation period (Fig. 5A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-PB22 and BB-22 dose-dependently reduced the acoustic response in mice. 5F-PB22 provoked an immediate impairment, especially at the highest doses, that persisted up to 5 h (Fig. 5A, significant effect of treatment ($F_{5,240}$=476.9, p<0.0001), time ($F_{7,240}$=113.2, p<0.0001) and time x treatment interaction ($F_{35,240}$=9.103, p<0.0001)). Similarly, BB-22 inhibited in a

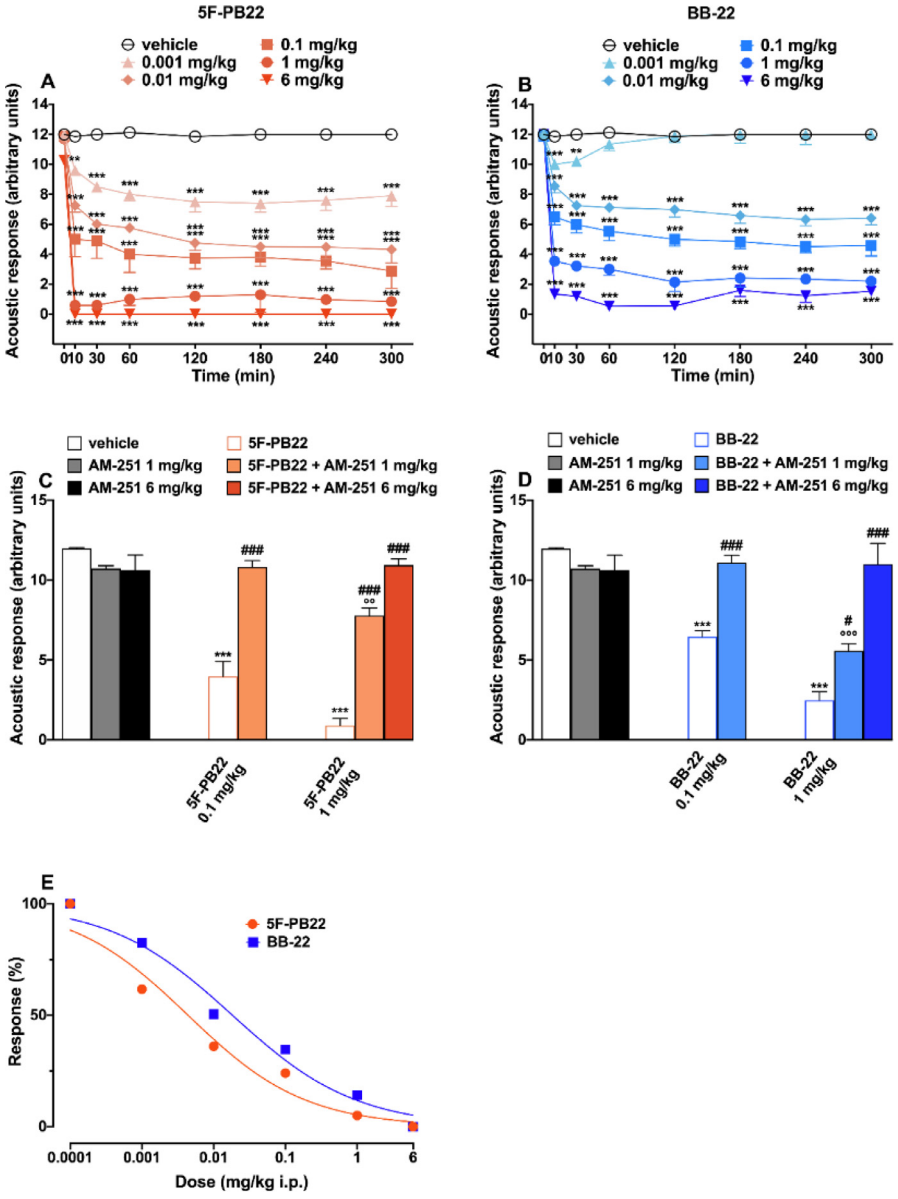

Fig. 5. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the acoustic response of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as arbitrary units and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ versus AM-251 (1 mg/kg); $^{\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus agonist.

transient (0.001 mg/kg i.p.) or prolonged manner (0.01-6 mg/kg i.p.) the acoustic response in mice (Fig. 5B; significant effect of treatment ($F_{5,240}$=882.5, p<0.0001), time ($F_{7,240}$=158.8, p<0.0001) and time x treatment interaction ($F_{35,240}$=20.66, p<0.0001)). In particular, 0.001 mg/kg of BB-22 induced a transient inhibition during the first 30 minutes after injection and then rapidly recovered to basal values. Pretreatment with AM-251 (1 mg/kg i.p.), which alone did not alter the response in mice (Fig. 5C e D), prevented the inhibition of visual object response induced by 0.1 mg/kg of 5F-PB22 (Fig. 5C; significant effect of treatment ($F_{4,19}$=28.70, p<0.0001)) and BB-22 (Fig. 5D; significant effect of treatment ($F_{4,19}$=29.33, p<0.0001)). Otherwise, the same doses of AM-251 did not totally prevent the impairment provoked by 1 mg/kg of the SCs. Higher dose of antagonist was required to totally prevent the effect. Pretreatment with 6 mg/kg of AM-251 prevented in fact the inhibition of the acoustic response induced by 1 mg/kg of 5F-PB22 (Fig. 5C; significant effect of treatment ($F_{5,22}$=71.16, p<0.0001)) and BB-22 (Fig. 5D; significant effect of treatment ($F_{5,22}$=40.39, p<0.0001)). 5F-PB22 appeared to be more potent in reducing acoustic response in mice, when compared to BB-22 (Fig. 5E; curves comparison ($F_{(1,64)}$=54.23, p < 0.0001)). ED_{50} values are presented in Table 3.

3.2.4. *Evaluation of the overall tactile reflex*

Overall tactile reflex did not change in vehicle-treated mice over the 5 h observation (Fig. 6A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Overall tactile reflexes were promptly inhibited by systemic administration of the highest doses of 5F-PB22 (Fig. 6A; significant effect of treatment ($F_{5,224}$=284.0, p<0.0001), time ($F_{7,224}$=39.40, p<0.0001) and time x treatment interaction ($F_{35,224}$=11.21, p<0.0001)) and BB-22 (Fig. 5B; significant effect of treatment ($F_{5,224}$=276.1, p<0.0001), time ($F_{7,224}$=37.86, p<0.0001, time x treatment interaction ($F_{35,224}$=8.9880, p<0.0001)), in a transient (0.1 mg/kg; i.p.) or prolonged manner (1-6 mg/kg; i.p.). Pretreatment with AM-251 (1 mg/kg i.p.), which alone did not alter the response in mice (Fig. 6C e D), did not prevent the effect induced by 1 mg/kg of 5F-PB22 (Fig. 6C; significant effect of treatment ($F_{5,22}$=20.21, p<0.0001)), which was however prevented by pretreatment with higher dose of antagonist (6 mg/kg i.p.). Otherwise, the impairment provoked by 1 mg/kg of BB-22 (Fig. 6D; significant effect of treatment ($F_{5,22}$=16.12, p<0.0001)) was prevented by pretreatment with both 1 and 6 mg/kg of AM-251. 5F-PB22 appeared to be more potent in reducing the overall tactile reflexes in mice, when compared to BB-22 (Fig. 6E; curves

