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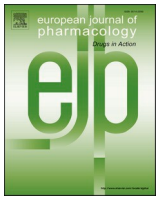

Pharmacological characterization of a new synthesis compound with antagonistic action on CB1 receptors: Evidence from the animal experimental model

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ABSTRACT

The endocannabinoid system is implicated in multiple physiological and pathological processes, making it a promising target for therapeutic intervention. CB1 receptor antagonists have shown potential in treating metabolic, neuropsychiatric, and addiction-related disorders. However, the adverse effects of CB1 antagonists like rimonabant have spurred the development of new compounds with improved safety profiles. This study investigates the pharmacological effects of a novel synthetic compound, 3-(5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-1H-pyrazol-3-yl)-2-phenylchinazolin-4(3H)-one (QD13), a quinazolinone derivative designed to antagonize CB1 receptor activity selectively. Molecular docking studies were conducted to assess the binding affinity of QD13 at the CB1 receptor, comparing it to the reference antagonist AM6538. The effects of QD13 were evaluated using the tetrad task in C57Bl/6 mice. The ability of QD13 to antagonize the effects of the CB1 agonist CP55,940 on locomotor activity, body temperature, catalepsy, and nociception were measured. Additionally, isolated ileum preparations were used to determine QD13's impact in counteracting the effects of CB1 agonists on gastrointestinal contractility. Docking analysis confirmed QD13's occupation of the CB1 receptor binding site, overlapping with AM6538, suggesting strong affinity. QD13 dose-dependently reversed CP55,940-induced hypomotility, catalepsy, analgesia, and hypothermia, with significant effects observed at 1 mg/kg and 3 mg/kg. Moreover, QD13 counteracted CP55,940-induced inhibition of ileal contractility, supporting its antagonistic activity in peripheral tissues. QD13 shows promising CB1 receptor antagonistic activity both centrally and peripherally. Its modulation of CB1 effects in the tetrad task and gastrointestinal assays suggests potential therapy for neuropsychiatric disorders, obesity, and addiction. Further studies are needed to clarify QD13's safety and efficacy.

1. Introduction

Designing and synthesizing new "molecular entities" with therapeutic potential is a crucial aspect of pharmaceutical research today. New molecular entities can be instrumental in treating several diseases for which current therapeutic options are either ineffective or unsatisfactory. In this context, there has been a growing interest in developing drugs that can modulate the endocannabinoid system (ECS). This system

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has captured the attention of the scientific community for its ability to regulate essential physiological functions, both in maintaining homeostasis and in response to various pathological conditions such as neurological, metabolic, and inflammatory disorders (Brancato et al., 2018; Di Marzo and Piscitelli, 2015; Zanettini, 2011), opening new therapeutic perspectives for diseases that remain difficult to treat with traditional therapies.

Drugs acting on the ECS primarily modulate cannabinoid type 1 (CB1) and type 2 (CB2) receptors or inhibit enzymes like FAAH and MAGL, enhancing the pharmacological activity of endocannabinoids like anandamide and 2-AG (Bajaj et al., 2021).

Among the most studied compounds, Cannabis Sativa derivatives such as Delta-8-tetrahydrocannabinol (Δ^8-THC), Delta-9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD) and cannabinol (CBN) have been widely characterized from a chemical and pharmacological point of view. These studies have led to their clinical use in different therapeutic settings (Mechoulam et al., 2002). For example, the combination of Δ^9-THC and CBN has been successfully used to treat chemotherapy-induced nausea and vomiting (Pertwee, 1997), as well as to manage chronic pain and muscle spasticity, conditions often associated with neurodegenerative diseases such as multiple sclerosis (Corey-Bloom et al., 2012). CBD, due to its anticonvulsant properties (Russo, 2017), has been approved for the treatment of rare and severe forms of epilepsy, such as Dravet and Lennox-Gastaut syndromes, providing a valid and effective therapeutic option for pathologies challenging to treat with conventional drugs (Devinsky et al., 2014; Thiele et al., 2018).

In recent years, there has been considerable interest in the role of cannabinoid receptor antagonists and reverse agonists in treating conditions such as neuroinflammation, gastrointestinal disorders, liver cirrhosis, asthma, and obesity (Lange and Kruse, 2005; Schindler et al., 2016). It has also been suggested that the use of these compounds may be beneficial in the treatment of "food addiction" and pathological addictions, including alcoholism and opioid abuse, such as heroin (Lavanco et al., 2018; Plescia et al., 2013; Richey and Woolcott, 2017). One of the first drugs developed with CB1 receptor antagonist activity was rimonabant. Although it demonstrated high potency and efficacy for oral administration, its clinical use has been limited due to the numerous side effects, including nausea, vomiting, depression, irritability, dizziness, anxiety, and hallucinations associated with it (Lange and Kruse, 2005; Richey and Woolcott, 2017). The emergence of this wide range of adverse effects has stimulated research into the synthesis of new compounds, both to create representative models of new molecules and as potential active ingredients capable of antagonizing the ECS, thus promising the treatment of different pathologies. In light of these concerns, we sought to design a structurally new CB1 receptor antagonist with good receptor affinity but potential for reduced psychiatric and metabolic side effects of the previous drugs like rimonabant. We focused on quinazolinone templates, which have proven to be versatile pharmacodynamics and have improved drug-likeness features in central nervous system (CNS)-active compounds (Abualassal et al., 2025; Haghighijoo et al., 2022). Our compound, 3-(5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-1H-pyrazol-3-yl)-2-phenyl-quinazolin-4(3H)-one (QD13), is a dual halogenated pyrazole appended to a phenyl-substituted quinazolin-4(3H)-one core, designed to enhance CB1R binding by favorable arm interactions, with optimization of arm-3 bulk to minimize inverse agonist activity (Table 1) (Hua et al., 2017; Lan et al., 1999; Lange and Kruse, 2005; Schindler et al., 2016).

Animal models provide a fundamental framework for characterizing new compounds through in vivo, ex vivo, and in vitro approaches and explaining their central and peripheral cannabinoidergic effects (Pertwee, 1997). They form an indispensable backbone for the identification, safety, efficacy, and mechanism of action, allowing them to be translated into clinical research.

Indeed, CB1 receptor antagonists have demonstrated potential in preclinical studies for addressing neuropsychiatric and neurodegenerative disorders. For instance, these compounds improve cognitive deficits in Alzheimer's disease models (de Bruin et al., 2010; Wolff and Leander, 2003) and reduce anhedonia in depression and anxiety models (Chagas et al., 2024; Hill and Patel, 2013). Additionally, they effectively decrease addictive behaviors by modulating dopaminergic pathways (Le Foll and Goldberg, 2005). The tetrad task is a widely accepted paradigm for evaluating cannabinoidergic activity in vivo. This behavioral assay evaluates four key effects of CB1 receptor activation: hypomotility, catalepsy, antinociception, and hypothermia. (Martin et al., 1991). CB1 receptor agonists typically induce significant effects across all domains, whereas antagonists can block these responses (Compton et al., 1992). This task provides a robust framework for characterizing receptor selectivity, potency, and efficacy, making it indispensable in preclinical cannabinoid research (Defrin et al., 2004; Plescia et al., 2018).

