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Novel analogs of PSNCBAM-1 as allosteric modulators of cannabinoid CB1 receptor

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Abstract

In this work, we explored the molecular framework of the known CB1R allosteric modulator PSNCBAM-1 with the aim to generate new bioactive analogs and to deepen the structure-activity relationships of this type of compounds. In particular, the introduction of a NH group between the pyridine ring and the phenyl nucleus generated the amino-phenyl-urea derivative **SN15b** that behaved as a positive allosteric modulator (PAM), increasing the CB1R binding affinity of the orthosteric ligand CP55,940. The functional activity was evaluated using serum response element (SRE) assay, which assesses the CB1R-dependent activation of the MAPK/ERK signaling pathway. **SN15b** and the biphenyl-urea analog **SC4a** significantly inhibited the response produced by CP55,940 in the low μM range, thus behaving as negative allosteric modulators (NAMs). The new derivatives presented here provide further insights about the modulation of CB1R binding and functional activity by allosteric ligands.

Graphical Abstract



Keywords

Allosteric modulator; CB1 receptor; Endocannabinoid system; MAPK/ERK signaling pathway; Serum response element

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1. Introduction

The endocannabinoid system (ECS) is abundantly expressed throughout the body and it consists of cannabinoid receptors, their endogenous ligands (endocannabinoids), and the enzymes involved in the metabolism of endocannabinoids.^{1, 2} Since the discovery of the cannabinoid receptors (CB1R and CB2R), several studies have been extensively devoted to their synthetic ligands. In fact, the direct activation of these receptors results in several beneficial effects, in the brain and in the periphery.^{3–7} CB1R is the most expressed G-protein coupled receptor (GPCR) in brain, where it transduces the endocannabinoid signaling and thereby acts as a modulator of neurotransmitter release.⁸ In the periphery, CB1R regulates metabolic processes including substrate storage and mobilization.^{9, 10} On the other hand, CB2R is mainly localised in peripheral immune cells and tissues, though recent studies showed its presence in CNS, and it has been investigated for treating pain and inflammation.^{11–14}

CB1R is shown to be involved in regulation of several physiological functions, such as food intake and energy balance, cardiovascular and reproductive functions, immune modulation and cell apoptosis.^{15–17} Therefore, CB1R has been suggested as an important target of small-molecule pharmacotherapeutics, for ameliorating some pathological conditions such as pain, nausea, obesity, type 2 diabetes, glaucoma and substance abuse.^{9, 10}

Several CB1R agonist and antagonists/inverse agonists have been reported in literature with different potencies and selectivities, showing effects in several preclinical disease models.^{3, 18–20} However, the orthosteric action of CB1R agonists and antagonists/inverse agonists induces important side effects.^{21, 22} In particular, CB1R orthosteric agonists produce psychotropic effects, addiction and cognitive impairment.^{3, 23} On the other hand, CB1R orthosteric antagonists/inverse agonists are associated with undesirable side effects, such as nausea, anxiety, depression, and in some cases, suicidal tendencies.^{21, 24}

Recently, it has been suggested that CB1R contains (an) allosteric binding site(s) topologically distinct from the orthosteric site.^{25–29} Compared to orthosteric ligands, allosteric modulators offer several advantages. In fact, they show greater subtype selectivity due to the higher sequence divergence at extracellular allosteric binding sites, respect to the conserved orthosteric domains; furthermore, allosteric modulators show tissue selectivity, because they exert effects only where endocannabinoids are present; finally, the effect of allosteric modulators is saturable because of their dependence on endogenous ligands.^{30, 31} For these reasons, allosteric modulators might offer an alternative strategy to pharmacologically modulate CB1R without producing unwanted side effects associated with CB1R orthosteric agonists and antagonists/inverse agonists.

Allosteric modulators can be classified as positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs), depending on the type of modulation on the affinity and/or efficacy of the orthosteric agonists.³² Various classes of compounds have been reported as CB1R allosteric modulators, such as the indole derivatives (e.g., the NAM Org27569),²⁵ the urea derivatives (e.g., the NAM PSNCBAM-1),²⁷ and other molecules (e.g. the PAMs RTI-371³³ and ZCZ011³⁴) (Figure 1).

Furthermore, recent works reported natural PAMs and NAMs of CB1R, such as the lipoxin A4, the peptide pepcan-12 (RVD-hemopessin), and pregnenolone (Figure 2).^{26, 28, 29}

PSNCBAM-1 was discovered by Prosidion Limited in 2007 through an high throughput screening of a small library.²⁷ This compound showed a pharmacological profile similar to Org27569, increasing the binding of CB1R agonists but decreasing their functional responses in several *in vitro* assays. In an *in vivo* study of acute feeding model, PSNCBAM-1 showed to decrease food intake and body weight.²⁷ Several analogs of PSNCBAM-1³⁵⁻³⁷ have been reported and structure-activity relationship studies showed that alkyl substitution at the 2-aminopyridine moiety is important for CB1R allosteric modulation; in particular, tertiary amine substitution is more favorable than secondary. Furthermore, the 4-position on the phenyl group tolerates structural modifications, but electron-withdrawing groups such as cyano or CF₃ are preferred,³⁵ and the substitution of the urea group with other spacers such as carbamate, methylated ureas or cyclic ureas, reduces the activity.³⁶

In an effort to deepen the knowledge on structural requirements for CB1R allosteric modulation within the PSNCBAM-1 template, we decided to introduce further modifications on the structure of PSNCBAM-1 obtaining compounds **SC4a**, **SC33**, **SN15b** and **FG45a** (Figure 3).

In particular, we investigated the role of the pyridine nitrogen atom by replacing it with a carbon atom (**SC4a**, **FG45a**). The derivative **SC4a** was previously reported³⁶ and its pharmacological activity was determined by cAMP Hunter assay and CNR1 PathHunter assay (β -arrestin), but competitive radioligand experiments were not conducted. **SC4a** was prepared in our laboratory following a different synthetic route respect to that reported in Ref. 36, with an overall comparable yield. Moreover, we verified the importance of the urea group by replacing one NH group with a methylene group, thus obtaining the carboxamide derivative **SC33**. Finally, we evaluated the influence due to the introduction of a NH group between the pyridine ring and phenyl nucleus (**SN15b**, **FG45a**). For all these compounds, the 4-chlorophenyl group and the 2-pyrrolydyl substituent have been selected based on previous results obtained for PSNCBAM-1 analogs.³⁵

In this work we reported the synthesis of compounds **SC4a**, **SC33**, **SN15b** and **FG45a** and their biological evaluation on the cannabinoid receptors, on the major metabolic enzymes of endocannabinoids, fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL) and on anandamide (AEA) uptake. Furthermore, the activity of the target compounds at CB1R was assessed in serum response element (SRE) assay.

2. Methods

2.1. Chemical synthesis

The synthesis of compound **SC4a** and **SC33** is described in Scheme 1. Commercial 1,3-dibromobenzene **1** was subjected to a monoamination with pyrrolidine in the presence of a catalytic system constituted by tris(dibenzylideneacetone)dipalladium(0) (Pd₂dba₃), 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene (BINAP) and *t*-BuONa (mol ratio: 0.05:0.15:2) in

toluene, affording the intermediate **2**. The subsequent Suzuki cross-coupling reaction with 3-nitrophenylboronic acid, in the presence of Pd(OAc)₂, P(Ph)₃ and aqueous Na₂CO₃ in 1,2-dimethoxyethane (DME), afforded compound **3**. Then the nitro group was reduced with Raney nickel and hydrazine hydrate in EtOH and the amino derivative **4** thus obtained was treated with 4-chloro-phenylisocyanate in anhydrous CHCl₃, affording the desired urea derivative **SC4a**. The treatment of **4** with (4-chlorophenyl)acetyl chloride in the presence of Et₃N and DMAP in anhydrous CH₂Cl₂ afforded the final amide derivative **SC33**.

