European Journal of Medicinal Chemistry 101 (2015) 651–667

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Research paper

Synthesis, pharmacological evaluation and docking studies of pyrrole structure-based CB_2 receptor antagonists

Giulio Ragusa [a, 1], María Gómez-Cañas [b, c, d, e, 1], Paula Morales [f, 1], Dow P. Hurst [g], Francesco Deligia [a], Ruth Pazos [b, c, d, e], Gerard A. Pinna [a], Javier Fernández-Ruiz [b, c, d, e], Pilar Goya [f], Patricia H. Reggio [g], Nadine Jagerovic [f, ***], Moisés García-Arencibia [b, c, d, e, **, 2], Gabriele Murineddu [a, *]

[a] Dipartimento di Chimica e Farmacia, Università degli Studi di Sassari, via F. Muroni 23/A, 07100 Sassari, Italy
[b] Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Investigación en Neuroquímica, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain
[c] Campus de Excelencia Internacional (CEI-Moncloa), Madrid, Spain
[d] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[e] Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain
[f] Instituto de Química Médica, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain
[g] Center for Drug Discovery, Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro, NC 27402, USA

ARTICLE INFO

Article history:
Received 18 December 2014
Received in revised form
23 June 2015
Accepted 23 June 2015
Available online 15 July 2015

Keywords:
Bioisosterism
Synthesis
Cannabinoid receptors
CB_2 antagonism
Docking studies

ABSTRACT

During the last years, there has been a continuous interest in the development of cannabinoid receptor ligands that may serve as therapeutic agents and/or as experimental tools. This prompted us to design and synthesize analogues of the CB_2 receptor antagonist *N*-fenchyl-5-(4-chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-1*H*-pyrazole-3-carboxamide (SR144528). The structural modifications involved the bioisosteric replacement of the pyrazole ring by a pyrrole ring and variations on the amine carbamoyl substituents. Two of these compounds, the fenchyl pyrrole analogue **6** and the myrtanyl derivative **10**, showed high affinity (K_i in the low nM range) and selectivity for the CB_2 receptor and both resulted to be antagonists/inverse agonists in [^{35}S]-GTPγS binding analysis and in an *in vitro* CB_2 receptor bioassay. Cannabinoid receptor binding data of the series allowed identifying steric constraints within the CB_2 binding pocket using a study of Van der Waals' volume maps. Glide docking studies revealed that all docked compounds bind in the same region of the CB_2 receptor inactive state model.

1. Introduction

The endocannabinoid system is an intercellular communicating system, active in the brain and in the periphery, that comprises the G-protein coupled cannabinoid receptors CB_1 and CB_2, some signalling lipids, and the proteins responsible for their synthesis and inactivation (reuptake and degradation) [1–3].

It appears well demonstrated that the CB_2R (CB_2 receptors) have a different distribution than CB_1R (CB_1 receptors) in the body, the former being preferentially found in the peripheral tissues (e.g. immune tissues, bone) and the latter widely distributed in the central nervous system (CNS) [4]. Recently, CB_2R gene transcripts and proteins have also been discovered in the CNS, preferentially located in glial elements [5], particularly when they become activated by different types of insults. However, they can be found only in small amounts in the CNS. In contrast, CB_1R are abundant in most neuronal cells, in concordance with their key role in regulation of synaptic processes [6].

The absence of psychoactive effects, given the poor presence of CB_2R in neuronal subpopulations, has increased the interest in

* Corresponding author.
** Corresponding author. Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Investigación en Neuroquímica, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain.
*** Corresponding author.
E-mail addresses: nadine@iqm.csic.es (N. Jagerovic), moisesgar@med.ucm.es (M. García-Arencibia), muri@uniss.it (G. Murineddu).
[1] These three authors share the first position within the authorship.
[2] Present address: Department of Biochemistry and Molecular Biology, Universidad de Las Palmas de Gran Canaria, ULPGC, Las Palmas, Spain.

http://dx.doi.org/10.1016/j.ejmech.2015.06.057
0223-5234/

developing selective CB_2R ligands. Selective agonists of this receptor may be useful for the treatment of neuropathic and inflammatory pain [7,8], and also for their anti-inflammatory/neuroprotective properties in a number of neurodegenerative disorders [9]. In addition, the selective activation of the CB_2R may be also useful for the treatment of certain types of cancer [10,11], as well as immune disorders [12]. By contrast, CB_2R antagonists may be useful for bone disorders [13] as these receptors are expressed in osteoblasts, osteoclasts, and osteocytes, in which they play a role in the regulation of specific activities of these cells in the process of bone formation/resorption. Particular attention has been paid to the process of osteoclast formation and bone resorption, which has been found to be inhibited by CB_2R antagonists and enhanced by CB_2R activation. However, the role of CB_2R in bone formation/resorption is rather controversial, and other authors reported opposite results using also pharmacological (agonists *vs* antagonists) and genetic (studies in CB_2R-deficient mice) strategies [14].

This biopharmacological potential prompted us to design and synthesize deaza-analogues of the potent CB_2 ligand SR144528 [15] (Fig. 1). To this end, structure–activity relationships (SAR) studies were conducted on two different regions of the template compound, SR144528, by (a) replacing the pyrazole ring of the phenylpyrazole moiety with a phenylpyrrole ring system (**6**), and (b) substituting the amine carbamoyl group on the pyrazole ring with other amines (**7**–**22**, **25** and **26**). The objective of this work was also to prepare a rigid analogue (**39**) incorporating the phenylpyrrole backbone into a tricyclic ring system for conformational restriction purpose.

Previously, as part of a project with Seltzman's group, Reggio [16] published studies on the fenchyl-pyrrole derivative of SR144528 (compound **6**). Meanwhile Seltzman prepared this pyrrole derivative by cycloaddition of tosylbenzylisocyanide with ethyl acrylate; in our group we had an ongoing project on the preparation of this pyrrole and derivatives by cycloaddition of acetophenone oxime with methyl propiolate. The study presented here showed a large structure variation on the amine carbamoyl substituent. The *in vitro* binding affinities for CB_2R and CB_1R were measured for all compounds (**6**–**22**, **25**, **26** and **39**) and selected compounds **6** and **10** were also tested for their functional activities in [^{35}S]-GTPγS binding analysis. To further study their interaction mode with CBRs, molecular docking studies were carried out. The results of these studies are reported below.

2. Results and discussion

2.1. Chemistry

The desired compounds (see Table 1) were prepared according to the reactions depicted in Schemes 1–3. A series of SR144528 analogues **6**–**22** was prepared (Scheme 1) from the 1*H*-pyrrole-3-carboxylic acid **5**. The key intermediate **5** was synthesized by a four-step synthesis starting from the commercial ketone **1**, whose reaction with hydroxylamine hydrochloride led to the corresponding oxime **2**. Further Michael addition of **2** with methyl propiolate, followed by thermal cyclization under microwave irradiation of the resulting *O*-vinyl oxime (structure of intermediate not reported), gave the pyrrole ester **3**. And then, *N*-alkylation of **3** with 4-methylbenzyl chloride in presence of NaH (60% in mineral oil) gave the *N*-methylbenzyl-1*H*-pyrrole **4** which was subsequently saponified to provide the carboxylic acid **5**. Finally, activation of **5** with thionyl chloride followed by reaction with the appropriate amines afforded the desired compounds **6**, **8**–**22**.

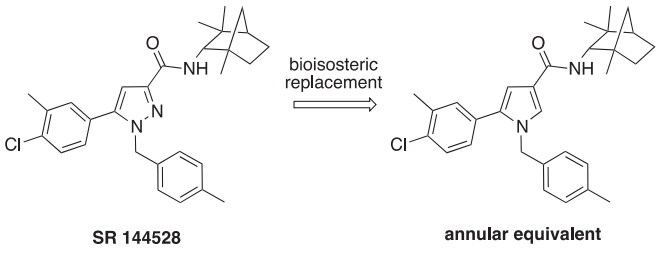


Fig. 1. Annular equivalent: pyrazole/pyrrole.

To evaluate the influence of the carboxy group of the fenchyl pyrrole **6** on receptor binding, this latter was replaced by a methylene group. The corresponding compound **7** was readily prepared by reduction of **6** with $LiAlH_4$ (Scheme 1). Furthermore, two other methylene compounds **25** and **26** were prepared using a different strategy as outlined in Scheme 2. Reduction of **5** to alcohol **23** with $LiAlH_4$ followed by oxidation to aldehyde **24** using MnO_2, and finally reductive amination in the presence of $NaCNBH_4$ led to compounds **25** and **26**.