Based on this foundation, the first aim of our study was to investigate the pharmacological properties of QD13, a novel synthetic compound designed to antagonize CB1 receptor activity. QD13 is a structurally optimized quinazolinone derivative designed to exhibit improved CB1 receptor binding, as suggested by in silico docking analysis (Plescia et al., 2018). To evaluate its in vivo effects, we utilized the established cannabinoid tetrad assay in C57Bl/6 mouse model, a standard model for assessing CB1-mediated pharmacological responses. This approach allowed us to measure key parameters of cannabinoid activity, including locomotion, nociception, body temperature, and catalepsy. By constructing the dose-response curve, we aimed to elucidate its pharmacodynamic properties and therapeutic potential.

Notably, previous studies have shown that cannabinoids regulate gastrointestinal contractility, ion transport, gastrointestinal secretion, epithelial scarring, and feeding behavior by activating CB1 receptors located in the sensory terminals of the vagus and the neurons of the enteric nervous system, both in humans and in different animal species (Behl et al., 2022; Burdyga et al., 2004; Fioramonti and Bueno, 2008;

Table 1
Comparative overview of CB1 antagonists vs. QD13 highlighting scaffold types, binding properties, and known issues.

Compound	Core Scaffold	Key Substituents (Arms 1–3)	Docking Score (kcal/mol)	CB1 Binding Mode	Known Issues	QD13 Differentiation
Rimonabant	Pyrazole	2,4-dichlorophenyl (Arm 1), piperidinyl (Arm 3)	~ −9.5 *(Lange and Kruse, 2005)*	Orthosteric, inverse agonist	Severe psychiatric side effects	QD13 replaces Arm 3 with bulky quinazolinone moiety to reduce inverse agonism
AM251	Pyrazole	Halogenated phenyl (Arm 1), piperidine (Arm 3)	~ −9.0 (Lan et al., 1999; Schindler et al., 2016)	Orthosteric, inverse agonist	Poor selectivity, CNS side effects	QD13 alters arm arrangement and polarity, potentially improving tolerability
AM6538	Rimonabant analog	Butylbenzene, dichlorobenzene, N-piperidinylacetamido	−9.2 (Hua et al., 2016, 2017*)*	High-affinity orthosteric antagonist	Limited in vivo data	QD13 shows 82 % overlap, similar binding pocket, simpler synthesis
QD13	Quinazolin-4 (3H)-one + Pyrazole	2,4-dichlorophenyl (Arm 1), 4-chlorophenyl (Arm 2), quinazolinone (Arm 3)	−8.8 *(Plescia et al., 2018; Docking and current data)*	Orthosteric, competitive antagonist	Evaluated in this study	Novel scaffold, favorable docking, dose-tolerable in vivo profile

Note: Docking scores are approximate and method-dependent; QD13's values were calculated using PyRx with CB1 PDB: 5TGZ.

Massa and Monory, 2006; Pertwee et al., 1996; Sanger, 2007). In vitro evidence indicates that in mouse ileum, CB1 receptor antagonists, such as rimonabant, could counteract the inhibition of spontaneous intestinal contractility induced by CB1 agonists (Baldassano et al., 2008; Pertwee et al., 1996). These results suggest that modulating the activity of cannabinoids in the gastrointestinal system could offer a therapeutic option for disorders such as irritable bowel syndrome, alterations in intestinal motility, and obesity (Massa and Monory, 2006). By blocking the activation of CB1 receptors, these compounds can modulate gastrointestinal secretion and motility, reducing inflammation and visceral pain (Di Marzo, 2006; Storr et al., 2004), and could be helpful in various therapeutic approaches.

Based on this evidence, the second objective of our study was to assess whether QD13 could counteract the effects of CB1 agonists on gastrointestinal contractility.

2. Materials & methods

2.1. Docking studies

The crystallographic structure of the CB1 receptor (PDB ID:5TGZ) was downloaded from the Protein Data Bank (PDB). It provided the Cartesian coordinates for the cannabinoid receptor CB1 in complex with AM6538, an antagonist obtained by a strategic modification of rimonabant (Fig. 1). This modification was designed to stabilize the ligand-receptor complex and facilitate crystallization of the CB1 protein. The Chimera package (UCSF) (Manca et al., 2013) was used to test the 5TGZ structure, identifying any missing or incongruous atoms and adding the missing hydrogen atoms. In addition, the irrelevant ligands and water molecules in the crystallographic structure were removed.

AM6538 was selected as the reference antagonist for docking studies because it is co-crystallized with the CB1 receptor, which allows modelling of ligand-receptor interactions to be reliably founded on empirical structural data. AM251 lacks a resolved co-crystal structure with CB1, and therefore reliable docking becomes increasingly impossible. Whereas AM251 was used in the in vivo assays for pharmacological benchmarking, AM6538 provided a structurally validated template for in silico comparison of QD13 within the orthosteric binding site.

The structure of ligand AM6538 was isolated from the protein-ligand complex using Chimera by selection and subsequent detachment of the ligand from the crystallographic receptor structure. The three-dimensional structure of the new quinazolinone compound QD13 (Fig. 2) was then generated using the Discovery Studio software (Accelrys Software Inc., San Diego, USA). The geometry of the compound has been optimized using the 'Clean' Option geometry software. The resulting structure was subsequently docked using PyRx-Phyton Prescription 0.7 software (Marinho et al., 2015), with which the

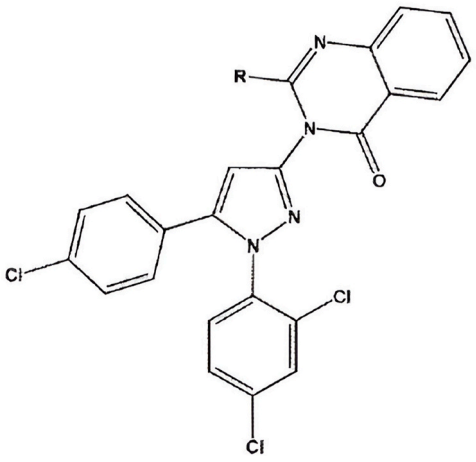

Fig. 2. Structure of the quinazolinon-3-(5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-1H-pyrazol-3-yl)-2-phenyl-chinazolin-4(3H)-one nucleus compound. R = C_6H_5.

structures of the optimized compounds, together with the reference AM6538, were docked in the active site of the CB1 receptor. A comparison of the crystallographic structure of AM6538 and the predicted structure via docking showed a high overlap, with an average quadratic deviation (RMSD) of 1.62 Å (Fig. 3), confirming the reliability of the generated model.

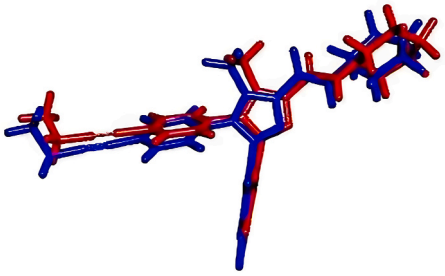

Fig. 3. Superposition of the crystallographic structure of reference AM6538 15 (blue) and that predicted through docking at the active site of the receptor (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