The synthesis of compound **FG45a** is described in Scheme 2. The intermediate **5** was obtained by treating **2** with commercial 1,3-diaminobenzene **6**, using the same reaction conditions described for the first step of Scheme 1. The subsequent reaction of **5** with 4-chloro-phenylisocyanate in anhydrous CHCl₃ afforded the desired urea derivative **FG45a**.

The synthesis of compound **SN15b** is described in Scheme 3. Commercially available 1,3-diaminobenzene **6** and 2,6-dibromopyridine were refluxed for 48 h in anhydrous toluene, in the presence of an excess of *t*-BuOK, obtaining the intermediate **7**, which was refluxed in an excess of pyrrolidine for 12 h, affording compound **8**. The subsequent reaction of **8** with 4-chloro-phenylisocyanate in anhydrous CHCl₃ afforded the desired urea derivative **SN15b**.

2.2. Biological assays

Cannabinoid receptors binding was evaluated by incubating the target compounds at different concentrations with membrane preparations obtained from CHO-K1 cells overexpressing *h*CB1R or *h*CB1R in presence of 0.5 nM of [³H]CP55,940.³⁸

The inhibitory activity of all four compounds and of compound PSNCBAM-1 on FAAH, MAGL and on AEA cell uptake was evaluated using U937 cell homogenate for metabolic enzymes and intact U937 cells for AEA uptake, in the presence of AEA and of [ethanolamine-1-³H]AEA (0.5 nM) as tracer.^{39, 40} Finally the functional activity of the new compounds was determined by SRE assay using HEK293 cells transfected with CB1R.⁴¹

3. Results and Discussion

Since the discovery of PSNCBAM-1, some SAR studies^{35–37} showed that CB1R allosteric modulators with improved affinity, efficacy and potency, could be generated from PSNCBAM-1 template. With the aim to extend the knowledge about the structural requirements of PSNCBAM-1 template as CB1R allosteric modulator, in this work we studied some derivatives designed by introducing various modifications on PSNCBAM-1 structure.

Firstly, with the purpose of studying the role of the pyridine nitrogen atom, we synthesized compound **SC4a** bearing a phenyl ring in replacement of the pyridine nucleus. Pharmacological characterization of **SC4a** showed its behavior as positive allosteric modulator for the orthosteric ligand [³H]CP55,940 at CB1R, inducing a dose-dependently binding increase by approximately 15–20%. The estimated EC₅₀ is 43 (5–520) nM (Fig. 4).

In the second step, with the aim to verify the importance of the urea group, we synthesized compound **SC33** characterized by a carboxamide group. The results of pharmacological studies showed that **SC33** does not influence [³H]CP55,940 binding to CB1R up to 10 μM, while only a minor binding (approx. 10%) seems to occur at 100 μM (Fig. 4).

Furthermore, we evaluated the influence of NH group placed between the pyridine ring and the phenyl nucleus, synthesizing compound **SN15b**. This derivative behaves as a positive allosteric modulator at CB1R for the orthosteric ligand [³H]CP55,940. The estimated EC₅₀ is 1.3 (0.41–5.8) μM and the binding is increased by 35% (Fig. 4).

Finally, we synthesized compound **FG45a** as an analog of **SC33** and characterized by the same spacer (NH) of **SN15b** between the two phenyl rings. **FG45a** behaves as a positive allosteric modulator by enhancing [³H]CP55,940 binding to CB1R with an EC₅₀ value of 13.5 (5.1–35.3) μM and reaching 182% of maximal binding (Fig. 4).

We also tested the progenitor compound PSNCBAM-1 which increased [³H]CP55,940 binding at CB1R by 15–20% with an EC₅₀ value of 270 (140–520) nM, thus showing a similar behavior as its analogs **SC4a**, **SN15b** and **FG45a** (Table 1 and Fig. 4).

Functional activity was evaluated using a serum response element (SRE) reporter assay. HEK293 cells were transiently transfected with both pGL4.33 [luc2P/SRE/Hygro] and *h*CB1R. The next day cells were treated with ligands for 5 h, following which transcriptional activation of the SRE was detected using luminescence from the luciferase reporter (Ref 41 and experimental procedures). The SRE assay assesses the contribution of MAPK/ERK signalling pathway and thus encompasses both G-protein dependent and G-protein independent signalling.⁴¹ Recent findings showed the roles of MAPK/ERK signaling pathways in human neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.⁴² Moreover the ERK signaling pathway plays a central role in several steps of cancer development.⁴³ As shown in Figure 5, all the compounds inhibited the response produced by CP55,940 at 33 nM (concentration corresponding to EC₈₀). **SC4a** and **SN15b** were the most potent, with IC₅₀ values of 291 (101–848) nM and 182 (92–357) nM, respectively, which were comparable to that of **PSNCBAM-1** (IC₅₀ value of 234 (161–337) nM). **SC33** and **FG45** were less potent, with IC₅₀ values of 1.9 (0.52–6.9) μM and 9.6 (0.36–25) μM, respectively.

Compounds were also tested for binding to CB2R and for interaction with the other major targets of the ECS. As shown in Figure 6, compound **SC4a** and **SC33** weakly bound to CB2R with *K_i* values of approximately 50 μM, while **SN15b** and **FG45a** did not significantly interact with the receptor up to 100 μM. The binding curves suggest that **SC4a** and **SC33** behave as weak orthosteric CB2R ligands and do not interact with a distinct allosteric pocket. PSNCBAM-1 did not show any significant binding to CB2R up to 10 μM.

In further experiments, all four compounds did not exhibit any significant inhibition of FAAH, MAGL and AEA cell uptake activity up to 10 μM. The progenitor compound PSNCBAM-1 was negative for MAGL and AEA cell uptake, but inhibited FAAH activity with an IC₅₀ value of 2.1 (1.9–2.4) μM (Table 1). Altogether, these data show that,

analogously to PSNCBAM-1, **SN15b** and **SC4a** are potent PAMs at CB1R for the orthosteric ligand [³H]CP55,940 with high selectivity over CB2R and the other targets of the ECS (Table 1). Furthermore all new compounds decreased the functional response in SRE assay showing to be NAMs.

4. Conclusion

With the aim to deepen the knowledge on structural requirements for CB1R allosteric modulation, we have designed and synthesized some analogs of urea derivative PSNCBAM-1, a known allosteric modulator of CB1R. The new compounds showed to be PAMs on binding affinity of orthosteric ligand (CP55,940) but NAMs in functional assays. This behavior is in line with the complex receptor pharmacology previously described for PSNCBAM-1.^{27, 44, 45} The obtained results showed that the pyridine nitrogen atom in PSNCBAM-1 is not an important feature for CB1R modulating activity, indeed the compound **SC4a**, concentration-dependently increased [³H]CP,55940 binding to CB1R. Furthermore, a spacer (NH group) between the pyridine ring and phenyl nucleus (**SN15b** and **FG45a**) is allowed for the modulation of CB1R activity. These compounds showed also high selectivity over CB2R and the other targets of the ECS. Moreover, all compounds inhibited the response produced by CP55,940 in SRE assay, indicating a behaviour as NAMs. In particular, **SC4a** and **SN15b** were the most potent, reducing the MAPK/ERK signalling pathway with an activity similar to that of PSNCBAM-1. The new urea derivatives presented here provide further insights regarding the modulation of CB1R binding and offer new opportunities for modulating specific signalling pathway associated with CB1R, avoiding unwanted side effects usually caused by CB1R orthosteric modulation.