Conformationally constrained analogue of the fenchyl pyrrole analogue **6** in which the C2′ of the di-substituted phenyl ring and the C4 of the pyrazole were linked by a methylene bridge was proposed. The target compound **39** was synthesized *via* the routes illustrated in Scheme 3. Benzoic acid derivative **27** was reduced to alcohol **28**, and then it was oxidized to aldehyde **29**. The Knoevenagel condensation of **29** with malonic acid in pyridine gave the derivative **30**, whose reduction to **31** followed by Sandmeyer reaction (**32**) and cyclization in presence of CH_3SO_3H yielded 4-methyl-5-chloro-1-indanone **33**. Subsequent bromination of **33** to **34** and its alkylation (**35**) with ethyl acetoacetate followed by treatment with NH_4OAc gave the tricyclic core **36**. Following the procedures used for the preparation of the series **6**, **8**–**22** starting from **5** as depicted in Scheme 1, *N*-alkylation of **36** with 4-methylbenzyl chloride (**37**) followed by saponification (**38**) and final reaction with fenchylamine, *via* acyl chloride, yielded the desired conformationally constrained compound **39**.

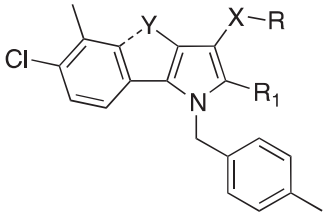
2.2. CB_1/CB_2 receptor binding studies

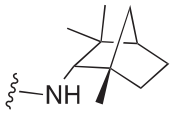
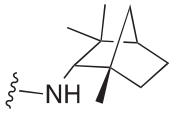
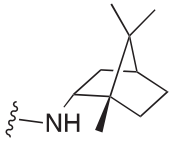
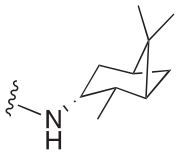
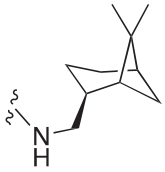
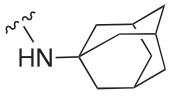
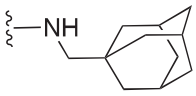
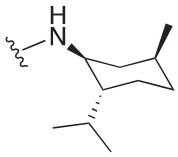
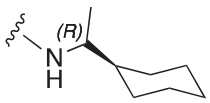
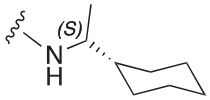
Radioligand binding assays have been used to evaluate the affinity of the new 5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrroles **6**–**22**, **25**, **26** and tricyclic congener **39** to CBRs expressed in membrane fractions of human CB_1 or CB_2 transfected cells. They were first subjected to a preliminary screening at a concentration of 40 μM, except for carboxamides **12**, **18**, **22** and amine **26**, which was performed at 10 μM, and for amine **25** and carboxamide **39**, performed at 5 μM, due to solubility reasons. A complete dose–response curve was generated for the compounds that displaced the radioligand by > 50% in the preliminary screen in at least one of the two receptors analysed. The CB_2R and CB_1R affinities of the new 5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrroles **6**–**22**, **25**, **26** and the tricyclic congener **39** are reported in Table 1. For comparison, the K_i values of the reference CB_2 ligand SR144528 have been reported.

Bioisosteric replacement of the pyrazole ring of the lead compound SR144528 by a pyrrole ring (**6**) retains an attractive CB_2R binding value (K_i = 5.7 nM) and CB_2 selectivity binding value (K_i CB_1/K_i CB_2 = 258), although not better than the parent compound (K_i = 0.6 nM, and K_i CB_1/K_i CB_2 = 667) [15]. This is consistent with the reduced, but not completely lost, affinity of the pyrrole **8** compared to its pyrazole counterpart (K_i = 164 and 29 nM [16], respectively). Thus, the replacement of the pyrazole *N*-1 nitrogen by CH did not drastically modify affinity for CB_2R.

As reported in the pyrazole series [16], we observed that the amide group plays a role in the interaction with the CB_2R.

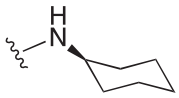
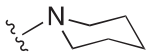
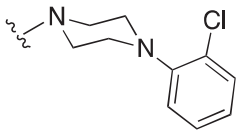
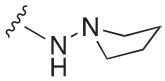
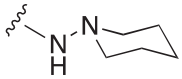
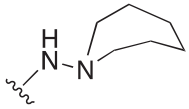
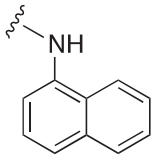
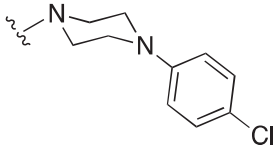
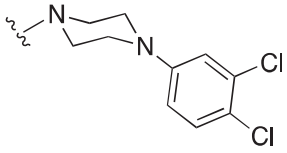
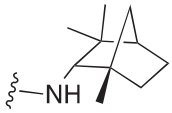
Table 1
Structures and binding data[a] for compounds **6**–**22**, **25**, **26**, **39** and **SR144528**.



Compd	X	Y	R	R_1	K_i CB_2 (nM)	K_i CB_1 (nM)
6	C=O	H		H	5.7 ± 0.7	1470 ± 179
7	CH_2	H		H	343 ± 24.1	ND[b]
8	C=O	H		H	164 ± 11.2	368 ± 45
9	C=O	H		H	>5000	ND
10	C=O	H		H	72.2 ± 9.4	>5000
11	C=O	H		H	>5000	>5000
12	C=O	H		H	>5000	>5000
13	C=O	H		H	1100 ± 91.5	>5000
14	C=O	H		H	>5000	>5000
15	C=O	H		H	492 ± 54.4	ND

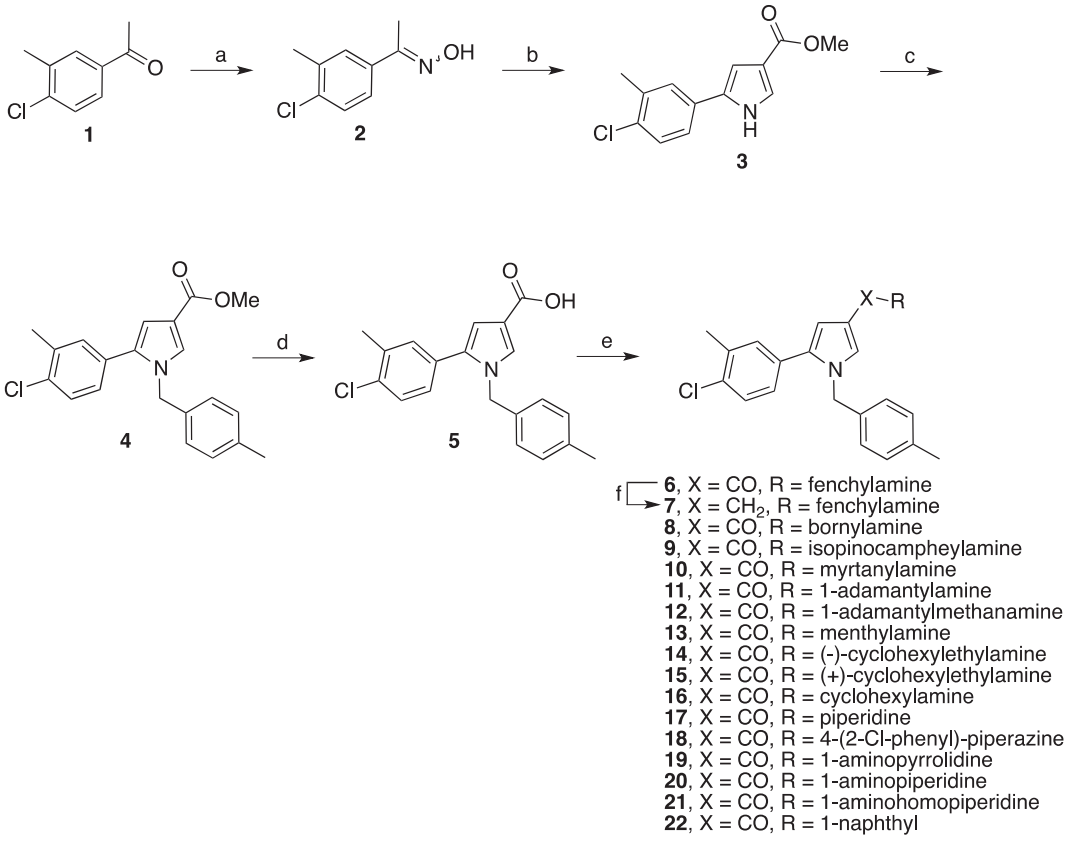
(*continued on next page*)

Table 1 (*continued*)

Compd	X	Y	R	R_1	K_i CB_2 (nM)	K_i CB_1 (nM)
16	C=O	H		H	244 ± 34.2	>5000
17	C=O	H		H	106 ± 7.2	3070 ± 15.5
18	C=O	H		H	1707 ± 226	>5000
19	C=O	H		H	>5000	>5000
20	C=O	H		H	>5000	>5000
21	C=O	H		H	575 ± 35	>5000
22	C=O	H		H	>5000	>5000
25	CH_2	H		H	>5000	>5000
26	CH_2	H		H	>5000	>5000
39	C=O	CH_2		CH_3	346 ± 117	>5000
SR144528 [15]					0.6	400