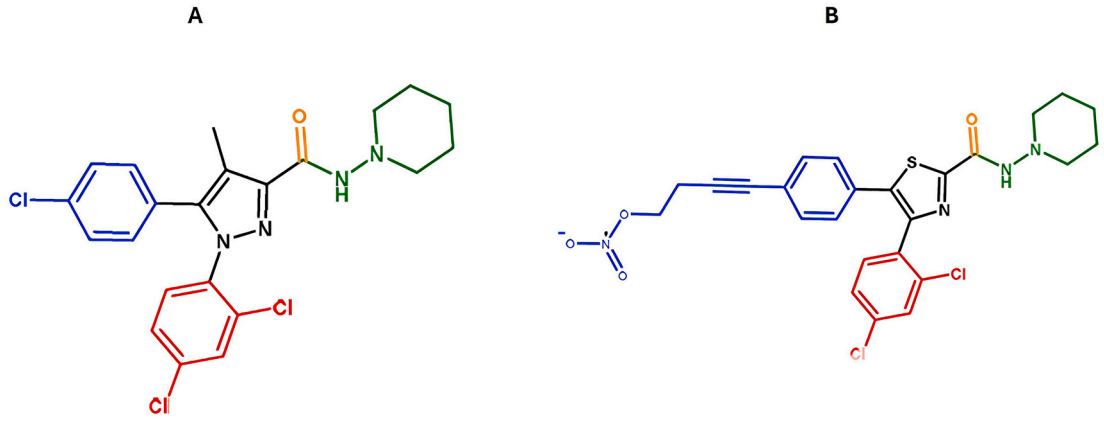

Fig. 1. Chemical structures of the Rimonabant (A) and the AM6538 (B). Arm 1 in red, arm 2 in blue and arm 3 in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2. Animals and experimental design

Male mice of strain C57BL/6 (Charles Rivers Laboratories Italia s.r.l), weighing 25–30g and about eight weeks old, are used for the experiments. The animals were housed in cages, with three birds per cage, and kept under controlled environmental conditions. The dark light cycle was 12 h (08:00–20:00 h), and the temperature and humidity of the room were maintained constant (22 ± 2 °C, 55 ± 10 %). The animals had free access to food and tap water. All mice used for the experiments were naïve and were divided into different experimental groups according to specific experimental protocols. In particular, to evaluate the efficacy of the new compound through the tetrad task, the animals were randomly assigned to the following experimental groups (n = 56): mice treated with CP55,940 (CP), mice treated with AM251 (AM), mice treated with AM251 plus CP55,940 (AM-CP), mice treated with different concentrations of the new compound 0.5, 1, 3, 5, 10 to which CP55,940 (QD13_0.5-CP), (QD13_1-CP), (QD13_3-CP), (QD13_5-CP), 10 (QD13_10-CP) were administered; mice treated with vehicle (Vh) (n = 8 mice per group for behavioural assays) (Fig. 4). During the test days, all animals were transported to the laboratory and allowed to set for an hour before the start of the experiments, which took place between 9:00 and 15:00.

This study was conducted following the current Italian legislation, law 26/2014, and the European Directive 2010/63/EU on protecting animals for scientific purposes. By the provisions of this decree, the research project was submitted and approved by the Committee for Animal Welfare of the University of Palermo and subsequently received the authorization of the Institutional Review Committee of the Ministry of Human Health, of Animal Health and the Ecosystem and International Relations, General Directorate of Animal Health of the Italian Ministry of Health (828/2024-PR to Fulvio Plescia).

2.3. Drugs

The agonist CP55,940 (Merck Life Scie$_{nc}$e, Milano, Italy) was administered at a dose of 0.1 mg/kg, and the known antagonist/inverse agonist AM251 (Merck Life Science, Milano, Italy) at a dose of 3 mg/kg, and the new experimental compound QD13 at a dose of 0.5, 1, 3, 5 and 10 mg/kg were administered acutely by intraperitoneal injection (*i.p.*). QD13 was synthesized in-house by the Department of Medicinal Chemistry, University of Palermo. The compound's structure and purity were confirmed by Elemental analysis data (C, H, N), IR, ^{1}H and ^{13}C NMR spectra. Animals not treated with these active compounds received a vehicle dose (1 ml/kg) to rule out that the effects observed were due to administration itself rather than the active compounds (Fig. 4). Before being administered, all compounds were solubilized in a vehicle solution containing 10 % ethanol, 5 % Tween 20 (Merck Life Science, Milan, Italy), 85 % saline, and fresh preparations.

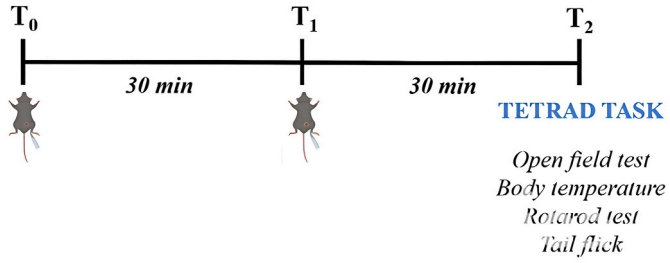

Fig. 4. Timing of administration preceding behavioral assessments. At the time (T_0), mice received a first dose of Vh (1 ml/kg), AM251 (3 mg/kg), or QD13 at doses of 0.5, 1, 3, 5, and 10 mg/kg. After 30 min (T_1), depending on the experimental group, the animals received a second administration of Vh (1 ml/kg) or CP55.940 (0.1 mg/kg).

2.4. Tetrad-task

2.4.1. Open field test

The animals were initially tested in the open field to assess their locomotor activity. The test was conducted in a room with moderate lighting (100 lx), using an automated video recording system, Any Maze (Ugo Basile, Italy). The arena used was a square box made of Plexiglas, with dimensions of 44 cm per side and a height of 20 cm. This system allowed us to simultaneously record several parameters, such as the total distance traveled (TDT), which measures motor activity. Each experimental session lasted 5 min, and the movement was recorded after the mice had been placed in the arena. The data collected was displayed and analyzed on a PC.

2.4.2. Body temperature

Body temperature was measured with a rectal probe connected to a Termoalert thermometer. Three measurements were made on each animal at 20-s intervals, for three measurements in 1 min. The final data was calculated as an average of the recorded values. Measurements were also repeated at 1, 2, and 3-h intervals.

2.4.3. Rotarod test

The mouse's ability to perform movements without falling, anticipating, or reacting promptly to possible imbalance factors was evaluated using the Rota-rod-unit (Ugo Basile, Italy). The animals had to walk on a rotating drum that increased speed from 4 rpm (revolutions per minute) to 40 rpm. The time taken for each animal to fall from the drum was recorded in seconds. No cut-off latency was used in the Rota-rod test to ensure that subtle deficits in motor coordination could be accurately detected (Plescia et al., 2018). All animals were trained to use Rota-rod for two consecutive days before the test day. Each animal has performed at least three test sessions in the two training days. The sessions ended when the animal fell off the rotating drum. The test was repeated if the animal failed to remain on the drum for at least 10 s. On the day of the test, a reliability test was conducted. Animals that did not stay on the rotating drum for 30 s in two separate tests did not meet the criteria and were excluded from the experiment. The time taken by the animals to fall from the rotation bar was measured 60 min after injection of the drug and reported as an average of the two studies.

2.4.4. Hot-water immersion tail-flick test

In the tail-flick test, nociception was measured through the tail-flick latency by immersion in hot water. In this dynamic evaluation, the tail was immersed for 2 cm in a precisely controlled water bath (Instruments srl, Bernareggio, MI, Italy) maintained at an optimum temperature of 52 ± 0,5 °C. A pronounced tail movement determined the latency of response. A time limit of 10 s has been strictly adhered to protect the animals and minimize tissue damage. This method ensures reliable and impact information on nociceptive behavior (Desroches et al., 2014).

