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Novel pyrazole derivatives as neutral $CB_1$ antagonists with significant activity towards food intake

Ilaria Manca, Andrea Mastinu, Francesca Olimpieri, Matteo Falzoi, Monica Sani, Stefania Ruiu, Giovanni Loriga, Alessandro Volonterio, Simone Tambaro, Mirko Emilio Heiner Bottazzi, Matteo Zanda, Gérard Aimè Pinna, Paolo Lazzari

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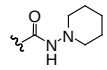
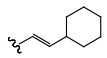
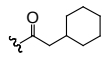
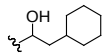
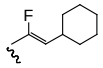
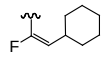
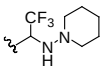
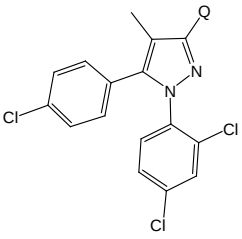
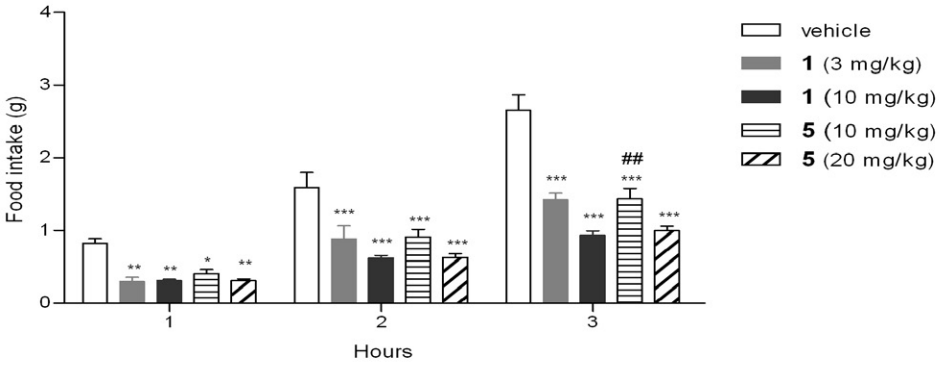
| **1** |  |
| **3** |  |
| **4** |  |
| **(±)-5** |  |
| **(Z)-6** |  |
| **(E)-7** |  |
| **(±)-8** |  |



Highlights

- Renew the importance of drugs based on $CB_1$ antagonists.
- Fluorinated compounds based on the lead rimonabant have been synthesized.
- Novel pyrazole derivatives showed neutral $CB_1$ antagonism behaviour.
- Coumpounds **±-5** and ***Z*-6** have significant in vivo activity towards food intake.

# Novel pyrazole derivatives as neutral $CB_1$ antagonists with significant activity towards food intake.

Ilaria Manca [a], Andrea Mastinu [b], Francesca Olimpieri [c], Matteo Falzoi [d,e], Monica Sani [f,g], Stefania Ruiu [b], Giovanni Loriga [b], Alessandro Volonterio [c,g], Simone Tambaro [a], Mirko Emilio Heiner Bottazzi [b], Matteo Zanda [g,h], Gérard Aimè Pinna [i], Paolo Lazzari [a,f,i,*]

[a] *Neuroscienze PharmaNess S.c.a r.l., Edificio 5, Loc. Piscinamanna, 09010 Pula (CA), Italy*
[b] *C.N.R. Istituto di Farmacologia Traslazionale, UOS Cagliari, Edificio 5, Loc. Piscinamanna, 09010 Pula (CA), Italy*
[c] *Dipartimento di Chimica, Materiali, e Ingegneria Chimica 'Giulio Natta', Via Mancinelli 7, 20131 Milano, Italy*
[d] *Consorzio ELPRO, Edificio 5, Loc. Piscinamanna, 09010 Pula (CA), Italy*
[e] *Dipartimento di Scienze della Vita e dell'Ambiente, Lab. Genetica, Via T.Fiorelli 1, 09126 Cagliari, Italy*
[f] *KemoTech S.r.l., Edificio 3, Loc. Piscinamanna, 09010 Pula (CA), Italy*
[g] *C.N.R. Istituto di Chimica del Riconoscimento Molecolare, Via Mancinelli 7, 20131 Milano, Italy*
[h] *Kosterlitz Centre for Therapeutics, Institute of Medical Sciences, College of Life Sciences and Medicine, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, Scotland, UK*
[i] *Dipartimento di Chimica e Farmacia, Università di Sassari, Via F. Muroni 23/A, 07100 Sassari, Italy*

[*] Corresponding author:
Paolo Lazzari, phone +39 07092433118, fax +39 07092433119, e_mail: paolo.lazzari@kemotech.it

**Abstract** – In spite of rimonabant's withdrawal from the European market due to its adverse effects, interest in the development of drugs based on $CB_1$ antagonists is revamping on the basis of the peculiar properties of this class of compounds. In particular, new strategies have been proposed for the treatment of obesity and/or related risk factors through $CB_1$ antagonists, i.e. by the development of selectively peripherally acting agents or by the identification of neutral $CB_1$ antagonists. New compounds based on the lead $CB_1$ antagonist/inverse agonist rimonabant have been synthesized with focus on obtaining neutral $CB_1$ antagonists. Amongst the new derivatives described in this paper, the mixture of the two enatiomers (±)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-(2-cyclohexyl-1-hydroxyethyl)-4-methyl-1*H*-pyrazole (**(±)-5**), and compound 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-[(*Z*)-2-cyclohexyl-1-fluorovinyl]-4-methyl-1*H*-pyrazole (**(*Z*)-6**), showed interesting pharmacological profiles. According to the preliminary pharmacological evaluation, these novel pyrazole derivatives showed in fact both neutral $CB_1$ antagonism behaviour and significant in vivo activity towards food intake.

**Keywords:** cannabinoids, $CB_1$ receptor, fluorinated ligands, food-intake.

## 1. Introduction

Different classes of cannabinoid $CB_1$ antagonists have been developed in the last two decades with principal focus on the discovery of new therapeutic strategies for obesity treatment [1-11]. $CB_1$ receptors ($CB_1R$), belonging to Class $\alpha$ G protein-coupled receptors (GPCRs), are in fact involved in appetite regulation and energy homeostasis, as well as in other physiological processes including intestinal motility, nociception, locomotive activity,

learning and memory [12]. Considering the wide distribution of $CB_1R$ at both peripheral level (i.e. gut, eye, liver, pancreas, adipose tissue, testis) and Central Nervous System [4], compounds able to interact with this cannabinoid receptor subtype have been also proposed as potential candidates for the treatment or prevention of other disorders and diseases such as pain [13], glaucoma [14], inflammation [15], drug and smoke addiction [16,17], vomiting [18,19], neurodegeneration (i.e. Parkinson's and Huntigton's diseases) [20,21], melanoma [22], and acute myocardial infarction [23]. In addition to obesity, $CB_1$ antagonists have been in particular proposed as therapeutic agents for smoking cessation [16], drug abuse [24-26], liver fibrosis [27], obesity-associated hepatic steatosis [28], sexual dysfunction [29], metabolic syndrome and dyslipidemia [30]. Cardiovascular and metabolic risk factor improvements due to the administration of $CB_1$ antagonists have been also highlighted in type 2 diabetes patients [31].

Despite initial enthusiasm in drug discovery for the identification and the selection of first $CB_1$ antagonist candidates able to reach the pharmaceutical market, with reference to the well known history concerning the $CB_1$ antagonist / $CB_1$ inverse agonist lead product *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (Acomplia®, rimonabant, SR141716A, **1**) (Figure 1) [32,33], industrial attention has been recently reduced on this class. In fact, Acomplia®, launched on the European market by Sanofi Aventis for the treatment of obesity, was soon blocked by European Medicines Agency (EMA) for side effects, i.e. anxiety and depression [34]. However, there is an increasing body of opinion that the use of cannabinoid derivatives as therapeutic agents should be reevaluated on the basis of the interesting properties of these compounds. In particular, for $CB_1$ antagonists, new strategies have been proposed for the treatment of obesity and/or related risk factors using cannabinoid agents with reduced or no side effects, i.e. by the development of selectively peripherally acting agents [35-37], or by the identification of neutral $CB_1$ antagonists [38].

It has been reported that $CB_1$ neutral antagonists and inverse agonists show different ways of interacting with the cannabinoid receptor. Particularly, hydrogen bond interaction with 3.28(192) is often found with inverse agonists [39-42]. Rimonabant $CB_1$ inverse agonism is in particular related to the capability of the carboxamide oxygen to form hydrogen bond with K3.28(192). $CB_1$ neutral antagonism is instead assured by avoiding hydrogen bond with K3.28(192). This has been confirmed by the neutral antagonism profile of VPSR (**2**) and VCHSR (**3**) compounds, the corresponding vinyl derivatives of **1** and *N*-(cyclohexyl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, respectively (Figure 1) [39]. The different behavior of $CB_1$ neutral antagonists and inverse agonists can be in part explained by the extended ternary complex model. According to this model, $CB_1$ agonists, neutral antagonists, and inverse agonists interact with this receptor subtype in different ways involving a ground or inactivate R state and an active R* state [39]. $CB_1$ agonist derivatives show higher affinity for R*, with a consequent receptor activation and shifting of the R / R* equilibrium towards the R*. $CB_1$ neutral antagonists show equal affinity for the two states, while $CB_1$ inverse agonists have higher affinity for R state with an R / R* equilibrium unbalanced towards the R state and a reduction of the signaling pathway activation. Equal affinity for R and R* has been evidenced by modeling studies for the biarylpyrazole scaffold of rimonabant, being this affinity due to the aromatic interaction with F3.36(200), Y5.39(275), and W5.43(279) of the transmembrane helix (TMH)3-4-5-6 aromatic microdomain [39-41]. In contrast, significant bias toward R state has been related to the carboxamide oxygen at the 3 position of pyrazole ring of rimonabant, as in the case of analogues bearing the same substituent, even if with a cyclohexyl instead of the piperidinyl ring.

Within the class of pyrazole based cannabinoids, the compound 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-(2-cyclohexyl-1-oxoethyl)-1*H*-pyrazole (**4**), namely ABD-395

(Figure 1), has been recently characterized and its $CB_1$ neutral antagonism behavior has been confirmed by both in vitro e in ex-vivo assays [43].

FIGURE 1

The evidence for $CB_1R$ inverse agonism in in vitro assays is generally related to the blockade of $CB_1$ agonist action and to the opposite effects determined by $CB_1$ inverse agonists when administered alone compared to those detected in the case of $CB_1$ agonists. In contrast to $CB_1$ agonists, $CB_1$ inverse agonists determine for example the increase of the $Ca^{2+}$ current in neurons expressing $CB_1R$ [42], the enhancement of the amplitude of electrically-evoked contractions of the mouse isolated vas deferens [43], the inhibition of basal $CB_1R$ activation by [$^{35}$S]GTP$\gamma$S binding assays, the rise of ongoing release of acetylcholine, noradrenaline and $\gamma$-aminobutyric acid in hippocampal slices [44,45]. In the case of $CB_1$ neutral antagonists, no effect is instead detected when they are administered alone. These compounds elicit their activity by counteracting the $CB_1$ agonist effects.

We have now synthesized the alcohol **(±)-5**, obtained as an equimolar mixture of enantiomers from the reduction of the ketone group of **4**, the fluorine derivatives (**(*Z*)-6** and **(*E*)-7**) of 5-(4-chlorophenyl)-3-(2-cyclohexylethenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole (**3**) and the racemic trifluoroethylamine analogue **(±)-8** of rimonabant (Figure 2), with the aim of obtaining new neutral $CB_1$ antagonists, that might be characterized by reduced side effects compared to the lead compound **1**. Neutral antagonism was previously highlighted for reference compounds **2**-**4** [39,43], and for the 5-(4-bromophenyl) analogues of both **4** and **5**, respectively ABD-399 and ABD-402 [43]**.**

FIGURE 2

The adopted medicinal chemistry strategies for the obtainment of the new potential $CB_1$ antagonist derivatives **5**-**8** with reduced or void side effects have been based on both the the well-established bioisosteric replacement of carboxamide group with vinyl fluoride and trifluoroethylamine moieties [46], and the neutral $CB_1$ antagonism highlighted for reference compounds **2**-**4** [39], as for the 5-(4-bromophenyl) analogue of **5** [43]. The new compounds were compared to rimonabant (**1**), VCHSR (**3**), and ABD-399 (**4**), both in terms of affinity to cannabinoid receptors and through intrinsic activity determination by ex-vivo assays (isolated organs) and in vitro tests based on the use of a specific cell line expressing $CB_1$ but not $CB_2$ receptors. Further investigations were carried out by preliminary [$^{35}$S]GTPγS binding assays with $CB_1$ transfected Chinese hamster ovary (CHO) cells. Moreover the capability of the new compounds to induce food intake reduction was determined by using an acute animal model.