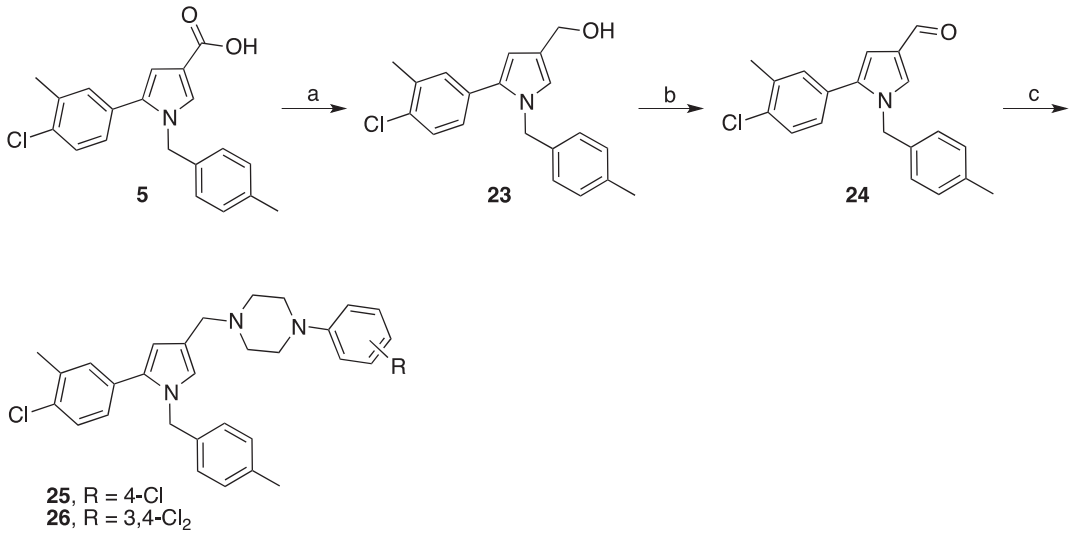
[a] Affinity of compounds for the CB_1R and CB_2R was assayed using RBHCB1M400UA and RBXCB2M400UA membranes respectively and [^{3}H]-CP-55,940 as radioligand. K_i values were obtained from three independent experiments carried out in triplicate and are expressed as mean ± standard error.
[b] ND, not determined.

Substitution of the carboxy group in pyrrole **6** by a methylene group (**7**) caused significant decrease in affinity. As well, pyrroles **25** and **26** that contain the methylene group resulted in loss of CB_2R binding. These data support the importance of hydrogen bond between the amide group and a residue (D(275)) of the inactive CB_2R binding site proposed by Kotsikorou et al. [16] for SR144528. Once confirmed the importance of the carbamoyl group, we focused our interest on the carbamoyl substituent. Curiously, few structural modifications have been reported in the literature on the fenchyl part of the pyrazole SR144528. One of these modifications was the substitution of the fenchyl by a bornyl group that causes a change in K_i (CB_2) value from 0.31 to 7.2 nM. In the pyrrole series, the same structural modification, compound **8**, lower the K_i (CB_2) value in the same extent (from 5.7 to 164 nM). Given the fact that much less is known about the influence of the carbamoyl substituent on CB_2 binding, we first compared compounds bearing as monoterpene moiety a bornyl (**8**), an isopinocampheyl (**9**) and a myrtanyl (**10**) group. It is worthy to note the variation of affinity and selectivity depending on the position of the carbamoylpyrrole on the fenchyl moiety (Table 1). Derivative **8** showed 4-fold increase in CB_1R affinity and reduced affinity towards CB_2R compared to the fenchyl analogue **6**, wheres the myrtanyl derivative **10** binds selectively to the CB_2R (K_i = 72.2 nM). The presence of isopinocamphene (**9**) as well as the bulky adamantanes (**11** and **12**) led

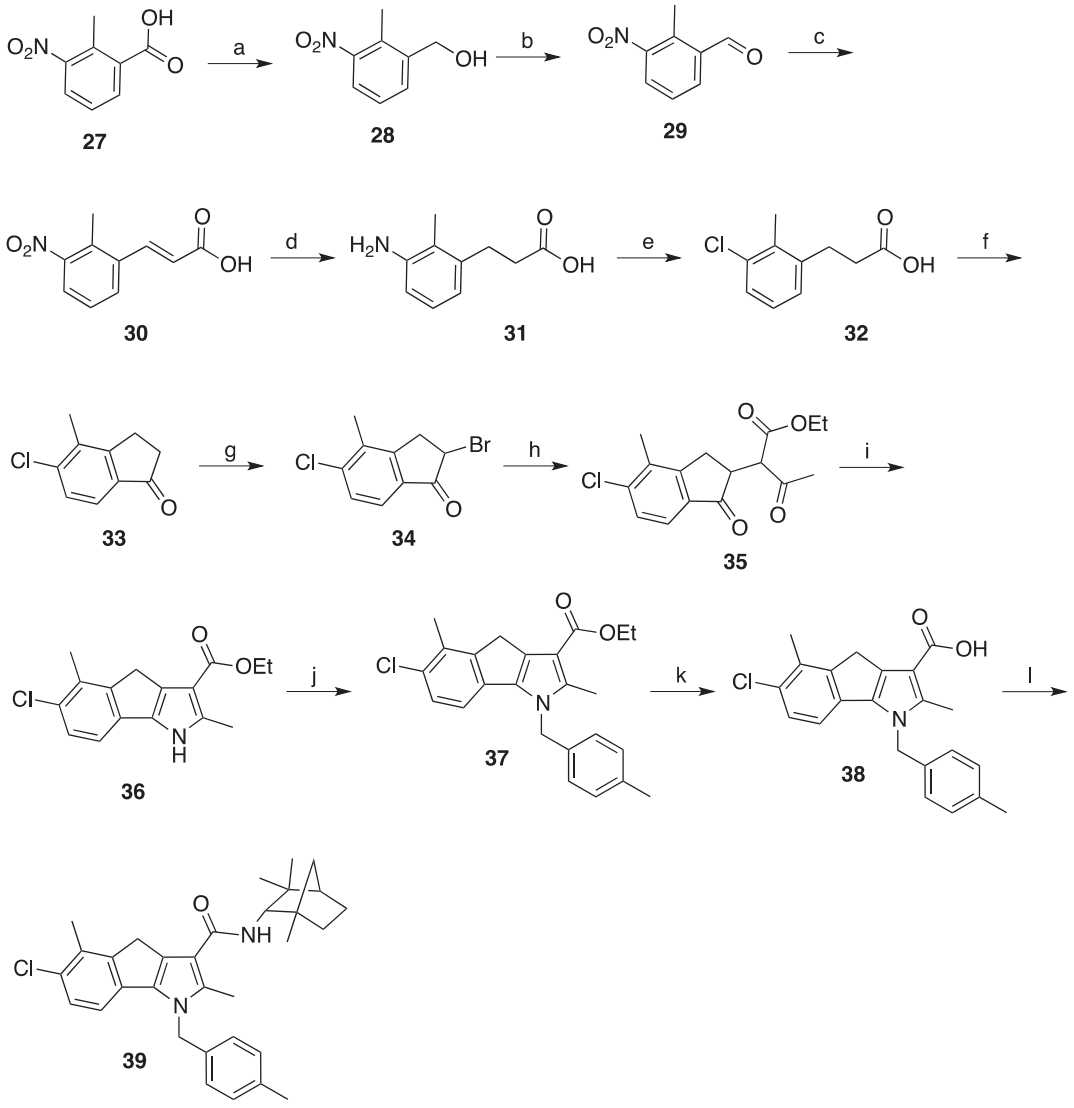


Scheme 1. *Reagents and conditions*: a) $NH_2OH{\cdot}HCl$, $AcONa{\cdot}3H_2O$, H_2O, EtOH, reflux, 2.5 h; b) (i) $HC{\equiv}CCO_2CH_3$, 1,4-diazabicyclo[2.2.2]octane (DABCO), toluene, MW, 80 °C, 10 min, (ii) MW, 170 °C, 45 min; c) (i) DMF_{an}, 60% NaH in mineral oil, r.t., 15 min, (ii) 4-Me−BnCl, THF_{anh}, r.t., 4 h; d) 10% $NaOH_{aq}$, reflux, 12 h; e) (i) $SOCl_2$, toluene, reflux, 4 h, (ii) CH_2Cl_2, TEA, $R{-}NH_2$, r.t., 2 h; f) $LiAlH_4$, THF_{anh}, r.t., 12 h.

to a total loss of affinity for CB receptors. Indeed, bulky substituents are not favourable for binding sites. To further explore the possible steric effects of the amine cyclohexyl substituents on CB receptors affinity, compounds **13**–**16** were evaluated. Among these, cyclohexyl derivative **16** showed the highest affinity for the CB_2R ($K_i = 244$ nM), whereas the introduction of the two enantiomers of cyclohexylethylamine furnished the compound **14**-(*R*), with no affinity for CBRs, and its *S*-enantiomer **15** with a K_i value of 492 nM for CB_2R. The influence of the nature of heterocyclic ring containing one or two nitrogen atoms on the carboxamide portion was also explored. *N*1-Piperidine (**17**) retained CB_2 affinity while *N*4-arylpiperazine (**18**) showed a clear decrease in CB_2 affinity. None of the piperazine derivatives (**18**, **25**, **26**) binds to CBRs except **18** with a K_i value in the micromolar range for CB_2R. Among the carbohydrazide



Scheme 2. *Reagents and conditions*: a) $LiAlH_4$, THF_{anh}, r.t., 4 h; b) MnO_2, CH_2Cl_2, reflux, 12 h; c) arylpiperazine, MeOH, AcOH, $NaCNBH_4$, 0–25 °C, 5–12 h.