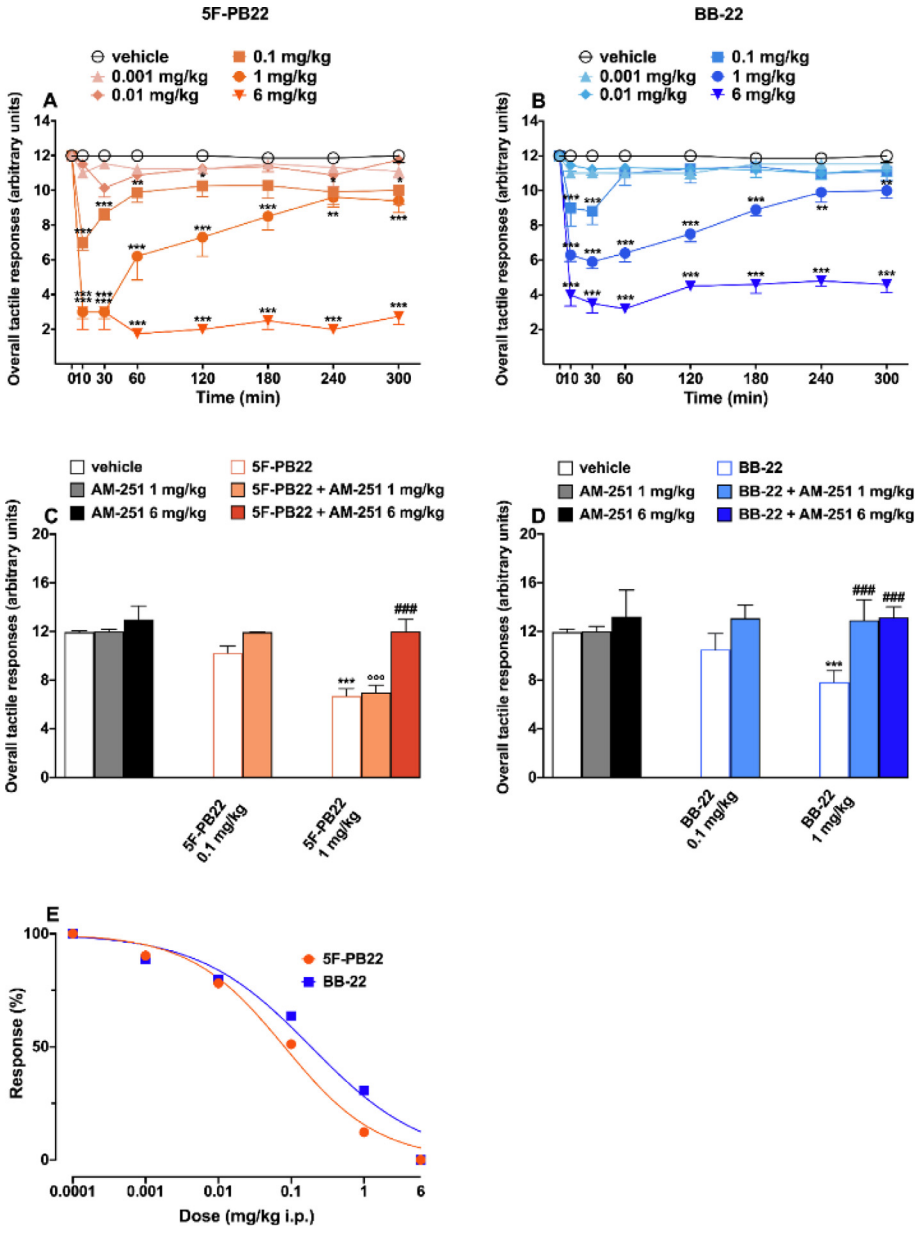

Fig. 6. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the overall tactile reflex of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as arbitrary units and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{°°°}p < 0.001$ versus AM-251 (1 mg/kg); $^{###}p < 0.001$ versus agonist.

comparison ($F_{(1,64)}$=33.90, $p < 0.0001$)). ED_{50} values are presented in Table 3.

3.2.5. *Evaluation of number of steps*

Number of steps was unchanged in vehicle-treated mice over the 5 hours observation (Fig. 7A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). 5F-PB22 (0.001-6 mg/kg; i.p.) systemic administration significantly reduced the number of steps in mice at all doses (Fig. 7A; significant effect of treatment ($F_{5,224}$=118.0, p<0.0001), time ($F_{7,224}$=32.17, p<0.0001) and time x treatment interaction ($F_{25,224}$=3.312, p<0.0001)). Specifically, 0.001 mg/kg of 5F-PB22 induced decrease of number of steps between 70 and 160 minutes after the injection, while the effect induced by doses of 0.1-6 mg/kg persisted up to 5 h. Differently, only highest doses (0.1-6 mg/kg; i.p.) of BB-22 reduced, in a significative and long lasting manner, the number of steps in mice (Fig. 7B; significant effect of treatment ($F_{5,224}$=118.4, p<0.0001), time ($F_{7,224}$=17.58, p<0.0001) and time x treatment interaction ($F_{25,224}$=3.020, p<0.0001)). Pretreatment with AM-251 (1 mg/kg; i.p.), which alone did not alter the response in mice (Fig. 7C e D), only partially prevented number of steps decrease induced by 0.1 mg/kg of 5F-PB22 (Fig. 7C; significant effect of treatment ($F_{4,25}$=15.08, p<0.0001). Otherwise, the effect induced by 0.1 mg/kg BB-22 was fully prevented by 1 mg/kg of AM-251 (Fig. 7D; significant effect of treatment ($F_{4,19}$=9.566, p=0.0002)). The same dose of AM-251 did not prevented the effect induced by 1 mg/kg of both 5F-PB22 (Fig. 9C; significant effect of treatment ($F_{5,22}$=20.84, p<0.0001)) and BB-22 (Fig. 7D; significant effect of treatment ($F_{5,22}$=18.12, p<0.0001)) on number of steps, which was however prevented by pretreatment with higher dose of antagonist (6 mg/kg; i.p.). 5F-PB22 appeared to be more potent in reducing the number of steps of mice, when compared to BB-22 (Fig. 7E; curves comparison ($F_{(1,64)}$=9.303, $p = 0.0033$)). ED_{50} values are presented in Table 3.

3.2.6. *Evaluation of time on rod*

Time on rod did not change in vehicle-treated mice in vehicle-treated mice over the 5 hours observation (Fig. 8A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Time on rod was significantly reduced by systemic adminis-

