European Journal of Medicinal Chemistry 203 (2020) 112606

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

PSNCBAM-1 analogs: Structural evolutions and allosteric properties at cannabinoid CB1 receptor

Serena Meini [a, 1], Francesca Gado [a, 1], Lesley A. Stevenson [b], Maria Digiacomo [a], Alessandro Saba [c], Simone Codini [c], Marco Macchia [a], Roger G. Pertwee [b], Simone Bertini [a, *], Clementina Manera [a]

[a] Department of Pharmacy, University of Pisa, Via Bonanno 6, 56126, Pisa, Italy
[b] School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, AB25 2ZD Aberdeen, Scotland, UK
[c] Department of Surgical Pathology, Molecular Medicine and of the Critical Area, University of Pisa, Via Savi 10, 56126, Pisa, Italy

ARTICLE INFO

Article history:
Received 15 April 2020
Received in revised form
17 June 2020
Accepted 18 June 2020
Available online 12 July 2020

Keywords:
Allosteric modulator
CB1 receptors
Endocannabinoid system
PSNCBAM-1
Diaryl urea
GTP
S

ABSTRACT

Allosteric modulation of the CB1Rs could represent an alternative strategy for the treatment of diseases in which these receptors are involved, without the undesirable effects associated with their orthosteric stimulation. PSNCBAM-1 is a reference diaryl urea derivative that positively affects the binding affinity of orthosteric ligands (PAM) and negatively affects the functional activity of orthosteric ligands (NAM) at CB1Rs. In this work we reported the design, synthesis and biological evaluation of three different series of compounds, derived from structural modifications of PSNCBAM-1 and its analogs reported in the recent literature. Almost all the new compounds increased the percentage of binding affinity of CP55940 at CB1Rs, showing a PAM profile. When tested alone in the [^{35}S]GTPγS functional assay, only a few derivatives lacked detectable activity, so were tested in the same functional assay in the presence of CP55940. Among these, compounds **11** and **18** proved to be functional NAMs at CB1Rs, dampening the orthosteric agonist-induced receptor functionality by approximately 30%. The structural features presented in this work provide new CB1R-allosteric modulators (with a profile similar to the reference compound PSNCBAM-1) and an extension of the structure-activity relationships for this type of molecule at CB1Rs.

1. Introduction

The endocannabinoid system (ECS) is ubiquitously distributed in the human organism and involved in important physiological and pathological mechanisms [1]. It consists of specific G protein-coupled receptors (cannabinoid receptor type 1 and 2, called CB1Rs and CB2Rs respectively) endogenous ligands, called endocannabinoids (the two main ones are anandamide and 2-arachidonoylglycerol) and it also comprises all the enzymes and transporters responsible for the biosynthesis, degradation and transport of endocannabinoids [1–3].

CB1Rs are mainly expressed in the central nervous system (CNS), although they can also be found in peripheral districts such as adipose tissue, cardiovascular system, gastro-intestinal tract, liver, pancreas, bone and skeletal muscle [4]. CB1Rs have a role in inhibiting the release of neurotransmitters in the CNS, affecting processes like memory, learning, motor functions and pain transmission. On the other hand, peripheral CB1Rs are involved in metabolism, energy balance, regulation of food intake and body weight gain [5–7]. CB2Rs are mostly present in immune cells where they regulate the release of pro-inflammatory cytokines [8,9], and they are also expressed in some brain regions [7,10,11].

In spite of their theorical usefulness, the use of CB1R orthosteric ligands in therapy is limited because of the onset of severe side effects. In particular, CB1R orthosteric agonists, although being effective in the treatment of pain, nausea, inflammation, cardiovascular disorders, multiple sclerosis and cancer, can also produce psychotropic effects, strong mood alteration, acute psychosis, and cognitive and motor impairments [12,13]. On the other hand, CB1R orthosteric antagonists/inverse agonists, besides their useful application in counteracting obesity, insulin resistance, osteoporosis, nicotine and alcohol addiction, can also give rise to

* Corresponding author.
E-mail address: simone.bertini@unipi.it (S. Bertini).
[1] S. M. and F. G. contributed equally.

https://doi.org/10.1016/j.ejmech.2020.112606
0223-5234/

depression, mood alterations and suicidal tendency [14–16].

For this reason, the need of an alternative therapeutic strategy in order to avoid these side effects and the discovery on CB1Rs of an allosteric binding site in 2005, led to further investigations in this field [17–19]. Allosteric modulators (AMs), binding to a topographically distinct site, stabilize a unique conformation of the receptor, influencing the binding affinity and/or the efficacy of orthosteric ligands [20–22]. They are characterized by a saturable effect, giving less risk of overdose, since the extent of their efficacy is dependent on the local concentration of endogenous ligands (endocannabinoids in the case of CBRs). This feature is also related to an improved selectivity, because AMs can provide an effect only in the tissues and at the time endocannabinoids are released, without interfering with the physiological signaling pathways [23]. Moreover, given the lower conservation of allosteric binding sites, the development of more selective compounds acting at these sites should be more easily achievable [20,24]. AMs can be classified as positive (PAMs) if they enhance the binding affinity and/or the efficacy of the orthosteric ligand, or negative (NAMs) if the binding affinity and/or the efficacy of the orthosteric ligand is decreased [25,26]. The well-known CB1R allosteric modulator PSNCBAM-1 (compound **1**, Fig. 1), characterized by a diaryl urea structure, showed divergent properties, affecting the binding affinity and the efficacy of orthosteric ligands in opposite directions at CB1R (PAM in binding and NAM in functionality) [27]. Given its usefulness in counteracting obesity, influencing food intake without affecting behavior, PSNCBAM-1 is a reference compound which can be taken as a starting point for structural modifications in order to achieve improved pharmacological properties [28].

To deepen the knowledge of the structure-activity relationships at CB1R for this class of compound, in this work we reported the design, synthesis and biological evaluation of three different series of compounds, derived from PSNCBAM-1 and its analogs reported in the recent literature [29,30]. The structure activity relationships studies performed up to date showed that the pyridinyl ring can be successfully replaced by a phenyl ring, giving the active compound **2** (Fig. 1) with a biological profile resembling the activity of PSNCBAM-1 at CB1R [29]. This consideration led us to the design of "A" series, comprising compounds **10**, **11** and **12** (Fig. 1). **10** and **11** are characterized by different substituents at the extremities of the molecule, to better understand the stereo-electronic requirements of these portions at CB1R. On the other hand, **12**, homologue of compound **3** (Fig. 1), recently reported in literature [29], was designed to establish if increasing the degrees of freedom of the molecule, could improve its activity. Another structural feature taken in account, is the insertion of an amine spatial linker in the biaryl system, similar to compounds **4** and **5** (Fig. 1), which displayed good properties as allosteric modulators with a behavior similar to that of PSNCBAM-1 [29]. On these bases, compounds **13** and **14** ("B" series, Fig. 1) were designed. Both compounds possess two pyridine rings to fully elucidate the importance of the nature of the biaryl system components. Another recent study proved that the pyrrolidinyl ring is not required for CB1R allosteric modulation and the pyridinyl ring could be replaced by other aromatic rings [30]. Some of the most promising derivatives are reported in Fig. 1 (compounds **6**–**9**). All these new derivatives displayed a PAM profile in binding assays and behaved as NAMs in functional assays, like the parental compound. Combining these structural features with the presence of the amine spatial linker, we designed compounds **15**–**19** ("C" series, Fig. 1). In the following paragraphs we described the synthetic routes of the new derivatives and their biological evaluation obtained by carrying out [^{3}H]CP55940 and [^{35}S]GTPγS binding assays.

2. Results and discussion

2.1. Chemistry

The synthetic procedure to obtain compounds of "A" series is reported in Scheme 1. 1,3-dibromobenzene (**20**) was heated at 80 °C in toluene with $Pd_2(dba)_3$, BINAP, *t*-BuONa and the suitable secondary cyclic amine, to afford intermediates **21** or **22**. These derivatives were subjected to a cross-coupling reaction with 3-nitrophenylboronic acid in presence of $Pd(OAc)_2$, PPh_3 and an aqueous solution of $NaHCO_3$ in DME at 95 °C, to obtain compounds **23** and **24**. The nitro group was then reduced with Ni-Raney and hydrazine hydrate in a mixture of ethanol and water at 50 °C affording derivatives **25** and **26** that were treated with the suitable phenylisocyanate to give the desired products **10** and **11**, respectively. For the preparation of **12**, the intermediate **25** was reacted with the acyl chloride **27** (synthesized from commercial 3-(4-chlorophenyl)propionic acid (**28**) and thionyl chloride), in the presence of Et_3N and DMAP in anhydrous DCM.

The synthesis of compounds of "B" series is reported in Scheme 2. Commercial 2,6-diaminopyridine (**29**) was reacted with commercial 2,6-dibromopyridine in presence of *t*-BuOK in refluxing

