Bioorganic & Medicinal Chemistry 28 (2020) 115672

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

New cannabinoid receptor antagonists as pharmacological tool

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ARTICLE INFO

Keywords:
Cannabinoid receptors
Antagonist
Indazole ether
Bone disease
Pharmacological tool

ABSTRACT

Synthesis and pharmacological evaluation of a new series of cannabinoid receptor antagonists of indazole ether derivatives have been performed. Pharmacological evaluation includes radioligand binding assays with [^{3}H]-CP55940 for CB1 and CB2 receptors and functional activity for cannabinoid receptors on isolated tissue. In addition, functional activity of the two synthetic cannabinoids antagonists **18** (PGN36) and **17** (PGN38) were carried out in the osteoblastic cell line MC3T3-E1 that is able to express CB2R upon osteogenic conditions. Both antagonists abolished the increase in collagen type I gene expression by the well-known inducer of bone activity, the HU308 agonist. The results of pharmacological tests have revealed that four of these derivatives behave as CB2R cannabinoid antagonists. In particular, the compounds **17** (PGN38) and **18** (PGN36) highlight as promising candidates as pharmacological tools.

1. Introduction

Identification and target validation are key steps of drug development and pharmacological research. To possess high selective and well-defined molecule mode of action, chemical probes are essential to study and characterize completely the action mechanism of any drugs. In this context, the cannabinoid system is a very interesting and intriguing biological system.

The endogenous cannabinoid system includes transmembrane cannabinoid CB1 and CB2 receptors, endogenous ligands (the endocannabinoids), and the processes responsible for their biosynthesis, cellular uptake, and metabolism.[1–3] This system has been implicated in a variety of biological processes, both in the central and peripheral nervous systems and in peripheral organs. As a consequence, an array of potential therapeutic targets is currently being studied including specific cannabinoid agonists, and antagonists/inverse agonists[4–8] as well as compounds that interrupt the synthesis, uptake or metabolism of the endocannabinoids.[9]

The CB1 receptor (CB1R) is one of the most abundant G protein–coupled receptors located within the mammalian brain and are present in highest concentration in olfactory and cortical brain regions, hippocampus, amygdale, basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex, and brainstem nuclei and in peripheral tissues such as testis, eye, urinary bladder, ileum, adipose tissue, liver, skeletal muscles and pancreas.[10–13]

Meanwhile, the CB2 receptor (CB2R) is mainly expressed in cells of the immune system in peripheral tissues, the thymus, tonsils, bone marrow, spleen, pancreas, peripheral nerve terminals, microglial cells, glioma and skin tumour cells.[10–12] It has been also described the existence of CB2R in the central nervous system (CNS) under both pathological[14] and physiological conditions.[15]

In relation to CB1R antagonists, the main principal applications that have been proposed for clinic are the treatment of obesity and metabolic syndrome, as well as the treatment of addictions.[16,17]

One of the main representative example is the SR1417A (rimonabant) a 1,2-diarylpyrazole derivative reported in 1994 by the Sanofi group as a selective CB1 antagonist.[18] However, rimonabant is now described as an inverse agonist at the CB1R, displaying negative intrinsic activity.[19]

In 2006, rimonabant was approved by the European Commission as

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https://doi.org/10.1016/j.bmc.2020.115672
Received 18 April 2020; Received in revised form 15 July 2020; Accepted 18 July 2020
Available online 29 July 2020

an anti-obesity agent. However, rimonabant was withdrawn by European Medicine Agency in 2008 because of its substantial CNS risk factors including depression and anxiety, including suicidal tendency. Therefore, the development of anti-obesity drugs targeting CB1R in the brain has been suspended and/or terminated globally, although there is some controversy over the origin of these side effects. According to a recently published meta-analysis, obesity increased the risk of depression and this increased the chances for developing obesity.[17] Other studies suggest that 50% of patients who are looking for anti-obesity treatment report depression symptoms.[20,21] If the adverse effects of rimonabant are attributed to its inverse agonist character, it was suggested that a neutral antagonist would be an alternative approach for treating obesity, related metabolic risk factors[22] or against drug addiction. Thus, Tudge *et al.*[23] have performed a clinical study with tetrahydrocannabivarin, a neutral antagonist, showing that treatment with this CB1 neutral antagonist increases neural responding to rewarding and aversive stimuli. This effect profile suggests therapeutic activity in obesity, perhaps with a lowered risk of depressive side effects. However, other authors suggest that neutral antagonism is a concept that probably does not exist in real world; therefore, apparent neutral antagonists are probably inverse agonists that have not yet been unmasked.[24]

As for the CB2R, the first selective antagonists, the pyrazole SR144528[25] and AM630[19] are two of the most commonly used CB2 selective antagonists and have been generally used as pharmacological tools in order to demonstrate specificity of CB2 selective agonists. However, SR144528 has been described both as an antagonist[25] and as an inverse agonist,[26,27] probably due to the different experimental protocols used. Regarding the therapeutics applications of antagonist CB2R, laboratory data suggest that antagonist/inverse agonists selective for the CB2R may have therapeutic potential. The expression of the receptor by immune cells, both in the periphery and in the CNS suggests that modulation of inflammations or allergies could be achieved with appropriate CB2R antagonists.[28]

For all these reasons, the description of alternative antagonists is very interesting, in order to characterize the action mechanism of new cannabinoids agonists. Encouraged by this challenge and continuing with our efforts in the design and development of new cannabinoids,[29,30] we proposed to develop cannabinoid antagonists.

Recently, we have published a new family of indazole ethers as a novel class of cannabinoid multitarget drugs with potential application for the treatment of Alzheimer's disease.[29] The agonist activity of these indazole ether derivatives is function of the substituents at positions 1, 3 and 5 of heterocyclic system. Continuing with the study of the properties of this family of indazole derivatives other substituents has been considered to obtain new indazole ethers behaving as cannabinoid antagonists.

In this work, we have addressed the synthesis and biological characterization of a family of indazole ethers that behave as pure CB2R antagonist. We have performed isolated tissue assays in order to characterize the biological profile of the cannabinoid. Later, we have studied the ability of these antagonists to revert the agonist effect of HU308 on primary osteoblasts assays.

2. Results and discussion

2.1. Chemistry

Based on previous results we performed the synthesis of a representative set of compounds (**1–22**) in order to found cannabinoid antagonists (Table 1).

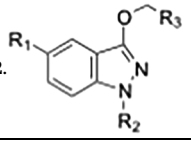
The synthetic strategy used depends of the desired substituents at positions N-1, C-5 and at the hydroxyl group of the indazole system. Thus, there are three possible strategies or synthetic routes that are presented in Scheme 1.

The **route A**, by direct reaction of the indazolol derivatives with the corresponding halides in acetone or butanone and caesium or potassium carbonate as base, is only useful to obtain disubstituted derivatives with identical group at both positions (N-1, O-3). For derivatives with different groups at these positions, the synthetic **route B** is a two-step procedure involving, first, the preparation of the indazolol derivatives with the benzyl or methyl substituents at position 1, and then introduction of the substituent at O-3 position using different arylalkyl or cycloalkylmethyl halides.