Scheme 3. *Reagents and conditions*: a) $NaBH_4$, THF_{anh}, CH_3SO_3H, r.t., 12 h; b) MnO_2, CH_2Cl_2, reflux, 12 h; c) $CH_2(COOH)_2$, pyridine, piperidine, reflux, 18 h; d) H_2, EtOH, Pd/C 10%, 30 psi, r.t., 12 h; e) $NaNO_2$, HCl, H_2O, CuCl, r.t., 12 h; f) CF_3SO_3H, 5–25 °C, 5 h; g) Br_2, AcOH, r.t., 4 h; h) $THF_{anh.}$, CH_3COCH_2COOEt, 60% NaH in mineral oil, r.t., 24 h; i) NH_4OAc, SiO_2, toluene, MW, 110 °C, 2.5 h; j) (i) DMF_{anh}, 60% NaH in mineral oil, r.t., 15 min, (ii) 4-Me–BnCl, THF_{anh}, r.t., 12 h; k) 10% $NaOH_{aq}$, reflux, 6 h; l) (i) $SOCl_2$, toluene, reflux, 4 h, (ii) CH_2Cl_2, TEA, fenchylamine, r.t., 4 h.

derivatives (**19**, **20** and **21**), the most interesting regarding CB_2R was **21** with a K_i value of 575 nM. Both, the introduction of the naphthalene system (**22**) and the substitution of the carboxamide function with *N*1-methylene-*N*4-aryl-piperazine (**25**, **26**) resulted in a loss of affinity for CBRs.

The conformationally restricted analogue (**39**) of the fenchyl compound **6** adopts a semi-planar geometry. Introduction of this conformational constrain led to a decrease in CB_2 affinity ($K_i = 343$ nM).

As commented in this study, we observed significant CB_2 affinity differences related to the nature of the carbamoyl substituent. Since this part of the corresponding binding site has been less explored, we underwent studies on the Van der Waals (VdW) volume map of the binding analogues and of the non-binding analogues.

2.3. Molecular modelling

Our previous studies of the binding site for the CB_2 antagonist, SR144528, have shown that the SR144528 amide functional group is critical to its CB_2 affinity. Glide docking studies suggested that the SR144528 amide hydrogen interaction with EC-3 loop residue, D(275), is the primary interaction for SR144528 at CB_2R, with aromatic stacking interactions in the TMH5/6 aromatic cluster of CB_2R also having importance [16]. Each compound in Table 1 for which there was measurable CB_2R affinity (**6**–**8**, **10**, **13**, **15**–**18**, **21**, **39**) was docked using Glide here in our CB_2R model of the inactive state. This CB_2R model was pre-equilibrated in a stearoyldocosahexaenoylphosphatidylcholine (SDPC) bilayer for 300 ns to allow it to adjust to a lipid environment [16]. Glide docking studies revealed that all of these compounds bind in the same region of CB_2R as SR144528. Table A-1 in the supporting data presents the docking information for all of the binding analogues. Below, we present the Glide docking study of compound **10** at CB_2R as an example of these results.

2.3.1. Conformational analysis

Fig. 2a illustrates the global minimum-energy conformer of **10** compared to that of SR144528. Fig. 2b provides a numbering

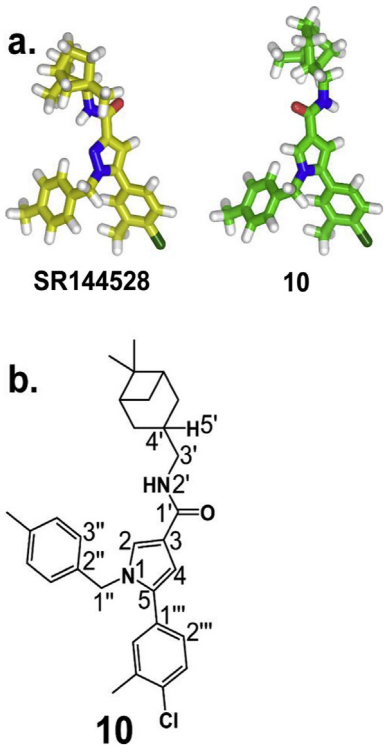


Fig. 2. (a) Global minimum energy conformer of SR144528 (left), and **10** (right). (b) Chemical drawing of **10** with atoms labeled to facilitate discussion of the conformational analysis.

system for **10** used in the discussion of dihedral angles below. The myrtanyl derivative, **10** generates a higher number of conformers compared to SR144528 due to the presence of an additional rotatable bond. However, the pyrrole ring and the amide of the global minimum energy conformer of **10** remain co-planar (C2–C3–C1′–N2′ = 177.8°). The methylbenzyl ring and the chloromethylphenyl ring of **10** adopt a relative position with respect to the pyrrole ring that is similar to SR144528. One major difference between the global minima for SR144528 and **10** is that the amide groups are oriented differently. Another difference between SR144528 and **10** is that the bulky myrtanyl ring in **10** is positioned towards the front face of the molecule and is almost perpendicular to the amide group (C1′–N2′–C3′–C4′ = −105.3° and N2′–C3′–C4′–H5′ = −54°).

2.3.2. Glide docking study

Glide docking studies were performed for all analogues with measurable binding affinities (i.e., SR144528 and analogues **6**, **7**, **8**, **10**, **13**, **15**, **16**, **17**, **18**, **21**, **39**). Because the docking positions and interaction sites are quite similar among these compounds, we show here the complete results for one of these, compound **10**, as an example. For information about Glide scores and Conformational Energy costs for the other analogues in this set, please see Supplementary data.

Analogue **10** was docked into our previously published model of the CB_2R inactive state [16]. Our recent dock of SR144528 in this CB_2 model revealed that SR144528 is a large ligand that modelling studies predict to span the entire CB_2 binding pocket with fenchyl ring near TMH1/2/7, amide functionality near TMH3/7, and aromatic moieties near TMH3/5/6. Fig. 3 presents Glide docking results for analogue **10** binding at CB_2R from an extracellular view. Here, EC-1 and -2 loops have been omitted for clarity. The EC-3 loop residue D(275) (shown in yellow) is the primary interaction site for **10**. All residues contributing energies of interaction of −2.0 kcal/mol or less, are shown with brown carbons. K3.28, shown with magenta carbons, contributes repulsive interactions.

Because the global minimum energy conformer of **10** has its amide group oriented differently than SR144528, a higher energy conformer of **10** (with the proper amide orientation) that is 0.4 kcal/mol above the global minimum was used for docking studies. Fig. 4c illustrates the final energy minimized **10**/CB_2R complex. In this complex, the amide hydrogen of **10** interacts with the EC-3 loop residue, D(275). The hydrogen bond heteroatom distance (N–O) and hydrogen bond (N–H–O) angle are 2.6 Å and 154°, respectively. The carboxamide and pyrrole are positioned closer to TMH2/3 and the myrtanyl ring is packed against TMH7. This orientation accommodates the steric bulk of the myrtanyl ring, but does introduce a closer unfavorable proximity of the carboxamide to K3.28. Like SR144528, **10** forms aromatic stacking interactions with W6.48(258) and W5.43(194). The chloromethylphenyl ring of compound **10** forms an offset parallel aromatic stack with W6.48(258) with a ring centroid to ring centroid distance of 4.3 Å with the 6-member ring and 5.5 Å with the 5-member ring. The chloromethylphenyl group also forms an aromatic T-stack with W5.43(194) (6-member ring) with a ring centroid to centroid distance of 6.1 Å and a ring plane to plane angle of 69°. Finally, the methylbenzyl ring forms an aromatic T-stack interaction with W5.43(194). The ring centroid to centroid distance is 6.3 Å with the 6-member ring and 6.9 Å with the 5-member ring and the ring plane to ring plane angle is 53°. The final docked conformational cost of **10** relative to its global minimum was calculated to be 4.5 kcal/mol. The net Glide score for **10** docked in CB_2R was found to be −7.5 kcal/mol. This result is consistent with the net Glide score for SR144528 (−9.2 kcal/mol; see Table A1) and is also consistent with the fact that **10** has a larger K_i at CB_2R (K_i = 72.2 nM) relative to SR144528 (K_i = 0.6 nM).

2.4. Reasons for loss of measurable binding at CB_2

The analogues that have no measurable binding in Table 1, fall into two categories: (1) analogues larger in size (analogues **9**, **11**, **12**, **14**, **22**, **25** and **26**) than analogues with measurable CB_2R binding and (2) analogues in which the electrostatic distributions may impact CB_2R affinity (**19** and **20**). These sets are considered separately below.