5. Experimental

5.1. Chemistry

Commercially available reagents were purchased from Sigma Aldrich or Alfa Aesar and used without purification. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AVANCE III™ 400 spectrometer (operating at 400 MHz). Chemical shift (δ) are reported in parts per million related to the residual solvent signal, while coupling constants (J) are expressed in Hertz (Hz). All synthesized compounds were analyzed by HPLC, showing a purity 95%. A Bechman HPLC instrument equipped with a System Gold Solvent Delivery module (Pumps) 125, System Gold UV/VIS Detector 166, Detector 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 H₂O/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., was used for compounds **SC4a**, **SC33b** and **FG45a**. For compound **SN15b** was chosen an isocratic elution with 100% of B. The flow rate was 1.0 ml/min. 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 aluminium silica gel (60 F254) plates that were visualized under a UV lamp ($\lambda = 254$ nm). Melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

5.1.1. 1-(3-Bromophenyl)pyrrolidine (2)—Commercial 1,3-dibromobenzene **1** (200.0 mg, 0.85 mmol), pyrrolidine (60.5 mg, 0.85 mmol) and 157.0 mg of a reagent constituted by tris(dibenzylideneacetone)dipalladium(0), BINAP and *t*-BuONa (mol ratio: 0.05:0.15:2) were suspended in toluene (2.5 mL) and allowed to react in a sealed tube, at 80 °C for 4.5 h, under magnetic stirring. After cooling to room temperature, the mixture was filtered under reduced pressure and the resulting solution evaporated *in vacuo*. The residue was dissolved in CHCl₃ and washed with a 10% aqueous solution of NaOH. The organic phase was dried over anhydrous Na₂SO₄, filtered, evaporated *in vacuo*, and purified by flash column chromatography on silica gel (petroleum ether), obtaining the intermediate **2** as a transparent oil (190.5 mg, 0.84 mmol). Yield: 99%. ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 7.05 (t, 1H, *J* = 8.0 Hz), 6.76–6.74 (m, 1H), 6.68 (m, 1H), 6.46 (m, 1H), 3.27–3.24 (m, 4H), 2.05–1.99 (m, 4H).

5.1.2. 1-(3'-Nitrobiphenyl-3-yl)pyrrolidine (3)—In a sealed tube, under a flux of nitrogen, were introduced DME (40.0 mL), Pd(OAc)₂ (158.1 mg, 0.24 mmol) and P(Ph)₃ (307.9 mg, 1.17 mmol). The mixture was left under magnetic stirring at room temperature for 15 min, allowing the *in situ* formation of the catalyst. Then, compound **2** (701.0 mg, 3.10 mmol), NaHCO₃ (775.0 mg, 9.23 mmol), H₂O (17.6 mL) and 3-nitrophenylboronic acid (571.5 mg, 3.42 mmol) were added. The mixture was allowed to react at 95 °C, overnight. After cooling to room temperature, the solvent was removed *in vacuo* and the residue partitioned between water and ethyl acetate. The organic phase was dried over anhydrous Na₂SO₄, filtered, evaporated *in vacuo*, and purified by flash column chromatography on silica gel (petroleum ether/CH₂Cl₂ 7:3), obtaining the intermediate **3** as an orange solid (491.0 mg, 1.83 mmol). Yield: 59%. ¹H NMR (CDCl₃, 400 MHz) 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* = 7.9 Hz), 7.33 (t, 1H, *J* = 7.8 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).

5.1.3. 3'-(Pyrrolidin-1-yl)biphenyl-3-amine (4)—Hydrazine hydrate (0.2 mL, 66 mmol) was added to a solution of **3** (63.8 mg, 0.29 mmol) in ethanol (4.0 mL). The mixture was stirred at 50 °C for 15 min. Then, an excess of Raney nickel (~ 60 mg) was added. After the bubbling ceased (the solution changed from orange to colorless), the mixture was cooled to room temperature and filtered through a celite pad. The filtrate was evaporated *in vacuo*, affording intermediate **4** (58.5 mg) as a white solid, which was used in the next step without further purification. ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 7.28 (t, 1H, *J* = 7.8 Hz), 7.22 (t, 1H, *J* = 7.8 Hz), 7.03–7.01 (m, 1H), 6.95–6.92 (m, 1H), 6.88–6.85 (m, 1H), 6.75–6.73 (m, 1H), 6.69–6.65 (m, 1H), 6.59–6.55 (m, 1H), 3.36–3.33 (m, 4H), 2.05–1.99 (m, 4H).

5.1.4. N-[3-(pyrrolidin-1-yl)phenyl]-1,3-diamino-benzene (5)—Obtained by treating **2** (714.0 mg, 3.16 mol) with commercial 1,3-diaminobenzene **6** (410 mg, 3.79 mmol) and 628.0 mg of a reagent constituted by tris(dibenzylideneacetone)dipalladium(0), BINAP and *t*-BuONa (mol ratio: 0.05:0.15:2) in toluene (7.9 mL), following the same reaction and work-up conditions used for the synthesis of **2**. The crude was purified by flash column chromatography on silica gel (petroleum ether/AcOEt 9:1), obtaining **5** as a yellow oil (342.0 mg, 1.35 mmol). Yield: 43%. ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 7.11 (t, 1H, *J* =

8.0 Hz), 7.02 (t, 1H, $J = 8.0$ Hz), 6.50–6.42 (m, 3H), 6.32–6.31 (m, 1H), 6.25–6.20 (m, 2H), 5.58 (bs, 1H), 3.66 (bs, 2H), 3.28–3.25 (m, 4H), 2.00–1.97 (m, 4H).

5.1.5. N-(6-bromopyridin-2-yl)benzene-1,3-diamine (7)—A mixture of 1,3-diaminobenzene **6** (2.60 g, 24.0 mmol), 2,6-dibromopyridine (7.11 g, 30.0 mmol), *t*-BuOK (6.16 g, 55.2 mmol) and anhydrous toluene (130.0 mL) was refluxed for 48 h, under magnetic stirring. 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 anhydrous Na₂SO₄, filtered, evaporated *in vacuo*, and purified by flash column chromatography on silica gel (petroleum ether/AcOEt 6:4), obtaining the intermediate **7** as a brown oil (147.0 mg, 0.56 mmol). Yield: 16%. ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 7.29 (td, 1H, $J = 7.9$ Hz, $J = 0.3$ Hz), 7.10 (td, 1H, $J = 7.7$ Hz, $J = 0.8$ Hz), 6.86 (dd, 1H, $J = 7.5$ Hz, $J = 0.6$ Hz), 6.81 (dd, 1H, $J = 8.2$ Hz, $J = 0.6$ Hz), 6.63–6.58 (m, 2H), 6.44–6.41 (m, 1H), 4.00 (bs, 2H).