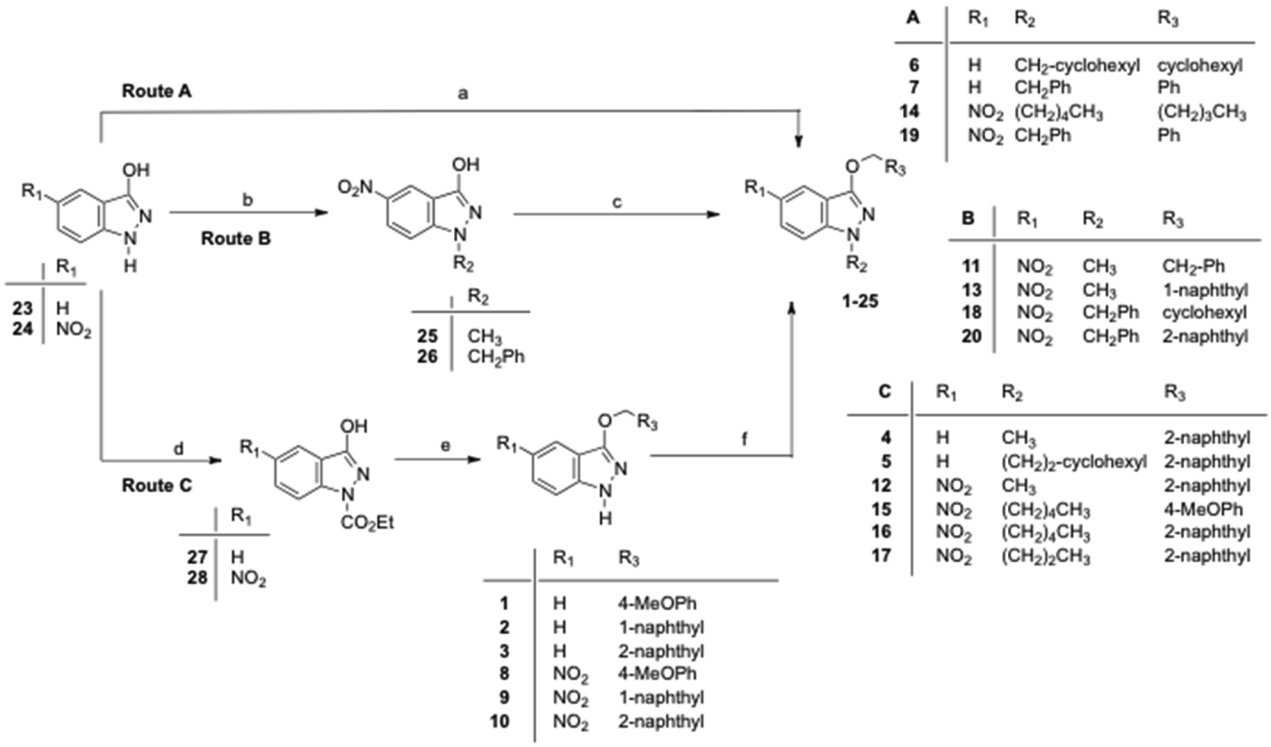
A more versatile and general synthetic route for the formation of substituted indazoles, the **route C**, comprises three steps involving

Table 1

Binding affinities (K_i, μM) and selectivity for CB1 and CB2 receptors of indazole derivatives **1–22**.



Compd	R_1	R_2	R_3	K_i CB1 (μM)	K_i CB2 (μM)	Selectivity K_i CB1/K_i CB2
1	H	H	4-MeOPh	21 ± 6	4.5 ± 0.8	4.7
2	H	H	1-naphthyl	1.6 ± 0.1	0.44 ± 0.08	3.6
3	H	H	2-naphthyl	21.7 ± 20.8	7 ± 2	3.1
4	H	CH_3	2-naphthyl	4 ± 2	10.1 ± 2.4	0.4
5	H	$(CH_2)_2$-cyclohexyl	2-naphthyl	0.15 ± 0.09	> 40	< 0.004
6	H	CH_2-cyclohexyl	cyclohexyl	0.2 ± 0.2	1.3 ± 0.6	0.2
7	H	CH_2-Ph	Ph	2.6 ± 0.9	3.5 ± 0.1	0.7
8	NO_2	H	4-MeOPh	> 40	1.8 ± 0.2	> 22.2
9	NO_2	H	1-naphthyl	> 40 (38%)	0.9 ± 0.2	> 44.4
10	NO_2	H	2-naphthyl	13.6 ± 5.4	5 ± 1	2.7
11	NO_2	CH_3	(CH_2)Ph	> 40 (25%)	1.2 ± 0.5	> 33.3
12	NO_2	CH_3	2-naphthyl	> 40	3.5 ± 2.5	> 11.4
13	NO_2	CH_3	1-naphthyl	≥40	0.25 ± 0.06	≥160.0
14	NO_2	$(CH_2)_4CH_3$	$(CH_2)_3CH_3$	1.3 ± 0.6	0.68 ± 0.04	1.9
15	NO_2	$(CH_2)_4CH_3$	4-MeOPh	0.8 ± 0.2	0.49 ± 0.06	1.6
16	NO_2	$(CH_2)_4CH_3$	2-naphthyl	1.6 ± 0.1	1.37 ± 0.06	1.2
17 (PGN38)	NO_2	$(CH_2)_2CH_3$	2-naphthyl	0.356 ± 0.006	0.47 ± 0.04	0.8
18 (PGN36)	NO_2	CH_2-Ph	cyclohexyl	> 40 (39%)	0.09 ± 0.03	> 444.4
19	NO_2	CH_2-Ph	Ph	1.2 ± 0.3	0.4 ± 0.1	3.0
20	NO_2	CH_2-Ph	2-naphthyl	> 10	0.4 ± 0.2	> 25.0
21	NH_2	$(CH_2)_2CH_3$	2-naphthyl	1.6 ± 0.2	2.9 ± 0.7	0.6
22	NH_2	CH_2-Ph	Ph	4 ± 1	1.6 ± 0.3	2.5



Reagents and conditions: a) R_2-X (X=Br or I), K_2CO_3 or Cs_2CO_3, acetone, reflux; b) benzyl bromide, sodium hydroxide, H_2O or methyl iodide, K_2CO_3, acetone, reflux c) Ph-$(CH_2)_2$Br or R_3-CH_2-X (X=Br or Cl), K_2CO_3, 2-butanone or Cs_2CO_3, acetone, reflux; d) ClCOOEt, pyridine, rt; e) 1) R_3-CH_2-X (X=Br or Cl), K_2CO_3, 2-butanone, reflux; 2) KOH / EtOH, rt; f) R_2-X (X=Br or I), K_2CO_3, 2-butanone, reflux

Scheme 1. Synthetic routes to prepare indazole derivatives **1**–**25**.

protection of position N-1, formation of the corresponding ether derivative, subsequent deprotection and introduction of the second desired arylmethyl or alkyl substituents at the N-1 position.

Following the **route A**, different N-1 substituted indazole ethers were prepared by reaction of indazolol **23** or 5-nitroindazolol **24**[31] with the corresponding halides in acetone or butanone and caesium or potassium carbonate as base (Table 1, Scheme 2). Thus, the synthesis of indazole ethers **6**[29] and 7 were carried out starting from **23** and cyclohexylmethyl or benzyl bromides, respectively. Similarly, the 5-nitroindazole ethers **14**[29] and **19**[32] were prepared by reaction of 5-nitro-3-indazolol **24** with the corresponding pentyl or benzyl halides, respectively.