2.5. Size considerations: Active Analogue Approach

Inspection of Table 1 suggests that enlargements of the amine carbamoyl substituent beyond a certain size results in analogues with no measurable CB_2R binding (K_i > 5000 nM) (analogues **9**, **11**, **12**, **14**, **22**, **25** and **26**). We hypothesized that the loss of binding for these analogues may be due to steric constraints within the CB_2R binding pocket. Therefore, to identify possible sterically occluded regions, we used a modified version of the Active Analogue Approach [17]. This approach identifies this region of space occupied by conformers of analogues with no measurable CB_2R affinity that is not occupied by conformers of analogues with measurable CB_2R affinity. Fig. 4a shows the union of the Van der Waals (VdW) volume maps for all accessible conformers of analogues with measurable CB_2R affinity (shown in green surface display). Fig. 4b shows the union of the Van der Waals (VdW) volume maps for all accessible conformers of analogues with no measurable CB_2R affinity (shown in red surface display). Fig. 4c illustrates, in red coloured grid, this volume of space occupied by atoms of analogues with no measurable CB_2R affinity that is not occupied by atoms of analogues with measurable CB_2R affinities. The global minimum conformation of the non-binding analogue **26** is shown in Fig. 4c as

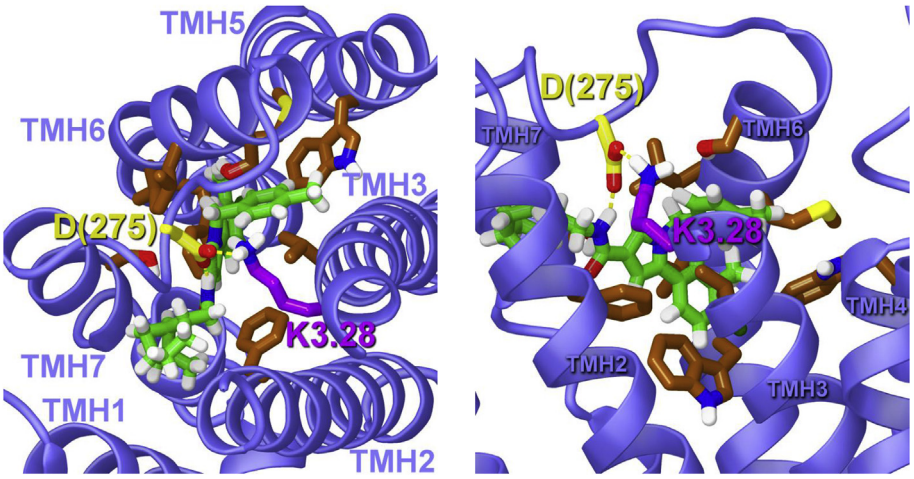


Fig. 3. (Left) Extracellular view of the analogue **10**/CB_2R complex. The EC-1 and -2 loops have been removed for clarity. The EC-3 loop residue D(275) (shown in yellow) is the primary interaction site for **10**. All residues contributing energies of interaction of −2.0 kcal/mol or less, are shown with brown carbons. K3.28 is shown in magenta. (Right) TMH2-3 side view of the analogue **10**/CB_2R complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

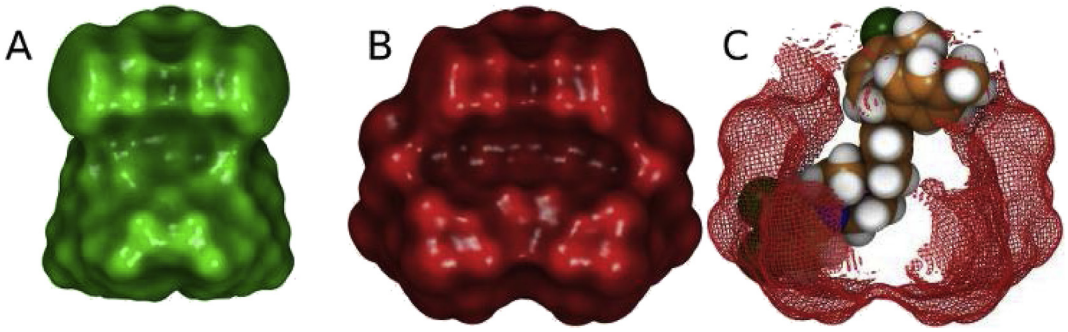


Fig. 4. (a) The union of the Van der Waals (VdW) volume maps for all accessible conformers of analogues with measurable CB_2R affinity is shown in green surface display. (b) The union of the Van der Waals (VdW) volume maps for all accessible conformers of analogues with no measurable CB_2R affinity is shown in red surface display. (c) The red coloured grid illustrates the calculated excluded volume. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a structural reference. It is clear here that the analogues with no measurable CB_2R affinities do project into space not occupied by analogues with measurable affinities. This is caused by the R groups extending further away from the plane of the central pyrrole ring than do the R groups of analogues with measurable affinities. In the context of the full CB_2R R bundle, this additional bulk prevents these analogues from binding at CB_2R due to steric overlaps with TMH7 that cannot be relieved.

2.6. Electrostatic effects

Compounds **19**, **20** and **21** form a unique set of analogues in

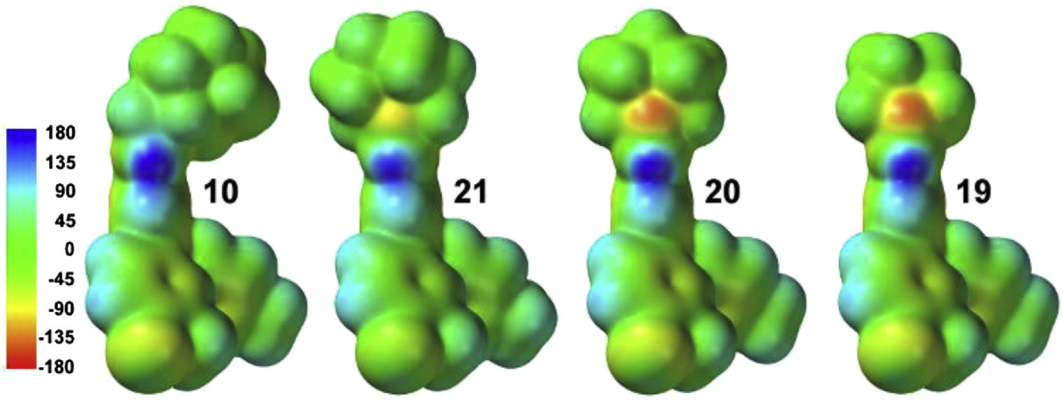


Fig. 5. The molecular electrostatic potential maps of compounds **10**, **21**, **20** and **19** are illustrated here. The positive dark blue regions correspond to the amide NH. The negative yellow-red regions in **21**, **20** and **19** correspond to the nitrogen adjacent to the amide NH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1 because each has a nitrogen atom directly attached to the amide NH. The result of such a substitution is that the electrostatic potentials of **19**, **20** and **21** will be different from high affinity compounds such as **6** or **10**. Because the primary interaction site for **6** or **10** is D(275) [16], (see above), we reasoned that the presence of a negative electrostatic potential region immediately adjacent to a negatively charged amino acid (D(275)) would be repulsive and lead to reduced binding affinity of these compounds for CB_2R. Fig. 5 shows a comparison of molecular electrostatic potential (MEP) maps for **10**, **21**, **20** and **19**. Global minimum energy conformers of each compound have the ring nitrogen's lone pair of electrons pointing in the same direction as the amide NH. Here the molecules are arranged such that the amide N–H is pointing towards the viewer. All analogue MEPs show a positive electrostatic potential (dark blue regions) that correspond to the amide hydrogen. However, the MEPs of **21**, **20** and **19** also show a negative potential region (red-yellow) immediately adjacent to the positive region. In each case, this corresponds to the ring nitrogen connected to the amide NH. The negative potential regions are stronger in **19** and **20** relative to **21**. The reason for this appears to be due to the size of the ring in which the nitrogen is incorporated. The rings (R substituent, see Table 1) in **19**, **20** and **21** progress in size from 5-, to 6-, to 7-membered rings. As the size of the rings increase, the charge on the ring nitrogen decreases, such that the negative potential region in **21** is significantly diminished relative to **19**. For analogues **19** and **20**, electrostatic repulsion of D(275) may diminish the ability of these analogues to interact at CB_2R, while the electrostatics of **21** may still allow interaction with D(275).

3. Determination of the functional activity at the CB_2 receptor

According to their binding values, compounds **6** (K_iCB_2 = 5.7 nM) and **10** (K_iCB_2 = 72.2 nM) have been chosen in order to determine their activity as agonist or antagonists on CB_2R. To this end, we conducted [^{35}S]-GTPγS binding studies, which demonstrated that they behave as antagonists/inverse agonists with values of IC_{50} of 171.4 ± 90.7 nM for compound **6** and of 1816.0 ± 70.2 nM for compound **10** (see a representative curve for compound **6** in Fig. 6). The differences of IC_{50} values for both compounds correlate with their differences in binding affinity, in both cases being in a range of 1–10.