5.1.6. N-[6-(pyrrolidin-1-yl)pyridin-2-yl]benzene-1,3-diamine (8)—A mixture of **7** (362.0 mg, 1.37 mmol) and pyrrolidine (2.17 mL) was refluxed for 12 h. After cooling to room temperature, pyrrolidine was removed *in vacuo*, obtaining a brown oil that was dissolved in CHCl₃ and washed with water. The organic phase was dried over anhydrous Na₂SO₄, filtered, evaporated *in vacuo*, and purified by flash column chromatography on silica gel (petroleum ether/AcOEt 6:4), obtaining the intermediate **8** as a white solid (153.0 mg, 0.60 mmol). Yield: 45%. ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 7.29 (t, 1H, $J = 7.9$ Hz), 7.06 (t, 1H, $J = 7.9$ Hz), 6.87–6.84 (m, 1H), 6.72 (dd, 1H, $J = 8.0$ Hz, $J = 1.9$ Hz), 6.43 (bs, 1H), 6.32 (dd, 1H, $J = 7.8$ Hz, $J = 1.9$ Hz), 6.12 (d, 1H, $J = 7.8$ Hz), 5.82 (d, 1H, $J = 8.1$ Hz), 3.47–3.42 (m, 4H), 3.20 (bs, 2H), 2.01–1.96 (m, 4H).

5.1.7. General procedure for the synthesis of urea derivatives SC4a, FG45a and SN15b—4-Chloro-phenylisocyanate (1 eq) was added to a solution of the suitable amine intermediate (1 eq) in the minimum amount of freshly distilled CHCl₃. The resulting mixture was left at room temperature, under magnetic stirring, for 12 h. The solvent was removed by evaporation *in vacuo* or by filtration and the residue purified.

5.1.8. 1-(4-Chlorophenyl)-3-[3'-(pyrrolidin-1-yl)biphenyl-3-yl]urea (SC4a)—Prepared from 4-chloro-phenylisocyanate (39.0 mg, 0.25 mmol) and intermediate **4** (58.5 mg, 0.25 mmol) dissolved in 10.2 mL of freshly distilled CHCl₃. Purification by flash column chromatography on silica gel (petroleum ether/AcOEt 7:3). **SC4a** (37.1 mg, 0.10 mmol). Yield: 39%. M. p.: 168–172 °C. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 8.84 (s, 1H), 8.79 (s, 1H), 7.72–7.69 (m, 1H), 7.49 (AA'XX', 2H, $J_{AX} = 8.9$ Hz, $J_{AA'XX'} = 1.6$ Hz), 7.43–7.41 (m, 1H), 7.35–7.30 (m, 1H), 7.33 (AA'XX', 2H, $J_{AX} = 8.9$ Hz, $J_{AA'XX'} = 1.6$ Hz), 7.26–7.22 (m, 2H), 6.81 (d, 1H, $J = 7.3$ Hz), 6.72–6.68 (m, 1H), 6.55 (dd, 1H, $J = 8.0$ Hz, $J = 1.7$ Hz), 3.30–3.27 (m, 4H), 1.99–1.96 (m, 4H). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 152.49, 148.07, 141.98, 141.15, 139.89, 138.71, 129.44, 129.16, 128.61, 125.32, 120.56, 119.75, 117.20, 116.71, 113.76, 110.96, 109.78, 41.33, 24.96. HPLC analysis: retention time = 20.62 min; peak area, 99% (280 nm).

5.1.9. 2-(4-Chlorophenyl)-N-[3'-(pyrrolidin-1-yl)biphenyl-3-yl]acetamide (SC33)

—The reaction was conducted under nitrogen atmosphere. Et₃N (29.3 mg, 0.29 mmol) and DMAP (35.4 mg, 0.29 mmol) were added to a solution of **4** (69.4 mg, 0.29 mmol) in anhydrous CH₂Cl₂ (2.0 mL). Then, the mixture was cooled to -5 °C (ice-NaCl bath) and (4-chlorophenyl)acetyl chloride (54.8 mg, 0.29 mmol) was added. The reaction mixture was left, under magnetic stirring, at room temperature for 24 h. The crude was washed twice with an aqueous saturated solution of NaHCO₃ and purified by flash column chromatography on silica gel (petroleum ether/AcOEt 8:2), obtaining the desired product **SC33** as a solid (24.8 mg, 0.06 mmol). Yield: 22%. M. p.: 138–140 °C. ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 7.68 (bs, 1H), 7.64 (bs, 1H), 7.52–7.47 (m, 1H), 7.40–7.30 (m, 4H), 7.30–7.23 (m, 2H), 6.86 (d, 1H, *J* = 7.6 Hz), 6.75–6.71 (m, 1H), 6.59 (dd, 1H, *J* = 8.1 Hz, *J* = 1.9 Hz), 3.67 (s, 2H), 3.35–3.32 (m, 4H), 2.04–2.01 (m, 4H). ¹³C NMR (CDCl₃, 400 MHz) δ (ppm): 168.66, 141.84, 137.90, 133.75, 133.02, 130.98, 129.67, 129.43, 129.36, 123.77, 118.85, 118.78, 44.21, 29.85, 25.56. HPLC analysis: retention time = 20.75 min; peak area, 98% (280 nm).

5.1.10. 1-(4-Chlorophenyl)-3-(3-[[3-(pyrrolidin-1-yl)phenyl]amino]phenyl)urea (FG45a)

—Prepared from 4-chloro-phenylisocyanate (31.8 mg, 0.21 mmol) and intermediate **5** (52.5 mg, 0.21 mmol) dissolved in 8.5 mL of freshly distilled CHCl₃. Purification by flash column chromatography on silica gel (CHCl₃) and subsequent crystallization from EtOH. **FG45a** (34.7 mg, 0.09 mmol). Yield: 42%. M. p.: 168–171 °C. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 8.73 (s, 1H), 8.60 (s, 1H), 7.97 (s, 1H); 7.46 (AA'XX', 2H, *J*_{AX} = 9.0 Hz, *J*_{AA'XX'} = 2.6 Hz), 7.36–7.35 (m, 1H), 7.31 (AA'XX', 2H, *J*_{AX} = 8.9 Hz, *J*_{AA'XX'} = 2.6 Hz), 7.07 (t, 1H, *J* = 8.0 Hz), 7.00 (t, 1H, *J* = 8.0 Hz), 6.79–6.77 (m, 1H), 6.66–6.64 (m, 1H), 6.36–6.30 (m, 2H), 6.08–6.07 (m, 1H), 3.22–3.19 (m, 4H), 1.94–1.92 (m, 4H). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 152.35, 148.57, 144.44, 143.78, 140.17, 138.80, 129.43, 129.25, 128.60, 125.20, 119.59, 110.64, 109.17, 106.04, 105.18, 104.29, 100.78, 47.24, 24.95. HPLC analysis: retention time = 16.53 min; peak area, 97% (280 nm).

5.1.11. 1-(4-Chlorophenyl)-3-[[6-(pyrrolidin-1-yl)pyridin-2-yl]amino]phenyl)urea (SN15b)

—Prepared from 4-chloro-phenylisocyanate (20.3 mg, 0.13 mmol) and intermediate **8** (33.8 mg, 0.13 mmol) dissolved in 5.4 mL of freshly distilled CHCl₃. Purification by crystallization from EtOH. **SN15b** (14.8 mg, 0.04 mmol). Yield: 28%. M. p.: dec. at 220 °C. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 8.81 (s, 1H), 8.67 (s, 1H), 8.54 (s, 1H), 7.88–7.82 (m, 1H), 7.50–7.47 (m, 2H), 7.44–7.42 (m, 1H), 7.33–7.31 (m, 2H), 7.28–7.24 (m, 1H), 7.11–7.07 (m, 1H), 6.81 (d, 1H, *J* = 8.1 Hz), 6.03 (d, 1H, *J* = 7.9 Hz), 5.77 (d, 1H, *J* = 7.6 Hz), 3.44–3.37 (m, 4H), 1.96–1.90 (m, 4H). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 155.99, 154.65, 152.31, 142.80, 139.52, 138.88, 138.03, 128.58, 125.11, 119.52, 111.76, 110.06, 107.86, 97.03, 95.56, 46.42, 25.01. HPLC analysis: retention time = 4.65 min; peak area, 95% (280 nm).