Interesting pharmacological profiles were determined, especially for compounds (±)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-(2-cyclohexyl-1-hydroxyethyl)-4-methyl-1*H*-pyrazole (**(±)-5**), and 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-[(*Z*)-2-cyclohexyl-1-fluorovinyl]-4-methyl-1*H*-pyrazole (**(*Z*)-6**). According to preliminary pharmacological assays, these new derivatives showed in fact both neutral $CB_1$ antagonism behaviour and significant in vivo activity towards food intake.

## 2. Chemistry

The racemic carbinol **(±)-5** was prepared by the method shown in Scheme 1. The method was based on previously reported procedure for the obtainment of the corresponding

ketone **4** [43], with revisions. Addition of the 4-chloropropiophenone (**9**) to a mixture of diethyl oxalate (**10**) and sodium ethoxide in absolute ethanol at room temperature afforded Claisen condensation product, the α,γ–diketoester **11** [47]. Subsequent reaction of **11** with 2,4-dichlorophenylhydrazine hydrochloride (**12**) in refluxing absolute ethanol afforded the ethyl 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxylate (**13**) [39,48]. Weinreb amide **14** [43] was synthesized from the reaction of **13** with *N*,*O*-dimethylhydroxylamine hydrochloride in the presence of trimethylaluminum. Grignard reaction of **14** with (cyclohexylmethyl)magnesium bromide (**15**) gave ketone **4** in good yield [43]. Finally, the carbonyl group of **4** was reduced by sodium borohydride at room temperature to obtain the racemic alcohol **(±)-5** (Scheme 1).

SCHEME 1

The preparation of derivatives **(*Z*)-6** and **(*E*)-7** is shown in Scheme 2. Weinreb amide **14** was reduced with $LiAlH_4$ to afford the aldehyde **16**, which was in turn condensed with diethylphosphite producing **17** in good yields. Fluorination of the hydroxy group of **17** using (diethylamino)sulfur trifluoride (DAST) afforded the fluoro derivative **18**. The final step to prepare the isomeric compounds **(*Z*)-6** and **(*E*)-7** consisted of the Horner-Wadsworth-Emmons olefination between the stabilized ylide derived from **18** and the cyclohexylcarboxaldehyde (**19**). The isomers **(*Z*)-6** and **(*E*)-7** were separated by flash chromatography in the 1.0:5.4 molar ratio.

SCHEME 2

Finally, the racemic trifluoromethyl derivative **8** has been synthesized as depicted in Scheme 3. The trifluoroethylalcohol **20**, obtained after trifluoromethylation of the aldehyde

**16** with the Ruppert-Prakash reagent [49], was oxidized to the corresponding ketone **21** via classical Swern oxidation [50-53]. Subsequently **21** was submitted to condensation with *N*-aminopiperidine in refluxing toluene, with azeotropic removal of water using pyridinium *p*-toluenesulfonate (PPTS) catalysis, affording the hydrazone **22** [54]. Such compound proved to be pretty labile, especially toward water. Thus, a number of different reducing agents, such as $LiAlH_4$ [55], borane-THF complex [56], and triethylsilane in trifluoroacetic acid (TFA) [57], were unsuccessfully tried. We achieved our best result using borane-trimethylamine complex in toluene in the presence of gaseous HCl, which successfully led to the synthesis of the racemic hydrazine hydrochloride **(±)-8** [58].

SCHEME 3

According to previously reported synthetic process [39], we have also synthesized the vinyl compound **3**, starting from key intermediate **13**.

## 3. Biology

### *3.1. Receptor binding assays*

The affinity of both the new and reference compounds for $CB_1R$ and $CB_2$ receptors ($CB_2R$) was determined according to the previously reported procedures [59]. Assays for the evaluation of $CB_1R$ and $CB_2R$ affinity were performed using mouse brain (minus cerebellum) and mouse spleen homogenates, respectively. [$^3$H]-(1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol ([$^3$H]CP-55,940) was employed as radio-labeled ligand. The experimental data ($IC_{50}$ values) were converted into

$K_i$ values [60]. The affinity of the new compounds **5**-**8** was compared with that of rimonabant **1** and the reference neutral $CB_1$ antagonists **3,4**.

*3.2. Intrinsic activity by in vitro assays*

Intrinsic activity of the new derivatives and reference compounds towards $CB_1R$ was evaluated through in vitro tests based on the determination of phosphorylated ERK 1/2 (P-ERK 1/2) expression in Mouse Neuroblastoma N1E-115 cell line, following the treatment of these cells with the cannabinoid compounds. As previously reported, this cell line expresses $CB_1$ but not $CB_2$ receptors, and its exposure to the cannabinoid agonists ACEA and WIN55-212,2 induces a rapid phosphorylation and activation of the ERK 1/2 [61]. This effect is counteracted by a pre-treatment of N1E-115 cells with a $CB_1$ antagonist such as rimonabant.

*3.3. Isolated organs (mouse vas deferens)*

The mouse isolated vas deferens is a nerve-smooth muscle preparation widely adopted in highly sensitive bioassays concerning $CB_1$ receptor modulation studies [62]. $CB_1$ agonists produce concentration-related decreases in the amplitude of electrically evoked contractions of the vas deferens. $CB_1$ antagonist derivatives determine parallel dextral shifts in $CB_1$ agonist log concentration-response curves in electrically stimulated tissues. Moreover it has been ascertained that $CB_1$ neutral antagonists and inverse agonists can be distinguished through this isolated organ technique. No significant increment in the amplitude of the electrically evoked contractions is detected in the case of neutral antagonists. In contrast, an increase of these contractions compared to the control is instead observed following the administration of inverse agonists [43]. According to these last reported behaviors, a preliminary indication of $CB_1$ neutral antagonism was evaluated

for the newly synthesized compounds in comparison with rimonabant and the reference neutral $CB_1$ antagonists **3** and **4**.

### *3.4. [$^{35}$S]GTPγS binding assays*

It has been reported that $CB_1$ agonist agents elicit [$^{35}$S]GTPγS binding stimulation through G protein-coupled receptor activation [43-45]. A concentration-response curve with a sigmoid shape is provided by following the action of $CB_1$ agonists on [$^{35}$S]GTPγS binding, expressed as stimulation percentage, as their concentration increases. In contrast, no substantial effect on GTP attachment to the receptors is caused by neutral $CB_1$ antagonist alone. In addition, $CB_1$ antagonist derivatives determine shifting to the right of the [$^{35}$S]GTPγS binding concentration-response curves of $CB_1$ agonists. $CB_1$ inverse agonist compounds show opposite effect to that detected in the case of $CB_1$ agonist derivatives. In particular, $CB_1$ inverse agonists exert a detach action towards GTP binding to the receptors, with corresponding negative values of [$^{35}$S]GTPγS binding stimulation percentage.

Preliminary [$^{35}$S]GTPγS binding assays were carried out to support intrinsic activity behaviour determined by isolated organ experiments. Assays were carried out using $CB_1$ transfected Chinese hamster ovary (CHO) cells. Novel compounds **5** and **(Z)-6**, and reference $CB_1$ ligands **1** and **4**, were assayed alone in order to evaluate their capability to stimulate or to inhibit GTPγS binding.

### *3.5. Food intake*

To preliminarily evaluate the potential application of the new $CB_1$ antagonist derivatives in the control of food intake and, potentially, in obesity treatment, compounds **(±)-5** and **(*Z*)-6** were tested in an acute in vivo assay based on the animal model previously reported by Di

Marzo [63]. C57Bl6/J mice were used. Activity of the new compounds after intraperitoneal dosing was compared with that of rimonabant and of the reference neutral $CB_1$ antagonists **3** and **4**.

## 4. Results and Discussion

### *4.1. Cannabinoid receptor affinities*

As first step of the study, radioreceptor binding assays were carried out in order to assure for the new derivatives both $CB_1$ affinity and selectively vs $CB_2R$. The affinity values expressed as $K_i$, of the reference compounds **1** and **3**,**4** were in accord to those previously reported by other authors [39,43]. As showed in Table 1, the substitution of *N*-(piperidinyl)-carboxamide group at position 3 of pyrazole ring of rimonabant (**1**) with the vinyl, fluorine-vinyl, trifluoroethylamine, ketone, and its corresponding alcohol, produced a reduction in $CB_1$ affinity. With the exclusion of compound **3**, $CB_1$ vs $CB_2$ selectivity was also negatively influenced by these replacements. However good $CB_1$ affinity and selectivity values were determined for both **(*Z*)-6** and the corresponding hydrogen analogue **3**, as well as for the ketone derivative **4**. The three compounds above showed $K_i$ values only one order of magnitude higher than that of the lead **1**. One further order of magnitude in $CB_1$ affinity was lost by the substitution of the carboxamide moiety of rimonabant with 1-hydroxy-2-cyclohexylethyl group of **(±)-5** or with 2,2,2-trifluoro-*N*-(piperidin-1-yl)ethylamine group of **(±)-8**. The drop in affinity observed for **(±)-8** is not surprising taking into account the well known importance of the 3-carboxamide oxygen as a hydrogen bond acceptor (HBA) in the binding mode of rimonabant-like ligands to the CB1R [10]. This confirms that a trifluoroethylamine function is not a good carboxamide replacement when the oxygen atom of the parent molecule plays an important HBA role, owing to the bad HBA properties of a

trifluoromethyl group [64]. The determined reduction in $CB_1$ affinity of **(±)-5** compared to that of the corresponding ketone **4**, was of the same entity of that reported for the analogues bearing a 4-bromophenyl instead of 4-chlorophenyl in position 5 of the pyrazole ring [43]. Moreover it is important to note as the separation feasibility of the two isomers (*E*) and (*Z*) of 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-(2-cyclohexyl-1-fluorovinyl)-4-methyl-1*H*-pyrazole, allowed to ascertain the relevance of the spatial geometry of the vinyl group to assure affinity towards $CB_1R$. Compound **6**, the *Z* isomer, showed in fact $CB_1$ affinity 16 fold higher than that of the corresponding *E* isomer (**7**).

TABLE 1

*4.2. Intrinsic activity by in vitro assays.*

Except compound **7** showing $K_i$ value for $CB_1R$ higher than 400 nM, all the new and reference compounds were assayed to determine or confirm their $CB_1$ antagonism. In common with rimonabant and in contrast to the reference cannabinoid agonist WIN55,212-2, no effect on the expression of P-ERK 1/2 was evidenced following N1E-115 cell line treatments with compounds **3**-**6** and **8** (Figure 3).

FIGURE 3

In addition the up-regulation of P-ERK 1/2 expression induced on the same cells by the administration of the reference cannabinoid agonist WIN55,212-2 (25 nM), was significantly counteracted through a pre-treatment step with the assayed compounds. The detected $CB_1$

antagonism effect of the tested derivatives was statistically significant starting from concentrations of 125-500 nM for compounds **3**, **4**, and **(*Z*)-6**, while a higher concentration (1 μM) was needed to have the same effect in the case of **(±)-5** and **(±)-8** which present lower affinity for $CB_1R$ (Figure 4).

FIGURE 4

*4.3. Isolated organs*

The effect of $CB_1$ agonists on mouse vas deferens is related to their action on naturally expressed prejunctional neuronal $CB_1R$ to inhibit release of the contractile neurotransmitters, noradrenaline and ATP, provoked by electrical stimulation [65]. Neutral antagonism of the new compounds for $CB_1R$ was preliminary evaluated by comparing the amplitude of the electrically evoked contractions in mouse vas deferens assays in the presence or in the absence of the derivatives (Figure 5). $CB_1$ inverse agonism of rimonabant (**1**) was confirmed by this study at both 0.1 and 1.0 μM. According to previously reported data by other authors [43], an increase in the amplitude of the evoked contractions was in fact detected with the reference compound at a concentration of 0.1 μM, with enhancement percentage higher than 90% at 1 μM. In contrast to rimonabant, no marked rise of these contractions compared to the control was instead detected following the administration of the compounds **3**-**8** at concentrations 0.1 - 100 μM (Figure 5). The same behavior was previously reported for both the reference derivative **4** and the corresponding 5-(4-bromophenyl) analogue of racemic compound **(±)-5**, being the effect of these compounds on isolated mouse vas deferens supported by the results obtained

through [$^{35}$S]GTPγS binding assays [43]. A $CB_1$R neutral antagonism profile of the new compounds was therefore suggested by the detected profiles.