To further confirm these properties, we also used an *in vitro* bioassay in which the CB_2R activity of titled compounds was assayed against LPS-induced inflammatory responses in cultures of mouse BV-2 microglial cell line. These cells express only CB_2R and selective agonists of this receptor reduce the intensity of this pro-

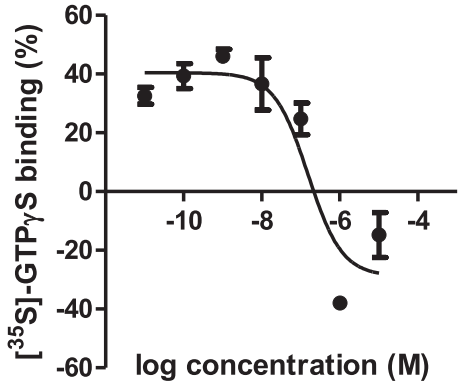


Fig. 6. Representative curve of the [^{35}S]-GTPγS binding for compound **6**.

inflammatory response. This response was quantitated by measuring the concentration of prostaglandin E2 (PGE2) using an ELISA immunoassay. Through this *in vitro* bioassay, it was possible to determine if the new compounds behave as CB_2 agonists, reducing the inflammatory response (by attenuating LPS-induced PGE2 release), or otherwise as antagonists/inverse agonists by reversing the effects of an agonist and, even, by increasing the inflammatory response produced LPS. This can be found in a control assay conducted with two well-known CB_2R ligands, the non-selective agonist WIN55,212-2, and the selective antagonist/inverse agonist SR144528. The data presented in Fig. 7 show how the stimulation of BV-2 cells with LPS produced a 3-fold increase in PGE2 release which was completely reversed by the co-incubation with WIN55,212-2. The complete blockade of WIN55,212-2 effects by SR144528 supports the fact that the PGE2 release is mediated through CB_2R activation.

Compounds **6** and **10** were examined in this bioassay and both of them behaved again as antagonists/inverse agonist of CB_2R. This was concluded from the observation that they were not able to reverse LPS-induced response, as did WIN55,212-2 in the control assay, but both were able to reverse the effect of WIN55,212-2 at the same extent as SR144528. In addition, both compounds elevated PGE2 levels when combined with LPS and, in particular, when combined with SR144528. These data are presented in Fig. 8.

4. Conclusions

In summary, a series of SR144528 derivatives were designed and synthesized as cannabinoid ligands. Among this pyrrole series, the closest structural SR144528 homologue (**6**) exhibited the best affinity for the CB_2R although not better selectivity *vs* CB_1R compared to SR144528. Structural modifications on the amine group of **6** could modulate the binding and selectivity for CBRs. Examination of the Van der Waals's volume maps of non-binding and binding derivatives allowed identifying steric constraints within the CB_2 binding site. Therefore, TMH7 represents the key region that may sterically block non-binding compounds. Each compound with measurable CB_2R affinity (**6**–**8**, **10**, **13**, **15**–**18**, **21**, **39**) was docked using Glide in our CB_2R model of the inactive state. These Glide docking studies revealed that all of these compounds bind in the same region of CB_2R as SR144528. Besides binding to CBRs,

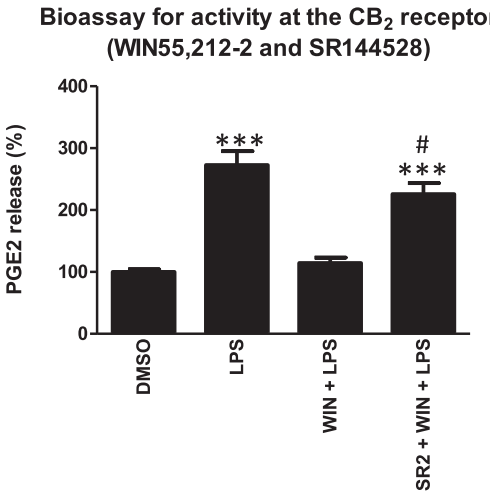


Fig. 7. Effects of WIN55,212-2 and SR144528 on the LPS-induced release of PGE2 in cultured BV-2 cells. Data were assessed by one-way analysis of variance ($F(3,34) = 29.98$, $p < 0.0001$; ***$p < 0.005$ *versus* controls (DMSO-exposed) or WIN + LPS; #$p < 0.05$ *versus* LPS).

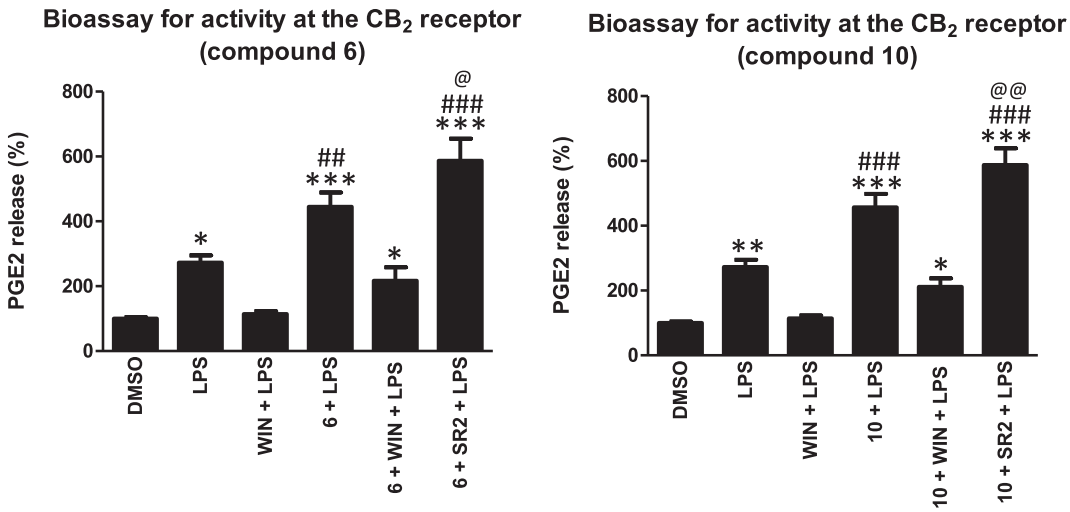


Fig. 8. Effects of compounds **6** or **10**, combined with WIN55,212-2 and/or SR144528, on the LPS-induced release of PGE2 in cultured BV-2 cells. Data were assessed by one-way analysis of variance ($F(5,52) = 24.26$, $p < 0.0001$ for compound **6**; $F(5,52) = 39.27$, $p < 0.0001$ for compound **10**; $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.005$ *versus* controls (DMSO-exposed) or WIN + LPS; ##$p < 0.01$, ###$p < 0.005$ *versus* LPS or compounds **6** or 10 + WIN + LPS; @$p < 0.05$, @@$p < 0.01$ *versus* compounds **6** or **10** + LPS).

functional studies on compounds **6** and **10** showed that they behaved as antagonists/inverse agonists of the CB_2R. Compounds **6** and **10** would deserve further investigation as potential therapeutic agents in those conditions in which the selective blockade of the CB_2R may have beneficial effects. An interesting possibility may be the treatment of certain bone disorders, as has been suggested in the Introduction.

5. Experimental section

5.1. General procedures

All reactions involving air or moisture-sensitive compounds were performed under argon atmosphere. Solvents and reagents were obtained from commercial suppliers and were used without further purification. Ketone **1** and amines for the synthesis of final compounds were purchased by Sigma–Aldrich®: fenchylamine [18] and benzyl alcohol **28** [19] was synthesized according to the literature procedure. Microwave irradiation experiments were carried out in a Biotage® Microwave Initiator Eight 2.5 in the standard configuration as delivered, including proprietary software. All experiments were carried out in sealed microwave process vials under normal absorption. After completion of the reaction, the vial was cooled down to 25 °C *via* air jet cooling before opening. Reaction temperatures were monitored by an IR sensor on the outside wall of the reaction. Hydrogenations were carried out in the 4560 Parr Apparatus using a H_2PEM-100 Parker Balston Hydrogen Generator. Flash column chromatography was performed automatically on Flash-master (Biotage®) with pre-packed Biotage® SNAP silica gel cartridges or manually on silica gel (Kieselgel 60, 0.040–0.063 mm, Merck®). Thin layer chromatography (TLC) was performed with Polygram SIL N-HR/HV_{254} pre-coated plastic sheets (0.2 mm) on aluminium sheets (Kieselgel 60 F254, Merck®). Melting points were obtained on a Köfler melting point apparatus and are uncorrected. IR spectra were recorded as nujol mulls on NaCl plates with a Jasco FT/IR 460 plus spectrophotometer and are expressed in ν (cm^{-1}). NMR experiments were run on a Varian Unity 200 spectrometer (200.07 MHz for ^1H, and 50.31 MHz for ^{13}C) and on a Bruker Avance III Nanoboy 400 system (400.13 MHz for ^1H, and 100.62 MHz for ^{13}C). Spectra were acquired using deuterated chloroform (chloroform-d) or deuterated dimethylsulfoxide (DMSO-d_6) as solvents. Chemical shifts (δ) for ^1H and ^{13}C NMR spectra are reported in parts per million (ppm) using the residual non-deuterated solvent resonance as the internal standard (for chloroform-d: 7.26 ppm, ^1H and 77.16 ppm, ^{13}C; for DMSO-d_6: 2.50 ppm, ^1H, 39.52 ppm, ^{13}C. Data are reported as follows: chemical shift (sorted in descending order), multiplicity (s for singlet, br s for broad singlet, d for doublet, t for triplet, q for quadruplet, m for multiplet), integration and coupling constants (J) in Hertz (Hz). LC/MS analyses were run on an Agilent 1100 LC/MSD system consisting of a single quadrupole detector (SQD) mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface and a photodiode array (PDA) detector. PDA range was 120–550 nm. ESI in positive mode was applied. Mobile phases: (A) MeOH in H_2O (8:2). Analyses were performed with: flow rate 0.9 mL/min; temperature 350 °C. All final compounds displayed ≥95% purity as determined by elemental analysis on a Perkin–Elmer 240-B analyser, for C, H, and N.