5.2. Biological assays

5.2.1. Radioligand binding assays on CB1R and CB2R—Cannabinoid receptors affinity was determined using membrane preparations obtained from CHO-K1 cells stably transfected with *hCB1* or *hCB2* as previously described.³⁸ Briefly, 20 mg of membrane

preparation were resuspended in 500 μL of binding buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl_2 , 0.5% fatty acid free BSA, pH 7.4) in silanized glass tubes. Membranes were incubated with the compounds at different concentrations (1 nM–100 μM) in presence of 0.5 nM of [^3H]CP55,940 (168 Ci $\cdot\text{mmol}^{-1}$) for 1.5 h at 30 $^\circ\text{C}$. Non-specific binding of the radioligand was determined in the presence of 10 μM of (*R*)-WIN55,512–2. Non-specific binding was around 10%. After the incubation time, membrane suspensions were rapidly filtered through a 0.05% polyethyleneimine presoaked 96-well microplate bonded with GF/B glass fiber filters (UniFilter-96GF/B, Perkin Elmer Life Sciences) under vacuum and washed 10 times with 167 μL of ice-cold washing buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl_2 , 0.5% fatty acid free BSA, pH 7.4). Filters were added to 3 mL of MicroScint 20 scintillation liquid and radioactivity was measured with the 1450 Micro Beta Trilux top counter. The non-specific binding was subtracted and results were expressed as [^3H]CP55,940 bound as % of binding in vehicle-treated samples.

5.2.2. FAAH, MAGL and AEA uptake assays—FAAH and MAGL activity assay and AEA uptake assays were performed as previously described.^{39,40} Briefly, FAAH and MAGL activity assays were performed using U937 cell homogenate (100 μg) which were diluted in 200 μL of Tris–HCl 10 mM, EDTA 1 mM, pH 8 containing 0.1% fatty acid-free BSA. Compounds were added at the screening concentration of 10 μM and incubated for 30 min at 37 $^\circ\text{C}$. Then, 100 nM of AEA containing 1 nM of [ethanolamine-1- ^3H]AEA as a tracer was added to the homogenates and incubated for 15 min at 37 $^\circ\text{C}$. The reaction was stopped by the addition of 400 μL of ice-cold $\text{CHCl}_3\text{:MeOH}$ (1:1), samples were vortexed and rapidly centrifuged at 16'000 \times g for 10 min. at 4 $^\circ\text{C}$. The aqueous phases were collected and the radioactivity was measured for tritium content by liquid scintillation spectroscopy. For AEA uptake assay 0.5×10^6 of intact U937 cells were suspended in 500 μL of serum-free medium in silanized glass tubes and preincubated with a screening concentration (10 μM) of the compounds for 20 min at 37 $^\circ\text{C}$. Then, the cells were incubated for 5 min at 37 $^\circ\text{C}$ with 100 nM of AEA and [ethanolamine-1- ^3H]AEA (0.5 nM) was added. The uptake process was stopped by transferring the tubes on ice and by rapid filtration over UniFilter®-96 GF/C filters pre-soaked with PBS supplemented with 0.2% BSA. Cells were washed three times with 100 μL ice-cold PBS supplemented with 1% fatty acid free BSA. After drying, 50 μL MicroScint 20 scintillation cocktail was added to the wells. The radioactivity was measured using a Trilux MicroBeta 1450.

5.2.3. Serum response element (SRE) assay—HEK293 cells were transiently transfected with *hCB1R* (5 ng/well) and pGL4.33 [luc2P/SRE/Hygro] vector (100 ng/well) reporter (Promega) plasmids using Lipofectamine 2000 as described by the manufacturer (Invitrogen).⁴⁶ Transfected HEK293 cells were seeded (60,000 cells per well) in 96-well plates. Five hours later, medium was changed to 1% FBS/DMEM. Cells were incubated overnight. The next day cells were treated with ligands for 5 h in serum-free DMEM medium at 37 $^\circ\text{C}$. After treatment, cells were lysed by 1X lysis buffer for 10 min. at room temperature. Plates were read to record bioluminescent after the injection of 40 μL Luciferin (250 μM) per well. Luminescence was measured in an Envision 2104 Multilabel Reader (Perkin Elmer). Luminescence values are given as relative light units. Concentration-effect curves for agonist-mediated receptor activation were analyzed by nonlinear regression

techniques using GraphPad Prism 5.0 software (GraphPad) and data were fitted to sigmoidal dose-response curves to obtain EC₅₀ values.

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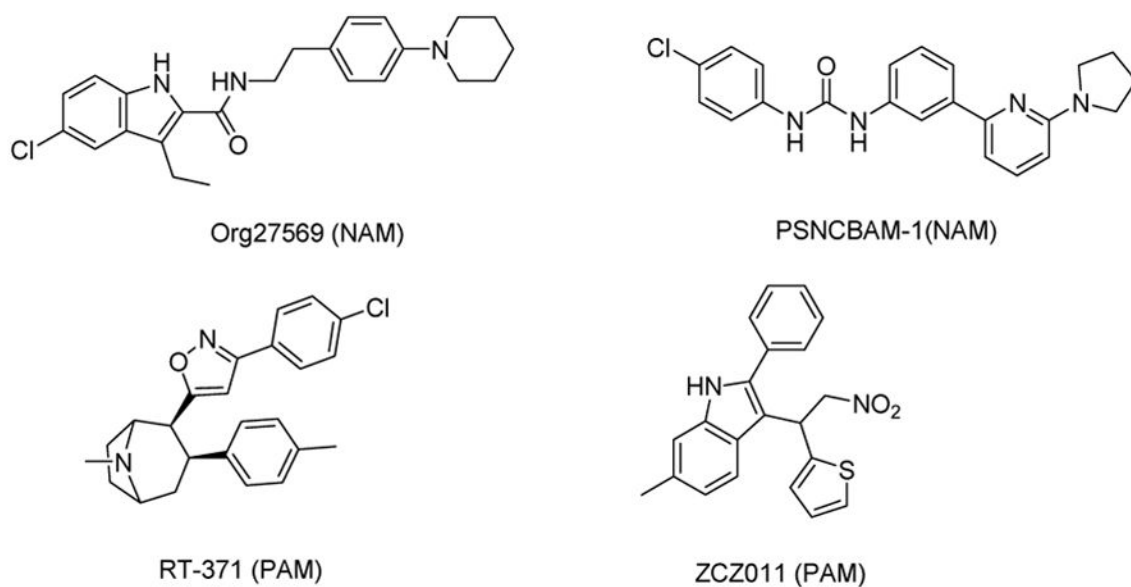


Figure 1.
Chemical structures of some CB1R allosteric modulators.