To synthesize 1,3-disubstituted indazoles with different groups at N-1 position and ether function the synthetic **route B** were followed starting from 5-nitro-3-hydroxyindazole **24**. Thus, the reaction of 5-nitroindazole **24** with methyl iodide or benzyl bromide afforded **25**[33] and **26**,[34] respectively. Then, subsequent O-alkylation of the indazoles **25** and **26** with the corresponding halides were carried out to obtain the 5-nitroindazoles **11**, **13**, **18**,[29] and **20**.[29]

Thus, the preparation of indazole ether **20** [29] was carried out with 2-naphthylmethyl bromide from 5-nitro-1-benzylindazole **26**. Following the same **route B**, the 1-methyl derivatives **11** and **13** were obtained from 5-nitro-3-indazolol **25** with phenethyl bromide or with 1-naphthylmethyl chloride in acetone, using K_2CO_3 as base, respectively. Similarly, the reaction of 1-benzyl-3-indazole **26** with cyclohexylmethyl bromide afforded the corresponding indazole ether **18** (PGN36).

As mentioned before the **route C** has been especially useful to the preparation of indazole ethers bearing aminoethyl groups at N-1 position.[29] Therefore, route C is the more versatile and suitable procedure to introduce any group and has been used for the preparation of the indazole ethers **4**, **5**, **12**, **15**–**17**. Thus, the first step is the reaction of ethyl chloroformate with the indazole derivatives **23** and **24** afforded the corresponding 1-ethoxycarbonyl derivatives **27**[34] and **28**,[32] respectively.

The second step is the formation of the ether function and subsequent deprotection. Thus, the reaction of **28** with the corresponding halides afforded the 5-nitro ether derivatives of 2-naphthylmethoxy **10**,[29] 1-naphthylmethoxy **9** and 4-methoxybenzyloxy **8**. Similarly, the preparation of 1-naphthyl, 2-naphthyl and 4-methoxyphenyl derivatives **2**, **3**, and **1**[29] were carried out in the same conditions from indazolol **27**. All these products were carried out using the procedure described for us.[29]

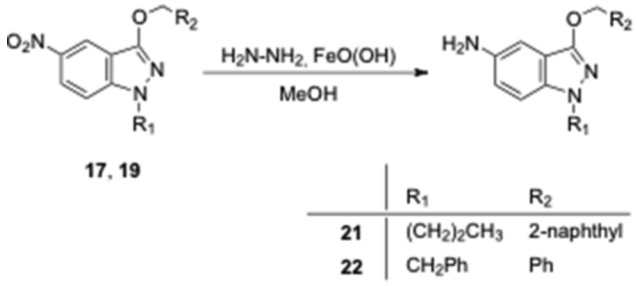
The last step of route C is to introduce the appropriate group in position N1 in order to obtain alkyl N1-substituted indazole ethers. Thus, the preparation of the 3-(2-naphthylmethoxy)indazoles **4** and **5** were carried out from **3**[29] with the corresponding alkyl halide to afford the 1-methyl derivative **4** and 1-cyclohexylethyl derivative **5**. Similarly, the reaction of the 5-nitro-2-naphthylmethoxyindazole **10**[29] with the corresponding alkyl iodides afforded the methyl, propyl and pentyl derivatives, **12**, **17** (PGN38) and **16**.[29] The 4-methoxybenzylderivative **15**[29] was carried from **8** with pentyl iodide.

Finally, the 5-amino derivative **21**, was obtained by catalytic reduction with ferric oxyhydroxide (FeO(OH))of 5-nitroderivative **17** (PGN38) with hydrazine, following the method described for 4-amino-3- benzyloxy-1-benzylindazole **18** [29] (Scheme 2).

2.2. Biological assays

2.2.1. In vitro binding studies in cannabinoid receptors.

Radioligand displacement assays were used to evaluate the affinity of the new compounds **1**–**22** using membranes from cells (HEK293EBNA) transfected with the CB1R or the CB2R and [^{3}H]-CP55940 as radioligand. Several indazole ethers showed low solubility at concentrations greater than 10 μM under test conditions (see material and methods section), therefore precluding the determination of their quantitative value of K_i for these compounds.



Scheme 2. Synthetic route for 5-amino indazole ethers.

All synthesized compounds together with their binding affinity for the CB1R and CB2R are gathered in Table 1. Examination of these data indicates that all of the evaluated derivatives bind to the CB2R, except the 1-cyclohexylmethyl derivative **5**. The obtained data shown that the 3-cyclohexylmethoxy derivative **18** (PGN36) is the most potent and the derivative with greatest affinity in CB2R. In relation to CB1R, all 5*H* derivatives assays showed some affinity. Regarding, the 5-nitroindazoles **8**, **12**, and **13** not exhibit affinity to 40 μM. However, the 1-alkyl-5-nitro derivatives **15** and **17** showed the lowest values of K_i.

Regarding CB2 selectivity, it is interesting to mark several compounds as the 5-nitro compounds **8**, **11**, **20**, the 5*H*-indazole **4**, the 5-amine derivative **18** and especially the derivatives **9** and **18** (PGN36) and the 1-methyl-indazoles **12**, **13**, due to interesting pharmacological profiles that they will be discussed later.

Receptors binding studies were performed using membrane fractions of human CB1R or CB2R transfected cells (HEK293EBNA). K_i value of WIN55212-2 is 36.2 nM in CB1 receptor. K_i values of WIN55212-2 and HU308 are 3.7 and 11.2 nM in CB2, respectively.

2.2.2. Cannabinoid activity: isolated tissue assays

According to the objectives, all derivatives that showed activity as cannabinoid ligands have been studied in detail in isolated tissues. Thus, the functional activity of the new compounds **1**–**22** has been tested on mouse vas deferens (MVD), a tissue commonly used to study and characterize cannabinoid effects.[29,30,35,36] In MVD cannabinoid agonists, acting at prejunctional cannabinoid receptors, reduce ATP and noradrenaline release and inhibit the electrically evoked smooth muscle contractions. In this tissue, cannabinoid receptor antagonists oppose the inhibitory effect of cannabinoid receptor agonists in a competitive and surmountable manner. CB1 and CB2-like cannabinoid receptors seem to be involved in this effect.[37–41]

The ability of indazoles **1**–**22** to inhibit the effect of WIN 55212-2 in this tissue was investigated. Variations produced by **1**–**22**, on the inhibition of electrically induced contractions evoked by WIN 55212-2, are shown in Table S1. Compounds **1–3**, **5–8**, **10**, **11**, **14**, **16**, **and 19–21** were devoid of antagonist properties when added to the organ bath 10 min before the addition of WIN 55212-2 at increasing concentrations. However, cannabinoids **4**, **15**, **17** (PGN38), **22** and, specially, selective CB2R indazoles **9**, **12**, **13**, and **18** (PGN36), antagonized the effect of WIN 55212-2 as shown in Figs. 1 and 2. These results indicate that in the MVD compounds **9**, **12**, **13**, and **18** (PGN36) behave as cannabinoid receptor antagonists with similar or higher effect to the reference cannabinoid receptor antagonist AM251.

Considering that the indazole derivatives **4**, **9**, **12**, **13**, **15**, **17** (PGN38), **18** (PGN36), and **22** showed an interesting profile as potential cannabinoid receptor antagonists, they were tested at the same concentrations used to WIN 55212-2 (in the range from 10^{-7} to 1.82×10^{-5} M). The results of these experiments with AM251 and with the cited indazoles indicate that they did not induce significant modification of contractile responses at the tested concentrations and therefore, it could be established that these indazoles did not show any intrinsic activity (Fig. 3, Table S2).

The obtained data suggest that these indazoles behave as antagonists with a better profile than AM630 and that they do not display the inverse agonist activity described for other cannabinoid receptor antagonists and then, considering these results and those obtained in the binding studies, **9**, **12**, **13**, and **18** (PGN36) could be considered as neutral cannabinoid CB2R antagonists.