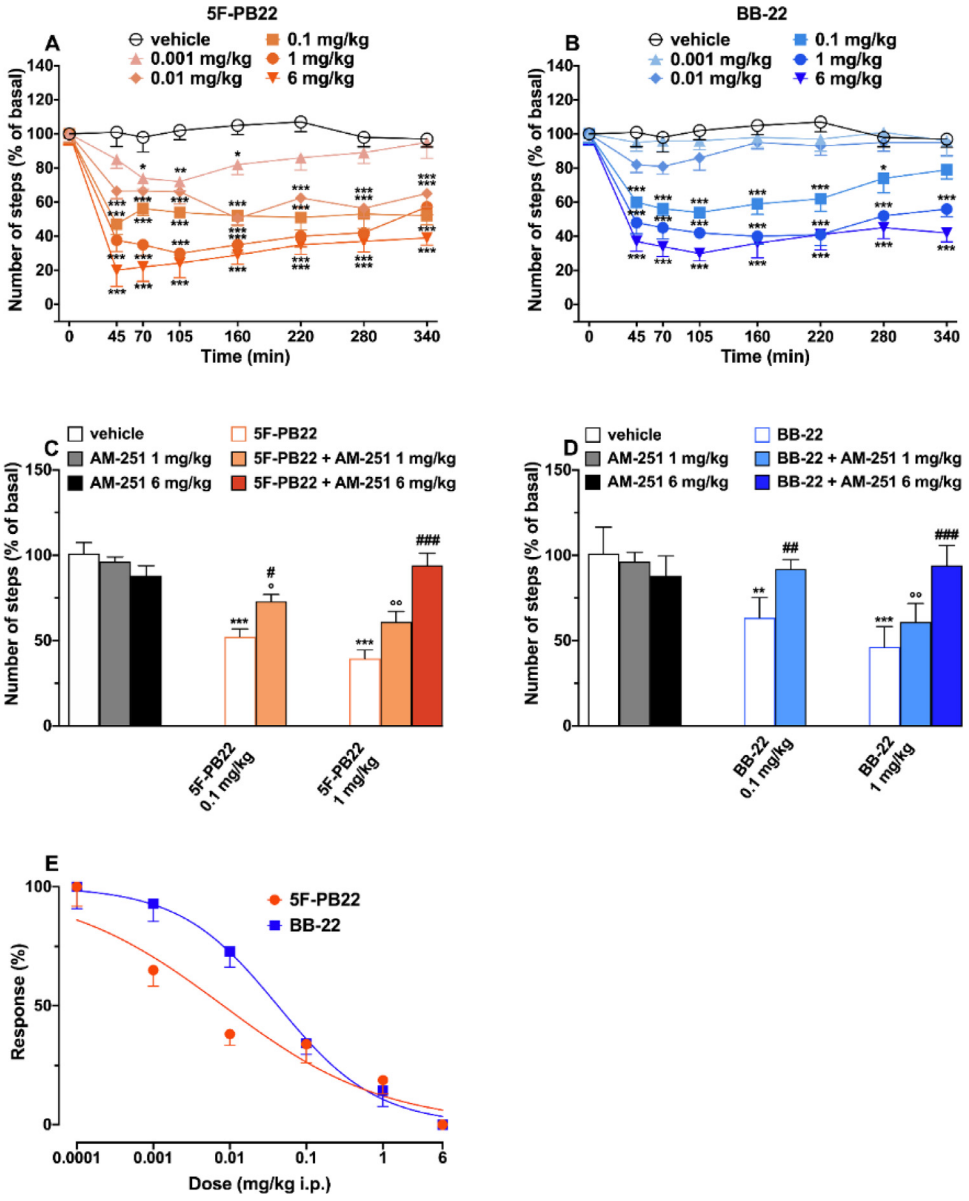

Fig. 7. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the number of steps of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as percentage of baseline and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ versus AM-251 (1 mg/kg); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus agonist.

tration of highest doses (1-6 mg/kg; i.p.) of 5F-PB22 (Fig. 8A; significant effect of treatment ($F_{5,224}$=64.73, p<0.0001), time ($F_{7,224}$=4.921, p<0.0001) and time x treatment interaction ($F_{35,224}$=1.990, p=0.0015)) and BB-22 (Fig. 8B; significant effect of treatment ($F_{5,224}$=29.98, p<0.0001), time ($F_{7,224}$=4.265, p=0.0002) and time x treatment interaction ($F_{35,224}$=0.9113, p=0.6154), in a transient (1 mg/kg; i.p.) or prolonged manner (6 mg/kg; i.p.). Specifically, BB-22 induced a transitory effect during the first 95 minutes, while that induced by 5F-PB22 persisted up to 210 minutes after the injection and both gradually recovered to basal level. Pretreatment with AM-251 (1-6 mg/kg; i.p.), which alone did not alter the response in mice (Fig. 8C and D), prevented the time on rod decrease induced by 1 mg/kg of both 5F-PB22 (Fig. 8C; significant effect of treatment ($F_{5,22}$=28.57, p<0.0001)) and BB-22 (Fig. 8D; significant effect of treatment ($F_{5,22}$=23.97, p<0.0001)). 5F-PB22 appeared to be as potent as BB-22 in reducing the time on rod (Fig. 8E). ED_{50} values are presented in Table 3.

3.2.7. Evaluation of core temperature

Core temperature did not change in vehicle-treated mice over the 5 h observation (Fig. 9A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Core temperature was reduced by systemic administration of the highest doses (0.1-6 mg/kg; i.p.) of 5F-PB22 (Fig. 9A; significant effect of treatment ($F_{5,196}$=266.8, p<0.0001), time ($F_{6,196}$=6.671, p<0.0001) and time x treatment interaction ($F_{30,196}$=10.40, p<0.0001)) and BB-22 (Fig. 9B; significant effect of treatment ($F_{5,196}$=148.3, p<0.0001), time ($F_{6,196}$=20.77, p<0.0001) and time x treatment interaction ($F_{30,196}$=6.991, p<0.0001)) in mice. Specifically, 0.1 mg/kg of 5F-PB22 briefly and transiently reduced core temperature during the first 50 minutes, while the effect of 1 and 6 mg/kg remained significantly different from control group (vehicle) for the entire duration (5 h) of the test. Similarly, 0.1 mg/kg of BB-22 induced significant core hypothermia only at 50 minutes of injection, but effect of 1 and 6 mg/kg was evident from 30 minutes and gradually recovered to basal level. Pretreatment with AM-251 (1 mg/kg; i.p.), which alone did not alter the core temperature of mice (Fig. 9C e D), did not prevent the effect induced by 1 mg/kg of 5F-PB22 (Fig. 9C; significant effect of treatment ($F_{5,22}$=29.23, p<0.0001)), which was however prevented by pretreatment with higher dose of antagonist (6 mg/kg; i.p.). Otherwise, the impairment provoked by 1 mg/kg of BB-22 (Fig. 9D; significant effect of treatment ($F_{5,22}$=25.64, p<0.0001)) was prevented by pretreatment with both 1 and 6 mg/kg of AM-251. 5F-PB22 appeared to be as potent as BB-22 in reducing core temperature of mice (Fig. 9E). ED_{50} values are presented in Table 3.