FIGURE 5

Mediated $CB_1$ activity of the novel compounds on isolated mouse vas deferens was ascertained by evaluating the capability of the synthesized derivatives to counteract the contraction inhibition elicited by the reference cannabinoid agonist WIN 55,212-2. According to previously reported behavior concerning $CB_1$ antagonists [66], compounds **3**-**8** affected the sigmoidal curve obtained with WIN 55,212-2, by shifting it towards right. As example of the detected results, the $CB_1$ antagonism behavior of compound **6** towards WIN 55,212-2 mouse vas deference activity has been reported in Figure 6.

FIGURE 6

*4.4. [$^{35}$S]GTPγS binding assays*

To further investigate intrinsic activity of the novel compounds, preliminary [$^{35}$S]GTPγS binding assays were carried out by using CB1 transfected Chinese hamster ovary (CHO) cells. Experiments were performed following the procedures by Greig at al. [43]. Derivatives **5**, **(Z)-6**, and reference compounds **1** and **4** were assayed.

The effect induced by different concentrations of each compound on [$^{35}$S]GTPγS binding is reported in Figure 7. The results are expressed as percentage of stimulation vs basal ± SEM. According to previously reported results: rimonabant showed an inverse agonism behavior by eliciting a significant reduction in [$^{35}$S]GTPγS binding at concentrations higher than $10^{-8}$ M (Figure 7a); in contrast to **1**, no significant effect was instead detected for the

reference neutral $CB_1$ antagonist compound **4** (Figure 7b). As in the case of compound **4**, both the novel derivatives **5** and **(Z)-6** didn't significantly affect [$^{35}$S]GTPγS binding, at least up to $10^{-5}$ M (Figure 7 c-d). $CB_1$R neutral antagonism profile of the new compounds detected by isolated organ assays was therefore further supported by the results reported in Figure 7.

FIGURE 7

*4.5. Food intake*

The activity of the new derivatives **(±)-5** and **(*Z*)-6**, rimonabant (**1**), and the reference compounds **3** and **4**, in food intake inhibition was evaluated according to the procedure by Di Marzo [63]. The results obtained during three hours from the presentation of food to the animals after a fast period of 18 hours are reported in Figure 8. The acute treatment of fasted C57Bl6/J mice with (*Z*)-**6** at the doses of 10 and 20 mg/kg, compared to the vehicle, induced a significant reduction of food intake starting from 3 and 2 hours from food availability, respectively (Figure 8 a). Rimonabant significantly affected food intake in the same animal model starting from the first hour of the evaluation. No significant variation was detected for **1** between the doses of 3 and 10 mg/kg, although a trend related to an increase of the effect with the dose was evidenced at third hour. It is important to note that no higher dosage was adopted for rimonabant (**1**) due to suffering state, locomotion blockage, and, in some case (one third of the tested mice), mortality, observed in the animals treated with this compound at 20 mg/kg. The extent of food intake inhibition for **(*Z*)-6** at 20 mg/kg was comparable to that detected in the case of rimonabant administration at 10 mg/kg. In contrast to the side effects related to **1** administration, no observable adverse effect was evidenced in the case of treatments with **(*Z*)-6** at 20 mg/kg.

Analogous behaviors were highlighted on food intake after treatments with compounds **4** and the racemic **(±)-5**, even if these compounds evidenced their effect earlier than **(*Z*)-6** (Figure 8 c-d). Considering the substantial lower affinity of **(±)-5** for $CB_1R$ if compared to those of the other assayed compounds, these results suggest a significantly higher availability of this derivative which might be ascribed to the presence of the hydroxyl substituent at 3 position of the pyrazole ring. Further studies must be performed in the future in order to separate the two enantiomers of **5**, and successively to verify the probable relevance of one of the two compounds in the reduction of food intake.

Starting from 2 hours of observation, the administration of compound **3** determined a significant inhibition of food intake relative to the vehicle (Figure 8 b). However the activity of **3** was significantly lower than that of **1** (10 mg/kg) and compounds **4**-**6**, especially at 20 mg/kg. The improved activity of **(*Z*)-6** compared to **3** suggests a possible enhancement of bioavailability as a result of introducing the vinylic fluorine atom. An alternative explanation of the detected in vivo behavior of **(*Z*)-6** compared to **3** could be related to the possible different metabolism of the two componds, which could be heavily influnced by the presence of fluorine atom in the case of **(*Z*)-6**.

FIGURE 8

## 5. Conclusion

We have described herein the synthesis of novel rimonabant derivatives with the goal of obtaining neutral $CB_1R$ antagonist compounds with reduced side effects relative to those of the reference compound **1**.

In particular, we have synthesized and tested the enantiomer mixture of alcohol derivative **(±)-5**, the keto parent compound **4**, the fluorine vinyl derivatives **(*Z*)-6** and **(*E*)-7**, and the racemic trifluoroethylamine rimonabant analogue **(±)-8**.

According to in vitro (radioreceptor binding assays and P-ERK 1/2 expression from N1E-115 cell line) and in ex vivo assays (isolated organs), the newly synthesized derivatives showed significant affinity for $CB_1R$ ($K_i$ values in the nanomolar range), with a biological profile compatible with that of neutral $CB_1R$ antagonist compounds.

In spite of their $CB_1$ affinities, which were lower than those of the reference compounds **3**-**4**, derivatives **(±)-5** and **(*Z*)-6** evidenced the most significant efficacy in the control of food intake in the adopted acute animal model. The entity of food intake inhibition for **(±)-5** and **(*Z*)-6** was comparable to that detected in the case of rimonabant, although at higher doses. However, it is important to note that no observable adverse effect was evidenced in the case of treatments with **(±)-5** and **(*Z*)-6** at doses up to 20 mg/kg, in contrast to the side effects highlighted in the case of administration of rimonabant at 20 mg/kg.

These results further support the relevance of the substituent at 3 position of the pyrazole ring for determining both $CB_1R$ affinity and neutral $CB_1$ antagonism or inverse $CB_1$ agonism. Moreover, the unexpectedly high in vivo potency and efficacy of the compounds may indicate improved bioavailability after i.p. administration.

In order to confirm and validate the improved properties of the new compounds compared to that of the lead compound **1**, their behavior will be further investigated, with particular emphasis to the identification of reduced or, better, void adverse effects as induction of anxiety and depression. Moreover, considering the interesting results obtained with **(±)-5**,

the separation and the characterization of the two enantiomers will be carried out in the future in order to identify the component with the best profile.

## 6. Experimental protocols

### *6.1. General methods*

Melting points were obtained on a Stuart SMP10 Melting point apparatus and are uncorrected. IR spectra were recorded on a Bruker Alpha-T FT-IR Spectrophotometer equipped with an Alpha Eco ATR as sampling module and are expressed in ν ($cm^{-1}$). All NMR spectra were taken on a Bruker Ultra Shield 400 Avance III NMR Spectrometer, with $^{1}H$, $^{13}C$ and $^{19}F$ observed at 400, 100 and 377 MHz, respectively. Chemical shifts for $^{1}H$ and $^{13}C$ NMR spectra are expressed in δ downfield from TMS ($Me_4Si$), for $^{19}F$ NMR spectra are expressed in δ from $CFCl_3$. Multiplicities are reported as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), qu (quintuplet), dd (doublet of doublets), m (multiplet). ESI mass spectra were recorded on a Bruker Esquire 3000 Plus Ion Trap mass spectrometer in the positive mode. All reactions involving air or moisture sensitive compounds were performed under highly pure nitrogen atmosphere. All reagents and solvents were purchased from Sigma-Aldrich Chemical Company and used as received. Combustion analyses of compounds used in biological testing were performed by Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari, Italy, and the analyses indicated by the symbols of the elements were within ± 0.4 % of the theoretical values.

### *6.2. Chemistry*

*6.2.1. Ethyl 4-(4-chlorophenyl)-3-methyl-2,4-dioxobutanoate* (**11**). Sodium metal (0.82 g, 35.58 mmol) was added in small portion to dry ethanol (30 mL) and stirred until all the sodium had reacted. Diethyl oxalate (**10**) (3.62 mL, 26.69 mmol) was added, followed by dropwise addition of a solution of 4-chloropropiophenone (**9**) (3.00 g, 17.79 mmol) in dry ethanol (40 mL). The mixture was stirred at room temperature for 18 h and then it was slowly poured into ice and 1N HCl was added. The resulting mixture was extracted with $Et_2O$, dried ($Na_2SO_4$), and concentrated to afford the analytically pure product in quantitative yield as a yellowish oil: $R_f$ = 0.23 (petroleum ether/EtOAc, 9:1); bp: 68-70℃ [55-65℃/700 mmHg] [46], IR : $\nu =$ 1680, 1730, 3440; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta =$ 1.30 (t, 3H, *J* =7.1 Hz), 1.45 (d, 3H, *J* =7.1 Hz), 4.28 (q, 2H, *J* =7.2 Hz), 5.00 (q, 1H, *J* =7.1 Hz), 7.50 (d, 2H, *J* =8.8 Hz), 7.93 (d, 2H, *J* =8.8 Hz). Anal. $C_{13}H_{13}ClO_4$ (C, H, N).

*6.2.2. Ethyl 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxylate* (**13**). A stirred mixture of diketoester **11** (3.00 g, 11.17 mmol) and 2,4-dichlorophenylhydrazine hydrochloride (**12**) (2.62 g, 12.28 mmol) in EtOH (90 mL) was heated under reflux for 16 h. The mixture was allowed to cool to room temperature, and the solvent was removed under reduced pressure to give a red-orange solid, which was purified by flash chromatography (petroleum ether/EtOAc, 9:1) to afford the analytically pure product as an orange solid (2.20 g, 50%). $R_f$ = 0.29 (petroleum ether/EtOAc, 9:1); IR : $\nu =$ 1724; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$=1.43 (t, 3H, *J* =7.1 Hz), 2.33 (s, 3H), 4.45 (q, 2H, *J* =7.2 Hz), 7.08 (bd, 2H, *J* =8.5 Hz), 7.29 (dd, 1H, *J* =2.2, 8.4 Hz), 7.30 (bd, 2H, *J* =8.5 Hz), 7.35 (d, 1H, *J* =8.4 Hz), 7.38 (d, 1H, *J* =2.2 Hz); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta =$ 9.8, 14.6, 61.1, 119.2, 127.2, 127.9, 129.0, 130.2, 130.9, 131.0, 133.2, 135.1, 136.0, 136.1, 143.0, 143.1, 162.8; ESI-MS *m/z*: 408.1 [ESMS *m/z*: 409.1 (M + 1)] [38,47].

*6.2.3. N-methoxy-N-methyl-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide* (**14**). Trimethylaluminum (2.44 mL of 2M solution in hexane, 4.88 mmol) was added dropwise to a suspension of dimethylhydroxylamine (0.48 g, 4.88 mmol) in dry $CH_2Cl_2$ (10 mL) at 0℃. The solution was stirred at 0℃ fo r 45 min and then at room temperature for 40 min. To this solution, the pyrazole ester **13** (1.00 g, 2.44 mmol) in $CH_2Cl_2$ (6 mL) was added dropwise. Stirring was continued for 16 h at room temperature. The reaction mixture was cooled to 0℃, and 10% HCl was carefully added dropwise. The mixture was extracted with $CH_2Cl_2$, washed with water, brine, dried over $Na_2SO_4$, and filtered. After evaporation of the solvent, the residue was purified by flash chromatography (petroleum ether/AcOEt, 7:3) affording 0.72 g (80%) of compound **14** as a white solid. $R_f$ = 0.49 (petroleum ether/AcOEt, 7:3); IR : $\nu =$ 1681; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 2.23 (s, 3H), 3.46 (s, 3H), 3.81 (s, 3H), 7.07 (bd, 2H, *J* =8.3 Hz), 7.16-7.34 (m, 4H), 7.43 (d, 1H, *J* =1.6 Hz). Anal. $C_{19}H_{16}Cl_3N_3O_2$ (C, H, N).