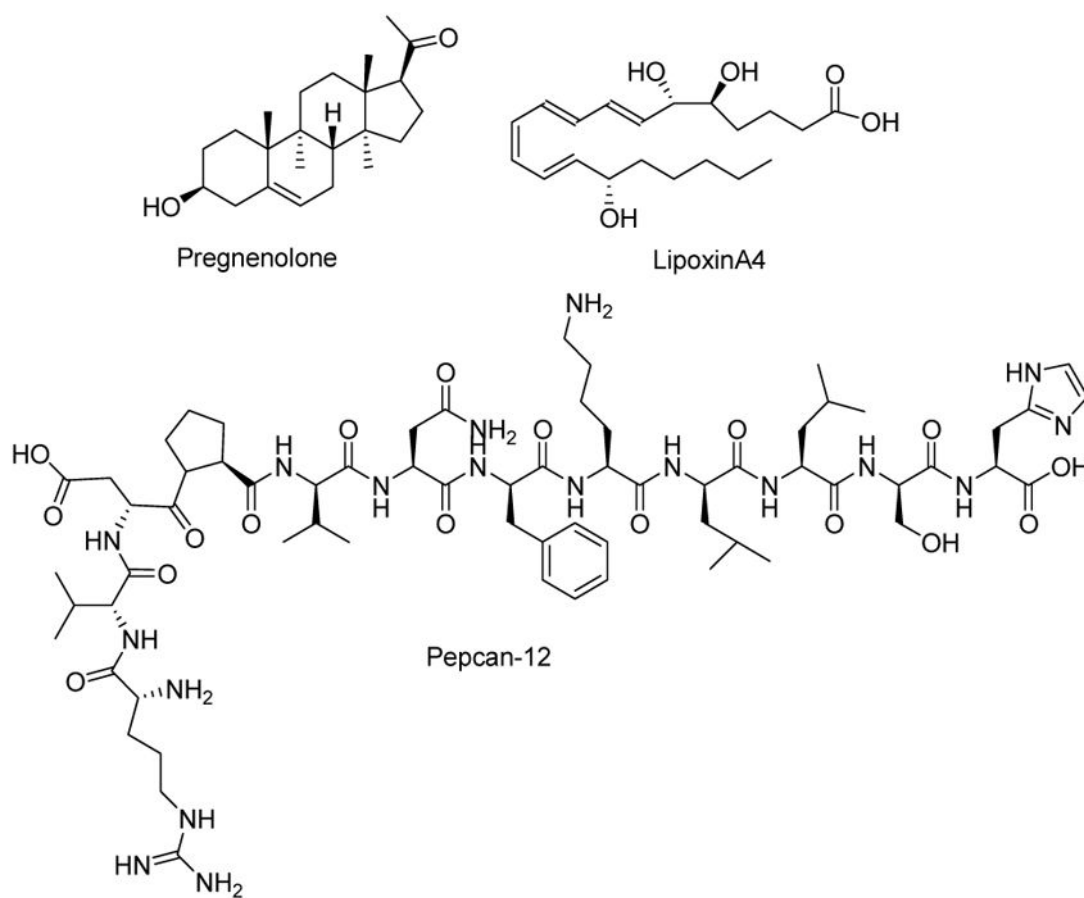


Figure 2.
Chemical structures of endogenous CB1R allosteric modulators.

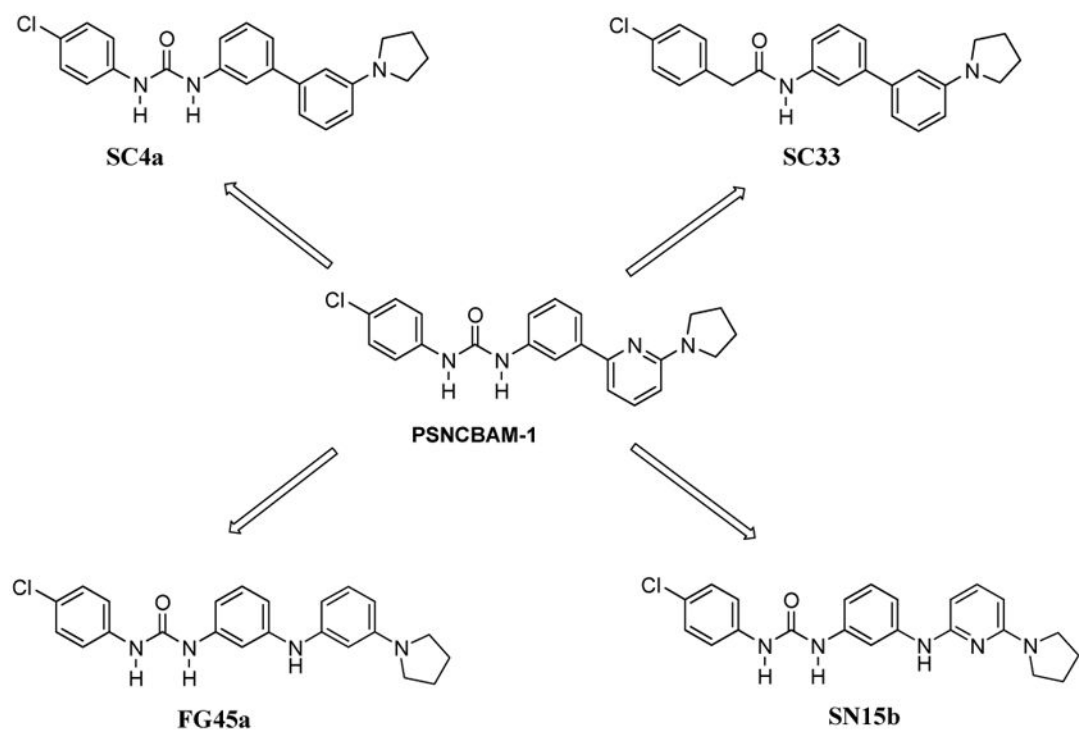


Figure 3.
Compounds **SC4a**, **SC33**, **SN15b** and **FG45a** structurally derived from urea derivative PSNCBAM-1.

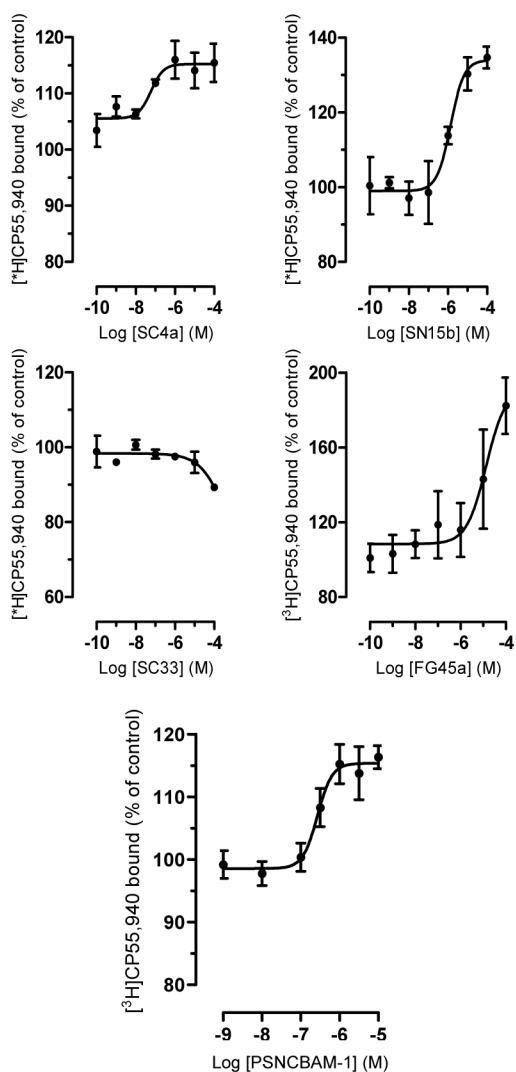


Figure 4.

CB1 receptor binding. Compounds **SC4a**, **SN15b**, **SC33**, **FG45a** and **PSNCBAM-1** were incubated at different concentrations with membrane preparations obtained from CHO-K1 cells overexpressing *hCB1R* in the presence of 0.5 nM of [³H]CP55,940. Data show mean values ± SD of at least 3 independent experiments each performed in triplicates.