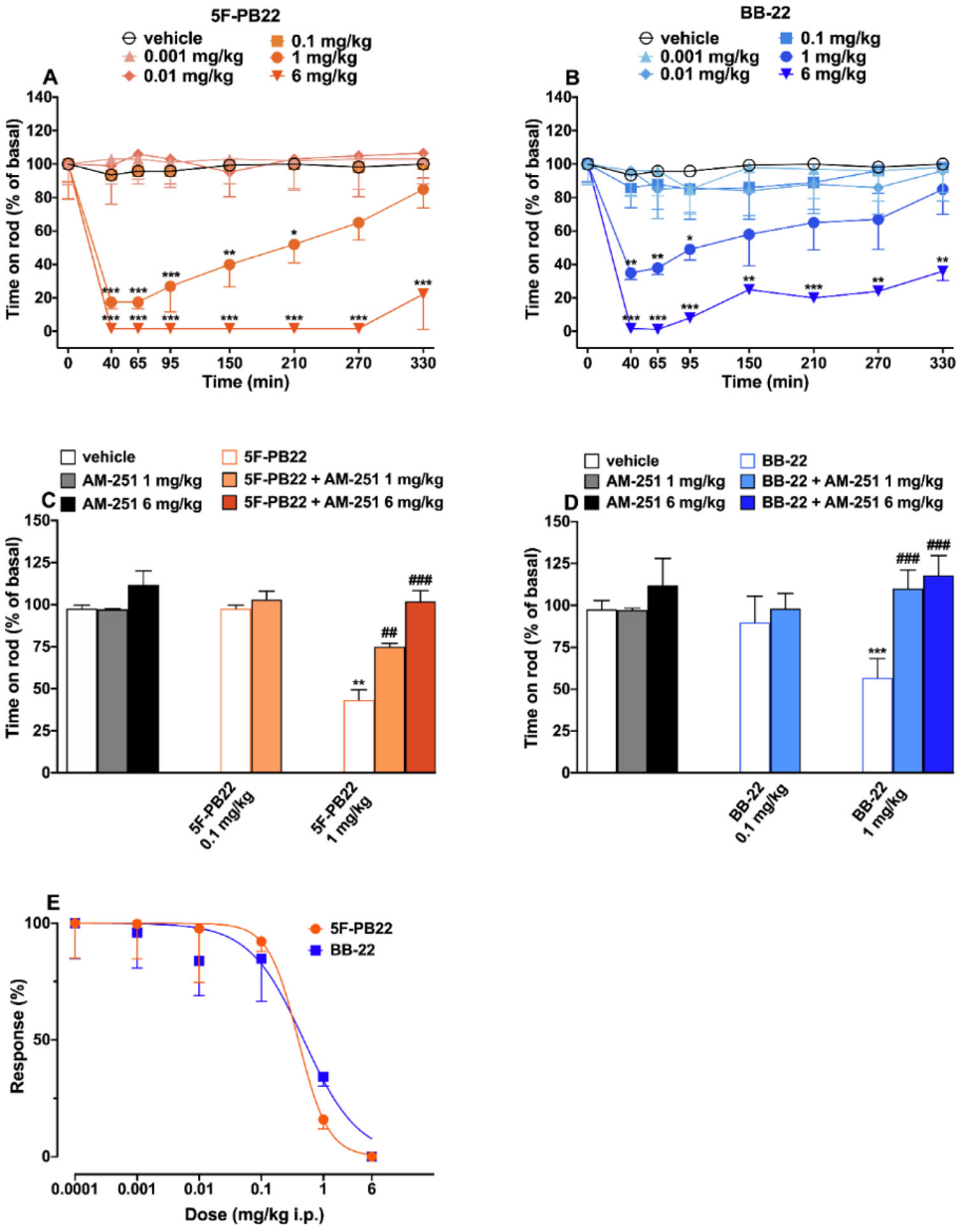

Fig. 8. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the time on rod in mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as percentage of baseline and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus agonist.

3.2.8. *Evaluation of breath rate*

Breath rate did not change in vehicle-treated mice over the 5 hours observation (Fig. 10A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of the highest doses (0.01-6 mg/kg; i.p.) of 5F-PB22 significantly reduced, in a brief and transient (0.01-0.1 mg/kg; i.p.) or prolonged manner (1-6 mg/kg; i.p.), the breath rate in mice (Fig. 10A; significant effect of treatment ($F_{5,224}$=276.4, p<0.0001), time ($F_{7,224}$=62.25, p<0.0001) and time x treatment interaction ($F_{35,224}$=14.03, p<0.0001)). Similarly, breath rate was reduced by doses of 0.1-6 mg/kg of BB-22 (Fig. 10B; significant effect of treatment ($F_{5,224}$=171.3, p<0.0001), time ($F_{7,224}$=47.90, p<0.0001) and time x treatment interaction ($F_{35,224}$=8.953, p<0.0001)). In particular, 0.1-1 mg/kg of BB-22 briefly and transiently reduced the breath rate, while effect of 6 mg/kg persisted up to 5 h after injection. Pretreatment with AM-251 (1 mg/kg; i.p.), which alone did not alter the response in mice (Fig. 10C and D), partially prevented the inhibitory effect induced by 1 mg/kg of the SCs. Otherwise, pretreatment with 6 mg/kg of antagonist prevented the breath rate decrease induced by 1 mg/kg of 5F-PB22 (significant effect of treatment ($F_{5,38}$=15.65, p<0.0001)) and BB-22 (significant effect of treatment ($F_{5,32}$=72.04, p<0.0001)). 5F-PB22 appeared to be as potent as BB-22 in reducing the breath rate of mice (Fig. 10E). ED_{50} values are presented in Table 3.

3.2.9. *Evaluation of pain induced by a mechanical stimulus*

Tail pinch test response did not change in vehicle-treated mice over the 5 hours observation (Fig. 11A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 5F-PB22 (0.001-6 mg/kg; i.p.) significantly increased the latency to the tail flick response evoked by the acute mechanical pain stimulus in mice (Fig. 11A; significant effect of treatment ($F_{5,196}$=389.1, p<0.0001), time ($F_{6,196}$=23.15, p<0.0001) and time x treatment interaction ($F_{30,196}$=7.105, p<0.0001)). Otherwise, BB-22 dose-dependently induced antinociception at the doses of 0.1-6 mg/kg (Fig. 11B; significant effect of treatment ($F_{5,196}$=162.2, p<0.0001), time ($F_{6,196}$=24.71, p<0.0001) and time x treatment in-