*6.2.4. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-(2-cyclohexyl-1-oxoethyl)-1H-pyrazole* (**4**). To a solution of compound **14** (0.50 g, 1.18 mmol) in dry THF (10 mL) the cyclohexylmethylmagnesium bromide (**15**) (7.06 ml of 0.5 M solution in THF, 3.53 mmol) was added dropwise at 0℃ under nitrogen. The react ion mixture was gradually warmed to room temperature and stirred at the same temperature for 24 h. Saturated aqueous $NH_4Cl$ was added dropwise at 0℃, the reaction mixture was diluted with $Et_2O$, and the layers were separated. The aqueous layer was extracted with $Et_2O$, and the combined organic layers were washed with water, dried ($Na_2SO_4$), and filtered. After evaporation of the solvent, the residue was purified by flash chromatography (petroleum ether/$Et_2O$ 9:1) affording 0.37 g (69%) of compound **4** as a white solid. $R_f$ = 0.5 (petroleum ether/$Et_2O$, 9:1); IR : $\nu =$ 1685; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$=0.98-1.11 (m, 2H), 1.14-1.36 (m, 4H),

1.61-1.81 (m, 4H), 1.97-2.11 (m, 1H), 2.32 (s, 3H), 2.94 (d, 2H, *J* =7.0 Hz), 7.05 (bd, 2H, *J* =8.6 Hz), 7.24-7.32 (m, 4H), 7.43 (d, 1H, *J* =1.9 Hz); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$= 9.8, 26.3, 26.4, 33.4, 34.3, 46.9, 118.0, 127.2, 127.9, 128.9, 130.4, 130.5, 130.9, 133.1, 134.9, 136.0, 136.1, 142.8, 149.6, 198.0; ESI (*m/z*) 485.1 [$M^+$+Na]. Anal. $C_{24}H_{23}Cl_3N_2O$ (C, H, N).

*6.2.5. (±)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-(2-cyclohexyl-1-hydroxyethyl)-4-methyl-1H-pyrazole* (**(±)-5**)**.** To a suspension of ketone **4** (0.15 g, 0.32 mmol) in MeOH (3 mL) was added sodium borohydride (25 mg, 0.67 mmol), and the mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with $CHCl_3$ and washed with water. The organic layer was dried over anhydrous $Na_2SO_4$, filtered, and concentrated to give 0.14 g (93%) of compound **5** as a white solid. $R_f$ = 0.27 (petroleum ether/AcOEt, 8:2); IR : $\nu$ = 3315; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 0.92-1.08 (m, 2H), 1.14-1.33 (m, 4H), 1.53-1.94 (m, 7H),1.95-2.05 (m, 1H), 2.12 (s, 3H), 4.93-5.02 (m, 1H), 7.06 (bd, 2H, *J* =8.6 Hz), 7.23-7.30 (m, 4H), 7.39 (bs, 1H); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$= 8.6, 26.2, 26.4, 26.6, 32.8, 34.1, 34.2, 44.7, 66.3, 112.6, 127.7, 128.1, 128.7, 130.2, 130.7, 130.8, 133.2, 134.4, 135.3, 136.5, 142.0, 155.2; ESI (*m/z*) 463.2 [$M^+$], 485.2 [$M^+$+Na]. Anal. $C_{24}H_{25}Cl_3N_2O$ (C, H, N).

*6.2.6. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carbaldehyde* (**16**). The Weinreb amide **14** (0.70 g, 1.65 mmol) dissolved in 7 mL of dry THF was added dropwise to a stirred suspension of $LiAlH_4$ (0.08 g, 1.98 mmol) in dry THF (10 mL) under nitrogen at 0℃ and the mixture was stirred for 20 minutes at the same temperature. The suspension became dark brown. The reaction mixture was quenched by addition of 10% HCl aq. and poured into brine. The mixture was extracted with $Et_2O$. The organic layer was dried over $Na_2SO_4$ and the solvent was removed by rotary evaporation, affording the

product **16** as a yellowish, viscous oil which formed crystals upon standing, in quantitative yield. $R_f$ = 0.29 (petroleum ether/$Et_2O$, 9:1); IR : $\nu$ = 1723; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 2.35 (s, 3H), 7.07 (d, 2H, *J* =8.3 Hz), 7.18-7.36 (m, 4H), 7.45 (bs, 1H), 10.13 (s, 1H).

*6.2.7. Diethyl [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]hydroxymethylphosphonate* (**17**). Triethylamine (0.23 mL, 1.68 mmol) was added to a stirred mixture of diethyl phosphite (0.44 mL, 3.82 mmol) in toluene (5 mL) at room temperature. After 5 minutes a solution of aldehyde **16** (0.57 g, 1.56 mmol) in toluene (5mL) was added. After 24 h at reflux, an aliquot of $K_2CO_3$ (0.43g, 3.12 mmol) was added to this mixture which was then strirred for further 2 h at the same temperature. The mixture was washed with water, extracted with $Et_2O$, dried with $Na_2SO_4$ and the solvent evaporated to give the crude product. Flash chromatography on silica gel (AcOEt/ petroleum ether, 7:3), gave 0.59 g (80%) of pure **17** as a viscous oil which became solid upon standing. $R_f$ = 0.22 (AcOEt/ petroleum ether, 7:3); IR : $\nu$ = 1245, 3590; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 1.27-1.40 (m, 6H), 2.18 (s, 3H), 4.10-4.27 (m, 4H), 5.17 (d, 1H, *J* =17.6 Hz), 7.07 (d, 2H, *J* =13.6 Hz), 7.25-7.30 (m, 4H), 7.41 (bs, 1H). Anal. $C_{21}H_{22}Cl_3N_2O_4P$ (C, H, N).

*6.2.8. Diethyl [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]fluoromethylphosphonate* (**18**). To a stirred solution of alcohol **17** (0.42 g, 0.83 mmol) in $CH_2Cl_2$ (2 mL) at −78°C was added DAST (0.13 mL, 1.00 mmol). After 1h at −78°C, the reaction mixture was poured carefully into a saturated $NaHCO_3$ solution. The aqueous layer was extracted with $CH_2Cl_2$. The combined organic phases where dried over $Na_2SO_4$, filtered and concentrated to give the crude product that was purified by flash chromatogarphy (petroleum ether/ AcOEt, 6:4) to give 0.33g (79%) of **18** as an orange oil.

$R_f$ = 0.40 (petroleum ether/ AcOEt, 6:4); IR : $\nu$ = 1246; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 1.32 (t, 3H, *J* =7.0 Hz), 1.37 (t, 3H, *J* =7.0 Hz), 2.23 (s, 3H), 4.07-4.24 (m, 2H), 4.24-4.34 (m, 2H), 5.89 (dd, 1H, *J* =9.2 and 44.5 Hz), 7.07 (d, 2H, *J* =8.2 Hz), 7.22-7.32 (m, 4H), 7.39 (bs, 1H). Anal. $C_{21}H_{21}Cl_3FN_2O_3P$ (C, H, N).

*6.2.9. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-[(Z)-2-cyclohexyl-1-fluorovinyl]-4-methyl-1H-pyrazole* (**(*Z*)-6**) *and 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-[(E)-2-cyclohexyl-1-fluorovinyl]-4-methyl-1H-pyrazole* (**(*E*)-7**). To a stirred solution of the phosphite derivative **18** (0.37 g, 0.73 mmol) dissolved in 2.32 mL of THF the cyclohexyl carboxaldehyde (**19**) (0.11 mmol) was added under nitrogen. The resulting mixture was cooled to –78℃, then 0.48 mL (0.95 mmol) of a solution 2M of lithium diisopropylamide (LDA) in THF/cyclohexane/ethylbenzene were added dropwise. The resulting solution was stirred at –78℃ for 30 minutes and then allowed to warm up to room temperature over 4 h and stirred at that temperature overnight. The reaction mixture was poured into water, and the water layer was extracted with ether. The combined organic materials were washed with 10% HCl aq. and then with water and brine, dried over $Na_2SO_4$ and concentrated at reduced pressure. The residue was purified by flash chromatogarphy (petroleum ether/ $Et_2O$, 96:4) to give 0.2g (59%) of **(*E*)-7** as a viscous, colorless oil and 0.04 g (11%) of **(*Z*)-6** as an oil that became solid upon standing. **Compound (*E*)-7**: $R_f$ = 0.46 (petroleum ether/ $Et_2O$, 96:4); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 1.06-1.36 (m, 4H), 1.53-1.73 (m, 4H), 1.74-1.85 (m, 2H), 2.13 (s, 3H), 2.49-2.64 (m, 1H), 5.42 (dd, 1H, *J* = 10.0 and 21.1 Hz), 7.07 (d, 2H, *J* =8.3 Hz), 7.15 (d, 1H, *J* =8.3 Hz), 7.23 (dd, 1H, *J*= 1.6 and 8.3 Hz), 7.30 (d, 2H, *J* =8.3 Hz), 7.45 (d, 1H, *J* =1.6 Hz); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$= 9.1, 9.2, 25.9, 26.0, 33.5, 33.6, 34.8, 34.9, 115.5, 117.6, 117.7, 127.6, 127.7, 128.9, 130.3, 130.4, 130.7, 133.1, 134.6, 135.3, 136.3, 141.3, 145.0, 145.4, 149.3, 151.7; ESI (*m/z*) 487.1 [$M^+$+Na]; $^{19}$F NMR

($CDCl_3$, 235 MHz,) $\delta$= –112.83 (s, 1F). Anal. $C_{24}H_{22}Cl_3FN_2$ (C, H, N). **Compound (*Z*)-6**: $R_f$ = 0.27 (petroleum ether/ $Et_2O$, 96:4); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 1.14-1.43 (m, 4H), 1.58-1.88 (m, 6H), 2.17 (s, 3H), 2.62-2.75 (m, 1H), 5.38 (dd, 1H, *J* = 9.4 and 38.5 Hz), 7.07 (d, 2H, *J* =8.6 Hz), 7.25-7.34 (m, 4H), 7.39 (d, 1H, *J*= 1.9 Hz). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$= 9.7, 9.8, 25.9, 26.0, 33.1, 33.2, 33.6, 33.7, 113.8, 115.0, 115.1, 127.6, 127.8, 128.8, 130.2, 130.8, 130.9, 133.1, 134.7, 135.6, 136.2, 142.3, 145.6, 145.9, 149.5, 152.0; ESI (*m/z*) 465.1 [$M^+$+H]; $^{19}$F NMR ($CDCl_3$, 235 MHz,) $\delta$= –122.19 (s, 1F). Anal. $C_{24}H_{22}Cl_3FN_2$ (C, H, N).

*6.2.10. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-(1-hydroxy-2,2,2-trifluoroethyl)-4-methyl-1H-pyrazole* (**20**). To a solution of **16** (390 mg, 1.1 mmol) in dry THF (6 mL), a 1 M tetrabutylammonium fluoride THF solution (55 µL, 0.055 mmol) was added at 0℃ under nitrogen atmosphere, followed by 2M trimethyl(trifluoromethyl)silane THF solution (1 mL, 2 mmol). The ice bath was then removed and the reaction was monitored by TLC analysis until completion. Additional 1M tetrabutylammonium fluoride THF solution (55 µL, 0.055 mmol) was then added at room temperature. After 15 min stirring, saturated $NH_4Cl$ aqueous solution was added and the mixture extracted with AcOEt. The combined organic layers were dried over anhydrous $Na_2SO_4$, concentrated under vacuum and the crude purified by flash chromatography (hexane/AcOEt, 8:2), yielding **20** as a pale yellow foam (407 mg, 85%). $R_f$ = 0.30 (hexane/AcOEt, 8:2); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 2.11 (s, 3H), 3.47 (d, 1H, *J* =7.9 Hz), 5.16 (m, 1H), 7.70 (m, 2H), 7.28 (m, 4H), 7.42 (d, 1H, *J* =2.0 Hz); $^{13}$C NMR ($CDCl_3$,101 MHz) $\delta$= 8.2, 67.4 (q, *J* = 33.5 Hz), 102.7, 114.2, 115.3, 122.8, 124.2 (q, *J* = 282.8 Hz), 125.6, 127.2, 127.4, 127.7, 127.7, 128.8, 129.0, 130.2, 130.5, 130.6,

130.7, 133.1, 134.9, 135.7, 135.8, 142.6, 146.0, 147.7; $^{19}$F NMR ($CDCl_3$, 235 MHz) $\delta$= –79.16 (d, 3F, $J$ =6.1 Hz). Anal. $C_{18}H_{12}Cl_3F_3N_2O$ (C, H, N).