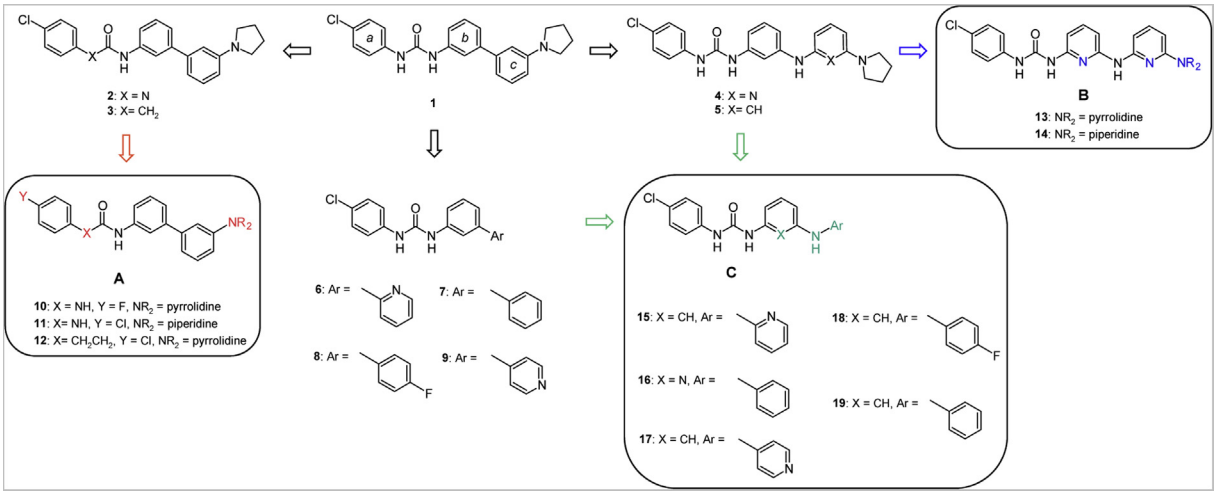
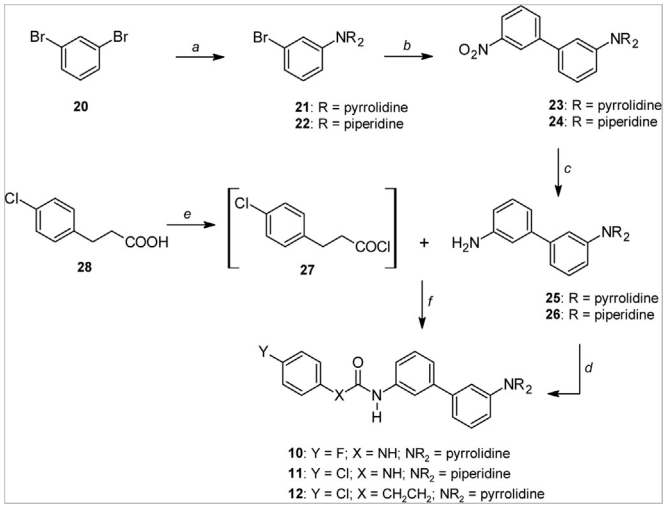
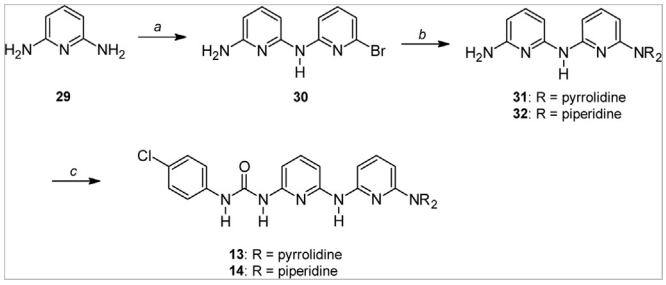


Fig. 1. Design of novel structural modifications of PSNCBAM-1 based on the derivatives recently reported in the literature [29,30].



Scheme 1. Synthetic route for the preparation of compounds of "A" series. Reagents and conditions: *a)* pyrrolidine or piperidine, $Pd_2(dba)_3$, BINAP, *t*-BuONa (mol ratio: 0.05:0.15:2), toluene, 80 °C, 4.5 h; *b)* 3-nitrophenylboronic acid, $Pd(OAc)_2$, PPh_3, aq. $NaHCO_3$, DME, 95 °C, overnight; *c)* Ni-Raney, $NH_2NH_2 \cdot H_2O$, abs. EtOH, 50 °C, 40 min–1 h; *d)* *p*-chlorophenylisocyanate or *p*-fluorophenylisocianate, anhydrous $CHCl_3$, rt, overnight; *e)* $SOCl_2$, rt, overnight; *f)* Et_3N, DMAP, anhydrous DCM, rt, overnight.



Scheme 2. Synthetic route for the preparation of compounds of "B" series. Reagents and conditions: *a)* 2,6-dibromopyridine, *t*-BuOK, anhydrous toluene, reflux, 48 h; *b)* pyrrolidine or piperidine, reflux, 12 h; *c)* *p*-chlorophenylisocianate, anhydrous $CHCl_3$, rt, overnight.

anhydrous toluene. The obtained intermediate **30** was refluxed with the suitable cyclic amine to afford compounds **31** and **32**, which were then reacted with *p*-chlorophenylisocyanate at room temperature in anhydrous chloroform to give the desired products **13** and **14**, respectively.

The synthetic route for the preparation of compounds of "C" series is described in Scheme 3. For the synthesis of compounds **15**, **18** and **19**, commercial 1,3-diaminobenzene (**33**) was refluxed with the suitable aryl halide in presence of *t*-BuOK in anhydrous toluene, to obtain intermediates **34**–**36**. These were then reacted with *p*-chlorophenylisocyanate at room temperature in anhydrous chloroform to give the desired compounds **15**, **18** and **19**.

For the synthesis of compound **16**, commercial 2,6-diaminopyridine (**29**) was reacted with iodobenzene in presence of *t*-BuOK and copper iodide, in anhydrous 1,4-dioxane at 100 °C, to afford the intermediate **37** that was treated with *p*-chlorophenylisocyanate as described above to give the desired compound **16**.

For the synthesis of compound **17**, commercial 1-bromo-3-nitrobenzene (**38**) was treated with 4-aminopyridine, xantphos and $Pd_2(dba)_3$ in anhydrous 1,4-dioxane in presence of Cs_2CO_3, at 95 °C, affording the derivative **39**, which was reduced with iron powder and ammonium chloride in a mixture of ethanol/water, at reflux, affording derivative **40**. This was then treated with *p*-chlorophenylisocyanate as described above to give the desired compound **17**.

2.2. [^{3}H]CP55940 binding assay (CB1R)

To evaluate CB1R binding, different concentrations of compounds **10**–**19** (from 1 nM up to 10 μM) were incubated with hCB1-CHO cell membrane preparations in presence of [^{3}H]CP55940 (0.700 nM), a high-affinity orthosteric CB1R and CB2R radioligand.

Almost all the compounds belonging to "A" series (characterized by a biphenyl moiety) and "B" series (characterized by a bipyridinyl system separated by an amine linker) (Figs. 2 and 3) resulted PAMs in binding assays, enhancing the binding of [^{3}H]CP55940 by approximately 70% (**10**), 60% (**13**) and 50% (**14**) up to 10 μM in a dose-dependent trend. Compound **11** displayed an enhancement of binding (about 40%) up to 1 μM, being completely ineffective at 10 μM. This behavior might be possibly due to a strong decrease of solubility which occurs when the concentration of **11** is raised to 10 μM. Compound **12**, the only amide derivative, did not influence the binding and behaved as a weak orthosteric ligand at 10 μM (Fig. S1 in Supplementary Data).

As to "C" series (Fig. 4), compounds **15**, **18** and **19** showed a PAM profile, dose-dependently increasing the binding percentage of [^{3}H]CP55940 by approximately 170% (**15**) and 90% (**18** and **19**). **15** displayed this trend up to 10 μM, whereas compounds **18** and **19** revealed a slow decrease of the activity above 1 μM. Finally, compounds **16** and **17** affected the binding of [^{3}H]CP55940 only at a neglectable extent (Fig. S2 in Supplementary Data).

2.3. [^{35}S]GTPγS assay

Different concentrations of compounds **10**, **11**, **13**–**15**, **18** and **19** (from 1 nM up to 10 μM) were tested in the [^{35}S]GTPγS assay, using hCB1-CHO membrane preparations, in order to determine their ability to affect CB1R functionality on their own. This evaluation was performed in order to establish if the compounds do produce a functional effect by themselves, since this behavior would preclude their pure allosteric nature.

Compounds **13** and **14** did not significatively alter the cellular response at CB1R up to 10 μM (Fig. 5).

On the other hand, compound **10**, **15** and **19** proved to decrease the cellular response by 45% (**10**) and approximately 30% (**15** and **19**) showing an inverse agonist profile (Fig. S3 in Supplementary Data).

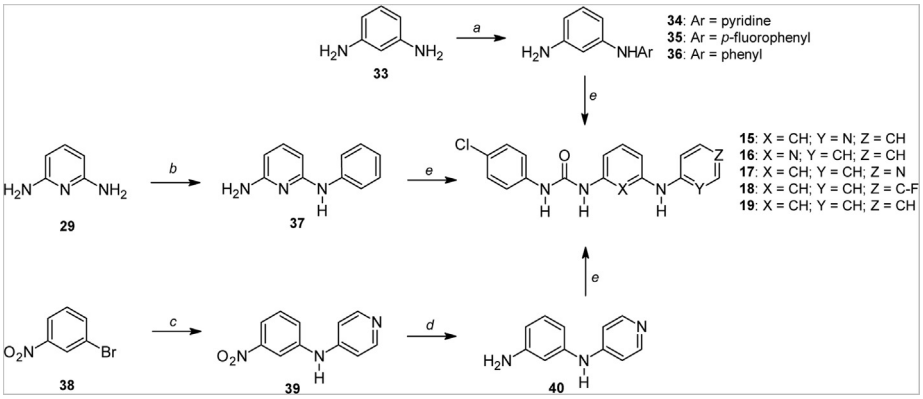
Compound **11** and **18** showed no significant alteration of receptor functionality up to 1 μM and a slight decrease only above this concentration, by approximately 30% and 35%, respectively (Fig. 6).

Because of the above results, compounds **11**, **13**, **14** and **18** (at a chosen concentration of 1 μM) were tested in [^{35}S]GTPγS functional assays, against different concentrations of CP55940 (from 0.1 nM up to 1 μM), to evaluate their ability to modulate the CB1R-mediated functional activity induced by the orthosteric agonist.

While **13** and **14** did not influence the activity of the orthosteric ligand (Fig. S4 in Supplementary Data), when CP55940 was co-administered with **11** and **18**, the receptor functionality was dampened by approximately 30%, suggesting that **11** and **18** behave as functional NAMs at CB1R. Compound **18**, albeit only to a minor extent, retained its activity in this assay also at 100 nM (Fig. 7).

2.4. [^{3}H]CP55940 binding assay (CB2R)

Compounds **11** and **18** were also tested in CB2R binding experiments (from 1 nM up to 10 μM) in the presence of [^{3}H]CP55940



Scheme 3. Synthetic route for the preparation of compounds of "C" series. Reagents and conditions: *a)* 2-brompyridine or 4-bromofluorobenzene or iodobenzene, *t*-BuOK, anhydrous toluene, reflux, 48 h; *b)* iodobenzene, *t*-BuOK, CuI, anhydrous 1,4-dioxane, 100 °C, 24 h; *c)* 4-aminopyridine, xantphos, $Pd_2(dba)_3$, Cs_2CO_3, anhydrous 1,4-dioxane, 95 °C, 24 h; *d)* Fe^0 powder, NH_4Cl, EtOH/H_2O, reflux, 4 h; *e)* *p*-chlorophenylisocianate, anhydrous $CHCl_3$, rt, overnight.