2.2.3. Tool as antagonist ligands

Endocannabinoids are present in the skeleton suggesting that skeletal endocannabinoid system plays an important role in the regulation of bone mass.[42,43] Bone cells express CBRs and the machinery for endocannabinoid metabolism, thereby indicating that endocannabinoids influence bone remodelling acting on CB1R and CB2R expressed on bone cells. It has been recently reported that bone remodelling is subjected to CNS,[44–47] which is also associated with the regulation of endocannabinoid brain levels.[48] However, the role of the CBRs in bone disorder is not clear due to the contradictory results of published works.[43] Different reasons could be responsible for these experimental inconsistencies, such as the species differences, off-target effects of CBR ligands at different concentrations, the activity of endogenous cannabinoids and the interactions of ligands with other receptors such as GPR55.[49] GPR55 is a G protein-coupled receptor that is activated by certain cannabinoids and by lysophosphatidylinositol (LPI). This receptor that has been expressed in human and mouse osteoclasts and osteoblasts is implicated in bone physiology by regulating osteoclast number and function.[50]

In this sense, having the biochemical tools necessary to get unravel the role of the cannabinoid ligands is of the utmost importance.

To confirm the antagonist activity of these compounds and therefore

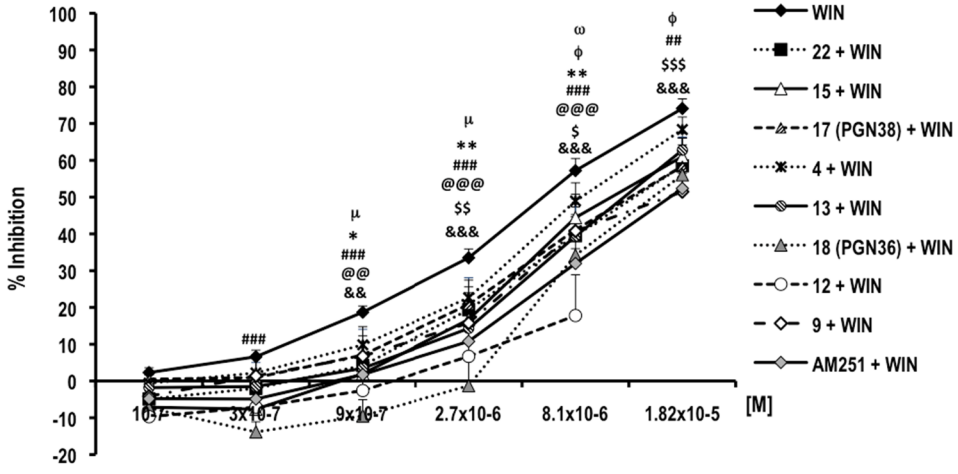


Fig. 1. Antagonist effect of the new derivatives in mouse isolated vas deferens (MVD). Lines show the % of inhibition (expressed as mean ± S.E.M., n = 6–8) of the electrically induced contractions of the MVD by addition of increasing concentrations of WIN 55,212–2 in control tissues (WIN) or in tissues incubated with the new compounds **4**, **9**, **12**, **13**, **15**, **17** (PGN38), **18** (PGN36), **22** or the cannabinoid receptor antagonist AM251 (10^{-6} M) that were added to the organ bath 10 min before each concentration of WIN. A two-way ANOVA followed by Bonferroni *post-hoc* test was used for statistical analysis. *vs.* WIN ($\mu,^{*,\omega,\phi,\$}p < 0.05$; $^{@,\&,\star,\$}p < 0.01$; $^{\#,@,\&,\$}p < 0.001$): ω**22** + WIN, μ**15** + WIN, ϕ**17** (PGN38) + WIN, *__13__ + WIN, $^{\#}$**18** (PGN36) + WIN, $^{@}$**12** + WIN, $^{\$}$**9** + WIN, $^{\&}$ AM251 + WIN.

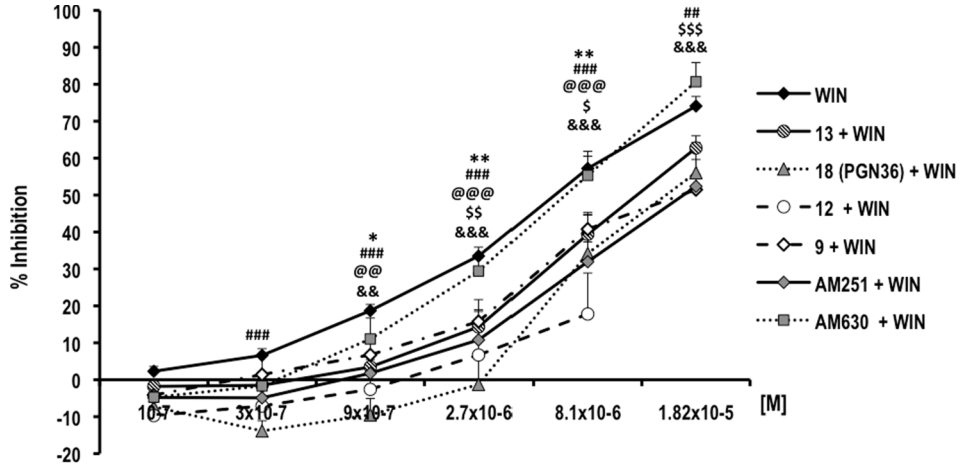


Fig. 2. Antagonist effect of indazoles **9, 12, 13, and 18** (PGN36) in mouse vas deferens (MVD). The graph shows the modification induced by the new indazoles on the inhibitory effect of WIN 55,212–2 (expressed as mean % of inhibition of the contractions ± S.E.M., n = 6–8) in tissues incubated with the new compounds or the cannabinoid receptor antagonists AM251 or AM630 (10^{-6} M) and in comparison with the effect of WIN in control tissues. A two-way ANOVA followed by Bonferroni *post-hoc* test was used for statistical analysis. *vs.* WIN ($^{*,\$}p < 0.05$; $^{@,\&,\star,\$}p < 0.01$; $^{\#,@,\&,\$}p < 0.001$): ***13** + WIN, $^{\#}$**18** (PGN36) + WIN, $^{@}$**12** + WIN, $^{\$}$**9** + WIN, $^{\&}$ AM251 + WIN.

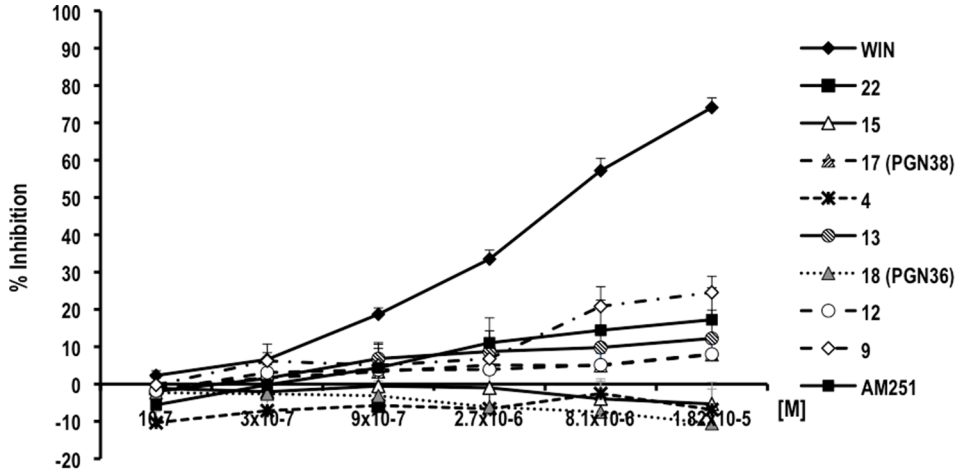


Fig. 3. Effect of WIN 55,212–2 (WIN), AM251, **4**, **9**, **12**, **13**, **15**, **17** (PGN38), **18** (PGN36) and **22** in isolated mouse vas deferens. Lines show the mean % ± S.E.M. (n = 6–8) of inhibition of the electrically induced contraction of this tissue by addition of increasing concentrations to the organ bath.

the ability to be used as biochemical tools, we used them in the mouse osteoblastic MC3T3-E1 cell line to study the osteoblast proliferation in assay on primary osteoblasts (obtained from the calvaria of new born mice). The osteoblastic cell line MC3T3-E1, a cellular system able to express CBR2 upon osteogenic conditions,[51] was used to study the effect of **17** (PGN38) and **18** (PGN36) on bone cell activity. The concentration of 10^{-6} M used of both CBR antagonists did not affect cell viability (Fig. 4).