*6.2.11. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-(1-oxo-2,2,2-trifluoroethyl)-1H-pyrazole* (**21**). To a solution of oxalyl chloride (550 µL, 6.3 mmol) in dry DCM (20 mL) dry DMSO (870 µL, 12.1 mmol) was slowly added drop-wise at -78°C under nitrogen atmosphere. After 15 min stirring, a solution of **20** (706 mg, 1.62 mmol) in dry DCM (5 mL) was slowly added and after further 15 min freshly distilled triethylamine (2.4 mL, 17.2 mmol) was added drop-wise. The cooling bath was then removed and the mixture was allowed to warm up to room temperature. The mixture was then concentrated under vacuum, diluted with water and finally extracted with AcOEt. The combined organic layers were dried over anhydrous $Na_2SO_4$, concentrated under vacuum and the residue purified by flash chromatography (DCM), affording 702 mg (99%) of product **21**. $R_f$ = 0.20 (hexane/AcOEt, 95:5); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$= 2.36 (s, 3H), 7.07 (d, 2H, $J$ =8.4 Hz), 7.34 (m, 4H), 7.44 (d, 1H, $J$ =1.8 Hz); $^{19}$F NMR ($CDCl_3$, 235 MHz,) $\delta$= –74.25 (s, 3F). Anal. $C_{18}H_{10}Cl_3F_3N_2O$ (C, H, N).

*6.2.12. (Z)-1-[5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]-2,2,2-trifluoro-N-(piperidin-1-yl)ethanimine* (**22**). To a solution of crude **21** (685 mg, 1.58 mmol) in toluene (20 mL) pyridinium *p*-toluenesulfonate (79 mg, 0.31 mmol) was added followed by N-aminopiperidine (258 µL, 2.39 mmol). The mixture was then refluxed 24h with a Dean-Stark trap to remove water. After the mixture was cooled to room temperature and concentrated under vacuum, the crude was purified by flash chromatography (hexane/AcOEt, 95:5), affording **22** as a pale yellow foam (407 mg, 50%). $R_f$ = 0.30

(Hex/AcOEt = 95:5); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$= 1.60 (m, 6H), 2.06 (s, 3H), 3.18 (t, 4H, *J* =5.3 Hz), 7.08 (d, 2H, *J* =8.5 Hz), 7.31 (m, 4H), 7.41 (d, 1H, *J* =1.9 Hz,); $^{13}$C NMR ($CDCl_3$, 101 MHz) $\delta$= 8.7, 23.7, 25.1, 54.3, 116.9, 117.9, 120.6, 122.0 (q, *J* =277.0 Hz), 123.3, 128.8, 129.0, 130.0, 130.3, 130.3, 130.4, 130.5, 130.7, 130.8, 133.0, 134.7, 135.7, 136.1, 141.6, 144.8; $^{19}$F NMR ($CDCl_3$, 235 MHz) $\delta$= –66.74 (s, 3F). Anal. $C_{23}H_{20}Cl_3F_3N_4$ (C, H, N).

*6.2.13. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]-2,2,2-trifluoro-N-(piperidin-1-yl)ethanamine hydrochloride* (**(±)-8**). A solution of **22** (203 mg, 0.39 mmol) and borane-trimethylamine complex (43 mg, 0.59 mmol) in toluene (6 mL) was saturated with gaseous HCl for about 40 min. After stirring for 4h at room temperature, the reaction was complete. Nitrogen was bubbled and the solvent removed under vacuum. The residue was purified by flash chromatography (hexane/AcOEt, 95:5) affording **(±)-8** as a white foam (150 mg, 70%). $R_f$ = 0.40 (hexane/AcOEt, 9:1); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$= 1.36 (m, 2H), 1.62 (m, 4H), 2.12 (s, 3H), 2.66 (m, 4H), 3.34 (bs, 1H), 4.63 (q, 1H, *J* =7.7 Hz), 7.08 (d, 2H, *J* = 7.3 Hz), 7.40 (s, 1H), 7.26 (dd, 4H, *J* =17.7 and 9.8 Hz,); $^{13}$C NMR ($CDCl_3$, 101 MHz) $\delta$= 8.6, 23.7, 26.1, 58.0, 59.5 (q, *J* =29.4 Hz), 114.6, 125.3 (q, *J* =281.9 Hz), 127.7, 127.9, 128.8, 130.3, 130.7, 130.8, 133.5, 134.8, 135.6, 136.5, 142.0, 147.3; $^{19}$F NMR ($CDCl_3$, 235 MHz) $\delta$= –75.03 (bs, 3F); ESI (*m/z*) 517.2 [$M^+$-HCl], 539.2 [$M^+$-HCl+Na]. Anal. $C_{23}H_{22}Cl_3F_3N_4 \cdot$ HCl (C, H, N).

*6.3. Pharmacology*

*6.3.1. Chemicals and Drugs for in vitro and in vivo assays.*

[$^3$H]-CP-55,940 (specific activity 180 Ci/mmol) was purchased from Perkin Elmer Italia. WIN 55,212-2 was obtained from Sigma-Aldrich (Milan, Italy). *N*-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (rimonabant) was purchased by KEMPROTECH Limited, Middlesbrough, UK.

*6.3.2. Animals*

All animal experiments were performed according to the UE guidelines for the care and use of experimental animals (CEE N° 86/609). Animals (Charles River, Calco, LC, Italy) were housed in the animal care quarters; temperatures were maintained at 22 ± 2 ℃ (humidity 55 ± 5%) on a 12 hrs light/dark cycle. Food and water were available ad libitum before to start with the experimental procedures.

*6.3.3. Receptor binding studies.*

Male CD1 mice weighing 30-35 g were killed by cervical dislocation and the brain (minus cerebellum) and spleen were rapidly removed and placed on an ice-cold plate. Brain tissues were used for $CB_1$ binding assays, while spleen tissues for $CB_2$ affinity evaluation. After thawing, tissues were homogenated in 20 vol. (wt/v) of ice-cold TME buffer (50 mM Tris-HCl, 1 mM EDTA and 3.0 mM $MgCl_2$, pH 7.4). The homogenates were centrifuged at 1,086 x g for 10 min at 4 ℃, and the resulting supernatants were centrifuged at 45,000 x g for 30 min.

For radioreceptor binding experiments, drugs were dissolved in dimethyl-sulfoxide (DMSO). DMSO concentration in the different assays never exceeded 0.1% (v/v) and was without effects.

[$^3$H]-CP-55,940 binding was performed by the previously described method [58]. Briefly, the membranes (30-80 μg of protein) were incubated with 0.5-1 nM of [$^3$H]-CP-55,940 for 1 h at 30 ℃ in a final volume of 0.5 ml of TME buffer containing 5 mg/ml of fatty acid-free

bovine serum albumin. Non-specific binding was estimated in the presence of 1 μM of CP-55,940. All binding studies were performed in glass tubes pre-treated with Sigma-Cote (Sigma Chemical Co. Ltd., Poole, UK), in order to reduce non-specific binding. The reaction was blocked by rapid filtration through Whatman(GF/C filters presoaked in 0.5% polyethyleneimine (PEI) using a Brandell 36-sample harvester (Gaithersburg, MD, USA). Filters were washed five times with 4 ml aliquots of ice cold Tris HCl buffer (pH 7.4) containing 1 mg/mL BSA. The filter bound radioactivity was measured in a liquid scintillation counter (Trisarb 2900, Packard, Meridien, USA) with 4 ml of scintillation fluid (Ultima Gold MV, Packard).

Protein determination was performed by means of Bradford protein assay using BSA as a standard according to the protocol of the supplier (Bio-Rad, Milan, Italy).

All experiments were performed in triplicate and results were confirmed in at least five independent experiments. Data from radioligand inhibition experiments were analyzed by nonlinear regression analysis of a Sigmoid Curve using Graph Pad Prism program. $IC_{50}$ values were derived from the calculated curves and converted to $K_i$ values as previously described [60].

*6.3.3. Intrinsic activity by in vitro assays.*

N1E-115 cells were grown at 37℃ in humidified 5% $CO_2$ in Dulbecco's Modified Eagle's Medium – high glucose supplemented with 10% Fetal Bovine Serum (FBS), 1 μg/ml Penicillin-Streptomycin, 2 mM L-Glutamine, 2.5 μg/mL Amphotericin B and 50 μg/mL Gentamicin. N1E-115 cells were treated with the compounds to be assayed and reference compounds. WIN55,212-2 was adopted as reference cannabinoid agonist to verify $CB_1R$ modulated effect by the synthesized compounds. Tested and reference compounds were dissolved in Dulbecco's Phosphate Buffered Saline (D-PBS) with 1% DMSO and treatments were performed in a volume of 10 μl/ml of cell suspension.

Cells lines from the European Collection of Cell Cultures (ECACC), growth medium, FBS, Penicillin-Streptomycin, L-Glutamine, Amphotericin B, Gentamicin, D-PBS and DMSO were purchased from Sigma-Aldrich (Milano, Italy).

P-ERK 1/2 expression was determined after N1E-115 cell treatment. Western blot analysis was adopted according to the previously reported procedure [60]. After appropriate time of exposure, cells were collected by centrifugation at 1000 x g and the resulting pellets were washed in ice-cold PBS buffer by centrifugation at 1000 x g. The pellet was lysed at 4 ℃ in buffer consisting in HEPES 20 mM, EDTA 0.2 mM, NaCl 125 mM, $MgCl_2$ 5 mM, glycerol 12%, Nonidet P-40 0.1%, Aprotinin 1 μg/mL, Pepstatin 0.7 μg/mL, Leupeptin 0.5 μg/mL, and phenylmethylsulfonyl fluoride (PMSF) 0.5 mM. The extracts were centrifuged at 10000 x g for 15 min at 4 ℃. The resulting supernatant was collected as total cell extracts. Extracted total proteins were quantified using a Quant-iT™ Protein Assay Kit (Invitrogen™) by a Qubit Quantitation platform system (Invitrogen™). Each western blot assay was carried out at fixed total protein amount of 40 μg. One-way ANOVA was performed as a statistical analysis using Graph Pad Prism program (San Diego).

To exclude potential $CB_1$ agonist compounds amongst those synthesized, in a first step P-ERK 1/2 was determined after 10 minute treatment of the N1E-115 cells with the compounds to be assayed. Successively to confirm the capability of the new derivatives to inhibit the $CB_1$ agonism activity, a 5 min pre-treatment with the $CB_1$ receptor antagonist rimonabant (1 μM) and with the compounds 3-8 (0.1-1.0 μM) was carried out before the exposure to the cells to the reference cannabinoid agonist WIN55,212-2 (25 nM). As shown by western blot analysis this compound displayed a significant induction of P-ERK 1/2 expression which is counteracted by rimonabant. The results were obtained from five independent experiments.

*6.3.4. Isolated organs assays.*

Vas deferens was obtained from male albino CD1 mice weighing 30 to 40 g. Tissue was mounted in a 10-ml organ bath at an initial tension of 0.5 g using previously described method [66]. The bath contained Krebs-Henseleit solution (118.2 mM NaCl, 4.75 mM KCl, 1.19 mM $KH_2PO_4$, 25.0 mM $NaHCO_3$, 11.0 mM glucose, and 2.54 mM $CaCl_2$), which was kept at 37℃ and bubbled with 95% $O_2$ and 5% $CO_2$. Isometric contractions were evoked by stimulation with 0.5-sec trains of three pulses of 110% maximal voltage (train frequency, 0.1 Hz; pulse duration, 0.5 msec) through platinum electrodes attached to the upper end of each bath and a stainless steel electrode attached to the lower end. Stimuli were generated by Grass S88K stimulator then amplified (multiplexing pulse booster 316S; Ugo Basile, Comerio, Italy) and divided to yield separate outputs to four organ baths. Contractions were monitored by computer using a data recording and analysis system (PowerLab 400) linked via preamplifiers (QuadBridge) to an F10 transducer (Biological Instruments, Besozzo, Italy). Each tissue was subject to several periods of stimulation. The first of these stimulation periods began after the tissue had equilibrated in the buffering medium but before drug administration, and continued for 10 min. The stimulator was then switched off for 15 min, after which the tissues were subjected to further periods of stimulation each lasting 5 min and separated by a stimulation-free period.

The compounds to be assayed were added once the contractile responses to electrical stimulation were reproducible. The results were obtained and reported as mean ± SEM from five independent experiments.

For isolated organ experiments, drugs were dissolved in dimethyl-sulfoxide (DMSO). DMSO concentration in the different assays never exceeded 0.1% (v/v) and was without effects.

To ascertain $CB_1$ mediated activity of the novel compound on mouse vas deferens, their counteracting effect on the response by the reference cannabionidergic reference

compound WIN 55,212-2 was investigated. The compounds to be assayed were added at fixed concentration 20 minutes before the first concentration of WIN 55,212-2. Inhibition of the electrically evoked twitch response was expressed in percentage and it was calculated by comparing the amplitude of the switch response after each addition of WIN 55,212-2 with that detected as basal. Data were plotted as Log antagonist concentration versus percentage of inhibition.