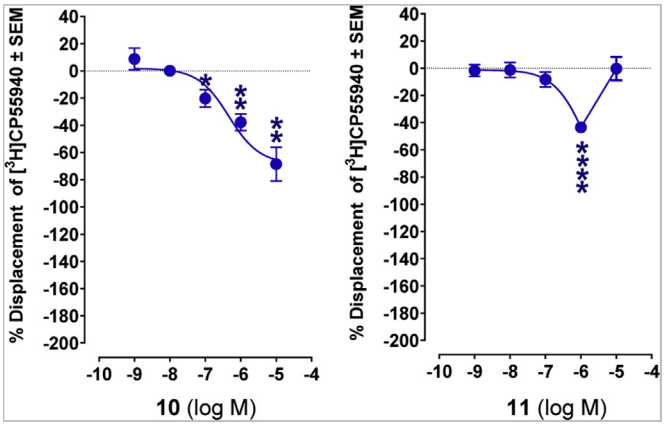


Fig. 2. Effects of compounds **10** and **11** ("A" series) (at 1 nM to 10 μM) on [^{3}H]CP55940 binding to CB1R. Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01; ****P < 0.0001) (one sample *t*-test). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

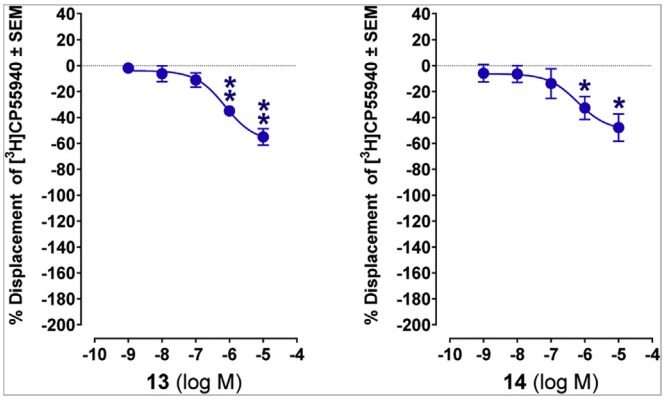


Fig. 3. Effects of compounds **13** and **14** ("B" series) (at 1 nM to 10 μM) on [^{3}H]CP55940 binding to CB1R. Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01) (one sample *t*-test). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

(0.700 nM), using hCB2-CHO cell membrane preparations, in order to assess their receptor subtype selectivity (Fig. 8). Derivative **11** produced a displacement of the orthosteric agonist above 1 μM, so further experiments in hCB2-CHO cells would be necessary in order to clarify its receptor subtype selectivity and its pharmacological effects. On the other hand, compound **18** did not affect the binding affinity of CP55940 at any concentration, displaying a high selectivity for CB1R over CB2R.

3. Conclusions

In this work we have reported the design, synthesis and biological evaluation of new derivatives of the known CB1R allosteric modulator PSNCBAM-1. The structural modifications are based on recent structural analysis described in literature, with the purpose of extending information about the structure activity relationships at CB1R of diaryl urea derivatives. As to the "A" series (characterized by a biphenyl system) compounds **10** and **11** behaved as PAMs in radioligand binding assays against CP55940, whereas **12**, the only amide derivative, did not display any detectable activity in this binding assay. These results suggest that the replacement of the chlorine atom on phenyl "*a*" with a fluorine atom (Fig. 1), combined with a biphenyl system, is tolerated and the substitution of pyrrolidine with piperidine does not remarkably change the activity. On the other hand, the urea group seems fundamental for the activity, as was previously claimed by Bertini et al. [29], and the increase of the degrees of freedom of this portion of the molecule does not provide any improvement. Compound **10** behaved as an inverse agonist in [^{35}S]GTPγS functional assays, whereas **11** inhibited the receptor functionality only above 10 μM, indicating that the strong electro-withdrawing (EWG) effect of fluorine might stabilize a receptor conformation endowed with higher constitutional activity, at least of this signaling pathway [31,32]. Moreover, **11** (1 μM) displayed a strong inhibition of the cellular response in the presence of CP55940, indicating a NAM behavior in functionality, with a profile similar to PSNCBAM-1. Regarding "B" series, where a bipyridinyl system is separated by an amine spatial linker, both compounds **13** and **14** behaved as PAMs in binding assay, not influencing the cellular response in [^{35}S]GTPγS functional assays either in the absence and in the presence of the orthosteric compound CP55940. This outcome confirms that the difference between pyrrolidine and piperidine on aryl ring "*c*" (Fig. 1) is negligible and might suggest that the presence of the bipyridinyl system together with the amine linker does not affect the influence on binding, but does stabilize a receptor conformation which cannot couple to G protein. Further investigations on this class of compounds should involve the functional assessment of other signaling pathways, such as β-arrestins recruitment and ERK phosphorylation, which could be differently influenced [33,34]. As

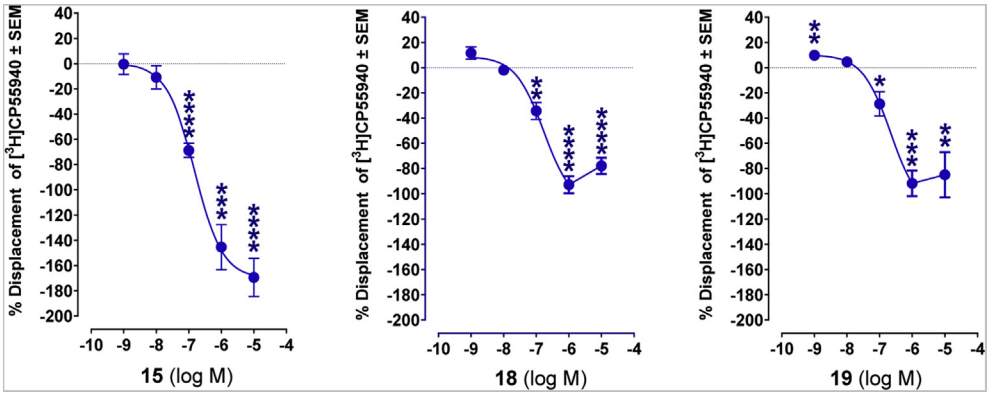


Fig. 4. Effects of compounds **15**, **18** and **19** ("C" series) (at 1 nM to 10 μM) on [^{3}H]CP55940 binding to CB1R. Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01; ***P < 0.001 ****P < 0.0001) (one sample *t*-test). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

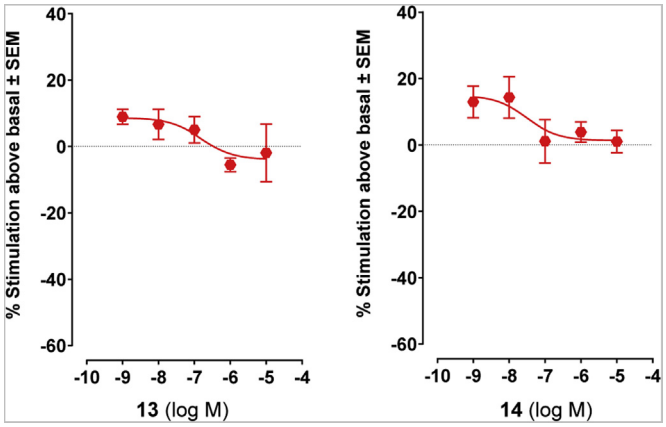


Fig. 5. [^{35}S]GTPγS assays performed with compounds **13** and **14** ("B" series). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

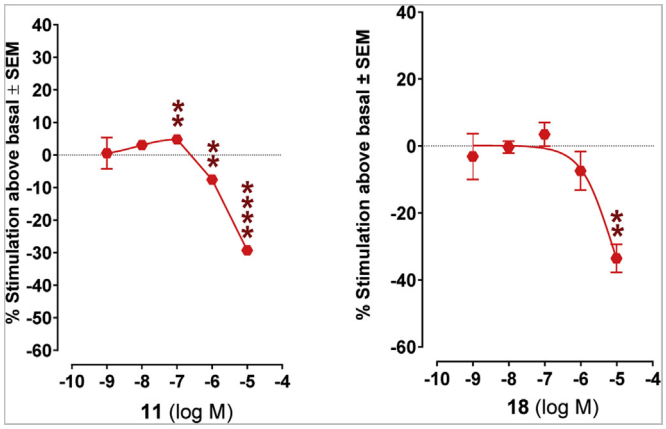


Fig. 6. [^{35}S]GTPγS assays performed with compound **11** ("A" series) and **18** ("C" series). Asterisks indicate mean values significantly different from zero (**P < 0.01; ****P < 0.0001) (one sample *t*-test). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

to compounds of "C" series, characterized by the presence of the amine spatial linker and the removal of the pyrrolidine moiety, only compounds **16** and **17** were ineffective in influencing the binding of CP55940 and the functionality at CB1R. **16** is the first derivative reported in the literature up to date, in which a pyridine ring replaces phenyl ring "*b*" (Fig. 1) and the results may indicate that a nitrogen atom is not tolerated at this position, in this kind of biaryl systems. A similar assumption can be made for **17**, which bears a nitrogen atom at position 4 of aryl ring "*c*" (Fig. 1). All the other compounds of "C" series behaved as PAMs in binding assays, but only **18** was unable to influence the receptor functionality on its own, decreasing the cellular response only in presence of CP55940. The fluorine atom, already related to a good activity both in terms of binding and of functionality and also to an increased metabolic stability by Nguyen et al. [30], might positively contribute thanks to its EWG effect combined with its small size, which makes it sterically comparable to a hydrogen with the ability to significantly change the lipophilicity and conformation of the molecule [31,32]. The remaining compounds of the series, **15** and **19**, behaved as inverse agonists in functional assays indicating that a biaryl system consisting of a phenyl and a pyridine separated by an amine linker (**15**) changes the kind of activity displayed by the compounds **4** and PSNCBAM-1 [29]. A similar behavior is displayed by **19**, which is directly derived from the combination of the structural features of **2** and **4** [29].

The new structural features analyzed in this work can be taken as starting points for further modifications of this kind of derivatives, providing an extension of the structural requirements at CB1R and potential novel strategies for developing new and improved allosteric modulators of CB1R.