Collagen type I gene expression was used as marker of bone activity. Neither **17** (PGN38) nor **18** (PGN36) were able to modify collagen type I gene expression (Fig. 5), indicating no effect of both compounds on bone activity. By contrast, HU308 10^{-8} M significantly increased the collagen type I gene expression compared with the control group but also with the other two concentrations used (10^{-6} M and 10^{-7} M, Fig. 6). This effect of HU308 on bone activity has been reported by some authors using similar concentrations to the used in our *in vitro* system.[52,53]

While cells treated only with HU308 10^{-8} M significantly increased the collagen type I gene expression, preincubations during the first 24 h with **17** (PGN38) 10^{-6} M or **18** (PGN36) 10^{-6} M totally abolished this effect showing similar values to the observed in the control group (Fig. 7).

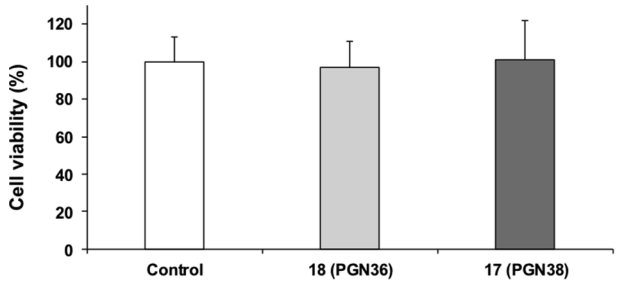


Fig. 4. Cell viability in MC3T3-E1 cells treated with **17** (PGN38) 10^{-6}M, **18** (PGN36) 10^{-6}M, and vehicle. Experiments were carried out three times in sextuplicate.

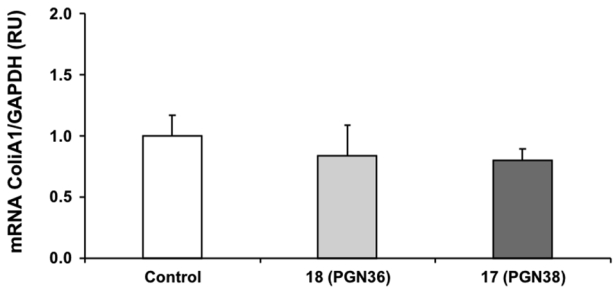


Fig. 5. Gene expression of Collagen type I in MC3T3-E1 cells treated with **17** (PGN38) 10^{-6}M, **18** (PGN36) 10^{-6}M, and vehicle. UR = relative units. Experiments were carried out three times in triplicates.

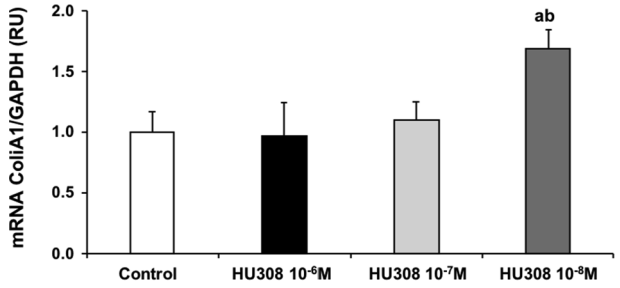


Fig. 6. Gene expression of Collagen type I in MC3T3-E1 cells treated with HU308 10^{-6}M, 10^{-7}M and 10^{-8}M. [a]p < 0.05 compared with control group; [b]p < 0.05 compared with HU308 10^{-7}M and 10^{-8}M. UR = relative units. Experiments were carried out three times in triplicates.

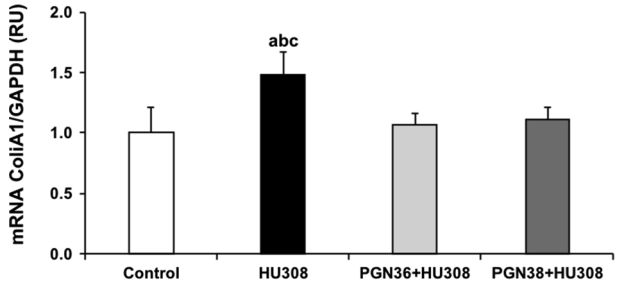


Fig. 7. Gene expression of Collagen type I in MC3T3-E1 cells pre-treated 24 h with **18** (PGN36) 10^{-6}M, **17** (PGN38) 10^{-6}M and HU308 10^{-8}M and 48 additional hours with HU308 10^{-8}M. [a]p < 0.05 compared with control group; [b]p < 0.05 compared with **18** (PGN36) 10^{-6}M; [c]p < 0.05 compared with **17** (PGN38) 10^{-6}M. Experiments were carried out three times in triplicates.

3. Conclusions

Indazole derivatives have been synthesized and identified as a new class of cannabinoid antagonists. Structural requirements for activity are the presence of one aromatic group, one H or alkyl group at position 1 and the nitro group at position 5. According to binding studies and tissues assays there are four mixed CB1/CB2 cannabinoid antagonists and four are selective agonist CB2R. In particular compound **18** (PGN36) showed greater affinity and selectivity by CB2R.

An interesting idea suggested about pharmacological application of cannabinoid antagonists is related to the treatment of certain bone disorders. Nevertheless, the performed studies have shown that cannabinoids antagonists either by CB1 or by CB2 action have no effect in MC3T3-E1 celular assays.

However, the selective CB2R cannabinoid agonist as HU308 stimulates osteoblast proliferation in assay on primary osteoblasts (obtained from the calvaria of new born mice) being completely abolish by the antagonist PGN36 and PGN38.

In conclusion, we present the CB2 antagonist **18** (PGN36) and the non-selective antagonist **17** (PGN38) that are able to reverse the effect of HU308 in assay on primary osteoblasts. Moreover, some antagonist described in this work as the compounds **13** (PGN8) and **9** (PGN70) already were used as pharmacological tool to demostrate that the agonist effect of WIN 55212-2 on multiple myeloma (MM) cells is mediated through CB2R.[54] Thus, the pro-apoptotic effect of WIN 55212-2 is inhibited through the pre-incubation with **13** (PGN8) in both U266 and RPMI cell lines.

Therefore, this new family of cannabinoid receptor antagonists are promising candidates as pharmacological tool.