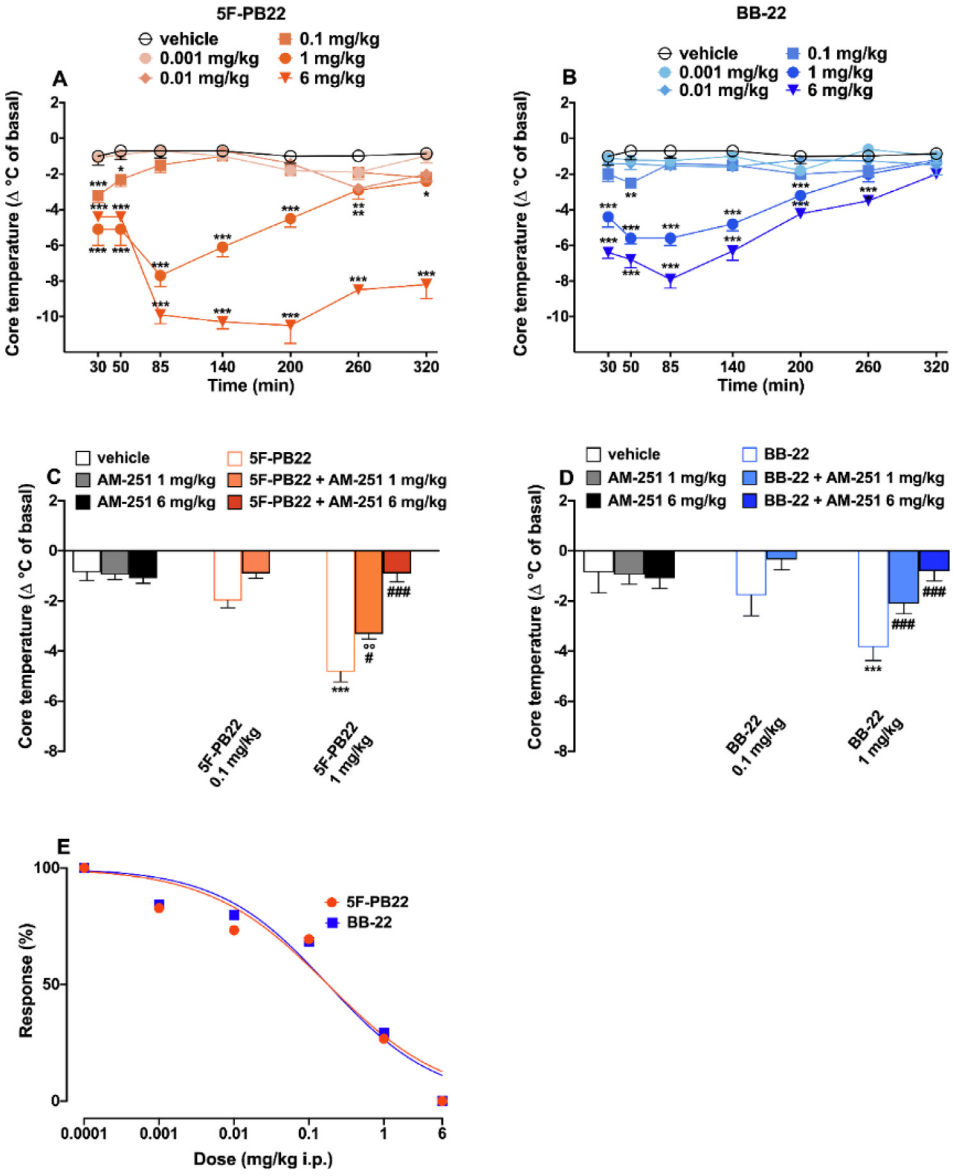

Fig. 9. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the core temperature of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as Δ°C of baseline and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{\circ\circ}p < 0.01$ versus AM-251 (1 mg/kg); $^{\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus agonist.

teraction ($F_{30,196}$=5.061, p<0.0001)). Specifically, 0.1 mg/kg of BB-22 briefly and transiently increased the latency to the tail flick response voked by the mechanical stimulus, while highest doses (1-6 mg/kg; i.p.) induced a long-lasting antinociceptive effect. Pretreatment with AM-251 (1 mg/kg; i.p.), which alone did not alter the response in mice (Fig. 11C e D), prevented mechanical antinociception induced by 0.1 mg/kg of both 5F-PB22 (Fig. 11C; significant effect of treatment ($F_{4,19}$=44.60, p<0.0001) and BB-22 (Fig. 11D; significant effect of treatment ($F_{4,19}$=16.60, p=0.0008)). Differently, the increase of latency to the tail flick response induced by 1 mg/kg of 5F-PB22 (Fig. 11C; significant effect of treatment ($F_{5,22}$=44.96, p<0.0001)) and BB-22 (Fig. 11D; significant effect of treatment ($F_{5,22}$=43.09, p<0.0001)) was only partially prevented by 1 mg/kg of AM-251. The effect was however abolished by pretreatment with the higher dose (6 mg/kg; i.p.) of the antagonist. 5F-PB22 appeared to be more potent in inducing acute mechanical antinociception, when compared to BB-22 (Fig. 11E; curves comparison ($F_{(1,64)}$=33.89, $p < 0.0001$)). ED_{50} values are presented in Table 3.

3.2.10. Evaluation of pain induced by a thermal stimulus

Tail withdrawal test response did not change in vehicle-treated mice over the 5 hours observation (Fig. 12A, B, C and D) and effect was similar to that observed in naïve untreated animals (data not shown). The latency to the tail flick response induced by the acute thermal pain stimulus in mice was increased by systemic administration of the highest doses (1-6 mg/kg; i.p.) of 5F-PB22 (Fig. 12A: significant effect of treatment ($F_{5,196}$=713.2, p<0.0001), time ($F_{6,196}$=180.7, p<0.0001) and time x treatment interaction ($F_{30,196}$=68.76, p<0.0001)) and BB-22 (Fig. 12B; significant effect of treatment ($F_{5.196}$=223.9, p<0.0001), time ($F_{6,196}$=66.04, p<0.0001) and time x treatment interaction ($F_{30,196}$=24.64, p<0.0001)). Thermal antinociceptive effect induced by 1 mg/kg of 5F-PB22 was not prevented by pretreatment with AM-251 (1 mg/kg; i.p.), which alone did not alter response of mice (Fig. 12C and D). However, pretreatment with higher doses of AM-251 (6 mg/kg; i.p.) prevented the antinociception induced by the same dose of 5F-PB22 (Fig. 12C; significant effect of treatment ($F_{5,22}$=32.34, p<0.0001)). Otherwise, pretreatment with both 1 and 6 mg/kg of AM-251 prevented the effect induced by BB-22 (Fig. 12D; significant effect of treatment ($F_{5,24}$=20.56, p<0.0001)). 5F-PB22 appeared to be as potent as BB-22 in inducing acute thermal antinociception (Fig. 12E). ED_{50} values are presented in Table 3.

4. Discussion

This study investigated for the first time pharmaco-toxicological effects induced by the acute systemic administration of quinolinyl ester indoles 5F-PB22 and BB-22 on CD-1 male mice and compared them

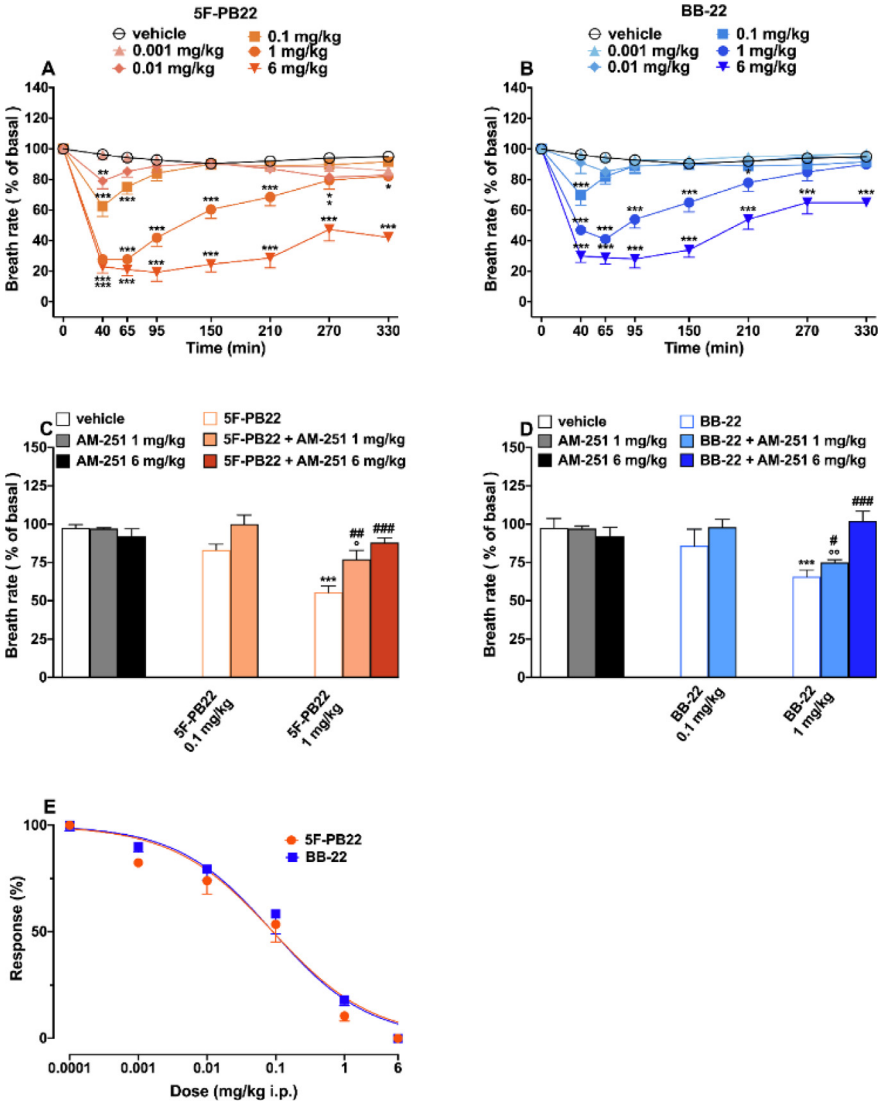

Fig. 10. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the breath rate of mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as percentage of baseline and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ versus AM-251 (1 mg/kg); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus agonist.

to evaluate their potency. Consistent with previous studies in rodents (Bilel et al., 2019; Canazza et al., 2016), we showed that the administration of increasing doses of 5F-PB22 and BB-22 causes the progressive onset of different pharmaco-behavioral effects in mice. In particular, both 5F-PB22 and BB-22 at lowest doses (0.001–0.01 mg/kg) mainly inhibited visual and acoustic sensorimotor responses. Increasing the dose (0.1 mg/kg), both compounds impaired sensorimotor responses, stepping activity, core temperature, breath rate and nociceptive response to mechanical stimuli. Similarly, both 5F-PB22 and BB-22 also reduced time on rod and increased thermal antinociception at the highest doses tested (1-6 mg/kg; Fig. 13).