*6.3.5. [$^{35}$S]GTPγS binding assays.*

To confirm neutral antagonism profiles by isolated organ assays, tests based on [$^{35}$S]GTPγS binding assays were performed according to previously reported procedure [43]. CHO cells transfected with the human recombinant $CB_1$ ($CB_1$ CHO cells) provided by PerkinElmer (Milano, Italy) were used. Intrinsic activity was preliminary evaluated using different concentrations of each compound alone. Briefly, an appropriate buffer solution (see details in Ref. 43), [$^{35}$S]GTPγS, the compound to be assayed, and $CB_1$ CHO cells were added in the wells of a 96-well plate. The assay was incubated at 37 ℃ for 1 h, and it was subsequently blocked by addition of ice-cold tris/BSA buffer, and filtration by 36-well Brandell (cell harvester) system and glass-fibre filters that have been soaked in Tris/BSA buffer at 4 ℃ for 24 hours. The wells were washed (1.2 ml of Tris/BSA for six times), and the filters were removed to be oven dried for 1 h. The paper portions of the filters were separated and they were transferred into vials with 5 ml of scintillation fluid (Microscint 20, PerkinElmer). The filters were soaked for 1 h, and radioactivity was then quantified by a liquid scintillation spectrometry (TopCount NXT PerkinElmer).

Net [$^{35}$S]GTPγS binding values were determined from experimental data by subtracting basal binding values. Statistical analysis was carried out by One-way ANOVA with Bonferroni post test (Graph Pad Prism 4 program), by comparing mean % stimulation values SEM to

the basal binding level (0.0 % stimulation). The results were obtained and reported as mean ± SEM from five independent experiments.

*6.3.6. Food intake.*

A widely described method was used for the screening of the new $CB_1$ antagonists in food intake animal model [63]. C57Bl6/J mice weighing 20-25 g were housed individually on a reverse light-dark cycle in a room with temperature and humidity control. The mice were fasted for 18 hrs before testing. In vivo assays were carried out dissolving compounds in saline solution containing Tween 80 and Ethanol (both at 2.5 wt%). Vehicle or $CB_1$ antagonists were administered by an intraperitoneal injection and 15 minutes later a known amount of food was presented. Food pellets were weighed after each hour for three hours. rimonabant was used as internal positive control in order to evaluate the efficacy of each compound.

Nine animals for each group were used in the study. Two-way ANOVA with Bonferroni post test was performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego CA).

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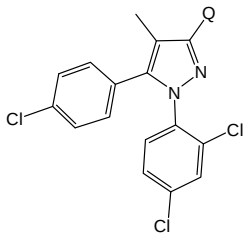
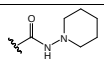
**Table 1.** Structure and binding data for compounds **1**, **3**-**8**




| | | Receptor affinity | | selectivity |
|---|---|---|---|---|
| **Compound** | **Q** | $K_i$**CB$_1$ (nM)**[a] | $K_i$**CB$_2$(nM)**[b] | $K_i$**CB$_1$/ $K_i$CB$_2$** |
| **1** |  | 1.5 ± 0.1 | 375.0 ± 24.0 | 0.004 |
| **3** |  | 11.2 ± 2.0 | 3797.0 ± 245.0 | 0.003 |
| **4** |  | 46.1 ± 1.0 | 2233.0 ± 390.0 | 0.021 |
| **(±)-5** |  | 175.0 ± 14.0 | 4200.0 ± 200.0 | 0.042 |
| **(*Z*)-6** |  | 25.8 ± 5.0 | 1950.0 ± 450.0 | 0.013 |
| **(*E*)-7** |  | 412.0 ± 3.6 | 4000.0 ± 100.0 | 0.103 |
| **(±)-8** |  | 287.0 ± 51.0 | >5000 | < 0.057 |

[a] Affinity of compounds for the $CB_1$ receptor was evaluated using mouse brain (minus cerebellum) homogenate and [$^3$H]-CP 55,940. [b] Affinity of compounds for the $CB_2$ receptor was assayed using mouse spleen homogenate and [$^3$H]-CP-55,940. $K_i$ values were obtained from five independent experiments carried out in triplicate and are expressed as mean ± standard error.

## Figure Captions

**Figure 1.** Structures of rimonabant (**1**), VPSR (**2**), VCHSR (**3**), and ABD-395 (**4**).

**Figure 2.** Novel compounds **5-8**.

**Scheme 1.** Reagents and conditions: a) Na, abs. EtOH, RT, 18 h; b) EtOH, reflux, 16 h; c) $Me_3Al$, $CH_3NHOCH_3 \bullet HCl$, $CH_2Cl_2$, RT, 16 h; d) THF, 0 °C; e) $NaBH_4$, MeOH, RT, 1 h.

**Scheme 2.** Reagents and conditions: a) $LiAlH_4$, THF, 0 °C, 20 min; b) $(EtO)_2P(O)H$, $Et_3N$, toluene, reflux, 24 h; c) DAST, $CH_2Cl_2$, –78 °C, 1 h; d) LDA, THF, –78 °C, 30 min.

**Scheme 3.** Reagents and conditions: a) trimethyl(trifluoromethyl)silane, TBAF sol THF, dry THF, 0 °C; b) oxalyl chloride, dry DMSO, triethylamine, dry $CH_2Cl_2$, -78 °C; c) *N*-aminopiperidine, PPTS, toluene, 140 °C, overnight; d) boranetrimethylamine complex, gaseous HCl, toluene, RT, 4h.

**Figure 3.** Dose response studies of P-ERK 1/2 expression following a 10 min exposure to $CB_1$ receptor agonist WIN55,212-2 (25 nM) or different concentrations of compounds **3** (a), **4** (b), **(±)-5** (c), **(*Z*)-6** (d) and **(±)-8** (e). Data are expressed as a mean percentage of vehicle. One-way ANOVA was performed as a statistical analysis; *$p < 0.05$ vs vehicle. On the top side of histograms are shown representative western blots of P-ERK expression.

**Figure 4**. Results of a competition study of P-ERK 1/2 expression mediate by a 5 min pretreatment with the $CB_1$ receptor antagonist/ inverse agonist rimonabant **1** or compounds **3**, **4**, **(±)-5**, **(*Z*)-6** and **(±)-8**, followed by a 10 min exposure to cannabinoid

receptor agonist WIN (WIN55,212-2 at the dose of 25nM). Inhibition of WIN action suggests antagonist activity of the new compounds. Data are expressed as a mean percentage of vehicle. *p < 0.05 vs vehicle; $^{\#}$p < 0.05 vs WIN55,212-2. On the top side of histograms are shown representative western blots of P-ERK expression.

**Figure 5.** Effect of vehicle containing DMSO, rimonabant **1**, and compounds **3**-**8**, on electrically evoked contractions of mouse vas deferens (see the text). The results are reported as mean ± SEM from five independent experiments. One-way ANOVA with Bonferroni post test was performed to determine the statistical difference: ***p<0.001, **p<0.01, *p<0.05 *vs* Vehicle; ###p<0.001, ##p<0.01, #p<0.05 *vs* rimonabant **1**.

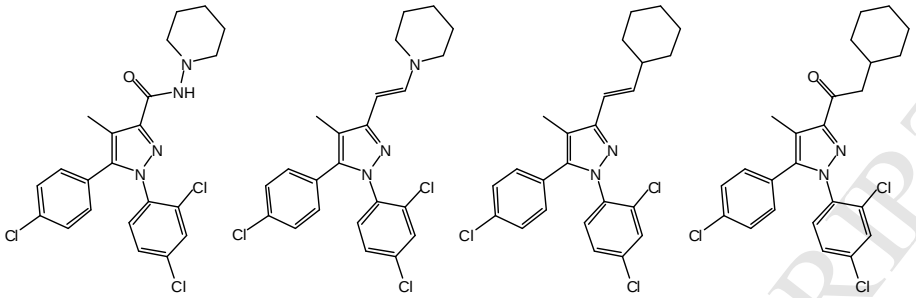
**Figure 6.** Concentration-response curves for WIN 55,212-2 on electrically evoked contractions of mouse vas deferens in presence of DMSO (vehicle; ■), and in presence of compound **6** at 0.1 μM (□), 1 μM (●), 10 μM (○). Each symbol represents the mean value ± S.E.M. of inhibition of electrically evoked contractions of mouse vas deferens expressed as a percentage of the amplitude of the twitch response measured before the first addition of WIN 55,212-2 to the organ bath. Compound **6** was added 20 min before the first addition of WIN 55,212-2.

**Figure 7.** Effect of various cannabinoid agents on [$^{35}$S]GTPγS binding in $CB_1$ CHO cells. Cells were incubated with different concentrations of each cannabinoid compound alone. The [$^{35}$S]GTPγS binding values are expressed as percentage of stimulation vs basal ± S.E.M. Each symbol represents the mean response ± SEM from five independent experiments. (a) rimonabant (**1**), (b) ABD-395 (**4**), (c) novel compound **(±)-5**, and (d) novel compound **(*Z*)-6**. Statistical analysis were carried out by One-way Anova with Bonferroni post test. Compound **1:** F (4,20) = 101.41, p < 0.0001; compound **4**: F (4,20) = 2.96, p =

0.05; compound **(±)-5**: F (4,20) = 8.03, p = 0.0005; compound **(*Z*)-6**: F (4,20) = 12.62, p < 0.0001.

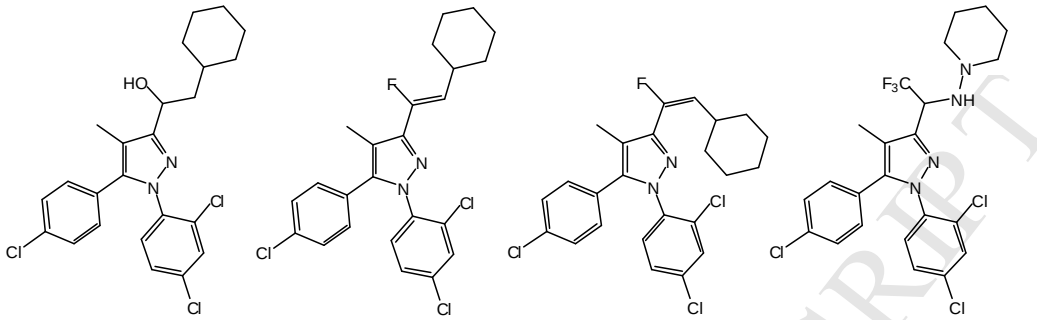
**Figure 8.** The figure shows the mean hourly food intake of fasted C57BL/6N mice i.p. treated with vehicle, rimonabant **1** at 3 and 10 mg/kg, or compound **(*Z*)-6** (a), compound **3** (b), compound **4** (c), compound **(±)-5** (d), each at 10 mg/kg and 20 mg/kg. Values are expressed as mean ± SEM. Two-way ANOVA was used for statistical analysis followed by Bonferroni post hoc test: ***p< 0.001, **p< 0.01, *p< 0.05 *vs* vehicle; $^{###}$p< 0.001, $^{##}$p< 0.01, $^{#}$p< 0.05 *vs* rimonabant. N= 9 for each group.