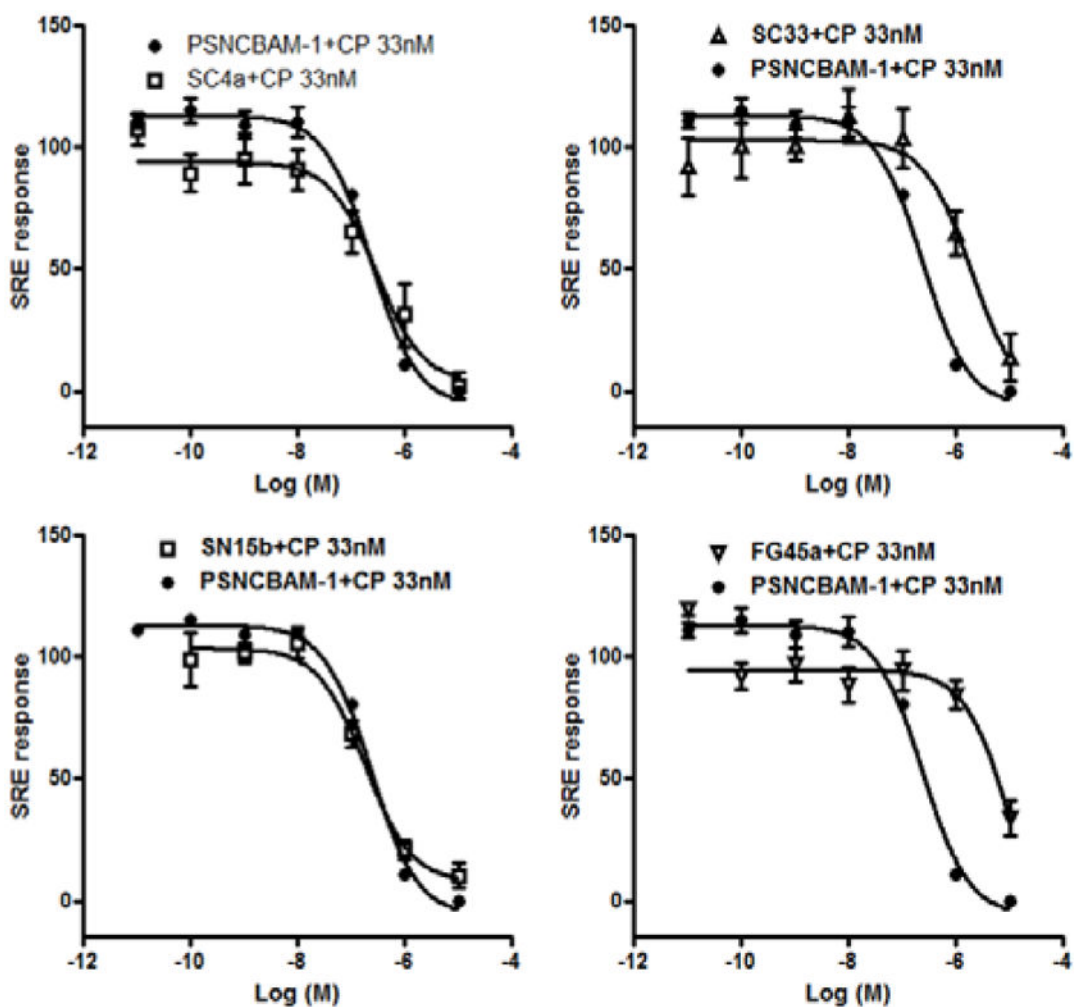


Figure 5. Compounds **SC4a**, **SN15b**, **SC33** and **FG45a** were found to decrease the SRE response induced by CB1 agonist CP55,940 (33 nM). PSNCBAM-1 was used as a reference compound. Data show mean values \pm SD of at least 3 independent experiments each performed in triplicates.

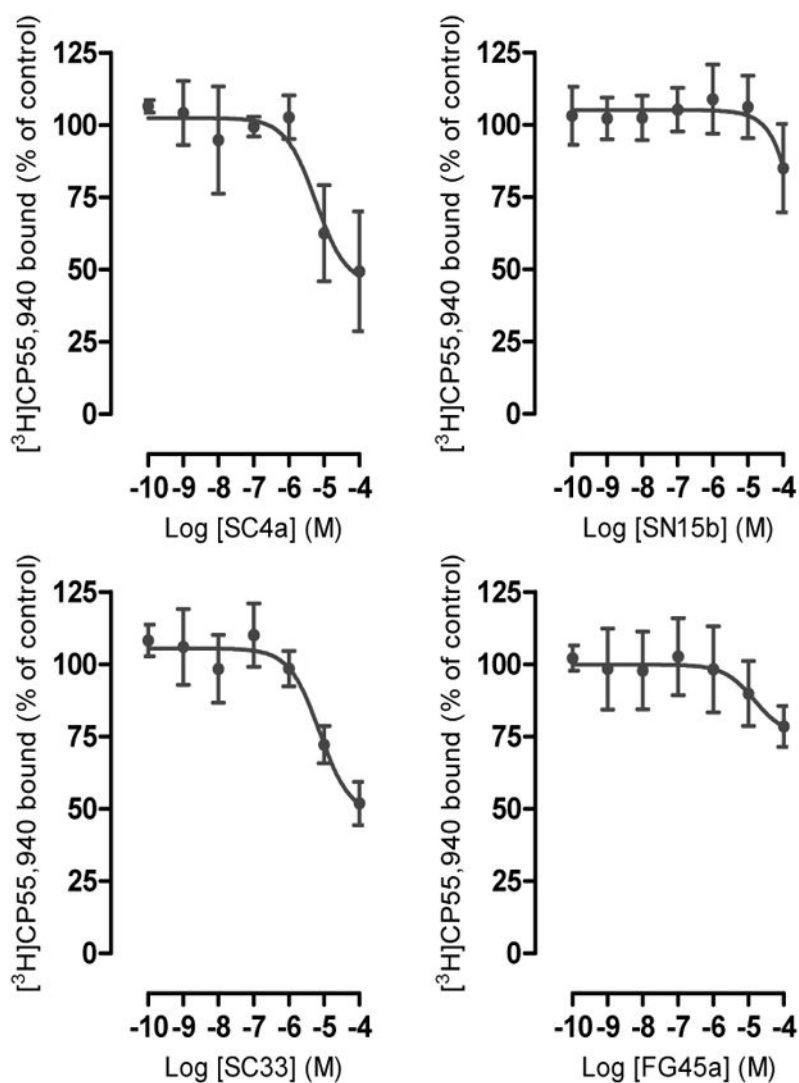
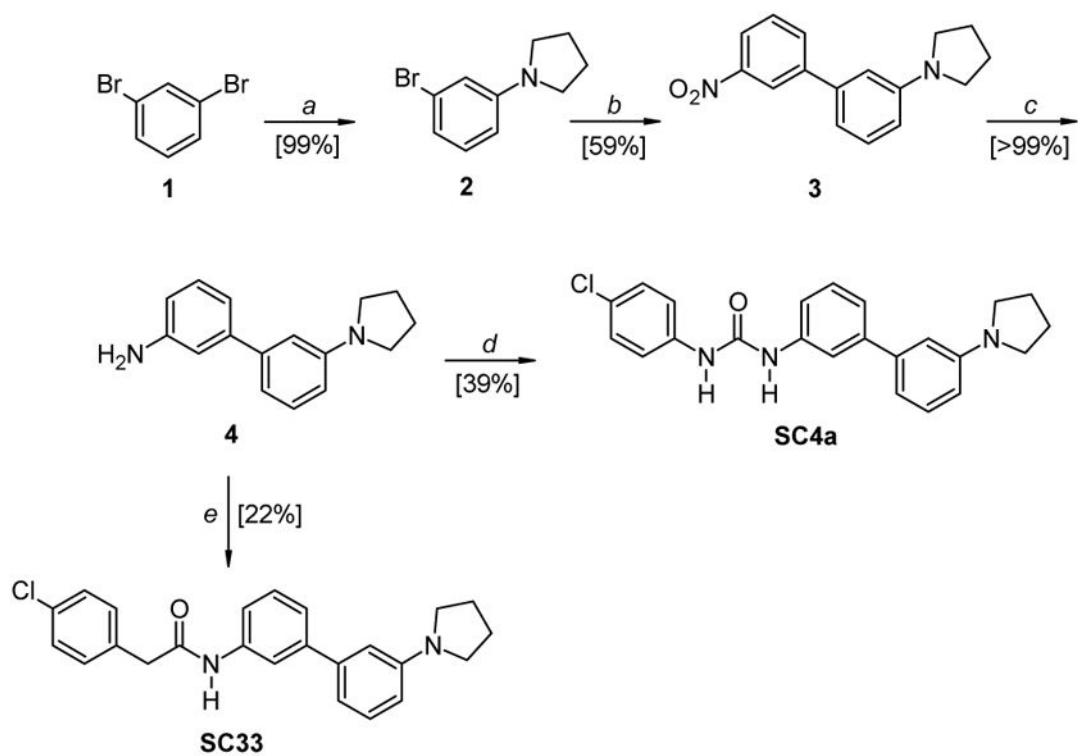
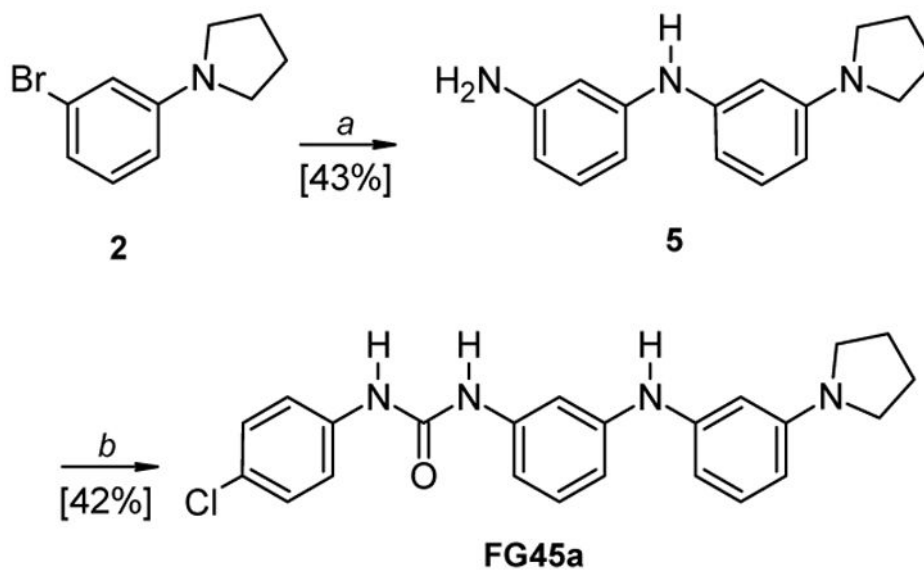