4. Experimental section

4.1. Chemistry

Commercially available reagents were purchased from Sigma Aldrich or TCI chemicals and used without purification, if not differently indicated in the procedures. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III™ 400 spectrometer (operating at 400 MHz or 100 MHz, respectively). Chemical shifts (δ) are reported in parts per million related to the residual solvent signal, while coupling constants (J) are expressed in Hertz (Hz). All final compounds were analyzed by HPLC, showing a purity in the range 96–99%. A Bechman HPLC instrument equipped with a System Gold Solvent Delivery module (Pumps) 125, System Gold UV/VIS Detector 166 (set to 278 nm) was employed. Analyses were performed on a reverse phase C18 column (Phenomenex 250 × 4.6 mm, 5 mm particle size, Gemini). The mobile phase was constituted by a mixture of H_2O/AcOH (0.1% v/v) (eluent A) and ACN (eluent B). A gradient starting from 50% of B, changing to 100% of B over 20 min, and returning to the initial conditions over 10 min,

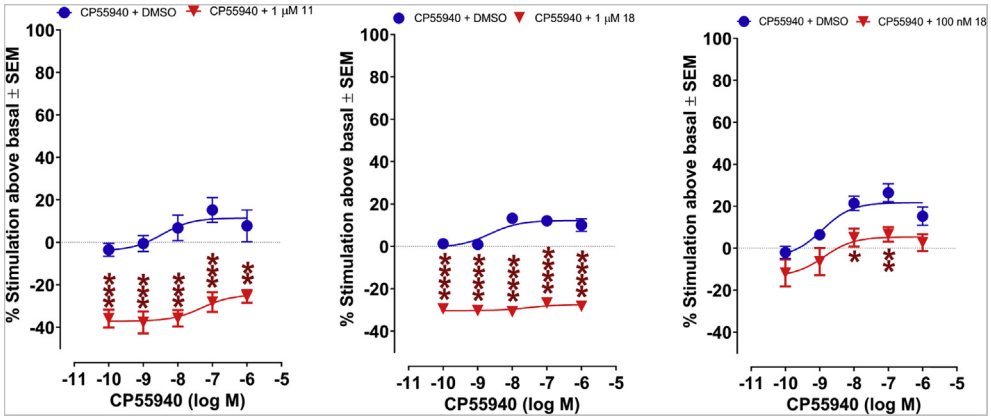


Fig. 7. CB1R [^{35}S]GTPγS experiments with compounds **11** ("A" series) and **18** ("C" series) against increasing concentrations of CP55940. Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01; ***P < 0.001 ****P < 0.0001) (one sample *t*-test). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

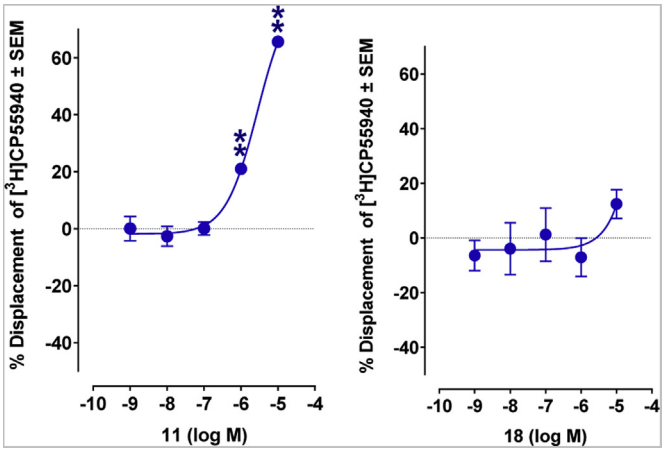


Fig. 8. Effects of compounds **11** ("A series) and **18** ("C" series) (at 1 nM to 10 μM) on [^{3}H]CP55940 binding to CB2R. Asterisks indicate mean values significantly different from zero (**P < 0.01) (one sample *t*-test). Data are expressed as the mean ± SEM of six independent experiments, each performed in duplicate.

was used for compounds **10**, **12**, **13**, **15**–**19**. For compounds **11** and **14** was chosen an isocratic elution with 80% of B and 20% of A. The flow rate was 1.0 mL/min. Retention times (HPLC, t_R) are given in minutes. Mass experiments were performed with an AB Sciex API 3000 triple quadrupole mass spectrometer, equipped with a TurboIonSpray source. Compounds were analyzed both in positive and negative mode; in Q1 scan mode, for the detection of pseudomolecular ions $[M+H]^+$ and $[M-H]^-$; to obtain structural information, MS-MS experiments were performed in product ion scan mode. Samples were dissolved in DMSO and diluted 1:1000 with an ACN/H_2O 50:50 (v/v) solution. The instrumental parameters used were: Curtain Gas: 12.0; Ion Spray Voltage: 5500 V; Temperature: 450 °C; Declustering Potential: 20.0; Entrance Potential: 10.0. Evaporation was carried out *in vacuo* using a rotating evaporator. Silica gel flash chromatography was performed using silica gel 60 Å (0.040–0.063 mm; MERK). Reactions was monitored by TLC on Merck aluminum silica gel (60 F254) plates that were visualized under a UV lamp ($\varepsilon = 254$ nm). Melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

4.1.1. General procedure A

The suitable phenylisocyanate (1.0 equiv) was added to a solution of aryl amine (1.0 equiv) and anhydrous chloroform (10.0 mL per 0.239 mmol of aryl amine) in a round bottom flask, under nitrogen atmosphere. The mixture was left under magnetic stirring overnight at room temperature.

4.1.2. General procedure B

The suitable phenylisocyanate (1.2 equiv) was added to a solution of aryl amine (1.0 equiv) and THF (2.7 mL per 0.440 mmol of aryl amine) in a round bottom flask. The mixture was refluxed for 1 h, then cooled to room temperature and the solvent was removed under reduced pressure. The residue was triturated with ethanol.

4.1.3. General procedure C

A mixture of the suitable aryl amine (1.0 equiv), *t*-BuOK (2.3 equiv) and the proper amount of aryl halide (1.0 equiv) were refluxed in anhydrous toluene (100.0 mL per 18.5 mmol of aryl amine) for 48 h, under inert atmosphere. After cooling to room temperature, the solvent was removed *in vacuo*, the residue dissolved in AcOEt and washed with water. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure.

4.1.4. General procedure D

In a round bottom flask, the suitable nitroarene (1.0 equiv) and hydrazine hydrate (0.14 equiv) were suspended in absolute ethanol (4.0 mL per 0.24 mmol of nitroarene). The mixture was stirred at 50 °C for 15 min to promote the complete solubilization of the reagents. Then, an excess of an aqueous suspension of Raney nickel was added (about 200 mg per 0.24 mmol of nitroarene) and the reaction, which changed from orange to colorless, was left under magnetic stirring at 50 °C until the bubbling ended. The mixture was filtered through a celite pad and washed with ethanol. The solution collected was evaporated under reduced pressure to obtain the desired product.

4.1.5. General procedure E

In a sealed tube, under nitrogen flux, commercial triphenylphosphine (0.4 equiv) and $Pd(OAc)_2$ (0.08 equiv) were dissolved in DME (5.0 mL per 0.3 mmol of triphenylphosphine) and the mixture was stirred for 15 min at room temperature. A solution of the suitable aryl halide (1.0 equiv) in DME (6.0 mL per 0.79 mmol of aryl halide) was added to the reaction tube, followed by the addition of $NaHCO_3$ (3.0 equiv), distilled water (4.5 mL per 0.79 mmol of aryl halide) and 3-nitrophenylboronic acid (1.0 equiv). The reaction mixture was stirred at 95 °C, overnight. After the removal of DME under reduced pressure, the residue was partitioned between AcOEt and distilled water. The organic phase was dried over

Na_2SO_4, filtered and evaporated under reduced pressure to obtain a dark oil.

4.1.6. General procedure F

In a sealed tube, commercial 1,3-dibromobenzene (1.0 equiv) was suspended in toluene (2.1 mL per 0.85 mmol of 1,3-dibromobenzene), followed by the addition of the suitable cyclic secondary amine (1.0 equiv) and the suitable amount of a reagent (157.2 mg of reagent per 0.85 mmol of starting material) constituted by tris(dibenzylideneacetone)dipalladium(0), BINAP and *t*-BuONa (mol ratio: 0.05:0.15:2). The reaction mixture was stirred at 80 °C for 4.5 h. After cooling to room temperature, the mixture was filtered under reduced pressure and the solution collected was evaporated under reduced pressure. The resulting residue was diluted with $CHCl_3$ and washed with a 10% aqueous solution of NaOH. After anhydrification over Na_2SO_4, the organic phase was filtered and evaporated under reduced pressure.

4.1.7. General procedure G

The suitable aryl halide (1.0 equiv) was refluxed with the suitable cyclic secondary amine (19.3 equiv) for 12 h. After cooling to room temperature, the excess of unreacted amine was removed under reduced pressure. The brown oil obtained was dissolved in $CHCl_3$ and washed with distilled water. The organic phase was then dried over Na_2SO_4, filtered and evaporated under reduced pressure.

4.1.8. 1-(3-bromophenyl)pyrrolidine (21)

Compound 21 was prepared from 1,3-dibromobenzene (0.10 mL, 0.85 mmol) and pyrrolidine according to general procedure F. Purification by flash column chromatography on silica gel, eluting with petroleum ether. 21 (190.5 mg, 0.843 mmol). Yield: 99%. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.06 (t, 1H, J = 8.0 Hz), 6.78–6.76 (m, 1H), 6.69–6.71 (m, 1H), 6.50–6.49 (m, 1H), 3.28–3.25 (m, 4H), 2.03–1.99 (m, 4H).

4.1.9. 1-(3-bromophenyl)piperidine (22)

Compound 22 was prepared from 1,3-diaminobenzene (0.50 mL, 4.24 mmol) and piperidine according to general procedure F. Purification by flash column chromatography on silica gel, eluting with petroleum ether and petroleum ether/AcOEt 9:1. 22 (676.3 mg, 2.81 mmol). Yield: 66%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.08 (t, 1H, J = 8.0 Hz), 7.04–7.03 (m, 1H), 6.92–6.89 (m, 1H), 6.84–6.81 (m, 1H), 3.17–3.14 (m, 4H), 1.71–1.66 (m, 4H), 1.61 (m, 2H).

4.1.10. 1-(3′-nitro[1,1′-biphenyl]-3-yl)pyrrolidine (23)

Compound 23 was prepared from 21 (179.1 mg, 0.793 mmol) according to general procedure E. Purification by flash column chromatography on silica gel (petroleum ether/CH_2Cl_2 7:3). 23 (115.0 mg, 0.43 mmol). Yield: 54%. ^{1}H NMR ($CDCl_3$) δ (ppm): 8.46–8.45 (m, 1H), 8.19–8.16 (m, 1H), 7.94–7.91 (m, 1H), 7.58 (t, 1H, J = 8.0 Hz), 7.33 (t, 1H, J = 8.0 Hz), 6.89 (d, 1H, J = 8.0 Hz), 6.75–6-74 (m, 1H), 6.65–6.63 (m, 1H), 3.38–3.35 (m, 4H), 2.07–2.03 (m, 4H).