**Figure 1**



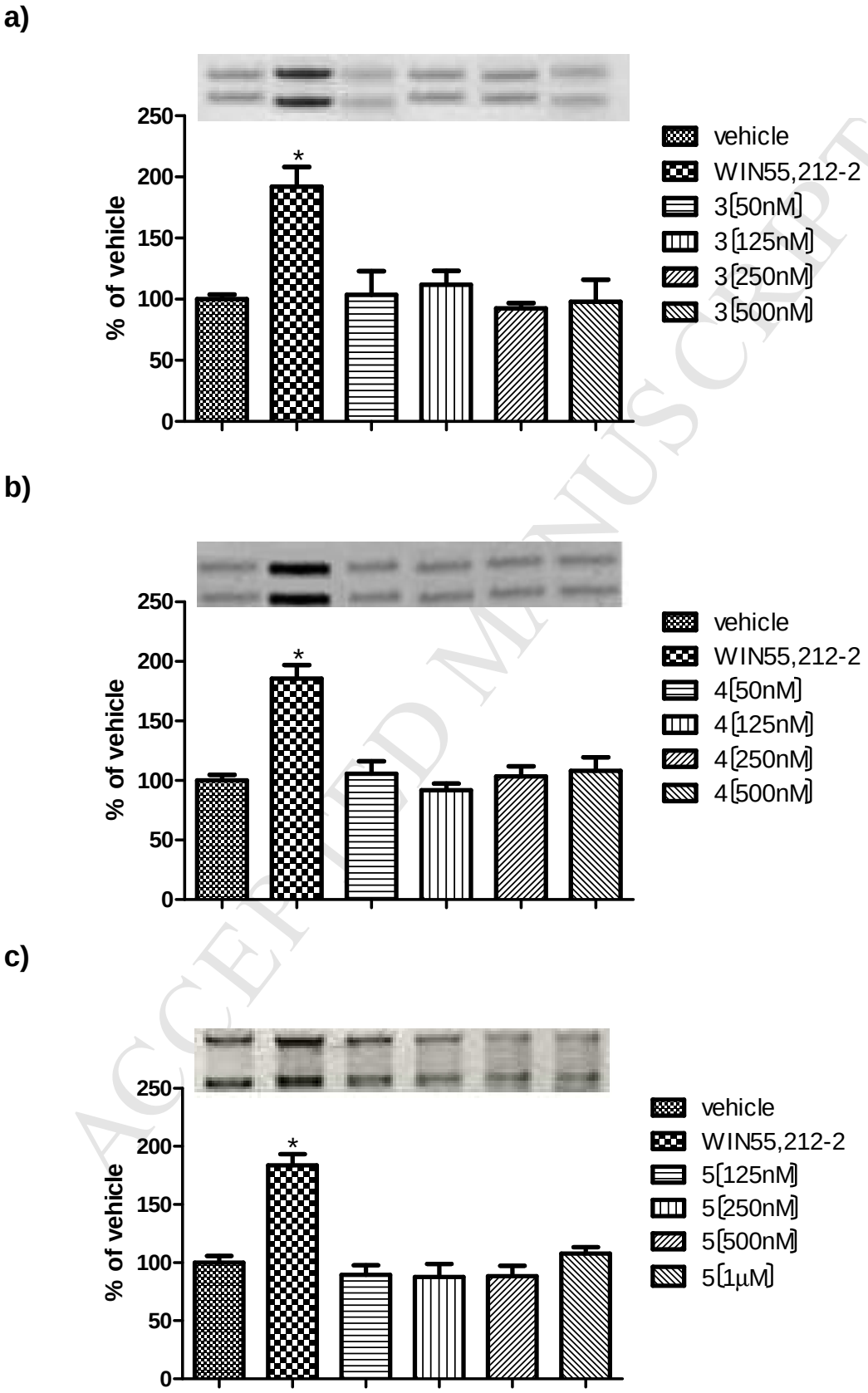

**1** **2** **3** **4**

**Figure 2**

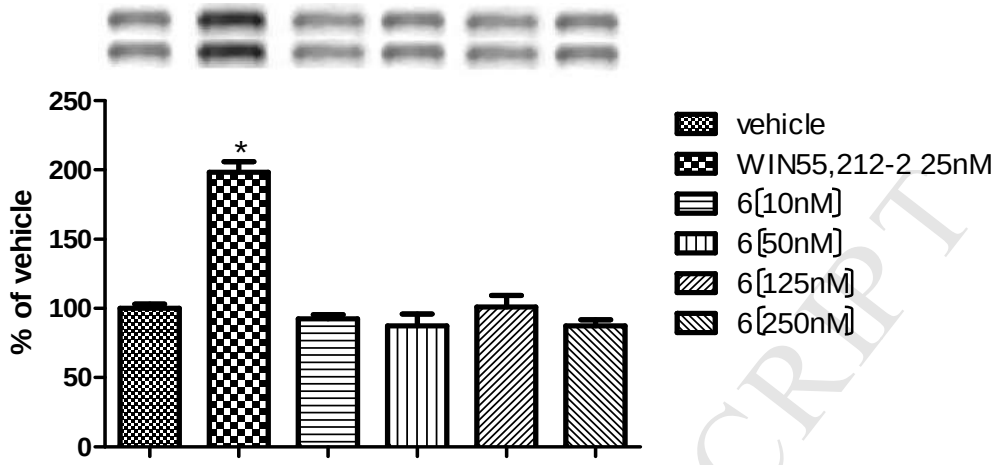



**(±)-5** ***Z*-6** ***E*-7** **(±)-8**

**Figure 3**



**d)**

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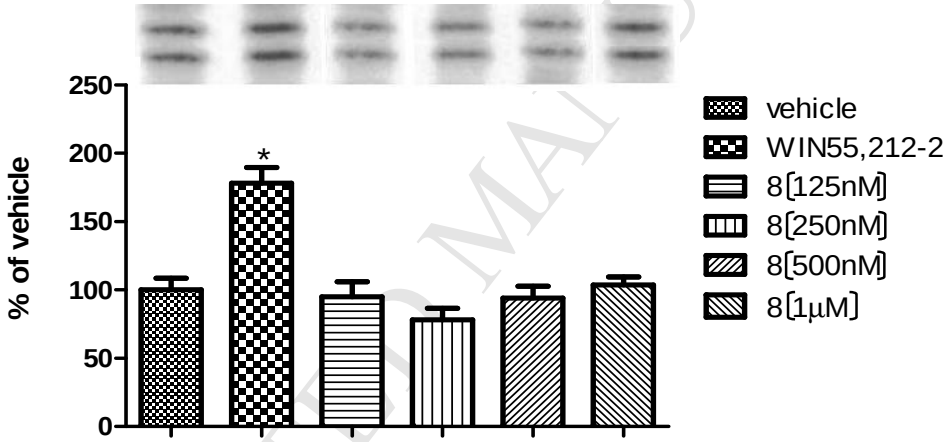

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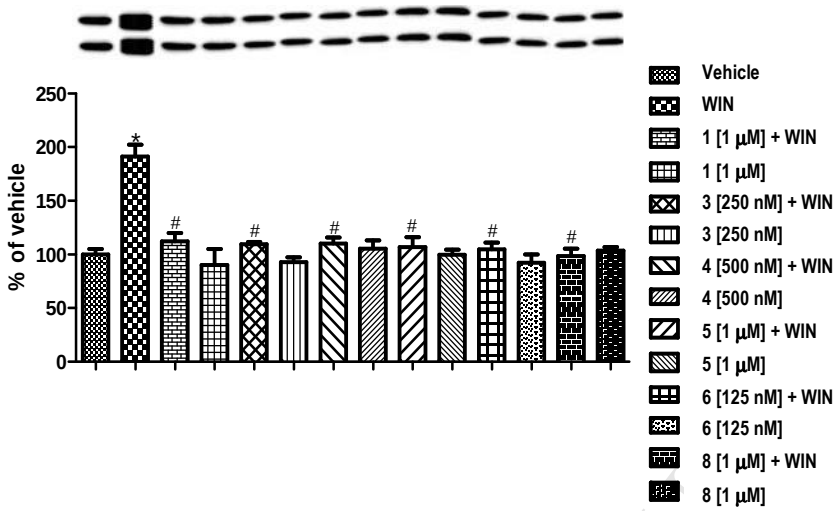

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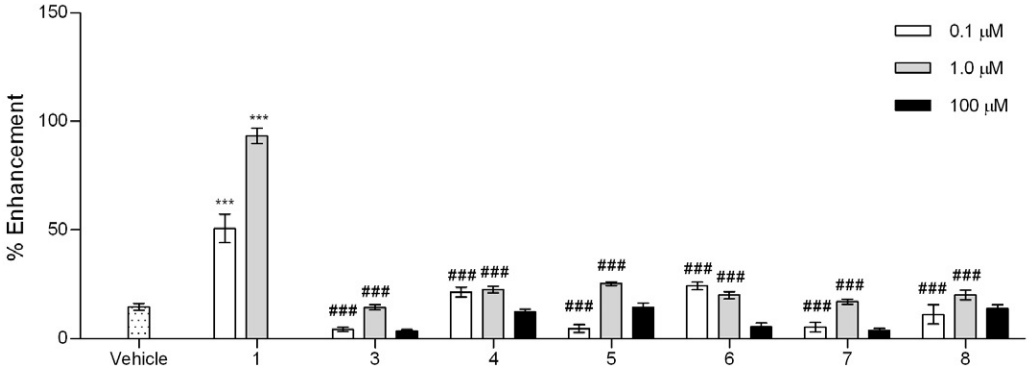

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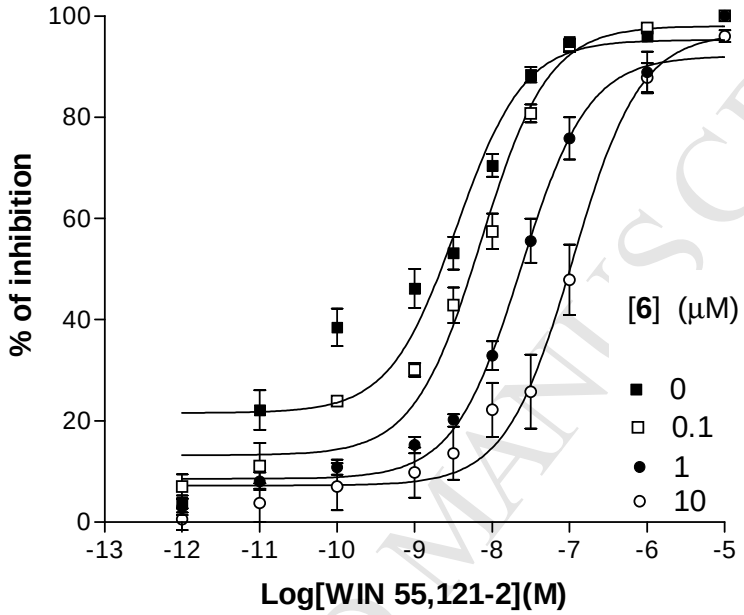