2.4.5. Separation of the ileum

Experiments were performed using adult male mice C57BL/6 killed by cervical dislocation (n = 5 per group for isolated ileum experiments). The abdomen was immediately opened, and the ileum was removed and placed in Krebs solution of the following composition (mM): NaCl 119; KCl 4.5; MgSO4 2.5; NaHCO3 25; KH2PO4 1.2, CaCl2 2.5, glucose 11.1. Segments (15 mm in length) were suspended in a four-channel organ bath containing 8 ml of oxygenated (95 % O2 and 5 % CO2) Krebs solution maintained at 37 °C. The distal end of each segment was tied to organ holders, and the proximal end was secured with a silk thread to an isometric force transducer (FORT 25, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). The mechanical activity was digitized on an A/D converter, visualized, recorded, and analyzed on a personal computer using the PowerLab/400 system (Ugo Basile, Italy). Longitudinal preparations were subjected to an initial tension of 500 mg and were allowed to equilibrate for at least 30 min. Rhythmic spontaneous

contractions developed in all preparations.

After the equilibration time, the responses of the preparations to cumulative concentrations of (30–300 nM) were examined. Drugs were added to the bath at increasing concentrations, and each concentration was left in contact with the tissue for 8 min. CP responses were tested in a different series of experiments. In the presence of (0.1 μM), a CB_1 antagonist was added to the organ bath 30 min before CP and dissolved in ethanol. Control experiments using the solvent alone showed none affected the tissue responses studied.

2.5. Statistical analysis

The data obtained from the tetrad task concerning motor activity, body temperature, motor coordination, and nociception were analyzed using a one-way ANOVA followed by Tukey's posthoc test ($\alpha = 0.05$). All data are reported with average values ± SEM. Only data that reached a value of $p < 0.05$ or more were considered statistically significant.

3. Results

3.1. Docking study

The results obtained from the docking analysis of QD13 and the reference compound AM6538 showed that the newly synthesized compound occupies the same binding space at the CB1 receptor as the reference antagonist AM6538.

AM6538 is oriented to form van der Waals interactions with the residues Phe102, Met103, Ile105, Ser123, Phe170, Phe174, Leu193, Val196, Thr197, Phe268, Leu276, Trp279, Trp356, Leu359, Met363, Phe379, Met384, Cys386, Leu387 and electrostatic interactions with residues Gly166, Ser167, Ser383, Ser390. The Ser383 residue stabilizes AM6538 by a hydrogen bond with the ligand carbonyl, confirming a critical key binding interaction (Fig. 5).

The analysis of the docking of the QD13 found that it occupies a position superimposed to that of AM6538 in the active site, interacting with circa 82 % of the amino acids involved in binding the reference antagonist (Table 2). The main interactions between QD13 and the CB1 receptor include residues responsible for van der Waals and those establishing electrostatic interactions. Fig. 6 shows the key amino acids involved in QD13 binding to the CB1 receptor (5TGZ).

3.2. Open field

All animals were subjected to open-field testing to evaluate newly synthesized molecules' ability to counteract the effects induced by the CP55,940 agonist on locomotor activity. The results obtained from the statistical analysis, conducted utilizing a one-way ANOVA, showed a significant impact of the treatment ($F_{(7\text{-}48)} = 5.76$; $p < 0.001$). In particular, post-hoc analysis using the Tukey test showed that administration of the known agonist CP55,940 (CP group) induced a significant reduction in locomotor activity ($q = 4.45$ $p < 0.05$) compared with Vh. The effect was reversed by administration of the antagonist/inverse agonist AM251 to the AM-CP group ($q = 6.94$; $p < 0.001$). When the results were analyzed to understand whether and at what dose the newly synthesized compound was able to counteract the effects of administration of the agonist, the results showed a significant increase in the total distance traveled in groups QD13_1-CP ($q = 6.33$; $p < 0.01$) and QD13_3-CP ($q = 7.14$; $p < 0.001$) when compared with their respective control (Fig. 7A). No statistically significant differences were observed in locomotor activity of animals belonging to groups QD13_0.5-CP ($q = 3.64$; $p = 0.19$), QD13_5-CP ($q = 2.75$; $p = 0.53$) and QD13_10-CP ($q = 2.75$; $p = 0.07$).

3.3. Body temperature

The ability of the newly synthesized compound to antagonize the

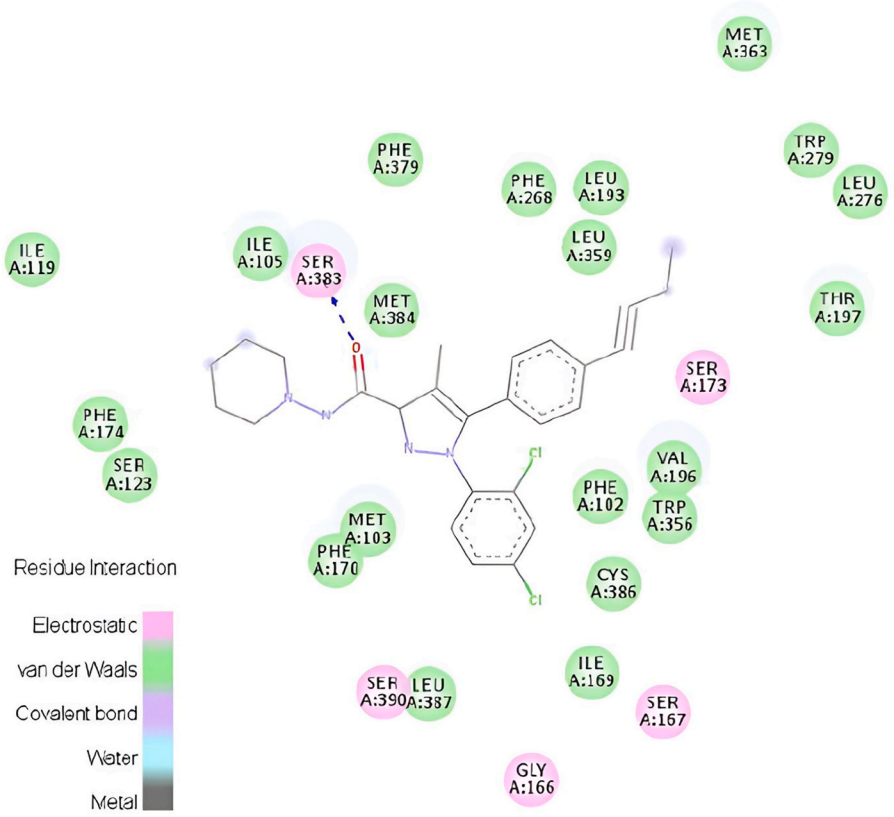

Fig. 5. Interactions of the compound AM6538 with the CB1 receptor (5 TG).

Table 2
Amino acids interact with AM6538 and QD13 at the receptor site.