Furthermore, tested compounds induced neurological alterations such as convulsions, hyperreflexia and myoclonias. These effects were observed immediately after the administration of the highest dose tested of both compounds (6 mg/kg), even though 5F-PB22 already induced such neurological responses at a dose of 1 mg/kg. This is in agreement with our previous data on different synthetic cannabinoid receptors agonists (Canazza et al., 2016; Ossato et al., 2015; Vigolo et al., 2015) and clinical reports showing the occurrence of seizure-like activity in humans after the recreational use of 5F-PB22 and BB-22 (Abouchedid et al., 2017; Schep et al., 2015). Noteworthy, pre-treatment with 1 mg/kg of the CB_1 receptor antagonist/inverse agonist AM-251 did not abolish all the effects induced by the administration of 1 mg/kg of these substances. Higher dose of AM-251 was required to observe the full prevention of the effect induced by 1 mg/kg of 5F-PB22 and BB-22, suggesting the great potency of these compounds and that they exert their action via acting on CB_1 cannabinoid receptor.

As can be seen from our results of *in vitro* binding experiments, 5F-PB22 and BB-22 retain picomolar affinity for both CD-1 murine and human CB_1 receptors. In particular, 5F-PB22 (Ki=0.027 nM) shows higher affinity than BB-22 (Ki=0.394 nM) in CD-1 mice preparation. In a similar way, 5F-PB22 displays higher affinity (Ki=0.055 nM) than BB-22 (Ki=0.736 nM) on human CB_1 receptors. Importantly, both substances show higher affinity when compared to the well-known synthetic cannabinoid JWH-018 both in human (Ki=9.46 nM) and mice (Ki=5.73 nM) receptors. As assumed for other similar compounds (Canazza et al., 2016; Vigolo et al., 2015), the high affinity could justify their potency value (5F-PB22 IC_{50}=0.048 nM and BB-22 IC_{50}=0.762 nM) in inhibiting cyclic AMP formation when compared to JWH-018 (IC_{50}=14.49 nM).

Although these quinolinyl ester indoles induced similar effects in behavioral tests, 5F-PB22 appeared to be more potent according to *in vitro* data. This evidence is consistent with previous study showing that small structural changes can lead to potence and effectiveness disparities (Bilel et al., 2020; Canazza et al., 2016; Ossato et al., 2016; Vigolo et al., 2015; Wiley et al., 2014). Therefore, it could be related to the pharmacokinetic profile of these substances, in addition to their pharmacodynamic features. Indeed, it has been shown that the presence of fluo-

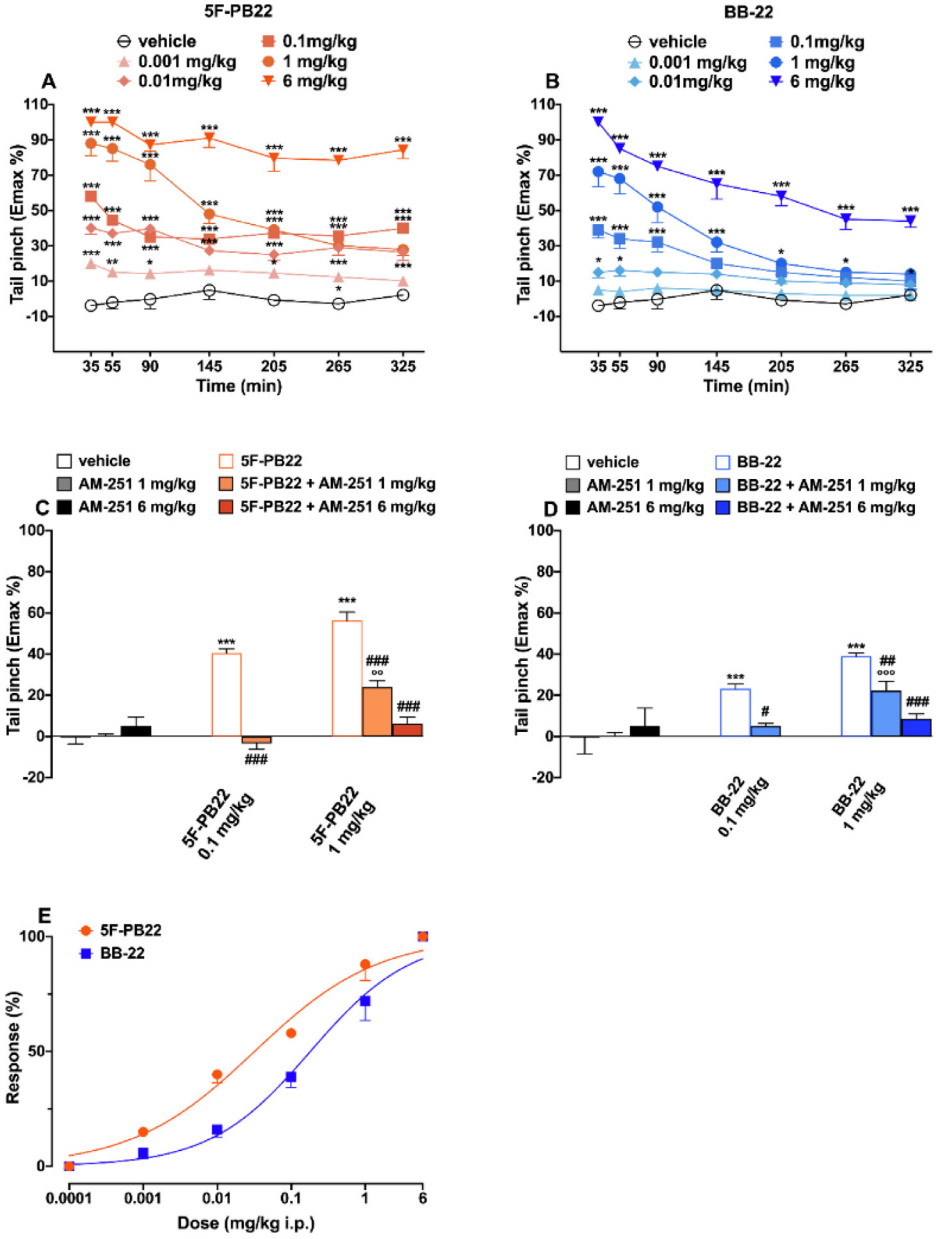

Fig. 11. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the tail pinch test in mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as Emax% and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle; $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ versus AM-251 (1 mg/kg); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus agonist.

rine increases compounds affinity for the CB_1 receptors (Banister et al., 2015; Wiley et al., 2014; Nikas et al., 2004) and also results in increased lipophilicity (Schifano et al., 2015). Thus, this latter can promote blood brain barrier penetration (Schifano et al., 2015) and influence blood concentration and urinary excretion of SCs (Kakehashi et al., 2020). Furthermore, it has been shown that two of the main metabolites of 5F-PB22 retained activity (Cannaert et al., 2016). Relying on this study, we cannot however exclude that this response profile can be related to the possible interaction between SCs and non-cannabinoid receptors (Wang et al., 2020; Pertwee, 2010; Ross et al., 2008).