4. Materials and methods

4.1. Synthesis

4.1.1. General

All starting materials were purchased from common commercial suppliers, mostly Sigma-Aldrich and Alfa Aesar, and were used without further purification. All the reactions were monitorized by TLC. Analytical thin-layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} plates and the compounds were visualized under UV light (λ = 254 or 365 nm). ^{1}H NMR spectra (300 MHz) and ^{13}C NMR spectra (75 MHz) were recorded on a Bruker Avance 300 spectrometer and are reported in ppm. The signal of the solvent was used as reference. Flash column chromatography was carried out using Merck silica gel 60 (230–400 mesh). HPLC-MS was performed using a Waters 2695 apparatus with a diodo array UV/Vis detector Waters 2996 and coupled to a Waters micromass ZQ using a Sunfire C_{18} (4.6 × 50 mm, 3.5 mM) column at 30 °C, with a flow rate of 1 mL / min. The mobile phases used were with different gradients of CH_3CN with 0.1% of formic acid in H_2O. The initial conditions, time of gradient (gt) and time of retention (rt) are specified in each case. Electrospray in positive mode was used for ionization. The sample injection volume was set 5 μL of a solution of 1 mg / mL CH_3CN. Melting points were determined with a Reichert-Jung Kofler apparatus. Elemental analysis were performed on a Heraeus CHN-O Rapid Analysis apparatus. The purity of all compounds was > 95% prior to biological testing. 1*H*-3-indazol-ol (**23**) was purchased on Alfa Aesar and used without further purification. 5-nitro-3-indazol-ol (**24**) was prepared from the procedure reported by Pfannstiel.[31] 1-benzyl-3-benzyloxy-5-nitroindazole (**19**) and 1-ethoxycarbonyl-5-nitro-3-indazolone (**28**) were prepared from the procedure reported by Arán et al.[32] 1-benzyl-5-nitro-3-indazolone **(26)** and 1-ethoxycarbonyl-3-indazolone (**27**) were prepared from the procedure reported by Palazzo.[34] The compounds **11** and **25** were synthesized by methods described in reference [33]. The compounds **1–3**, **6–10**, **14**, **15**, **16**, **18**, **20**, **and 22** were described in reference [29].

4.1.2. General procedure for the synthesis of the products 4, 5, 12, 17

To a suspension of 1*H*-3-indazolylether derivative in butanone, K_2CO_3 were added. The suspension was stirred and heated to reflux and the corresponding halide in excess was added and the reaction was maintained at reflux until the complete elimination of starting material. Then, it was cooled and filtered to remove inorganic salts. The remaining solvent was evaporated under reduced pressure and the product was purified by crystallization or by silica gel column chromatography using the appropriate solvents. Reaction times, conditions and specific treatments are described individually for each compound.

4.1.3. 1-Methyl-3-(2-naphthylmethoxy)indazole (4)

From **3** (0.30 g, 1.10 mmol), methyl iodide (0.18 mL, 3.40 mmol) and K_2CO_3 (0.33 g, 2.34 mmol) in 2-butanone (20 mL). The final product was purified by chromatography column using as eluent methylene chloride: hexane (1:2, 2:1). Reaction time: 24 h. Yield: (0.06 g, 52%). Oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.04 (bs, 1H, Ar); 7.96–7.91 (m, 3H, Ar); 7.66–7.62 (m, 2H, 4-H, Ar); 7.55–7.47 (m, 3H, 7-H, Ar); 7.38 (t, *J* = 7.8 Hz, 1H, 6-H); 7.03 (t, *J* = 7.8 Hz, 1H, 5-H); 5.55 (s, 2H, CH_2); 3.87 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 155.6 (C-3); 142.0 (C-7a); 127.3 (C-6); 120.1 (C-5); 118.9 (C-4); 112.5 (C-3a); 108.5 (C-7); 70.7 (CH_2); 35.1 (CH_3); 134.5 (Ar); 133.3 (Ar); 133.1 (Ar); 128.2 (Ar); 128.1 (Ar); 127.7 (Ar); 126.8 (Ar); 126.1 (Ar); 126.0 (Ar); 125.8 (Ar). HPLC-MS (ES^+)**:** CH_3CN/H_2O 20:80, gt: 18 min; rt: 16.50, $[M + H]^+$ = 289.3.

4.1.4. 1-(2-Cyclohexyl)ethyl-3-(2-naphthylmethoxy)indazole (5)

From **3** (0.48 g, 1.85 mmol), 2-(cyclohexyl)ethyl bromide (0.30 mL, 1.92 mmol) and K_2CO_3 (0.62 g, 4.5 mmol) in 2-butanone (50 mL). The final product was purified by chromatography column using as eluent

methylene chloride: hexane (1:2, 5:1). Reaction time: 96 h. Yield: (0.42 g, 60%). Oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.05 (bs, 1H, Ar); 7.95–7.91 (m, 3H, Ar); 7.70–7.62 (m, 2H, Ar, 4-H); 7.54–7.48 (m, 3H, 7-H, Ar); 7.37 (t, 1H, 6-H); 7.02 (t, 1H, 5-H); 5.59 (s, 2H, O-CH_2); 4.23 (t, J = 7.1 Hz, 2H, N1-CH_2); 1.78–1.57 (m, 7H, CH_2Cy); 1.30–1.15 (m, 4H, Cy); 0.98–0.90 (m, 2H, Cy). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 155.4 (C-3); 141.2 (C-7a); 126.9 (C-6); 120.1 (C-5); 118.8 (C-4); 112.5 (C-3a); 108.5 (C-7); 70.7 (O-CH_2); 46.3 (N1-CH_2); 35.9 (C, Cy); 34.1 (CH_2Cy); 32.1 (2C, Cy); 25.5 (C, Cy); 25.2 (2C, Cy); 134.6 (Ar); 133.3 (Ar); 133.1 (Ar); 128.1 (Ar); 128.0 (Ar); 127.7 (Ar); 127.0 (Ar); 126.1 (Ar); 126.0 (Ar); 125.9 (Ar). HPLC-MS (ES^+): CH_3CN/H_2O 10:90, gt: 8 min; rt: 7.45, $[M + H]^+$ = 385.4.

4.1.5. 1-Methyl-3-(2-naphthylmethoxy)-5-nitroindazole (12)

From **10** (0.15 g, 3.93 mmol), methyl iodide (0.90 g, 4.01 mmol) and K_2CO_3 (2.57 g, 18.57 mmol), in 2-butanone (60 mL). The final product was purified by chromatography column using as eluent methylene chloride: hexane (1:1, 5:1). Reaction time: 24 h. Yield: (0.10 g, 58%). mp. 131–132 °C (2-propanol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.72 (d, J = 2.2 Hz, 1H, 4-H); 8.24 (dd, J = 9.3 Hz, J = 2.2 Hz, 1H, 6-H); 7.99 (bs, 1H, Ar); 7.92–7.85 (m, 3H, Ar); 7.64–7.61 (m, 1H, Ar); 7.53–7.48 (m, 2H, Ar); 7.24 (d, J = 9.2 Hz, 1H, 7-H); 5.60 (s, 2H, CH_2); 3.95 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 157.5 (C-3); 143.0 (C-7a); 140.9 (C-5); 122.6 (C-6); 118.7 (C-4); 112.0 (C-3a); 108.5 (C-7); 71.19 (CH_2); 35.6 (CH_3) 133.6 (Ar); 133.2 (Ar); 128.4 (Ar); 128.0 (Ar); 127.8 (Ar); 127.4 (Ar); 126.3 (Ar); 126.2 (Ar); 125.7 (Ar). HPLC-MS (ES^+)**:** CH_3CN/H_2O 10:90, gt: 8.00 min, rt: 6.16, $[M + H]^+$ = 334.1.