4.1.11. 1-(3′-nitro[1,1′-biphenyl]-3-yl)piperidine (24)

Compound 24 was prepared from 22 (89.8 mg, 0.37 mmol) according to general procedure E. Purification by two subsequent flash column chromatographies on silica gel (petroleum ether/AcOEt 9:1 and petroleum ether/CH_2Cl_2 8:2). 24 (73.8 mg, 0.263 mmol). Yield: 70%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 8.44–8.43 (m, 1H), 8.19–8.17 (m,1H), 7.91–7.89 (m,1H), 7.58 (t, 1H, J = 7.9 Hz), 7.36 (t, 1H, J = 7.9 Hz), 7.15–6.98 (m, 1H), 7.07–6.98 (m, 2H), 3.26–3.23 (m, 4H), 1.78–1.71 (m, 6H).

4.1.12. 3′-(pyrrolidin-1-yl)[1,1′-biphenyl]-3-amine (25)

Compound 25 was prepared starting from 23 (63.8 mg, 0.24 mmol) according to general procedure D. 25 (58.5 mg, 0.233 mmol). Yield: 97%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.30 (t, 1H, J = 7.8 Hz), 7.23 (t, 1H, J = 7.8 Hz), 7.05–7.02 (m, 1H), 6.95–6.93 (m, 1H), 6.89–6.86 (m, 1H), 6.76–6.75 (m, 1H), 6.70–6.67 (m, 1H), 6.59–6.56 (m,1H), 3.36–3.33 (m, 4H), 2.06–2.01 (m, 4H).

4.1.13. 3′-(piperidin-1-yl)[1,1′-biphenyl]-3-amine (26)

Compound 26 was prepared from 24 (73.8 mg, 0.263 mmol) according to general procedure D. 26 (64.6 mg, 0.229 mmol) Yield: 87%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.31–7.25 (m, 1H), 7.22–7.19 (m,1H), 7.14–7.10 (m,1H), 7.03–6.96 (m, 2H), 6.95–6.88 (m, 2H), 6.68–6.64 (m,1H), 3.21–3.16 (m, 4H), 1.74–1.66 (m, 6H).

4.1.14. N-(4-fluorophenyl)-N′-[3′-(pyrrolidin-1-yl)[1,1′-biphenyl]-3-yl]urea (**10**)

Compound **10** was prepared from **25** (68.0 mg, 0.285 mmol), following general procedure A. Purification by flash column chromatography on silica gel (petroleum ether/AcOEt 7:3). **10** (28.7 mg, 0.076 mmol). Yield: 27%. M. p.: 185 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 8.74 (m, 2H), 7.70–7.69 (m, 1H), 7.49–7.45 (m, 2H), 7.43–7.41 (m, 1H), 7.33 (t,1H, J = 7.7 Hz), 7.23 (AA'XX', 2H, J_{AX} = 8.0 Hz, $J_{AA'XX'}$ = 1.5 Hz), 7.12 (AA'XX', 2H, J_{AX} = 8.0 Hz, $J_{AA'XX'}$ = 1.5 Hz), 6.82–6.80 (m, 1H), 6.71–6.69 (m, 1H), 6.56–6.53 (m, 1H), 3.30–3.27 (m, 4H), 1.99–1.96 (m, 4H). ^{13}C NMR: (DMSO-d_6) δ (ppm): 157.34 (d, J = 228.0 Hz), 152.65, 148.02, 141.93, 141.14, 140.01, 135.98 (d, J = 2.0 Hz), 129.40, 129.10, 120.38, 119.97 (d, J = 7.0 Hz), 117.09, 116.61, 115.22 (d, J = 22.0 Hz), 113.73, 110.91, 109.75, 47.30, 24.93. MS: the experiment in full scan mode showed: m/z 376 ($[M+H]^+$, 100); MS–MS of the ion at m/z 376 showed: m/z 265 ($[M+H]^+$ – *p*-Cl-C_6H_4-NH, 100), 239 ($[M+H]^+$ – *p*-Cl-C_6H_4-NHCO, 18). HPLC, t_R 15.23.

4.1.15. N-(4-chlorophenyl)-N′-[3′-(piperidin-1-yl)[1,1′-biphenyl]-3-yl]urea (**11**)

Compound **11** was prepared from **26** (59.1 mg, 0.234 mmol), following general procedure A. Purification by two subsequent flash column chromatographies on silica gel ($CHCl_3$/MeOH 99:1). **11** (53.4 mg, 0.131 mmol). Yield: 56%. M. p.: 99 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 8.94 (bs, 1H), 8.88 (bs, 1H), 7.70–7.68 (m, 1H), 7.50 (AA'XX', 2H, J_{AX} = 9.0 Hz, $J_{AA'XX'}$ = 2.6 Hz), 7.44–7.42 (m, 1H), 7.36–7.34 (m, 1H), 7.32 (AA'XX', 2H, J_{AX} = 9.0 Hz, $J_{AA'XX'}$ = 2.6 Hz), 7.30–7.26 (m, 1H), 7.25–7.22 (m, 1H), 7.09–7.08 (m, 1H), 6.99–6.92 (m, 2H), 3.21–3.18 (m, 4H), 1.67–1.61 (m, 4H), 1.57–1.55 (m, 2H). ^{13}C NMR: (DMSO-d_6) δ (ppm): 152.46, 152.06, 141.60, 141.05, 139.88, 138.65, 129.37, 129.13, 128.53, 125.29, 120.51, 119.73, 117.24, 116.92, 116.70, 115.07, 114.03, 48.52, 25.19, 23.86. MS: the experiment in full scan mode showed: m/z 406 ($[M+H]^+$, 100); MS–MS of the ion at m/z 406 showed: m/z 279 ($[M+H]^+$ – *p*-Cl-C_6H_4-NH, 100). HPLC, t_R 4.70.

4.1.16. 3-(4-Chlorophenyl)-N-[3′-(pyrrolidin-1-yl)biphenyl-3-yl] propanamide (**12**)

In a vial, commercial 3-(4-chlorophenyl)propionic acid **28** (119.0 mg, 0.645 mmol) was dissolved in thionyl chloride (1.56 mL, 0.645 mmol) under nitrogen flux. The vial was sealed and left under magnetic stirring at room temperature, overnight, to allow the formation of the acyl chloride (**27**), then the excess of unreacted thionyl chloride was removed under nitrogen flux. In a round bottom flask under nitrogen atmosphere, triethylamine (0.1 mL, 75.2 mg, 0.745 mmol) and DMAP (90.5 mg, 0.741 mmol) were added to a solution of **25** (70.1 mg, 0.745 mmol) in the minimum amount of anhydrous CH_2Cl_2. The flask was cooled to −5 °C (ice-NaCl bath) and the crude acyl chloride was added dropwise. After 24 h at room temperature, under magnetic stirring, the reaction was heated at 40 °C for 6 h. The reaction mixture was partitioned between CH_2Cl_2 and an aqueous saturated solution of $NaHCO_3$.

After anhydrification over $MgSO_4$, the organic phase was filtered and evaporated. Purification by two subsequent flash column chromatographies on silica gel (petroleum ether/AcOEt 7:3 and $CHCl_3$/acetone 8:2). **12** (22.0 mg, 0.054 mmol). Yield: 8%. M. p.: 133 °C. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.62 (bs, 1H), 7.51–7.50 (m, 2H), 7.34–7.33 (m, 2H), 7.28–7.22 (m, 3H), 7.15–7.12 (m, 2H), 6.84–6.82 (m, 1H), 6.71 (m, 1H), 6.57–6.54 (m, 1H), 3.33–3.30 (m, 4H), 3.02–2.99 (m, 2H), 2.64–2.60 (m, 2H), 2.01–1.98 (m, 4H). ^{13}C NMR: ($CDCl_3$) δ (ppm): 170.23, 148.31, 143.27, 141.76, 139.29, 138.08, 132.12, 129.87, 129.55, 129.26, 128.74, 123.45, 118.86, 118.79; 114.64, 111.02, 110.49, 47.78, 30.87, 29.79, 25.55. MS: the experiment in full scan mode showed: *m/z* 405 ($[M+H]^+$, 100); MS–MS of the ion at *m/z* 405 showed: *m/z* 279 ($[M+H]^+$ – *p*-Cl-C_6H_4-CH_2, 25), 197 ($[M+H]^+$ – *p*-Cl-C_6H_4-CH_2CH_2 – pyrrolidine, 67). HPLC, t_R 15.23.

4.1.17. N^2-(6-bromopyridin-2-yl)pyridine-2,6-diamine (30)

Compound 30 was obtained from 2,6-diaminopyridine (2.60 g, 24.0 mmol, 1 equiv) and 2,6-dibromopyridine (1.25 equiv) according to general procedure C. The crude product was purified by flash column chromatography on silica gel (petroleum ether/AcOEt 6:4). 30 (147.0 mg, 0.560 mmol). Yield: 30%. ^{1}H NMR: ($CDCl_3$) δ (ppm) 8.40 (bs, 1H), 7.52 (dd, 1H, J = 8.0 Hz, J = 0.4 Hz), 7.37 (m, 2H), 6.94 (dd, 1H, J = 7.6 Hz, J = 0.4 Hz), 6.59 (dd, 1H, J = 7.6 Hz, J = 0.4 Hz), 6.09 (dd, 1H, J = 8.0 Hz, J = 0.4 Hz), 4.87 (bs, 2H).

4.1.18. N^2-[6-(pyrrolidin-1-yl)pyridin-2-yl]pyridine-2,6-diamine (31)

Compound 31 was prepared from 30 (0.768 g, 2.90 mmol) and pyrrolidine according to general procedure G. Purification by flash liquid column chromatography on silica gel, eluting with petroleum ether/AcOEt 4:6. 31 (0.550 g, 2.14 mmol). Yield: 74%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.37–7.25 (m, 3H), 7.07 (bs, 1H), 6.36 (d, 1H, J = 7.6 Hz), 6.01 (dd, 1H, J = 8.0 Hz, J = 0.8 Hz), 5.87 (d, 1H, J = 8.0 Hz), 4.30 (bs, 2H), 3.47–3.44 (m, 4H), 1.99–1.96 (m, 4H).