4.1. Sensorimotor responses

In line with our previous studies (Bilel et al., 2020; Canazza et al., 2016; Ossato et al., 2016; Vigolo et al., 2015), 5F-PB22 and BB-22 induced a significant impairment of visual, acoustic, and tactile responses in mice. However, 5F-PB22 appeared to induce a deeper impairment when compared to BB-22. Pretreatment with AM-251 prevented the effects, strengthening the hypothesis that these substances could act on CB_1 receptors located in circuitries designated for sensorimotor responsiveness (Gomez-Nieto et al., 2014; Reig and Silberberg., 2014; Yoneda et al., 2013; Dasilva et al., 2012; Hemelt and Keller., 2008; Tzounopolus et al., 2007; Price et al., 2003). Relying on this study, these findings might also be related to auditory and visual sensory altered perceptions observed for other SCs in humans (Yeruva et al., 2019) as suggested for Δ^9-THC (Winton-Brown et al., 2011). Furthermore, we have previously showed that SCs affected the prepulse inhibition of the acoustic startle reflex in rodents (Bilel et al., 2020; Bilel et al., 2019), confirming that the use of these substances might results in severe information processing and sensory impairments likewise reported by users after the intake of 5F-PB-22 (https://psychonautwiki.org/wiki/5F-PB-22).

4.2. Motor assessment

5F-PB22 and BB-22 also reduced stimulated (drag and accelerod test) motor activity of mice, as already pointed out for different synthetic compounds (Canazza et al., 2016; Vigolo et al., 2015). Except for a tran-

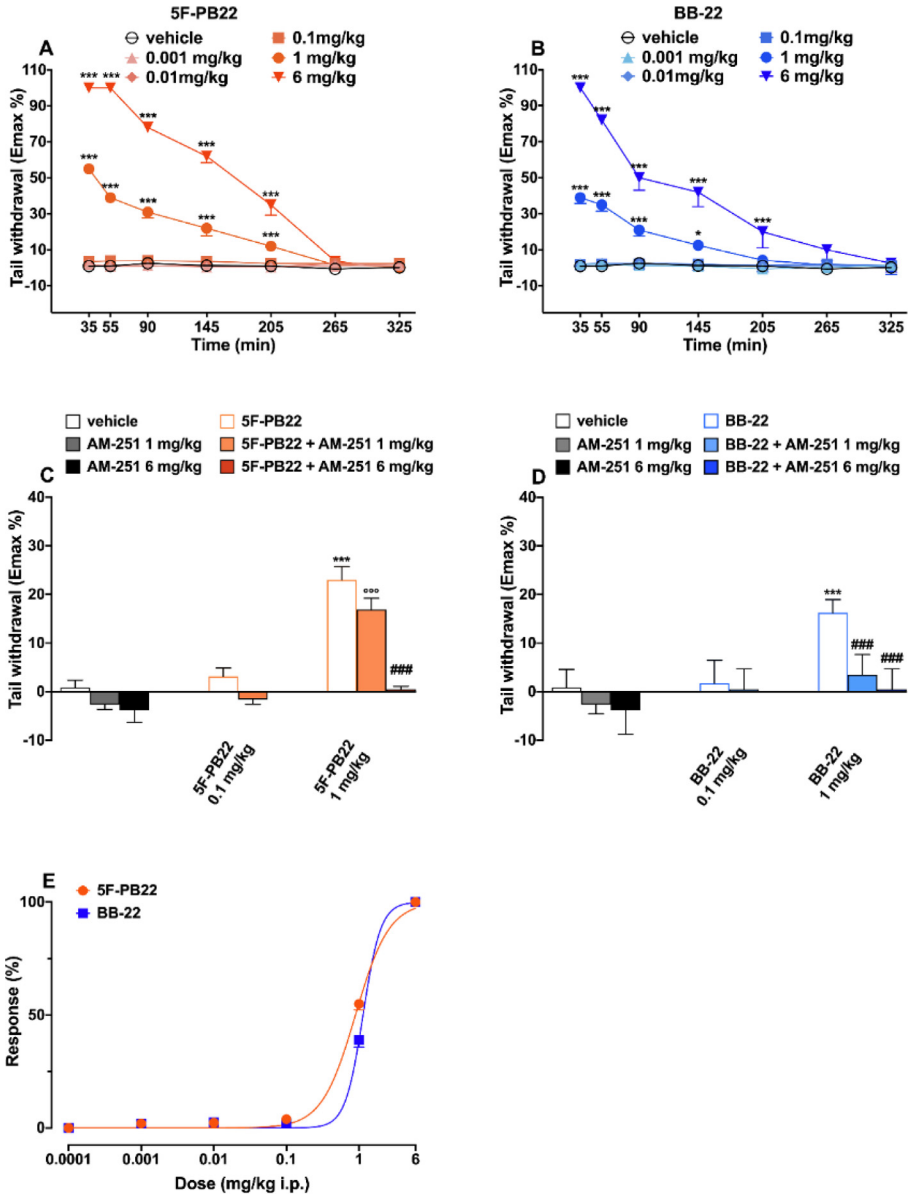

Fig. 12. Effect of 5F-PB22 (0.001-6 mg/kg i.p.; A) and BB-22 (0.001-6 mg/kg i.p.; B) on the tail withdrawal test in mice, interaction with the selective CB_1 receptor antagonist AM-251 (C and D) and comparison of the ED_{50} curves (E). Data are expressed as Emax% and represent the mean ± SEM of 6 or 4 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparison for the dose-response curve of each compound at different time-points. The analysis of the total average effect of each compound and AM-251 was performed with one-way ANOVA followed by Tukey's test. ED_{50} curves were compared performing the F test. $^{*}p < 0.05$, $^{***}p < 0.001$ versus vehicle; $^{°°°}p < 0.05$ versus AM-251 (1 mg/kg); $^{###}p < 0.001$ versus agonist.

sitory decrease of the number of steps induced by 5F-PB22 at the lowest dose tested, both substances altered the sensory responses of mice at doses (0.001-0.01 mg/kg) that did not affect their motor capacity. Thus, confirming that the sensory impairment is not only related to a disruption of motor function and suggesting that sensorimotor and motor responses are mediated by different process (Ossato et al., 2015). It has been showed that SCs, as well as Δ^9-THC (De Giacomo et al., 2020), may regulate motor activity by acting on CB_1 receptors located in the cerebellum and basal ganglia (Funada et al., 2020; Morera-Herreras et al., 2012; Rodriguez de Fonseca et al., 1998). Specifically, they may modulate motor activity by affecting dopaminergic motor circuits or central glutamate neurotransmission (Funada et al., 2020; Morera-Herreras et al., 2012).