5.1.1. Synthesis of (E,Z)-1-(4-dichloro-3-methylphenyl)ethanone oxime (2)

A mixture of ketone **1** (500 mg, 2.97 mmol, 1 eq), $NH_2OH{\cdot}HCl$ (1.7 eq) and $AcONa{\cdot}3H_2O$ (3 eq) in 60% aqueous ethanol solution (1.74 mL) was refluxed for 2.5 h. The suspension was cooled at room temperature and the resulting precipitate was filtered, washed (H_2O), and then solved in Et_2O. The organic solution was dried (Na_2SO_4) and concentrated to give **2** as white solid (520 mg, 95.5%). $R_f = 0.33$ (petroleum ether/EtOAc 9:1); mp 104–106 °C; ^1H NMR ($CDCl_3$) δ 2.27 (s, 3H), 2.40 (s, 3H), 7.31–7.47 (m, 2H), 7.49 (s. 1H), 8.51 (br s, 1H, OH, exch. with D_2O).

5.1.2. Synthesis of methyl-5-(4-chloro-3-methylphenyl)-1H-pyrrole-3-carboxylate (3)

To a stirred solution of 1,4-diazabicyclo[2.2.2]octane (DABCO) (0.1 eq) and oxime **2** (549 mg, 2.99 mmol, 1 eq) at −5 °C in dry toluene (5.25 mL) methyl propiolate (1 eq) was dropwise added. The reaction mixture was allowed to warm to room temperature and was subjected to a two-stage microwave irradiation sequence (stage 1, 80 °C, 10 min; stage 2, 170 °C, 45 min). The mixture was concentrated under reduced pressure, and the residue was purified by gradient-flash chromatography (petroleum ether/EtOAc 95:5–7:3) to afford the pyrrole ester **3** as an orange solid (149 mg,

20%). R_f = 0.28 (petroleum ether/EtOAc 8:2); mp 135–138 °C; ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H), 3.84 (s, 3H), 6.88 (s, 1H), 7.24–7.36 (m, 3H), 7.47 (s, 1H), 8.76 (br s, 1H, NH, exch. with D_2O).

*5.1.3. Synthesis of methyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxylate (**4**)*

To a solution of pyrrole-ester **3** (299 mg, 1.20 mmol, 1 eq) in anhydrous DMF (4 mL) under N_2, was added portionwise 60% NaH in mineral oil (1.2 eq). The solution was stirred at room temperature for 15–20 min, then a solution of 4-methyl-benzyl chloride (1 eq) in anhydrous THF (1.2 mL) was added dropwise: the resulting mixture was stirred at room temperature for 3 h. The solution was poured in H_2O (8 mL) and extracted with CH_2Cl_2, which was washed (H_2O), dried (Na_2SO_4) and concentrated under reduced pressure to furnish an oily brown residue, whose flash chromatography purification (petroleum ether/EtOAc 95:5) gave derivative **4** as a white solid (254 mg, 60%). R_f = 0.55 (petroleum ether/EtOAc 8:2); mp 154–156 °C; ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H), 2.35 (s, 3H), 3.80 (s, 3H), 5.03 (s, 2H), 6.63 (d, 1H, J = 1.6 Hz), 6.90 (d, 2H, J = 8.0 Hz), 7.00–7.16 (m, 4H), 7.30 (d, 1H, J = 8.0 Hz), 7.35 (d, 1H, J = 1.8 Hz).

*5.1.4. Synthesis of 5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxylic acid (**5**)*

A solution of pyrrole ester **4** (400 mg, 1.13 mmol) in a 10% hydroalcoholic NaOH solution (11.75 mL, 60% EtOH) was refluxed overnight. The solution was cooled at room temperature and acidified with 37% HCl. The precipitate was filtered, washed (H_2O) and air-dried to yield the analytically pure acid **5** (320 mg, 83.5%) as a grey. R_f = 0.18 (petroleum ether/EtOAc 8:2); mp 191–192 °C; ^{1}H NMR ($CDCl_3$) δ 1.80 (br s, 1H, OH, exch. with D_2O), 2.33 (s, 3H), 2.36 (s, 3H), 5.05 (s, 2H), 6.67 (s, 1H), 6.91 (d, 2H, J = 7.8 Hz), 6.98–7.32 (m, 5H), 7.42 (s, 1H).

*5.1.5. General procedure for the synthesis of carboxamides **6**, **8**–**22***

A mixture of the acid **5** (98 mg, 0.29 mmol, 1 eq) and thionyl chloride (3 eq) in toluene (2.38 mL) was refluxed for 4 h. The solvent and the excess of $SOCl_2$ were removed under reduced pressure and the resulting dark solid in CH_2Cl_2 (15 mL) was dropwise added to a solution of requisite amine or hydrazine (1.5 eq) and Et_3N (1.5 eq) in CH_2Cl_2 (15 mL) at 0 °C. The mixture was refluxed for 4 h. The mixture was then poured into a separatory funnel and brine was added. The aqueous layer was separated and extracted with CH_2Cl_2. The combined organic layer were washed (H_2O), dried (Na_2SO_4) and concentrated under reduced pressure. The analytically pure product was isolated by flash chromatography purification.

*5.1.6. N-(1)-(S)-Fenchyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**6**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(1)-(*S*)-fenchylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **6** (96.5 g, 70%) as beige solid. R_f = 0.44 (petroleum ether/EtOAc 8:2); mp 155–156 °C; IR 1633 (C=O), 3354 (NH); ^{1}H NMR ($CDCl_3$) δ 0.84 (s, 3H), 1.09 (s, 3H), 1.16 (s, 3H), 1.20–1.35 (m, 2H), 1.45–1.55 (m, 1H), 1.63–1.71 (m, 4H), 2.32 (s, 3H), 2.34 (s, 3H), 3.81 (d, 1H, J = 8.0 Hz), 5.03 (s, 2H), 5.78 (d, 1H, J = 8.0 Hz, NH, exch. with D_2O), 6.38 (s, 1H), 6.92 (d, 2H, J = 8.0 Hz), 7.06 (d, 1H, J = 8.0 Hz), 7.11 (d, 2H, J = 8.0 Hz), 7.16–7.21 (m, 1H), 7.31 (d, 2H, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$) δ 19.67 (CH_3), 20.06 (CH_3), 21.04 (CH_3), 21.25 (CH_3), 26.01 (CH_2), 27.34 (CH_2), 30.78 (CH_3), 39.36 (CH), 42.64 (CH_2), 48.14 (CH), 48.56 (CH), 51.04 (CH_2), 62.78 (CH), 106.73 (CH), 119.97 (C), 125.39 (CH), 126.74 (CH ×2), 127.66 (CH), 129.09 (CH), 129.51 (CH ×2), 130.72 (C), 131.66 (CH), 133.98 (C), 134.23 (C), 134.51 (C), 136.23 (C), 137.55 (C), 164.87 (C=O); MS (ESI): $C_{30}H_{35}ClN_2O$ requires *m*/*z* 475, found 476 $[M + 1]^+$; Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.85; H, 7.43; Cl 7.46; N, 5.90. Found: C, 75.88; H, 7.45; Cl 7.43; N, 5.93.

*5.1.7. N-(R)-(+)-Bornyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**8**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(*R*)-(+)-bornylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **8** (73 mg, 53%) as a yellow solid. R_f = 0.58 (petroleum ether/EtOAc 7:3); mp 96–100 °C; IR 1635 (C=O), 3350 (NH); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H), 0.89 (s, 3H), 0.99 (s, 3H), 1.23 (d, 2H, J = 8.8 Hz), 1.38–1.60 (m, 3H), 1.62–1.85 (m, 2H), 2.32 (s, 6H), 4.42 (t, 1H, J = 8.4 Hz), 5.04 (s, 2H), 5.8 (d, 1H, J = 8.4 Hz, NH, exch. with D_2O), 6.42 (s, 1H), 6.91 (d, 2H, J = 7.4 Hz), 7.00–7.20 (m, 4H), 7.28–7.32 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.72 (CH_3), 18.70 (CH_3), 18.86 (CH_3), 20.04 (CH_3), 21.05 (CH_3), 28.09 (CH_2), 28.43 (CH_2), 37.88 (CH_2), 44.93 (CH), 48.13 (C), 49.56 (C), 50.94 (CH_2), 53.32 (CH), 106.89 (CH), 119.92 (CH), 125.35 (CH), 126.59 (CH ×2), 127.57 (CH), 129.04 (C), 129.46 (CH ×2), 130.65 (C), 131.57 (CH), 133.89 (C), 134.29 (C), 134.47 (C), 136.18 (C), 137.48 (C), 164.46 (C=O); MS (ESI): $C_{30}H_{35}ClN_2O$ requires *m*/*z* 475, found 476 $[M + 1]^+$; Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.88; H, 7.45; Cl 7.43; N, 5.93. Found: Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.85; H, 7.43; Cl 7.46; N, 5.90. Found: C, 75.87; H, 7.45; Cl 7.44; N, 5.91.