**Figure 7**

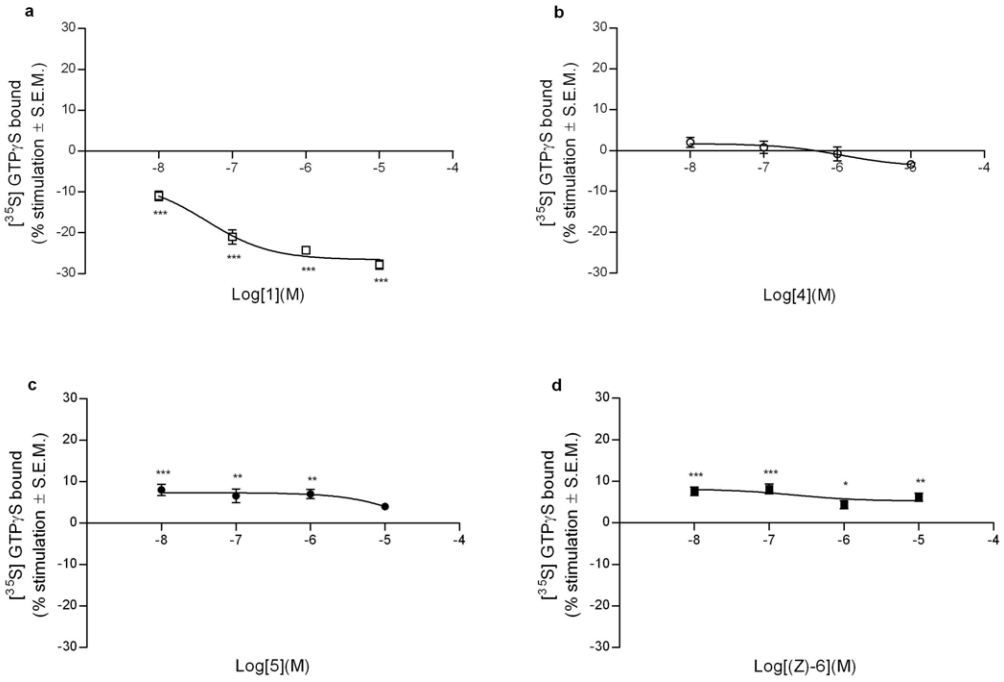
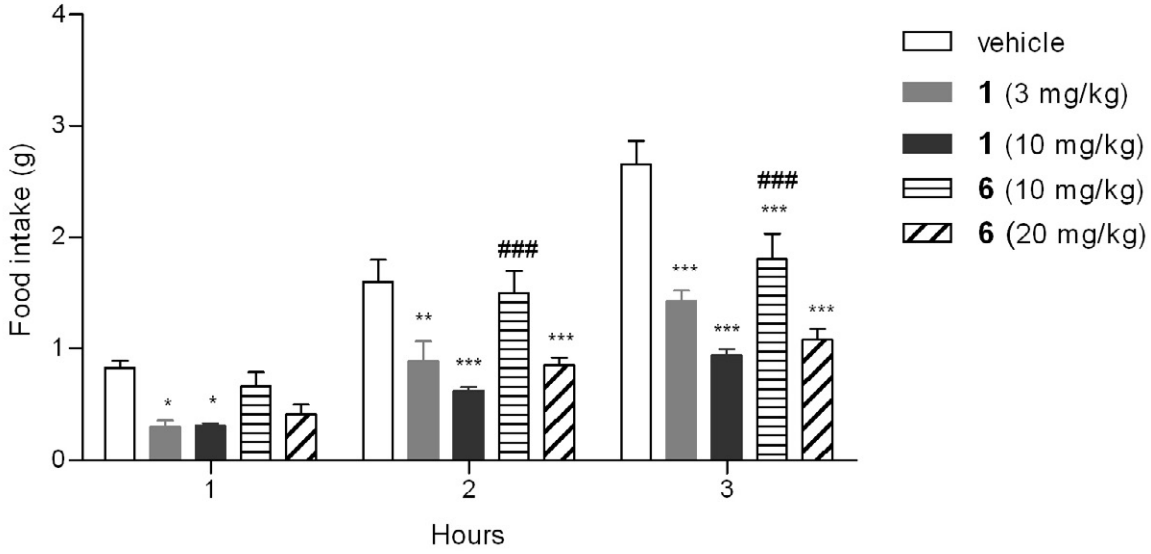
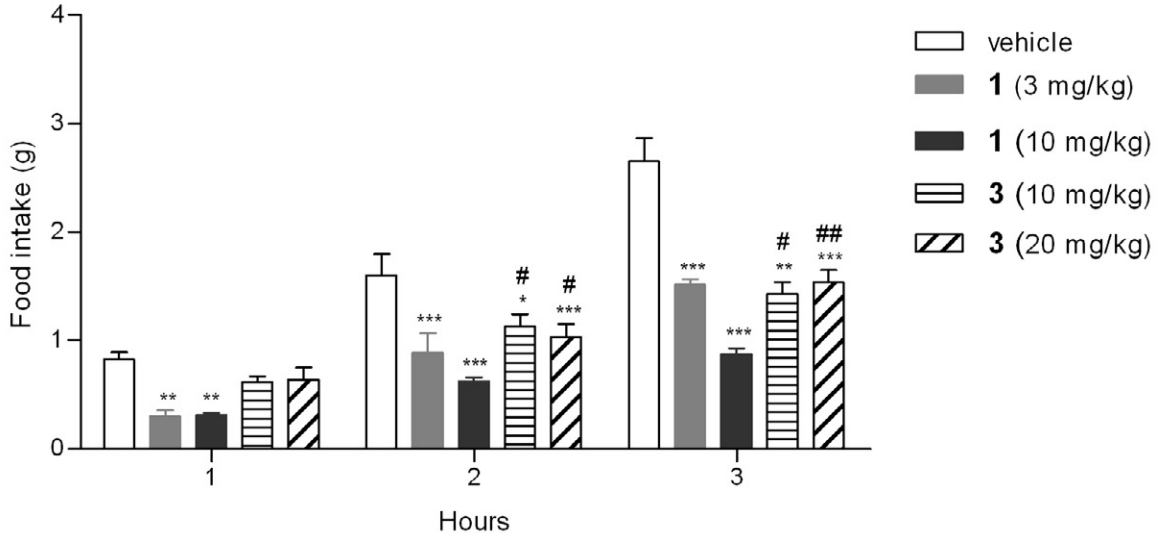


**Figure 8**

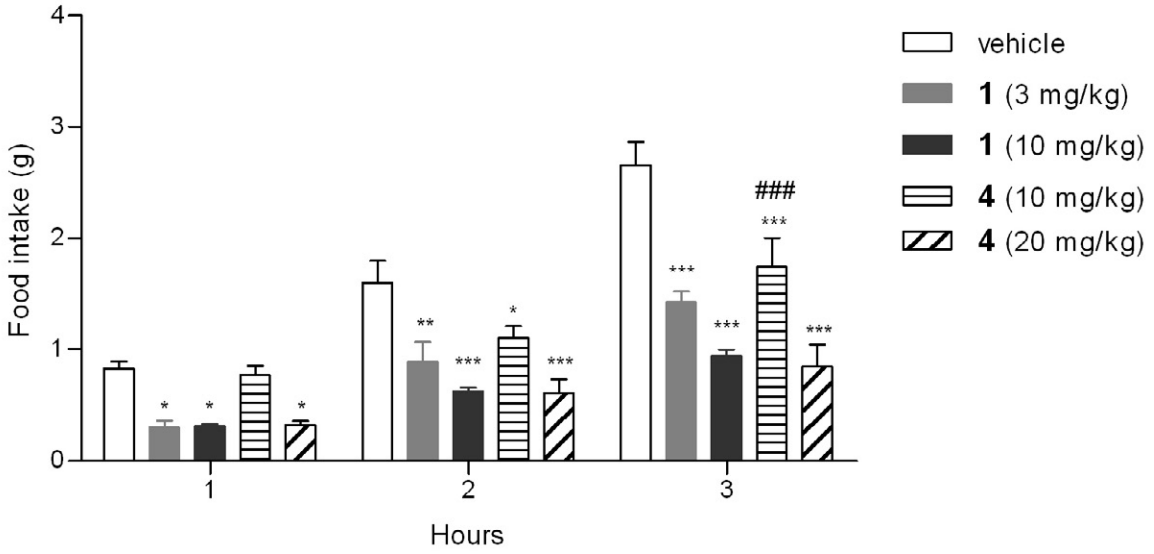
**a)**


Food intake (g)
Hours
vehicle
1 (3 mg/kg)
1 (10 mg/kg)
6 (10 mg/kg)
6 (20 mg/kg)

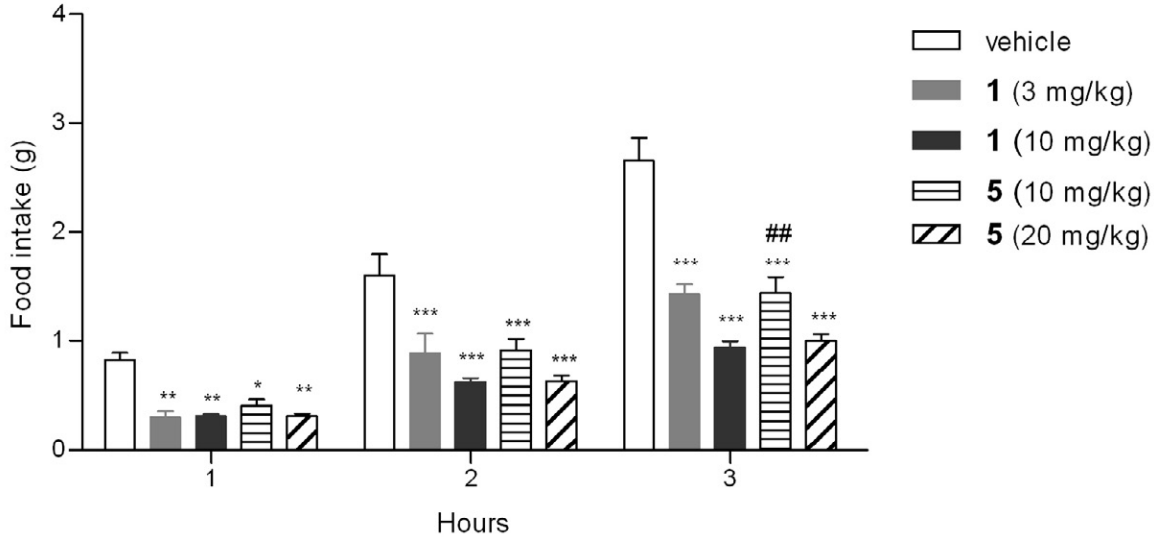
**b)**


Food intake (g)
Hours
vehicle
1 (3 mg/kg)
1 (10 mg/kg)
3 (10 mg/kg)
3 (20 mg/kg)

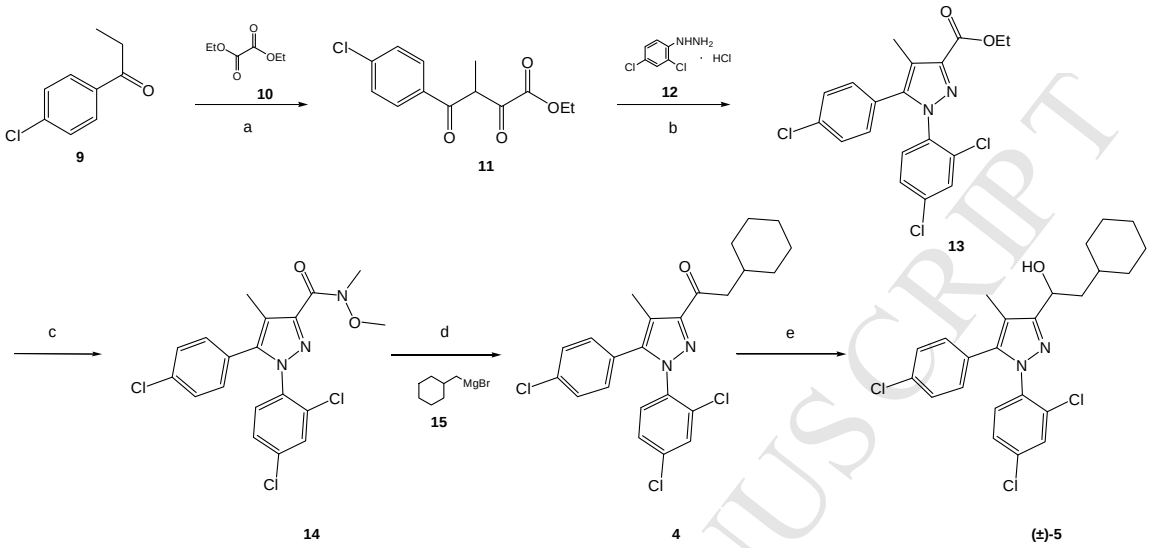
**c)**


Food intake (g)
Hours
vehicle
1 (3 mg/kg)
1 (10 mg/kg)
4 (10 mg/kg)
4 (20 mg/kg)
###

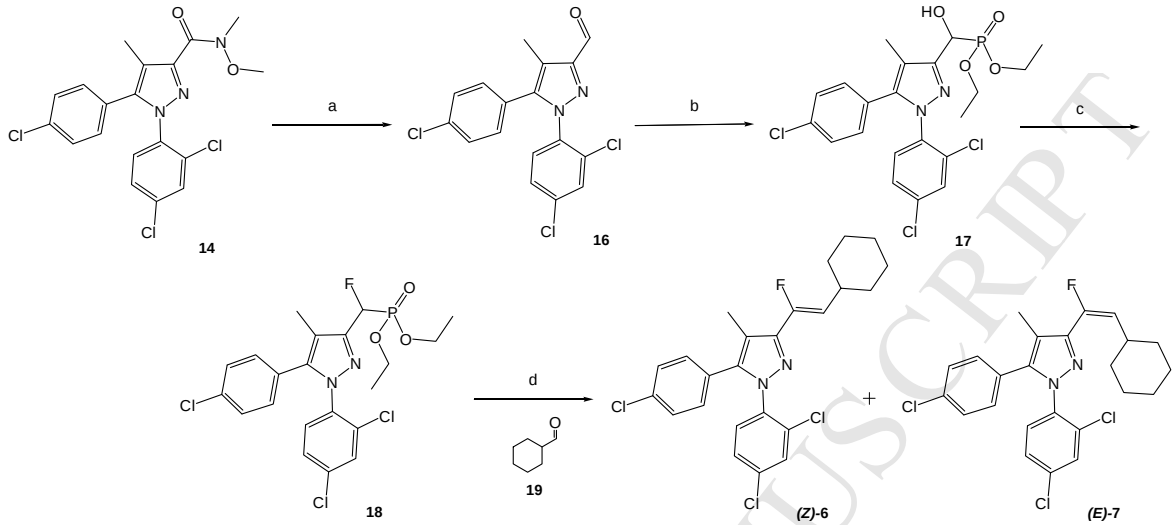
**d)**


Food intake (g)
Hours
vehicle
1 (3 mg/kg)
1 (10 mg/kg)
5 (10 mg/kg)
5 (20 mg/kg)
##

## Scheme 1



## Scheme 2



## Scheme 3