AM6538	QD13
PHE 102	PHE 102
MET 103	MET 103
ILE 105	ILE 105
–	–
SER 123	SER 123
–	–
GLY 166	GLY 166
SER 167	SER 167
–	ILE 169
PHE 170	PHE 170
–	–
PHE 174	–
–	–
–	PHE 189
LEU 193	LEU 193
VAL 193	VAL 193
THR 197	THR 197
–	ILE 267
PHE 268	PHE 268
–	PRO 269
LEU 276	–
TRP 279	–
TRP 356	TRP 356
LEU 359	LEU 359
MET 363	–
PHE 379	PHE 379
–	ALA 380
SER 383	SER 383
MET 384	MET 384
CYS 386	CYS 386
LEU 387	LEU 387
SER 390	SER 390
%	82

effects induced by CP55,940 on body temperature was evaluated through a one-way variance analysis, which found a significant effect of treatment ($F_{(7\text{-}48)} = 9.91$; $p < 0.001$). Post-hoc analysis conducted by the multiple comparison test Tukey showed a significant reduction in body temperature in the CP group ($q = 9.36$ $p < 0.001$) compared to the Vh group. In addition, a reversal of the body temperature decrease induced by the administration of CP55,940 was observed both in the AM-CP group ($q = 9.01$; $p < 0.001$), treated with AM251 and in the QD13_1-CP ($q = 5.20$; $p < 0.05$), QD13_3-CP ($q = 7.40$; $p < 0.001$) and QD13_10-CP ($q = 5.21$; $p < 0.05$) groups, to which the new compound was administered at concentrations of 1, 3 and 10 mg/kg respectively when compared with the corresponding control group. No statistically significant differences were found in the comparison between groups QD13_0.5-CP ($q = 3.63$; $p = 0.198$) and QD13_5-CP ($q = 3.34$; $p = 0.282$), treated with the compound at concentrations of 0.5 and 5 mg/kg respectively when compared with the control group (Fig. 7B).

3.4. Rotarod test

Animals from the various experimental groups were subjected to the Rota-rod test to evaluate the efficacy of the new compound in countering the effects induced by the CB1 agonist on motor coordination and balance. The statistical analysis performed employing an ANOVA at one-way on latency time - defined as the period taken by the animals to fall from the rotating cylinder - revealed a significant effect of the treatment ($F_{(7\text{-}48)} = 6.45$; $p < 0.001$). The post-hoc analysis conducted with the Tukey test showed a significant reduction in latency time in the CP group ($q = 5.63$; $p < 0.01$) compared to the Vh group. In addition, in the group where AM251 or other compounds with potential antagonistic activity were administered, a substantial increase of latency time was observed in the AM-CP group ($q = 6.94$; $p < 0.001$), QD13_1-CP ($q = 4.73$; $p < 0.05$), QD13_3-CP ($q = 4.52$; $p < 0.05$) and QD13_10-CP ($q = 7.38$; $p < 0.001$) groups, compared to the control group. In contrast, no

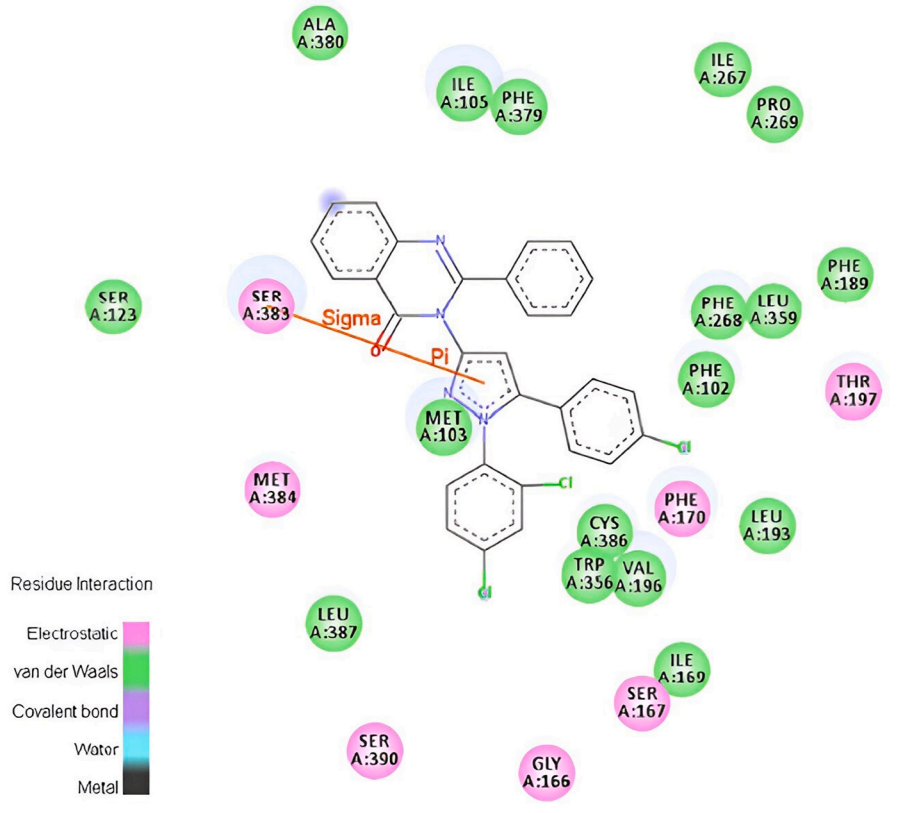

Fig. 6. Interactions of compound QD13 with the CB1 receptor (5 TG).

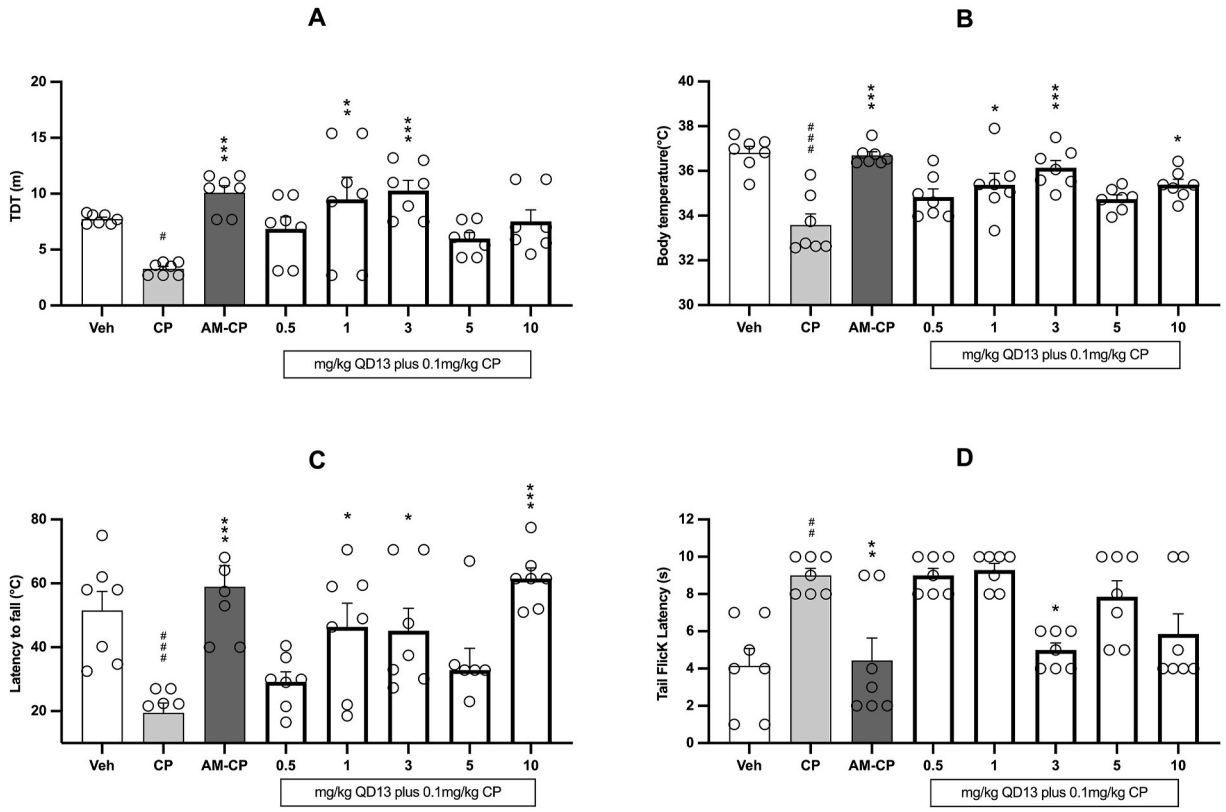

Fig. 7. Evaluation of the pharmacological efficacy of the new compound in counteracting cannabinoid-induced effects in the tetrad task (n = 8 per group for behavioral assays). The new compound was able to reverse the effects induced by the administration of the known agonist CP55.940 in the four paradigms analyzed: locomotor activity (A), body temperature (B), motor induration (C), and nociception (D). Each value represents an average of ±SEM of 7 mice. $^{###}p < 0.001$; $^{##}p < 0.01$ and $^{#}p < 0.05$ vs vehicle. ***p < 0.001; **p < 0.01 and *p < 0.05 vs CP.

statistically significant differences were found in the groups QD13_0.5-CP (q = 1.71; p = 0.926) and QD13_5-CP (q = 2.36; p < 0.78), although a positive trend was observed in both groups compared to the control group (Fig. 7C).