4.1.6. 3-(2-Naphthylmethoxy)-5-nitro-1-propylindazole (17)

From **10** (0.17 g, 0.52 mmol), 1-propyl iodide (0.10 mL, 1.02 mmol) and K_2CO_3 (0.35 g, 2.53 mmol) in 2-butanone (30 mL). The product was isolated by recrystallization from 2-propanol. Reaction time: 48 h. Yield: (0.10 g, 55%). mp. 95–97 °C (2-propanol). ^{1}H NMR (300 MHz, $CDCl_3$) 8.71 (d, J = 2.2 Hz, 1H, 4-H); 8.22 (dd, J = 9.2 Hz, J = 2.2 Hz, 1H, 6-H); 7.99 (bs, 1H, Ar); 7.91–7.85 (m, 3H, Ar); 7.65–7.62 (m, 1H, Ar); 7.52–7.49 (m, 2H, Ar); 7.24 (d, J = 9.1 Hz, 1H, 7-H); 5.61 (s, 2H, O-CH_2); 4.19 (t, J = 6.9 Hz, 2H, N1-CH_2); 1.94 (q, 2H, CH_2); 0.92 (t, J = 7.3 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 157.8 (C-3); 142.1 (C-7a); 141.1 (C-5); 122.8 (C-6); 119.2 (C-4); 112.3 (C-3a); 108.9 (C-7); 71.5 (O-CH_2); 50.9 (N1-CH_2); 23.4 (CH_2); 11.7 (CH_3); 134.1 (Ar); 133.6 (Ar); 128.7 (Ar); 128.4 (Ar); 128.1 (Ar); 127.6 (Ar); 126.7 (Ar); 126.6 (Ar); 126.2 (Ar). HPLC-MS (ES^+): CH_3CN/H_2O 15:95, gt: 8.00 min, rt: 6.87, $[M + H]^+$ = 362.4.

4.1.7. 1-Methyl-3-(1-naphthylmethoxy)-5-nitroindazole (13)

To a solution of **25** (0.06 g, 0.19 mmol) in acetone (60 mL), was added Cs_2CO_3 (0.06 g, 0.40 mmol). The suspension was stirred and heated to reflux and 1-naphthylmethyl chloride (0.06 g, 0.32 mmol) was added. The reaction was maintained at reflux until the complete elimination of starting material. Then, it was cooled and filtered to remove inorganic salts. The remaining solvent was evaporated under reduced pressure and the product was purified by chromatography column using as eluent methylene chloride: hexane (1:1, 5:1). Reaction time: 24 h. Yield: (0.04 g, 67%). mp 152–155 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.64 (d, J = 2.2 Hz, 1H, 4-H); 8.24 (dd, J = 9.1 Hz, J = 2.1 Hz, 1H, 6-H); 8.15 (d, 1H, Ar); 7.98–7.87 (m, 2H, Ar); 7.70 (d, 1H, Ar); 7.60–7.48 (m, 3H, Ar); 7.24 (d, J = 9.2 Hz, 1H, 7-H); 5.89 (s, 2H, CH_2); 3.99 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 157.5 (C-3); 143.0 (C-7a); 140.8 (C-5); 122.6 (C-6); 118.7 (C-4); 112.0 (C-3a); 108.5 (C-7); 69.5 (CH_2); 35.6 (CH_3); 133.8 (Ar); 131.7 (Ar); 131.6 (Ar); 129.4 (Ar); 128.7 (Ar); 127.8 (Ar); 126.6 (Ar); 126.0 (Ar); 125.3 (Ar). HPLC-MS (ES^+)**:** CH_3CN/H_2O 10:90, gt: 8.00 min, rt: 6.12, $[M + H]^+$ = 334.1.

4.1.8. 1-Benzyl-3-cyclohexylmethoxy-5-nitroindazole (18)

To a solution of **26** (0.10 g, 0.38 mmol) in 2-butanone (60 mL), was added K_2CO_3 (0.14 g, 0.98 mmol). The suspension was stirred and heated to reflux and cyclohexylmethyl bromide (0.10 mL, 0.70 mmol) was added. The reaction was maintained at reflux until the complete elimination of starting material. Then, it was cooled and filtered to remove inorganic salts. The remaining solvent was evaporated under reduced pressure and the product was purified by silica gel column chromatography using as eluent a mixture methylene chloride: hexane (1:1, 5:1). Reaction time: 24 h. Yield: (0.08 g, 60%). mp. 72–75 °C (2-propanol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.67 (d, 1H, 4-H); 8.17 (dd, 1H, 6-H); 7.30 (d, 1H, 7-H); 7.29–7.15 (m, 5H, Ar); 5.41 (s, 2H, N1-CH_2); 4.20 (d, 2H, O-CH_2); 1.93–1.09 (m, 11*H*, CyHex). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 158.7 (C-3); 143.1 (C-5); 141.3 (C-7a); 137.6 (C, Ar); 129.3 (2C, Ar); 128.4 (C, Ar); 127.5 (2C, Ar); 123.0 (C-6); 119.2 (C-4); 113.2 (C-3a); 109.2 (C-7); 71.5 (O-CH_2); 53.3 (N1-CH_2); 38.0 (CH_2); 30.1 (2C, CH_2); 26.9 (CH_3); 26.2 (2C, CH_2). HPLC-MS (ES^+): CH_3CN/H_2O 10:90, gt: 8 min; rt: 7.07, $[M + H]^+$ = 366.4.

4.1.9. 5-Amino-3-(2-naphthylmethoxy)-1-propylindazole (21)

To a suspension of **17** (44 mg, 0.12 mmol), and FeO(OH) (3 mg, 0.03 mmol) in methanol (15 mL) is added monohydrated hydrazine (3.0 mL, 7.9 mmol) under Argon atmosphere. The suspension was stirred and heated at 70 °C until the complete elimination of starting material. Then, the suspension is filtered over zelite to eliminate the catalyst. After evaporate the solvent under reduced pressure, the crude was suspended on water and extracted with diethylic ether. The organic solvent was removed at reduced pressure to obtain **21** as oil. Reaction time: 3 h. Yield: (0.028 g, 70%). Oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.97 (bs, 1H, Ar); 7.88–7.84 (m, 3H, Ar); 7.63 (dd, J = 8.2 Hz, J = 1.5 Hz, 1H, Ar); 7.50–7.47 (m, 2H, Ar); 7.09 (d, J = 8.8 Hz, 1H, 7-H); 6.93 (d, J = 2.1 Hz, 1H, 4-H); 6.84 (dd, J = 8.8 Hz, J = 2.2 Hz, 1H, 6-H); 5.56 (s, 2H, O-CH_2); 4.10 (t, J = 7.0 Hz, 2H, N1-CH_2); 3.18 (bs, 2H, NH_2); 1.86 (m, 2H, CH_2); 0.89 (t, J = 7.4 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 153.5 (C-3); 137.8 (C-5); 136.4 (C-7a); 118.2 (C-6); 111.9 (C-3a); 108.5 (C-7); 102.0 (C-4); 69.7 (O-CH_2); 49.2 (N1-CH_2); 22.2 (CH_2); 10.4 (CH_3); 133.8 (Ar); 132.3 (Ar); 132.1 (Ar); 127.1 (Ar); 127.0 (Ar); 126.7 (Ar); 125.9 (Ar); 125.1 (Ar); 125.0 (Ar); 124.9 (Ar). HPLC-MS (ES^+): CH_3CN/H_2O 10:90, gt: 5.00 min, rt: 3.90, $[M + H]^+$ = 333.1.