4.1.19. N^2-[6-(piperidin-1-yl)pyridin-2-yl]pyridine-2,6-diamine (32)

Compound 32 was prepared from 30 (0.837 g, 3.17 mmol) and piperidine according to general procedure G. Purification by flash liquid column chromatography on silica gel, eluting with petroleum ether/AcOEt 6:4. 32 (0.231 g, 0.900 mmol). Yield: 30%. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.38–7.33 (m, 2H), 6.99 (dd,1H, J = 8.0 Hz, J = 0.8 Hz), 6.97 (bs, 1H), 6.57 (d,1H, J = 7.6 Hz), 6.17 (d, 1H, J = 8.0 Hz), 6.03 (dd, 1H, J = 7.6 Hz, J = 0.4 Hz), 4.28 (bs, 2H), 3.51–3.49 (m, 4H), 1.64–1.63 (m, 6H).

4.1.20. N-(4-chlorophenyl)-N'-(6-{[6-(pyrrolidin-1-yl)pyridin-2-yl]amino}pyridin-2-yl)urea (13)

Compound 13 was prepared from 31 (0.114 g, 0.440 mmol) according to general procedure B. 13 (0.0392 g, 0.0955 mmol). Yield: 22%. M. P.: dec. 242 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 10.7 (bs, 1H), 9.31 (bs, 1H), 9.23 (bs, 1H), 7.62–7.55 (m, 4H), 7.38–7.31 (m, 3H), 6.77 (d, 1H, J = 7.6 Hz), 6.52 (d, 1H, J = 7.6 Hz), 5.93 (d, 1H, J = 8.0 Hz), 3.37–3.35 (m, 4H), 1.93–1.90 (m, 4H). ^{13}C NMR (DMSO-d_6) δ (ppm): 152.31, 152.23, 150.90, 139.67, 138.50, 138.45, 137.96, 128.62, 128.39, 126.11, 121.27, 119.85, 104.64, 98.27, 97.92, 46.37, 24.94. MS: the experiment in full scan mode showed: *m/z* 409 ($[M+H]^+$, 100); MS–MS of the ion at *m/z* 354 showed: *m/z* 282 ($[M+H]^+$ – *p*-Cl-C_6H_4-NH, 100), 256 ($[M+H]^+$ – *p*-Cl-C_6H_4-NHCO, 79). HPLC, t_R 4.76.

4.1.21. N-(4-chlorophenyl)-N'-(6-{[6-(piperidin-1-yl)pyridin-2-yl]amino}pyridin-2-yl)urea (14)

Compound 14 was prepared from 32 (0.0912 g, 0.355 mmol) according to general procedure B. 14 (0.0116 g, 0.0274 mmol). Yield: 8%. M. P.: dec. 244 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 7.56 (t, 1H, J = 8.0 Hz), 7.53 (AA'XX', 2H, J_{AX} = 8.8 Hz, $J_{AA'XX'}$ = 2.0 Hz), 7.39 (t, 1H, J = 8.0 Hz), 7.33 (AA'XX', 2H, J_{AX} = 8.8 Hz, $J_{AA'XX'}$ = 2.0 Hz), 7.27 (d, 1H, J = 7.6 Hz), 6.84 (d, 1H, J = 8.0 Hz), 6.71 (d, 1H, J = 7.6 Hz), 6.30 (d, 1H, J = 8.4 Hz), 3.62–3.58 (m, 5H), 1.78–1.74 (m, 5H). ^{13}C NMR (DMSO-d_6) δ (ppm): 157.95, 152.40, 152.37, 152.15, 150.92, 139.55, 139.00, 138.06, 128.42, 126.00, 120.99, 104.53, 102.51, 99.59, 98.63, 45.65, 25.10, 24.31. MS: the experiment in full scan mode showed: *m/z* 421 ($[M-H]^-$, 35), 385 ($[M-H]^-$ – Cl, 100); MS–MS of the ion at *m/z* 421 showed: *m/z* 294 ($[M-H]^-$ – *p*-Cl-C_6H_4-NH, 20), 268 ($[M-H]^-$ – *p*-Cl-C_6H_4-NHCO, 100), 126 [*p*-Cl-C_6H_4-$NH]^-$, 10). HPLC, t_R 4.97.

4.1.22. N^1-(pyridin-2-yl)benzene-1,3-diamine (34)

Compound 34 was obtained from 1,3-diaminobenzene (1.00 g, 9.25 mmol, 1 equiv) and 2-bromopyridine (0.5 equiv) according to general procedure C. Purification by flash column chromatography on silica gel (*n*-hexane/AcOEt 1:9). 34 (50.0 mg of a mixture containing approximately 85% of the desired product. Percentage ratio calculated comparing corresponding integration of ^{1}H NMR peaks). Yield: 2%. ^{1}H NMR: (CD_3OD) δ (ppm): 8.05–8.03 (m,1H), 7.54–7.50 (m, 1H), 7.02 (t, 1H, J = 8.0 Hz), 6.90 (t, 1H, J = 2.0 Hz), 6.86–6.84 (m, 1H), 6.73–6.69 (m, 2H), 6.41–6.38 (m, 1H).

4.1.23. N^1-(4-fluorophenyl)benzene-1,3-diamine (35)

Compound 35 was obtained from 1,3-diaminobenzene (2.00 g, 18.5 mmol) and 4-bromofluorobenzene (3.24 g, 18.5 mmol) according to general procedure C. Purification by flash column chromatography on silica gel (*n*-hexane/AcOEt 9:1 and AcOEt). 35 (52.0 mg, 0.257 mmol). Yield: 1%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.06–7.02 (m, 3H), 7.00–6.95 (m, 2H), 6.37 (dd, 1H, J = 8.0 Hz, J = 1.2 Hz), 6.31 (t, 1H, J = 2.0 Hz), 6.25–6.23 (m, 1H), 5.50–5.47 (bs, 1H), 3.69–3.59 (bs, 2H).

4.1.24. N^1-phenylbenzene-1,3-diamine (36)

Compound 36 was obtained from 1,3-diaminobenzene (1.00 g, 9.25 mmol) and iodobenzene (1.89 g, 9.25 mmol) according to general procedure C. Purification by flash column chromatography on silica gel (petroleum ether/AcOEt 7:3). 36 (33.0 mg, 0.179 mmol). Yield: 2%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.29–7.24 (m, 2H), 7.09–7.03 (m, 3H), 6.93 (t, 1H, J = 7.2 Hz), 6.48–6.46 (m, 1H), 6.43–6.42 (m, 1H), 6.29–6.26 (m, 1H), 5.64 (bs, 1H), 3.55 (bs, 2H).

4.1.25. N^2-phenyl-2,6-diaminopyridine (37)

Commercial *t*-BuOK (1.37 g, 12.2 mmol), iodobenzene (1.0 mL, 10.7 mmol) and CuI (227.0 mg, 1.19 mmol) were added to a solution of 2,6-diaminopyridine (1.00 g, 9.16 mmol) crystalized from toluene, in anhydrous 1,4-dioxane (18.0 mL), under nitrogen atmosphere. The reaction mixture was stirred at 100 °C for 24 h. Then, it was diluted with AcOEt and brine (30 mL) to allow the partition of the crude product. The organic phase was anhydrified over Na_2SO_4, filtered and evaporated under reduced pressure. The purification was performed by flash column chromatography on silica gel (Et_2O). 37 (84.0 mg, 0.453 mmol). Yield: 5%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 7.33–7.28 (m, 4H), 7.27–7.26 (m, 1H), 7.03–7.00 (m, 1H), 6.32 (bs, 1H), 6.26 (d, 1H, J = 8.0 Hz), 5.98 (dd, 1H, J = 8.0, J = 0.4 Hz), 4.28 (bs, 2H).

4.1.26. N-(3-nitrophenyl)pyridin-4-amine (39)

200 mg (2.12 mmol) of 4-aminopyridine were dissolved in 7.1 mL of freshly distilled 1,4-dioxane. Then, 358.0 mg (1.77 mmol) of 1-bromo-3-nitrobenzene, 1.44 g (4.43 mmol) of Cs_2CO_3, 162.0 mg (0.177 mmol) of xantphos and 81.0 mg (0.089 mmol) of $Pd_2(dba)_3$ were added to the solution and the reaction mixture was heated under magnetic stirring at 95 °C for 24 h. After cooling to room

temperature, the mixture was partitioned between distilled water and AcOEt. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure, to obtain an orange-brown solid. Purification by flash liquid column chromatography on silica gel, eluting with AcOEt/MeOH 9:1. 39 (311.0 mg, 1.45 mmol). Yield: 82%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 8.41–8.39 (m, 2H), 8.04–8.03 (m, 1H), 7.94–7.92 (m, 1H), 7.54–7.48 (m, 2H), 6.91–6.89 (m, 2H), 6.28 (bs, 1H).

4.1.27. N^1-(pyridin-4-yl)benzene-1,3-diamine (40)

Compound 39 (150.0 mg, 0.697 mmol) was dissolved in a mixture of EtOH/H_2O (8.6 mL: 4.3 mL). After the addition of 430.0 mg (0.697 mmol) of iron powder and 215.0 mg (4.02 mmol) of NH_4Cl, the reaction mixture was stirred at reflux for 4 h. After cooling to room temperature, the reaction mixture was filtered over a celite pad. The filtrate was evaporated *in vacuo* and the residue was dissolved in $CHCl_3$ and washed with 10% aqueous NaOH. The organic phase was dried over Na_2SO_4, filtered and evaporated. 40 (86.0 mg, 0.464 mmol). Yield: 67%. ^{1}H NMR: ($CDCl_3$) δ (ppm): 8.27–8.25 (m, 2H), 7.12 (t, 1H, J = 7.9 Hz), 6.81–6.80 (m, 2H), 6.58–6.55 (m, 1H), 6.51 (t, 1H, J = 2.1 Hz), 6.45 (ddd, 1H, J = 8.0 Hz, J = 2.2 Hz, J = 0.8 Hz), 5.98 (bs, 1H), 3.71 (bs, 2H).