3.5. Tail flick

All experimental groups were subjected to the tail-flick test to assess whether the new compound could counteract the analgesic effect induced by the administration of the known agonist, thus increasing the central nervous system's response to a pain stimulus. The results obtained from the statistical analysis conducted employing an ANOVA at one-way on tail-flick latency time showed a significant effect of the treatment ($F_{(7\text{-}48)} = 8.04$; $p < 0.001$). The post hoc analysis, performed using Tukey's multiple post-test comparisons, showed a significant increase in tail-flick latency time in the CP group ($q = 6.27$; $p < 0.01$) and a reduction in latency in the AM-CP group ($q = 5.90$; $p < 0.01$) when compared with the respective control groups. In addition, a significant reduction in the latency time of tail-flick was observed in the group receiving the new compound at the dose of 3 mg/kg, QD13_3-CP ($q = 5.16$; $p < 0.05$), compared to the group treated with only CP55,940, suggesting a dose antagonist effect specific to the newly synthesized compound. In contrast, no statistically significant differences were found in latency times for the administration of the new compound at other concentrations: QD13_0.5-CP ($q = 0.00$; $p > 0.99$), QD13_1-CP ($q = 0.369$; $p > 0.99$), QD13_5-CP ($q = 1.48$; $p = 0.97$) and QD13_10-CP ($q = 4.06$; $p = 0.10$), from comparison with the CP group (Fig. 7D).

3.6. Effects on isolated mouse ileum segments

CP55,940 (30–300 nM) produced a concentration-dependent inhibitory effect, consisting of a decrease in the mean amplitude of spontaneous contractions, with changes in the resting tone at higher concentrations. At the highest concentrations, CP55,940 reduced the spontaneous contractions by about 90 %. The inhibitory effect of CP55,940 was significantly antagonized by the QD13 cannabinoid CB1 antagonist (0.1 μM) (Fig. 8).

4. Discussion

The first objective of our study was to assess the ability of the newly synthesized compound QD13 to bind to the cannabinoid receptor CB1. To this end, the molecular structure of QD13 was compared with that of the known antagonist, AM6538, and then the interaction with the binding site of the agonist CP55,940 on the CB1 receptor was analyzed.

The molecular docking study found that compound QD13 occupies, similarly to AM6538, the same binding site as CP55,940 (Fig. 9). This finding is significant as it indicates a potential direct competition between QD13 and the CP55,940 agonist, suggesting that the new compound may exert antagonistic action on the CB1 receptor. The comparison between QD13 and the antagonist AM6538 allowed us to clarify the similarities between the binding modes of these two compounds. In particular, the antagonist AM6538 is oriented so that the 2,4-dichlorobenzene (Fig. 10, in red), butyl benzene (Fig. 10, in blue), and N-piperidininlacetamido (Fig. 10, in green) groups represent arm 1, arm 2 and arm 3 respectively. Similarly, the compound QD13 binds equally, with its three arms occupying their respective CB1 receptor binding cavities (Fig. 11). In particular, the 2,4-dichlorophenyl group is in arm 1, and the 4-chlorophenyl group is in arm 2, respectively linked to the pyrazole nucleus in positions 1 and 5. The quinazolinone group occupies the zone called arm 3 instead, which, following Hua's hypothesis (Hua et al., 2016), represents a bulky group essential on arm 3 for CB1

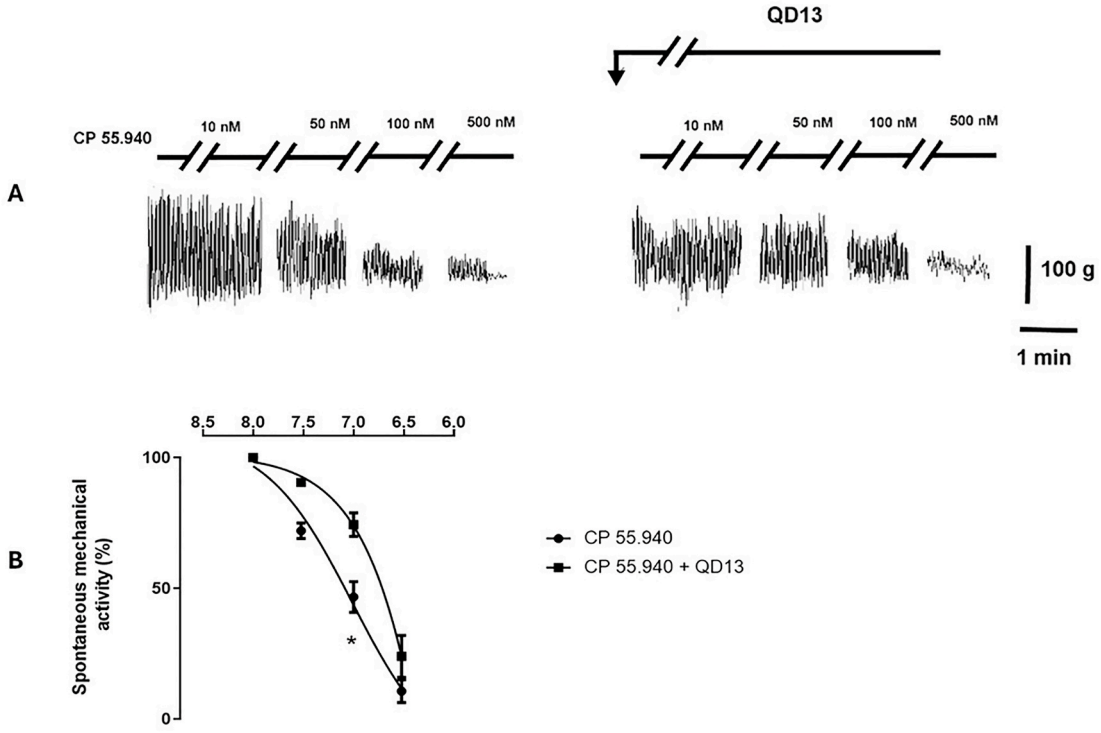

Fig. 8. Representative tracing showing the inhibitory effects induced by CP 55,940 in control conditions and after treatment of the preparation with QD13 (A). Concentration-response curves for the inhibitory effects induced by CP 55,940 in control conditions and after treatment of the preparation with QD13 (0.1 μM), cannabinoid CB1 receptor antagonist (B) (n = 5 per group for isolated ileum experiments). The inhibitory response is expressed as a percent of the resting activity. Each value is mean ± S.E.M. of at least four experiments. *$P < 0.05$ compared control value.