4.3. Physiological responses

The present study demonstrated that both tested compounds altered physiological parameters (core temperature and breath rate) of animals and pretreatment with AM-251 abolished their effects, suggesting again the involvement of CB_1 receptors. Specifically, the highest doses (1-6 mg/kg) of 5F-PB22 and BB-22 induced hypothermia in mice. Despite 5F-PB22 induced a higher maximal decrease of the core temperature than that induced by BB-22, the latter appeared to be as potent as 5F-PB-22 in lowering body temperature. Our results agree with previous in vivo studies on the non-halogenated analogue of 5F-PB22 (PB-22; Schreiber et al., 2019) and further SCs (Schindler et al., 2017; Canazza et al., 2016; Ossato et al., 2015; Vigolo et al., 2015; De Vry et al., 2004). As supposed for other compounds (Schindler et al., 2017; Vigolo et al., 2015), the response might be mediated by CB_1 receptors highly expressed in the CNS (Central Nervous System; Ovadia et al., 1995; Pertwee, 2010). In particular, they likely exert their action in the preoptic anterior hypothalamus (Rawls et al., 2002). However, 5F-PB22 and BB-22 possess indole-derived structures (Carlier et al., 2018; WHO, 2017) and recent studies have revealed that such compounds (i.e., AM-2201 and JWH-018) enhance $5HT_1A$ receptor-mediated hypothermia in rodents (Yano et al., 2020; Elmore et al., 2018). Thereby, this could be considered as a non-cannabinoid mechanism which contribute to the effects (Yano et al., 2020) and possibly explain the similar hypothermic profile of tested compounds.

Moreover, both 5F-PB22 and BB-22 similarly decreased the breath rate of mice provoking a prolonged depressive effect at highest dose

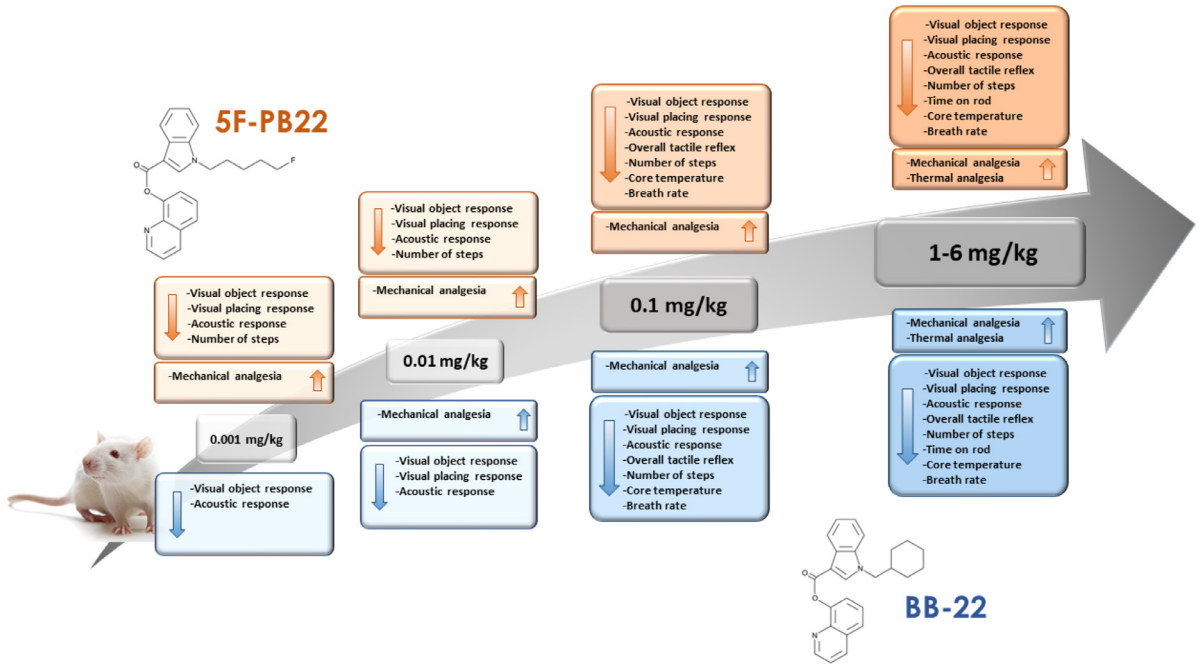

Fig. 13. Schematic comparison of the progressive appearance of pharmacological and behavioral effects induced by the administration of increasing doses of 5F-PB22 (0.001-6 mg/kg) in respect to BB-22 (0.001-6 mg/kg) in CD-1 male mice.

administered (6 mg/kg). Consistent with our findings, previous studies have shown that Δ^9-THC (Graham and Li, 1973; Rosenkrantz et al., 1974; Philips et al., 1971) and SCs induced acute respiratory depression in rodents (Bilel et al., 2019; Schmid et al., 2003). Although the effect is probably centrally mediated (Pfitzer et al., 2004), an additional peripheral action should be considered since a study suggested that CB_1 receptors found on airway nerves mediated effects on bronchial responsiveness of rodents (Calignano et al., 2000).

4.4. Pain threshold

5F-PB22 and BB-22 increased the threshold to acute mechanical stimulus in mice starting from the lowest doses tested, while the threshold to acute thermal pain stimulus was increased by systemic administration of the highest doses (1-6 mg/kg). Furthermore, 5F-PB22 appeared to be more potent in reducing the nociception to acute mechanical stimuli. This evidence is in line with our previous studies on further SCs (Bilel et al., 2019; Canazza et al., 2016; Vigolo et al., 2015; Ossato et al., 2015) and suggest that such compounds can induce their antinociceptive effect via acting on different response pathways to pain induced by mechanical (Martin et al., 1996) and thermal (Hohmann et al., 1999) stimuli. The effect was moreover prevented by the administration of AM-251, in contrast to the involvement of CB_2 in altering nociceptive responses in mice recently suggested by Wang and colleagues (Wang et al., 2020). Actually, further studies have pointed out an important role for both central (Dogrul et al., 2012) and peripheral (Yu et al., 2010; Agarwal et al., 2007; Lichtman and Martin, 1991) CB_1 receptors, which are located in mid- and hindbrain regions, and spinal cord of rodents (Woodhams et al., 2017; Klinger-Gratz et al., 2018; Tsou et al., 1998).

Conclusions

The present study disclosed the overall progressive pharmacological and behavioral effects induced by the increasing doses administration of the quinolinyl ester indoles 5F-PB22 and BB-22 in mice (Fig. 13), highlighting the ability of both compounds to primarily disrupt visual and acoustic sensorimotor responses. Thus, the responsiveness related to doses considered as "threshold dosage" by users may enhance the sensorimotor disruption that can likely contribute to the severe general impairment typically observed in drivers affected by the consumption of SCs. With increasing dose, impaired sensorimotor and motor responses, core temperature, breath rate and nociceptive threshold were then observed confirming that the abuse of these compounds can be cause of concern for public health and regulatory system. Our results moreover underlined that the difference of the chemical structures of the two SCs results in disparities involving both the pharmacological activity and adverse effects.

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Ethical statements

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Project activated in collaboration with the Presidency of the Council of Ministers-DPA Anti-Drug Policies (Italy).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Giorgia Corli: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Micaela Tirri:** Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Raffaella Arfè:** Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Sabrine Bilel:** Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Beatrice Marchetti:** Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Adolfo Gregori:** Resources, Writing – review & editing. **Fabiana Di Rosa:** Resources, Writing – review & editing. **Fabrizio Vincenzi:** Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Fabio De-Giorgio:** Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Pier Andrea Borea:** Writing – review & editing. **Katia Varani:** Conceptualization, Validation, Formal analysis, Resources, Writing – review & editing, Visualization, Project administration. **Matteo Marti:** Conceptualization, Validation, Formal analysis, Resources, Writing – review & editing, Visualization, Project administration, Supervision, Funding acquisition.

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