4.1.28. N-(4-chlorophenyl) –N'-{3-[(pyridin-2-yl) amino]phenyl} urea (15)

Compound 15 was prepared from 34 (43.0 mg, 0.232 mmol) according to general procedure A. 15 (55 mg, 0.169 mmol). Yield: 73%. M. p.: 190 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 9.02 (s, 1H), 8.77 (s, 1H), 8.65 (s, 1H), 8.15–8.14 (m, 1H), 7.80 (s, 1H), 7.58–7.54 (m, 1H), 7.49–7.47 (m, 2H), 7.34–7.29 (m, 3H), 7.15 (t, 1H, J = 8.0 Hz), 7.06–7.04 (m, 1H), 6.83 (d, 1H, J = 8.4 Hz), 6.75–6.72 (m, 1H). ^{13}C NMR: (DMSO-d_6) δ (ppm): 155.92, 152.36, 147.19, 142.14, 139.73, 138.81, 137.19, 128.65, 125.21, 119.58, 119.49, 114.25, 112.06, 110.60, 110.48, 107.83. MS: the experiment in full scan mode showed: *m/z* 337 ([M-H]$^-$, 21); MS–MS of the ion at *m/z* 337 showed: *m/z* 210 ([M-H]$^-$ – *p*-Cl-C_6H_4-NH, 100), 126 ([*p*-Cl-C_6H_4-NH]$^-$, 14). HPLC, t_R 4.60.

4.1.29. N-(6-anilinopyridin-2-yl)-N'-(4-chlorophenyl)urea (16)

Compound 16 was prepared from 37 (80.0 mg, 0.432 mmol) according to general procedure A. Purification by flash column chromatography on silica gel ($CHCl_3$/CH_3OH 95:5). 16 (17.0 mg, 0.050 mmol). Yield: 11%. M. p.: 187 °C. ^{1}H NMR: (CD_3OD) δ (ppm): 7.91 (s, 1H), 7.50 (t, 1H, J = 8.0 Hz), 7.37–7.36 (m, 2H), 7.27–7.23 (m, 2H), 7.12–7.10 (m, 2H), 7.03–7.01 (m, 1H), 6.96–6.94 (m, 2H), 6.43–6.37 (m, 2H). ^{13}C NMR: (CD_3OD) δ (ppm): 155.78, 152.59, 142.19, 141.11, 138.51, 130.45, 129.38, 129.00, 123.83, 122.62, 122.19, 106.48, 104.27, 102.13. MS: the experiment in full scan mode showed: *m/z* 337 ([M-H]$^-$, 21); MS–MS of the ion at *m/z* 337 showed: *m/z* 210 ([M-H]$^-$ – *p*-Cl-C_6H_4-NH, 11), 184 ([M-H]$^-$ – *p*-Cl-C_6H_4-NHCO, 100), 126 [*p*-Cl-C_6H_4-NH]$^-$, 24). HPLC, t_R 9.97.

4.1.30. N-(4-chlorophenyl)-N'-{3-[(pyridin-4-yl)amino]phenyl} urea (17)

Compound 17 was prepared from 40 (43.0 mg, 0.232 mmol) according to general procedure A. 17 (67.0 mg, 0.198 mmol). Yield: 85%. M. p.: 220 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 8.80 (s, 1H), 8.79 (s, 1H), 8.74 (s, 1H), 8.19–8.18 (m, 2H), 7.48 (AA'XX', 2H, J_{AX} = 9.0 Hz, $J_{AA'XX'}$ = 2.6 Hz), 7.48–7.46 (m, 1H), 7.33–7.31 (AA'XX', 2H, J_{AX} = 9.0 Hz, $J_{AA'XX'}$ = 2.6 Hz), 7.23 (t, 1H, J = 8.0 Hz), 7.02–7.00 (m, 1H), 6.93–6.91 (m, 2H), 6.81–6.79 (m, 1H). ^{13}C NMR: (CD_3OD) δ (ppm): 155.00, 153.80, 149.84, 142.07, 141.75, 139.53, 131.00, 129.98, 128.91, 121.91, 116.95, 115.80, 113.39, 110.74. MS: the experiment in full scan mode showed: *m/z* 337 ([M-H]$^-$, 23); MS–MS of the ion at *m/z* 337 showed: *m/z* 210 ([M-H]$^-$ – *p*-Cl-C_6H_4-NH, 100), 184 ([M-H]$^-$ – *p*-Cl-C_6H_4-NHCO, 68), 126 [*p*-Cl-C_6H_4-NH]$^-$, 24). HPLC, t_R 2.13.

4.1.31. N-(4-chlorophenyl) –N'-{3-[(4-fluorophenyl) amino] phenyl} urea (18)

Compound 18 was prepared from 35 (49.0 mg, 0.242 mmol) according to general procedure A. 18 (55.0 mg, 0.154 mmol). Yield: 64%. M. p.: 160 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 8.74 (s, 1H), 8.62 (s, 1H), 8.13 (s, 1H), 7.46 (AA'XX', 2H, J_{AX} = 9.0 Hz, $J_{AA'XX'}$ = 2.8 Hz), 7.31 (AA'XX', 2H, J_{AX} = 9.0 Hz, $J_{AA'XX'}$ = 2.8 Hz), 7.25–7.24 (m, 1H), 7.11–7.08 (m, 5H), 6.83–6.81 (m, 1H), 6.62–6.60 (m, 1H). ^{13}C NMR: (DMSO-d_6) δ (ppm): 156.40 (d, J = 235.0 Hz), 152.35, 144.45, 140.41, 139.65 (d, J = 2.0 Hz), 138.77, 129.47, 128.64, 125.25, 119.64, 119.16 (d, J = 7.0 Hz), 115.67 (d, J = 22.0 Hz), 109.93, 109.55, 105.62. MS: the experiment in full scan mode showed: *m/z* 354 ([M-H]$^-$, 21); MS–MS of the ion at *m/z* 354 showed: *m/z* 227 ([M-H]$^-$ – *p*-Cl-C_6H_4-NH, 100), 201 ([M-H]$^-$ – *p*-Cl-C_6H_4-NHCO, 96), 126 [*p*-Cl-C_6H_4-NH]$^-$, 15). HPLC, t_R 10.15.

4.1.32. N-(3-anilinophenyl)-N'-(4-chlorophenyl)urea (19)

Compound 19 was prepared starting from 36 (27.0 mg, 0.147 mmol) according to general procedure A. 19 (8.0 mg, 0.025 mmol). Yield: 17%. M. p.: 170 °C. ^{1}H NMR: (DMSO-d_6) δ (ppm): 8.74 (s, 1H), 8.62 (s, 1H), 8.16 (s, 1H), 7.46 (AA'XX', 2H, J_{AX} = 8.8 Hz, $J_{AA'XX'}$ = 2.8 Hz), 7.31 (AA'XX', 2H, J_{AX} = 9.2 Hz, $J_{AA'XX'}$ = 2.6 Hz), 7.34–7.30 (m, 1H), 7.25–7.21 (m, 2H), 7.13–7.07 (m, 3H), 6.86–6.80 (m, 2H), 6.69–6.66 (m, 1H). ^{13}C NMR: (DMSO-d_6) δ (ppm): 152.32, 143.91, 143.29, 140.30, 138.73, 129.36, 129.09, 128.82, 128.59, 125.21, 119.81, 116.99, 110.60, 109.77, 106.27. MS: the experiment in full scan mode showed: *m/z* 336 ([M-H]$^-$, 23); MS–MS of the ion at *m/z* 336 showed: *m/z* 209 ([M-H]$^-$ – *p*-Cl-C_6H_4-NH, 100), 183 ([M-H]$^-$ – *p*-Cl-C_6H_4-NHCO, 25), 126 [*p*-Cl-C_6H_4-NH]$^-$, 5). HPLC, t_R 11.99.

4.2. Biological assays

4.2.1. Cell membrane preparations

CHO cells stably transfected with cDNA encoding human cannabinoid CB1Rs or CB2Rs were maintained at 37 °C and 5% CO_2 in Gibco Ham's F-12 nutrient mix supplied by Fisher Scientific UK Ltd. that was supplemented with 2 mM L-glutamine, 10% FBS, and 0.6% penicillin–streptomycin, all also supplied by Fisher Scientific UK Ltd., and with the disulfate salt of G418 [(2R,3S,4R,5R,6S)-5-amino-6-{[(1R,2S,3S,4R,6S)-4,6-diamino-3-{[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4 (methylamino)oxan-2-yl]oxy}-2-hydroxycyclohexyl]oxy}-2-[(1R)-1-hydroxyethyl]oxane-3,4-diol; 600 mg mL^{-1}] supplied by Sigma-Aldrich UK. All cells were exposed to 5% CO_2 in their respective media and were passaged twice a week using nonenzymatic cell dissociation solution. To prepare the membranes, cells were scraped from flask, centrifuged at 1800 rpm for 5 min and the pellet obtained was frozen at −20 °C. To perform a radioligand binding assays, cells were defrosted, diluted with 500 μL of Tris buffer (composed by 50.0 mM of Tris-HCl and 50 mM of Tris-base).

4.2.2. Radioligand displacement assay

The total assay volume was 500 μL and for the procedure [^{3}H] CP55940 (0.700 nM) and Tris binding buffer (0.1% BSA in Tris buffer, with a pH of 7.4) were employed. Different concentrations of the compounds (from 1 nM to 10 μM, obtained from 10.00 mM stock solutions in DMSO) were incubated at 37 °C with hCB1- or hCB2-CHO cell membranes (50.0 μg of protein per tube) and DMSO vehicle (0.1% per tube) for 60 min. The assay was terminated by adding ice-cold Tris binding buffer and filtrating under vacuum (24-well sampling manifold, Brandel Cell Harvester; Brandel Inc, Gaithersburg, MD, USA). The filters used (Brandel GF/B filters) had

been soaked in Tris binding buffer at 4 °C for at least 24 h. The filtration was carried out by washing each reaction well with 1.200 mL of Tris binding buffer for six times. After oven-drying for 60 min, the filters were placed in 3.00 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK) and subjected to liquid scintillation spectrometry in order to quantify the radioactivity. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μM unlabeled CP55940.

4.2.3. [^{35}S]GTPγS assays

The total assay volume was 500 μL and for the procedure [^{35}S]GTPγS (0.1 nM), GDP (30 μM), GTPγS (30 μM and binding buffer (50 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EDTA and 1 mM DTT (dithiothreitol), with a pH of 7.4) were employed. Different concentrations of the compounds (from 1 nM to 10 μM, obtained from 10.00 mM stock solutions in DMSO) were incubated at 30 °C with hCB1-CHO cell membranes (50.0 μg of protein per tube) and DMSO vehicle (0.1% per tube) for 90 min. The assay was terminated by adding ice-cold Tris binding buffer and filtrating under vacuum (24-well sampling manifold, Brandel Cell Harvester; Brandel Inc, Gaithersburg, MD, USA). The filters used (Brandel GF/B filters) had been soaked in Tris binding buffer at 4 °C for at least 24 h. The filtration was carried out by washing each reaction well with 1.200 mL of Tris binding buffer six times. After oven-drying for 60 min, the filters were placed in 3.00 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK) and subjected to liquid scintillation spectrometry in order to quantify the radioactivity. The basal binding was obtained by measuring the radioactivity in the absence of cannabinoids and in the presence of 320 μM of GDP. NSB was measured in the presence of 10 μM of unlabeled GTPγS.