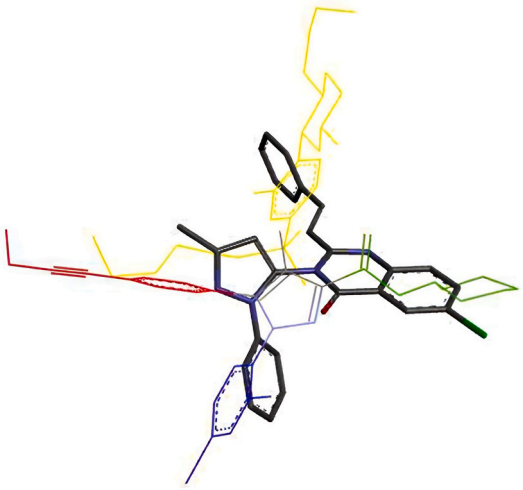

Fig. 9. Spatial arrangement of QD13, AM6538, and CP55.940 at the CB1 receptor binding site. The structure of AM6538 is colored in red, blue, and verse according to the three arms, compound CP55.940 in yellow and compound QD13 in grey. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antagonism. The quinazolinone group also has a phenyl nucleus bound at position 2 that overlaps in the same space occupied by the CP55,940 agonist, suggesting potential competitive interactions at the receptor's active site. These results support the idea that QD13 binds to the CB1 receptor similarly to other known antagonists but with structural characteristics that could give it a specific and potentially superior action in blocking receptor activation. Compared to earlier CB1 antagonists such as AM251 and rimonabant, QD13's molecular structure has novel substitutions at the arm-1 and arm-3 binding sites, based on new structure-activity relationship data[16-19]. These substitutions are intended to preserve CB1 binding with reduced CB1 inverse agonist activity, which has been linked to the psychiatric side effects of earlier drugs. The docking supports this, with QD13 binding to a large part of the orthosteric binding site but varying in electrostatic interaction profiles, suggesting a potentially different pharmacological footprint.

Based on the evidence obtained from docking studies concerning the alleged antagonistic activity of the new compound, the effects of QD13 administration at five different dosages (0.5, 1, 3, 5 and 10 mg/kg) were evaluated in vivo to determine at which concentration the new compound was able to counteract the effects induced by the administration of CP55,940. Our data showed that QD13, administered at doses of 1 and 3 mg/kg 30 min before the known agonist, blocked the behavioral consequences induced by the activation of the cannabinoid system.

In particular, when the new compound's ability to counteract the effects induced by CP55,940 on motor activity and coordination was assessed, the total distance traveled in the open field increased, and latency time in rota-rod improved, similar to that observed in the group treated with the antagonist/inverse agonist AM251 and CP55,940.

Activation of the CB1 receptor by agonist compounds such as THC can induce significant alterations in both activity and motor coordination (Bosier et al., 2010; Calapai et al., 2022). This effect is probably due to the activation of CB1 receptors in the cerebellum, basal ganglia and motor cortex, which are key brain areas for regulating motor activity (Castillo et al., 2012; Glass et al., 1997; Pertwee, 2006). Preclinical studies have shown that such activation in the basal ganglia reduces the release of neurotransmitters, such as GABA and glutamate, negatively affecting motor activity and coordination (Benarroch, 2007; Gerdeman and Lovinger, 2001; Mariani et al., 2023; van der Stelt and Di Marzo, 2003). In humans, acute administration of THC has been shown to cause a slowing of reaction times and a reduction in fine motor coordination, as observed in precision-demanding activities such as driving (Hart, 2001). In addition, inhibition of CB1 receptors by specific antagonists such as rimonabant showed opposite effects, improving coordination to

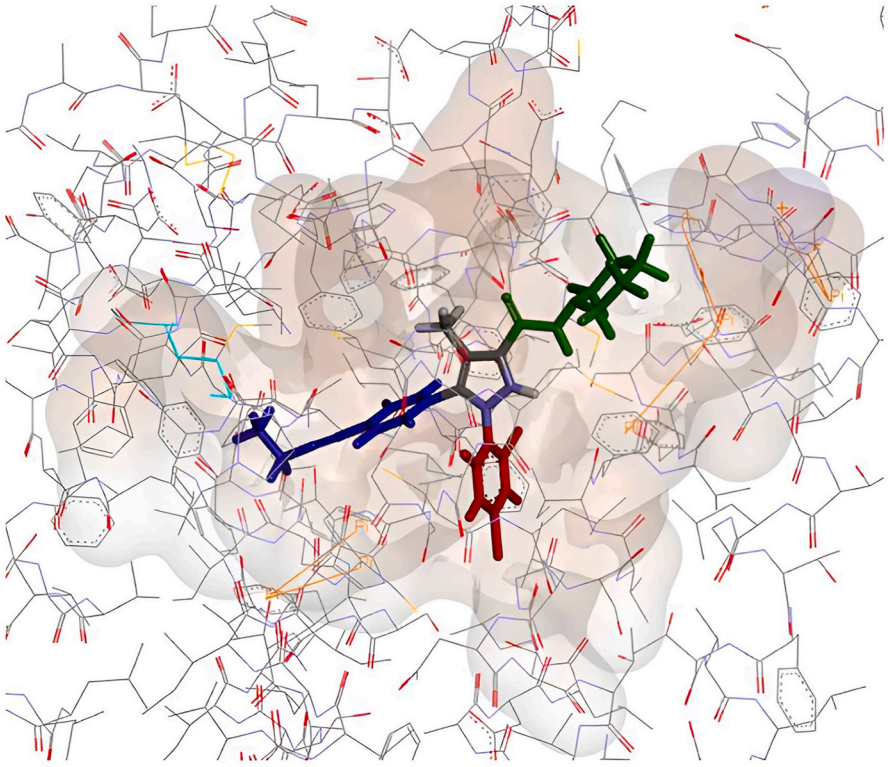

Fig. 10. Docking CB1 – AM6538.

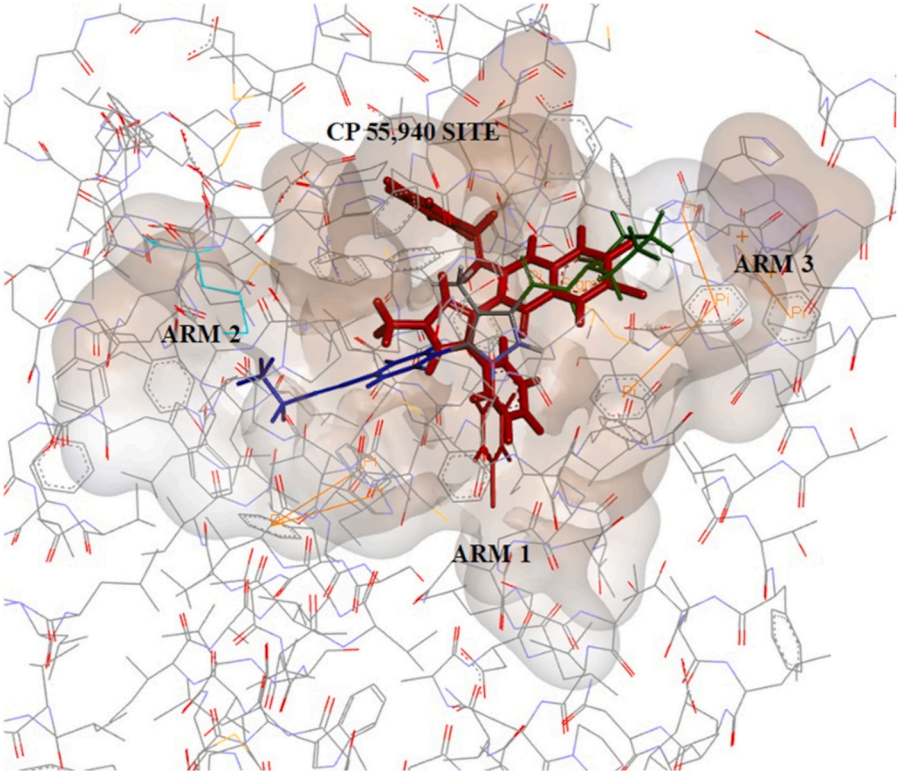

Fig. 11. Docking CB1- QD13 (red). Rimonabant was included for comparison. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