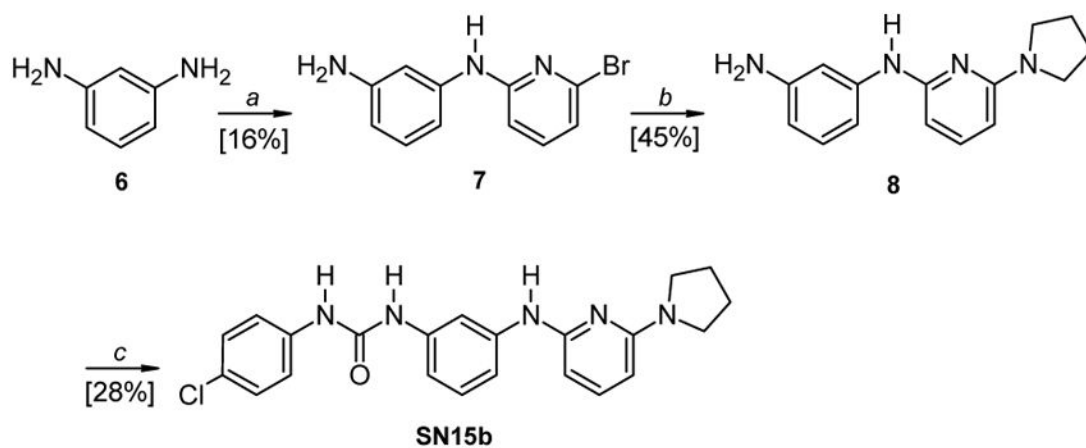
Figure 6. CB2 receptor binding. Compound **SC4a**, **SN15b**, **SC33** and **FG45a** were incubated at different concentrations with membrane preparations obtained from CHO-K1 cells overexpressing *hCB2* receptors in presence of 0.5 nM of [^3H]CP55,940. Data show mean values \pm SD of at least 3 independent experiments each performed in triplicates.

**Scheme 1.**

Reagents and conditions: *a*) pyrrolidine, Pd₂(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, P(Ph)₃, aq. NaHCO₃, DME, 95 °C, overnight; *c*) Raney nickel, NH₂NH₂·H₂O, EtOH, 50 °C, 40 min – 1 h; *d*) 4-chloro-phenylisocyanate, anhydrous CHCl₃, RT, 12 h; *e*) (4-chlorophenyl)acetyl chloride, Et₃N, DMAP, anhydrous CHCl₃, RT, 24 h.

**Scheme 2.**

Reagents and conditions: *a*) 1,3-diaminobenzene, Pd₂(dba)₃: BINAP: *t*-BuONa (mol ratio: 0.05:0.15:2), toluene, 80 °C, 4.5 h; *b*) 4-chloro-phenylisocyanate, anhydrous CHCl₃, RT, 12 h.

**Scheme 3.**

Reagents and conditions: *a*) 2,6-dibromopyridine, *t*-BuOK, anhydrous toluene, reflux, 48 h; *b*) pyrrolidine, reflux, 12 h; *c*) 4-chloro-phenylisocyanate, anhydrous CHCl₃, RT, 12 h.

Table 1

Summary data for **SC33**, **SN15b**, **SC4a** and **FG45a**.^a

Compound	EC ₅₀ (μM)	IC ₅₀ (μM)	CBIRBinding	CBIRSRE assay	CB2RBinding	FAAH	MAGL	AEA uptake
PSNCBAM-1	0.27 (0.14–0.52)	0.23 (0.16–0.34)	>10	>10	2.1 (1.9–2.4)	>100	>10	>10
SC33	>10	1.9 (0.52–6.9)	>100	>10	>10	>10	>10	>10
SN15b	1.3(0.41–5.8)	0.18 (0.09–0.36)	52.6(21.5–88.4)	>10	>10	>10	>10	>10
SC4a	0.043(0.005–0.52)	0.29 (0.10–0.84)	49.8(12.9–91.5)	>10	>10	>10	>10	>10
FG45a	13.5(5.1–35.3)	9.6 (0.36 – 25)	>100	>10	>10	>10	>10	>10

^aData show mean and 95% CI; n = 3.