5. Biological methods

5.1. Radioligand binding assays for CB1 and CB2 receptors

CB1/CB2 receptor binding studies of indazole ether derivatives **1–22** (Table 1) were performed using membrane fractions of human CB1/CB2 receptor transfected cells purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). HEK293EBNA membranes were resuspended in Tris buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL BSA fatty acid free, pH 7.4). Fractions of the final membrane suspension (about 0.415 mg/mL of protein for CB1 and about 0.18 mg/mL of protein for CB2) were incubated at 30 °C for 90 min with 0.54 nM [^{3}H]-CP55940 (139.6 Ci/mmol) for CB1 and 0.33 nM [^{3}H]-CP55940 (139.6 Ci/mmol) for CB2, in the presence or absence of several concentrations of the competing drug, in a final volume of 0.2 mL for CB1 and 0.6 mL for CB2 of assay buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL BSA fatty acid free, pH 7.4). Nonspecific binding was determined in the presence of 10 μM WIN 55,212–2. Silanized tubes were used throughout the experiment to minimize receptor binding loss due to tube adsorption. The reaction was terminated by rapid vacuum filtration with a filter mate Harvester apparatus (Perkin-Elmer) through Filtermat A GF/C filters presoaked in 0.05% polyethylenimine (PEI).

The filters were washed nine times with ice-cold buffer for CB1 (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL BSA fatty acid free, pH 7.4) for CB2 (50 mM Tris-HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 1 mg/mL BSA fatty acid free, pH 7.5), and bound radioactivity was measured with a 1450 LSC & Luminiscence counter Wallac

MicroBeta TriLux (Perkin-Elmer). The binding assay showed the appropriate sensitivity to CB1 and CB2 ligands. Thus, WIN 55,212–2 inhibited the binding with a *K*i value of 36.2 nM (CB1R) and WIN 55,212–2 and HU308 inhibited the binding with K_i values of 3.7 and 11.2 nM (CB2R), respectively. For all binding experiments, competition binding curves were analyzed by using an iterative curve-fitting procedure GraphPad,[55] which provided IC50 values for test compounds. K_i values were determined by the method of Cheng and Prusoff.[56,57]

5.1.1. Functional activity for cannabinoid receptors on isolated tissue

The functional activity of the compounds **1**–**22** for CBRs was evaluated on the mouse vas deferens preparation. This is a nerve-smooth muscle preparation that serves as a highly sensitive and quantitative functional *in vitro* bioassay for cannabinoid receptor agonists. These ligands induce concentration-related decreases in the amplitude of electrically evoked contractions of the vas deferens by acting on naturally expressed prejunctional neuronal cannabinoid receptors to inhibit release of the contractile neurotransmitters, noradrenaline and ATP, that is provoked by the electrical stimulation.[38]

For this study, male ICR mice weighing 25–30 g were used. Mouse vas deferens were isolated as described by Hughes.[58] Tissues were suspended in a 10 mL organ bath containing 5 mL of Krebs solution (NaCl 118; KCl 4.75; $CaCl_2$ 2.54; KH_2PO_4 1.19; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11 mM) that was continuously gassed with carbogen (95% O_2 and 5% CO_2). Tissues were kept under 0.5 g of resting tension at 37 °C and were electrically stimulated through two platinum ring electrodes. They were subjected to alternate periods of stimulation (trains of five rectangular pulses of 70 V, 15 Hz and 2 ms duration each were applied every minute) and rest (10 min). The isometric force was monitored by computer using a MacLab data recording and analysis system.

The effect of the synthetic cannabinoid agonist WIN 55212-2 and that of the new compounds (10^{-7}–1.82×10^{-5} M) was tested by constructing concentration-response curves in a step-by-step manner. Curves were carried out by the following protocol: WIN 55212-2 or the new compounds were added at a concentration to the organ bath 50 min after the beginning of electrical stimulation and their effect on the electrically induced contractions was evaluated 10 min after their addition. Then, the electrical stimulation was stopped, Krebs solution was replaced and the following concentration of the compound was added. This protocol was repeated for every concentration of the curve.

In order to check the antagonist profile of the new compounds, they were added to the organ bath at a concentration of 10^{-6} M 10 min before each addition of the increasing concentrations of WIN 55212-2 and their effect were compared with that of the cannabinoid antagonists AM251 and AM630. For some compounds, the antagonist effect was evaluated in experiments where the concentration-response curve of WIN 55212-2 ranged between 3×10^{-8} and 8.1×10^{-6} M.

5.1.2. Functional activity of cannabinoids on bone

The mouse osteoblastic MC3T3-E1 cell line (ATCC® CRL2593™) was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Vervier, Belgium) supplemented with 10% fetal bovine serum (Lonza), 100 μg/mL streptomycin (Lonza) and 100 U/mL penicillin (Lonza) and at 37 °C in a humidified 5% CO_2 atmosphere. Cells were grown to confluence and then were placed in 96-wells and 6-wells plates with the same culture medium.

5.1.3. Cell viability assay

To confirm whether the **18** (PGN36) and **17** (PGN38) compounds at 10^{-6} M concentrations were able to modify cell viability a MTT assay was carried out. MTT reduction only occurs in metabolically active cells. In 96-wells plates, cells were placed in a culture medium containing DMEM with 10% fetal bovine serum during 24 h. After that, the medium was replaced by DMEM culture medium supplemented with **18** (PGN36) 10^{-6} M, **17** (PGN38) 10^{-6} M or vehicle (control group) during 48 h.

5.1.4. Collagen type I gene expression

In 6-well plates, cells were placed in DMEM culture medium with 10% fetal bovine serum. After 24 h, unattached cells were removed, and the attached cells were cultured in osteogenic medium containing DMEM, 10% fetal bovine serum, 50 μg/mL L-ascorbic acid, 10^{-9} M dexamethasone, and 10 mM b-glycerophosphate (Saint Louis, MO, USA). The cells were treated with **18** (PGN36) 10^{-6} M, **17** (PGN38) 10^{-6} M, HU308 (10^{-6} to 10^{-8} M) and vehicle and maintained at 37 °C in a fully humidified atmosphere at 5% CO_2 in air during 72 h.

In order to evaluate if **18** (PGN36) and **17** (PGN38) antagonize the effect of the agonist HU308, cells were placed in 6-wells plates with DMEM culture medium supplemented with 10% fetal bovine serum. After 24 h, the attached cells were pre-treated with **18** (PGN36) 10^{-6} M, **17** (PGN38) 10^{-6} M and HU308 10^{-8} M and cultured in the same osteogenic medium. After 24 h, the medium of cells was replaced by the osteogenic medium containing only the agonist HU308 (10^{-8} M) except the control group that received vehicle. The cells were maintained at 37 °C in a fully humidified atmosphere at 5% CO_2 in air during 48 additional hours.

Total RNA from MC3T3-E1 cells was extracted using TRI reagent (Sigma-Aldrich). cDNA was synthesized from 2 mg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (qPCR) reactions were performed in triplicate using the Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA), FastStart Universal Probe Master (Roche Applied Science, Indianapolis, IN), and predeveloped assays (Applied Biosystems) for collagen type I and GAPDH. Relative quantification of target genes was performed by comparing threshold cycles using the ΔΔCTmethod, as described previously.[59]

Author contributions

The manuscript was realizad through contributions of all authors. All authors have approved this version of the manuscript.

Declaration of Competing Interest

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

Acknowledgements

This work was supported from Ministerio de Ciencia e Innovación under Grant RTI2018-096100B-100, Plan Nacional de I + D + I 2013-2016, ISCIII–FEDER (FIS16/00637), Plan de Ciencia, Tecnología e Innovación 2013-2017 and 2018-2022 del Principado de Asturias (GRUPIN14-028, IDI-2018-000152), RedInRen from ISCIII (RD16/0009/0017). Natalia Carrillo López was supported by GRUPIN14-028 and IDI-2018-000152. The authors would like to thank to Guadalupe Pablo for their excellent technical assistant.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115672.

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