some extent and increasing locomotor activity, especially when administered at high doses. These results suggest that QD13 can exert an antagonistic activity against the CB1 receptor, effectively blocking the receptor activation by the agonists and preventing the effects observed in the CP55,940 group.

Data analysis of the regulation of body temperature and pain

sensitivity modulation showed that the novel compound effectively counteracts the effects induced by the administration of the known agonist, thereby confirming its antagonist characteristics on CB1 receptors. In particular, the treatment with CP55,940 resulted in a significant reduction of body temperature, an effect attributable to the activation of the CB1 receptors located in the preoptic nucleus, whose stimulation results in a decrease of heat production and an increase of its dissipation, according to what has been observed in other preclinical studies (Rawls et al., 2002; Ripamonte et al., 2020). The recovery of body temperature observed in the group treated with the reference antagonist AM251 and in the group treated with the new compound confirms the latter's effectiveness in countering the CP55,940-induced hypothermic effect. This effect is consistent with that described for other antagonistic activity compounds (Plescia et al., 2018; Pryce and Baker, 2017), suggesting a marked affinity of the new compound for the CB1 receptor and the ability to neutralize agonistic effects restoring physiological parameters.

Similarly, the tail-flick test showed that CP55,940 can increase latency time in response to a painful stimulus, reflecting the ability of cannabinoid agonists to modulate pain perception through activation of CB1 receptors in key sites of the nociceptive system, such as the periaqueductal grey substance and the raphe nucleus (Milligan et al., 2020; Walker et al., 1999). The observation that administration of the AM251 antagonist or QD13 at 3 mg/kg dose considerably decreases latency time suggests the compound's selective potency to block CB1 receptors and facilitate the reinstatement of standard nociceptive transmission. The specificity of the antagonistic effect observed at 3 mg/kg, compared to other tested concentrations, may reflect the existence of an optimal therapeutic window, where the compound exerts its maximum activity without triggering compensatory mechanisms or paradoxical effects, as suggested by previous studies on modulators of the endocannabinoid system (Gobbi et al., 2005; Pertwee, 2006).

Overall, this difference in potency, despite similar receptor docking profiles, may be a result of a combination of pharmacodynamic and pharmacokinetic factors. Unlike the full inverse agonist AM251, QD13 may be a partial inverse agonist or neutral antagonist, leading to reduced efficacy in vivo. Additionally, QD13's quinazolinone scaffold may alter its lipophilicity, oral bioavailability, or CNS penetration relative to AM251. Metabolic stability and receptor binding kinetics differences could also be responsible for its decreased apparent potency.

Additionally, the dose–response curve observed with QD13 may be an inverted-U-shaped curve, a phenomenon generally accounted for in cannabinoid pharmacology [55]. This kind of phenomenon suggests that increased doses beyond a certain level may have decreased efficacy through receptor desensitization or opposing downstream signaling mechanisms.

Together, these descriptions offer mechanistic justification for the differential activity of QD13 and AM251, though structurally and computationally similar.

Finally, data on peripheral effects show that QD13 can counteract the activation of CB1 receptors even in extra-cerebral tissues. CB1 receptor activation modulates the spontaneous mechanical activity of the longitudinal muscle of the mouse ileum (Baldassano et al., 2008). In our preparations, the inhibitory effects, dependent on the concentration of the CB1 antagonist, were neutralized by QD13, mimicking the action observed with the traditional CB1 antagonist used to counteract the effects of anandamide.

Overall, the present research results show that the new compound QD13 has the potential to interact with the CB1 competitively. This finding, in line with the results obtained from molecular docking studies, such as those using other compounds like AM6538, is intriguing and warrants further investigation. The current research provides an additional contribution to the knowledge about the relevance of regulating the endocannabinoid system in the modulation of many physiological processes at central and peripheral levels. It also highlights how conformational diversity in quinazolinone core compounds associated with broad and meticulous pharmacological characterization may represent a good starting point for discovering new molecular entities with a high experimental and therapeutic potential.

Further research is needed to understand how QD13 interacts within the body, its safety, and its long-term implications for the endocannabinoid system. Understanding these aspects would enable us to translate preclinical data into clinical trials and open up novel therapeutic avenues for diseases currently unresponsive to conventional therapy.

5. Limitations

While our findings provide encouraging evidence for the potential of QD13 as a cannabinoid CB1 receptor antagonist, several study's limitations should be acknowledged.

First, although molecular docking indicated that QD13 binds within the orthosteric site of the CB1 receptor, this conclusion was based solely on Docking studies. Direct in vitro binding assays, e.g., radioligand displacement or cAMP inhibition assays, were not performed and are essential to confirm the affinity and competitive nature of QD13 binding. Therefore, we cannot conclusively affirm QD13's receptor binding kinetics or compare its affinity to established CB1 antagonists such as AM251 or rimonabant.

Second, the current study did not include QD13-only treatment groups in the behavioral assays. As a result, the intrinsic activity or baseline pharmacological effects of QD13 in the absence of CB1 agonist challenge remain undetermined. This omission limits our ability to interpret whether QD13 exhibits any partial agonist or inverse agonist activity.

Finally, the novelty claim is currently grounded in structural and preliminary functional evidence but needs comprehensive receptor profiling and comparative efficacy assessments to distinguish it from the plethora of CB1 antagonists. Moreover, although biological variability and narrow therapeutic windows may contribute to knowledge of the effects at certain doses, more refined pharmacokinetic/pharmacodynamic correlation studies are warranted to fully characterize QD13's therapeutic profile and receptor selectivity.

CRediT authorship contribution statement

Fabiana Plescia: Methodology, Investigation, Data curation. **Gianluca Lavanco:** Methodology, Investigation, Data curation. **Maria Grazia Zizzo:** Methodology, Investigation. **Cesare D'Amico:** Methodology, Investigation. **Luigi Bellocchio:** Writing – review & editing. **Ginevra Malta:** Visualization, Formal analysis. **Francesca Vaccaro:** Methodology, Investigation. **Emanuele Cannizzaro:** Visualization, Data curation. **Leila Dimino:** Methodology, Investigation. **Fulvio Plescia:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Data availability statement

Data is available on request from the authors.

Declaration of competing interest

This research was funded by NextGenerationEU – fondi MUR D.M. 737/2021 – "PRJ-1271" to Fulvio Plescia; NextGenerationEU – fondi MUR D.M. 737/2021 – "PRJ-0992" to Gianluca Lavanco. The funder had no further role in the study design, collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication. The Authors declare that they have no competing interests.

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Data availability

The data that has been used is confidential.

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