4.2.4. [^{35}S]GTPγS assay in presence of CP55940

The total assay volume was 500 μL and for the procedure [^{35}S]GTPγS (0.1 nM), GDP (30 μM), GTPγS (30 μM and binding buffer (50 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EDTA and 1 mM DTT (dithiothreitol), with a pH of 7.4) were employed. Each compound was incubated at 1 μM (obtained from 10.00 mM stock solutions in DMSO) in the presence of different concentrations of CP55940 (from 0.1 nM to 1 μM) at 30 °C with hCB1-CHO cell membranes (50.0 μg of protein per tube) and DMSO vehicle (0.1% per tube) for 90 min. The assay was terminated by adding ice-cold Tris binding buffer and filtrating under vacuum (24-well sampling manifold, Brandel Cell Harvester; Brandel Inc, Gaithersburg, MD, USA). The filters used (Brandel GF/B filters) had been soaked in Tris binding buffer at 4 °C for at least 24 h. The filtration was carried out by washing each reaction well with 1.200 mL of Tris binding buffer for six times. After oven-drying for 60 min, the filters were placed in 3.00 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK) and subjected to liquid scintillation spectrometry in order to quantify the radioactivity. The basal binding was obtained by measuring the radioactivity in absence of cannabinoids and in presence of 320 μM of GDP. NSB was measured in the presence of 10 μM of unlabeled GTPγS.

4.2.5. Data analysis

In binding assays, the results were calculated as percentage changes of [^{3}H]CP55940 basal binding in the presence of vehicle. In functional assays, the results were calculated as percentage changes of [^{35}S]GTPγS basal stimulation in the presence of vehicle. The sigmoidal log concentration-response curves, the values of EC_{50}, E_{max}, S.E.M. and 95% confidence intervals were calculated using GraphPad Prism 5.0 (GraphPad, San Diego, CA). The one sample *t*-test was employed and P values < 0.05 were considered as significant. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgments

This research was supported by grant from University of Pisa, Italy (Progetti di Ricerca di Ateneo, PRA_2018_20).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112606.

References

[1] L. Cristino, T. Bisogno, V. Di Marzo, Cannabinoids and the expanded endocannabinoid system in neurological disorders, Nat. Rev. Neurol. 16 (2020) 9–29.
[2] E.L. Scotter, M.E. Abood, M. Glass, The endocannabinoid system as a target for the treatment of neurodegenerative disease, Br. J. Pharmacol. 160 (2010) 480–498.
[3] P. Pacher, S. Bátkai, G. Kunos, The endocannabinoid system as an emerging target of pharmacotherapy, Pharmacol. Rev. 58 (2006) 389–462.
[4] R.G. Pertwee, A.C. Howlett, M.E. Abood, S.P. Alexander, V. Di Marzo, M.R. Elphick, P.J. Greasley, H.S. Hansen, G. Kunos, K. Mackie, R. Mechoulam, R.A. Ross, International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2, Pharmacol. Rev. 62 (2010) 588–631.
[5] S. Zou, U. Kumar, Cannabinoid receptors and the endocannabinoid system: signaling and function in the central nervous system, Int. J. Mol. Sci. 19 (2018) 833.
[6] C. Quarta, D. Cota, Anti-obesity therapy with peripheral CB_1 blockers: from promise to safe(?) practice, Int. J. Obes. (2020), https://doi.org/10.1038/s41366-020-0577-8. Ahead of print.
[7] P. Pacher, R. Mechoulam, Is lipid signaling through cannabinoid 2 receptors part of a protective system? Prog. Lipid Res. 50 (2011) 193–211.
[8] D. Chanda, D. Neumann, J.F.C. Glatz, The endocannabinoid system: overview of an emerging multi-faceted therapeutic target, Prostaglandins Leukot. Essent. Fatty Acids 140 (2019) 51–56.
[9] A.C. Howlett, M. Abood, CB1 and CB2 receptor pharmacology, Adv. Pharmacol. 80 (2017) 169–206.
[10] D.A. Kendall, G.A. Yudowski, Cannabinoid receptors in the central nervous system: their signaling and roles in disease, Front. Cell. Neurosci. 10 (2017) 294.
[11] M.D. Van Sickle, M. Duncan, P.J. Kingsley, A. Mouihate, P. Urbani, K. Mackie, N. Stella, A. Makriyannis, D. Piomelli, J.S. Davison, L.J. Marnett, V. Di Marzo, Q.J. Pittman, K.D. Patel, K.A. Sharkey, Identification and functional characterization of brainstem cannabinoid CB2 receptors, Science 310 (2005) 329–332.
[12] J. van Amsterdam, T. Brunt, W. van den Brink, The adverse health effects of synthetic cannabinoids with emphasis on psychosis-like effects, J. Psychopharmacol. 29 (2015) 254–263.
[13] F. Borgan, K. Beck, E. Butler, R. McCutcheon, M. Veronese, A. Vernon, O.D. Howes, The effects of cannabinoid 1 receptor compounds on memory: a meta-analysis and systematic review across species, Psychopharmacology 236 (2019) 3257–3270.
[14] R. Christensen, P.K. Kristensen, E.M. Bartels, H. Bliddal, A. Astrup, Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomized trials, Lancet 370 (2007) 1706–1713.
[15] E. Galaj, Z. Xi, Potential of cannabinoid receptor ligands as treatment for substance use disorders, CNS Drugs 33 (2019) 1001–1030.
[16] D. Jones, End of the line for cannabinoid receptor 1 as an anti-obesity target? Nat. Rev. Drug Discov. 7 (2008) 961–962.
[17] M.R. Price, G.L. Baillie, A. Thomas, L.A. Stevenson, M. Easson, R. Goodwin, A. McLean, L. McIntosh, G. Goodwin, G. Walker, P. Westwood, J. Marrs, F. Thomson, P. Cowley, A. Christopoulos, R.G. Pertwee, R.A. Ross, Allosteric modulation of the cannabinoid CB1 receptor, Mol. Pharmacol. 68 (2005) 1484–1495.
[18] F. Gado, S. Meini, S. Bertini, M. Digiacomo, M. Macchia, C. Manera, Allosteric modulators targeting cannabinoid CB1 and CB2 receptors: implications for drug discovery, Future Med. Chem. 11 (2019) 2019–2037.
[19] F. Gado, L. Di Cesare Mannelli, E. Lucarini, S. Bertini, E. Cappelli, M. Digiacomo, L.A. Stevenson, M. Macchia, T. Tuccinardi, C. Ghelardini, R.G. Pertwee, C. Manera, Identification of the first synthetic allosteric modulator of the CB2 receptors and evidence of its efficacy for neuropathic pain relief, J. Med. Chem. 62 (2019) 276–287.
[20] A. Christopoulos, Allosteric binding sites on cell-surface receptors: novel

targets for drug discovery, Nat. Rev. Drug Discov. 1 (2002) 198–210.
[21] S. Urwyler, Allosteric modulation of family C G-protein-coupled receptors: from molecular insights to therapeutic perspectives, Pharmacol. Rev. 63 (2011) 59–126.
[22] S. Hryhorowicz, M. Kaczmarek-Ryś, A. Andrzejewska, K. Staszak, M. Hryhorowicz, A. Korcz, R. Słomski, Allosteric modulation of cannabinoid receptor 1-current challenges and future opportunities, Int. J. Mol. Sci. 22 (2019) 5874.
[23] L.T. May, A. Christopoulos, Allosteric modulators of G-protein-coupled receptors, Curr. Opin. Pharmacol. 3 (2003) 551–556.
[24] R.A. Ross, Allosterism and cannabinoid CB1 receptors: the shape of things to come, Trends Pharmacol. Sci. 28 (2007) 567–572.
[25] L. Wang, B. Martin, R. Brenneman, L.M. Luttrell, S. Maudsley, Allosteric modulators of G protein-coupled receptors: future therapeutics for complex physiological disorders, J. Pharmacol. Exp. Therapeut. 331 (2009) 340–348.
[26] T.M. Bridges, C.W. Lindsley, G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms, ACS Chem. Biol. 3 (2008) 530–541.
[27] J. Horswill, U. Bali, S. Shaaban, J. Keily, P. Jeevaratnam, A. Babbs, C. Reynet, P. Wong Kai, In, PSNCBAM-1, a novel allosteric antagonist at cannabinoid CB1 receptors with hypophagic effects in rats, Br. J. Pharmacol. 152 (2007) 805–814.
[28] R. Dopart, S.S. Immadi, D. Lu, D.A. Kendall, Structural optimization of the diarylurea PSNCBAM-1, an allosteric modulator of cannabinoid receptor 1, Curr. Ther. Res. 92 (2020), 100574 (and references therein).
[29] S. Bertini, A. Chicca, F. Gado, C. Arena, D. Nieri, M. Digiacomo, G. Saccomanni, P. Zhao, M.E. Abood, M. Macchia, J. Gertsch, C. Manera, Novel analogs of PSNCBAM-1 as allosteric modulators of cannabinoid CB1 receptor, Bioorg. Med. Chem. 25 (2017) 6427–6434.
[30] T. Nguyen, N. German, A.M. Decker, T.L. Langston, T.F. Gamage, C.E. Farquhar, J.X. Li, J.L. Wiley, B.F. Thomas, Y. Zhang, Novel diarylurea based allosteric modulators of the cannabinoid CB1 receptor: evaluation of importance of 6-pyrrolidinylpyridinyl substitution, J. Med. Chem. 60 (2017) 7410–7424.
[31] P. Shah, A.D. Westwell, The role of fluorine in medicinal chemistry, J. Enzym. Inhib. Med. Chem. 22 (2007) 527–540.
[32] H.J. Bohm, D. Banner, S. Bendels, M. Kansy, B. Kuhn, K. Muller, U. Obst-Sander, M. Stahl, Fluorine in medicinal chemistry, Chembiochem 5 (2004) 637–643.
[33] T.P. Kenakin, Biased signalling and allosteric machines: new vistas and challenges for drug discovery, Br. J. Pharmacol. 165 (2012) 1659–1669.
[34] J.S. Smith, R.J. Lefkowitz, S. Rajagopal, Biased signalling: from simple switches to allosteric microprocessors, Nat. Rev. Drug Discov. 17 (2018) 243–260.