*5.1.8. N-(1R,2R,3R,5S)-Isopinocampheyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**9**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(1*R*,2*R*,3*R*,5*S*)-isopinocampheylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **9** (89.5 mg, 65%) as a brown solid. R_f = 0.225 (petroleum ether/EtOAc 8:2); mp 85–89 °C; IR 1634 (C=O), 3352 (NH); ^{1}H NMR ($CDCl_3$) δ 1.08 (s, 3H), 1.15 (d, 3H, J = 6.8 Hz), 1.23 (s, 3H), 1.57–1.62 (m, 1H), 1.83–1.86 (m, 2H), 1.92–1.97 (m, 1H), 2.32 (s, 3H), 2.33 (s, 3H), 2.37–2.43 (m, 1H), 2.64–2.70 (m, 1H), 4.44 (t, 1H, J = 7.2 Hz), 5.02 (s, 2H), 5.68 (d, 1H, J = 8.4 Hz, NH, exch. with con D_2O), 6.42 (s, 1H), 6.90 (d, 2H, J = 7.6 Hz), 7.03–7.20 (m, 3H), 7.28–7.30 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 20.07 (CH_3), 20.80 (CH_3), 21.08 (CH_3), 23.40 (CH_3), 28.06 (CH_2), 35.34 (CH_2), 37.54 (CH_2), 38.46 (C), 41.71 (CH_3), 46.61 (CH), 47.46 (CH), 47.89 (CH), 50.99 (CH_2), 107.14 (CH), 119.98 (C), 125.33 (CH), 126.68 (CH), 126.74 (CH), 127.14 (CH), 127.60 (CH), 129.09 (CH), 129.52 (CH ×2), 131.62 (CH), 133.91 (C), 134.36 (C), 134.52 (C), 135.48 (C), 136.21 (C), 137.54 (C), 163.88 (C=O); MS (ESI): $C_{30}H_{35}ClN_2O$ requires *m*/*z* 475, found 476 $[M + 1]^+$; Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.88; H, 7.45; Cl 7.43; N, 5.93. Found: Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.85; H, 7.43; Cl 7.46; N, 5.90. Found: C, 75.87; H, 7.46; Cl 7.47; N, 5.92.

*5.1.9. N-(1S,2R)-Myrtanyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**10**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(1*S*,2*R*)-myrtanylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **10** (91 mg, 66%) as a beige solid. R_f = 0.56 (petroleum ether/EtOAc 8:2); mp 72–76 °C; IR 1635 (C=O), 3354 (NH); ^{1}H NMR ($CDCl_3$) δ 0.88 (d, 1H, J = 8.0 Hz), 1.05 (s, 3H), 1.18 (s, 3H), 1.52–1.56 (m, 1H), 1.81–1.96 (m, 7H), 2.31 (s, CH_3), 2.31 (s, CH_3), 3.36–3.42 (m, 2H), 5.02 (s, 2H), 5.78–5.85 (br s, 1H, NH, exch. with D_2O), 6.39 (s, 1H), 6.89 (d, 2H, J = 8.0 Hz), 7.03 (d, 1H, J = 8.0 Hz), 7.08 (d, 2H, J = 8.0 Hz), 7.13 (s, 1H), 7.25–7.29 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 18.87 (CH_2), 19.03 (CH_3), 20.05 (CH_3), 22.18 (CH_3), 25.04 (CH_2), 27.00 (CH_3), 32.30 (CH_2), 37.70 (C), 40.39 (CH), 40.63 (CH), 42.80 (CH), 43.93 (CH_2), 49.96 (CH_2), 106.10 (CH), 118.83 (C), 124.25

(CH), 125.66 (CH ×2), 126.57 (CH), 128.05 (CH), 129.72 (CH ×2), 130.57 (C), 132.87 (CH), 133.20 (C), 133.29 (C) 133.46 (C), 135.15 (C), 136.49 (C), 163.43 (C=O); MS (ESI): $C_{30}H_{35}ClN_2O$ requires *m/z* 475, found 476 $[M + 1]^+$; Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.88; H, 7.45; Cl 7.43; N, 5.93. Found: Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.85; H, 7.43; Cl 7.46; N, 5.90. Found: C, 75.88; H, 7.45; Cl 7.45; N, 5.91.

5.1.10. N-(Adamantan-1-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**11**)

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-1-adamantylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **11** (64.5 mg, 47%) as a beige solid. $R_f = 0.41$ (petroleum ether/EtOAc 8:2); mp 125–127 °C; IR 1637 (C=O), 3354 (NH); ^{1}H NMR ($CDCl_3$) δ 1.67–1.73 (m, 7H), 2.08–2.12 (m, 8H), 2.32 (s, 3H), 2.33 (s, 3H), 5.01 (s, 2H), 6.34 (s, 1H), 6.89 (d, 2H, J = 8.0 Hz), 7.03 (d, 1H, J = 8.0 Hz), 7.09 (d, 2H, J = 8.0 Hz), 7.14 (s, 1H), 7.21 (br s, 1H, NH, exch. with D_2O), 7.29 (d, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 20.07 (CH_3), 21.08 (CH_3), 29.56 (5 ×CH), 36.47 (CH_2 ×2), 41.95 (CH_2 ×2), 50.96 (CH_2), 51.79 (CH_2), 107.16 (CH), 121.05 (C), 125.08 (CH), 126.66 (CH ×2), 127.60 (CH), 129.49 (CH ×2), 130.80 (C), 131.60 (CH), 133.87 (C), 134.39 (C), 135.90 (C), 136.17 (C), 137.50 (C), 163.76 (C=O); MS (ESI): $C_{30}H_{33}ClN_2O$ requires *m/z* 473, found 474 $[M + 1]^+$; Anal. calcd for $C_{30}H_{35}ClN_2O$: C, 75.88; H, 7.45; Cl 7.43; N, 5.93. Found: Anal. calcd for $C_{30}H_{33}ClN_2O$: C,76.17; H, 7.03; Cl, 7.49; N, 5.92. Found: C, 76.22; H, 7.09; Cl, 7.43; N, 5.90.

5.1.11. N-Adamantylmethane-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**12**)

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-1-adamantylmethanamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **12** (90.4 mg, 64%) as a brown solid. $R_f = 0.47$ (petroleum ether/EtOAc 7:3); mp 78–81 °C; IR 1633 (C=O), 3351 (NH); ^{1}H NMR ($CDCl_3$) δ 1.22–1.65 (m, 10H), 1.92–2.00 (m, 4H) 2.32 (s, 3H), 2.34 (s, 3H), 3.10 (d, 1H, J = 6.8 Hz), 5.03 (s, 2H), 5.81 (br s, 1H, NH, exch. with D_2O), 6.40 (s, 1H), 6.91 (d, 2H, J = 7.6 Hz), 6.96–7.18 (m, 4H), 7.20–7.33 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 20.00 (CH_3), 21.02 (CH_3), 28.19 (CH_2 ×3), 33.97 (C), 36.89 (CH_2 ×3), 40.21 (CH ×3), 50.62 (CH_2), 50.91 (CH_2), 106.96 (CH), 117.95 (C), 119.71 (CH), 125.37 (CH), 126.61 (CH ×2), 127.51 (C), 129.00 (CH), 129.41 (CH ×2), 130.77 (C), 131.52 (CH), 134.03 (C), 134.21 (C), 136.12 (C), 137.43 (C), 164.57 (C=O); MS (ESI): $C_{31}H_{35}ClN_2O$ requires *m/z* 487, found 488 $[M + 1]^+$; Anal. calcd for $C_{31}H_{35}ClN_2O$: C, 76.44; H, 7.24; Cl, 7.28; N, 5.75. Found: C, 76.47; H, 7.26; Cl, 7.30; N, 5.79.

5.1.12. N-Menthyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**13**)

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-menthylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **13** (105 mg, 76%) as a brown solid. $R_f = 0.42$ (petroleum ether/EtOAc 8:2); mp 98–102 °C; IR 1635 (C=O), 3350 (NH); ^{1}H NMR ($CDCl_3$) δ 0.83 (d, 6H, J = 7.6 Hz), 0.89 (d, 3H, J = 7.4 Hz), 0.98–1.25 (m, 2H), 1.45–1.79 (m, 5H), 1.83–2.18 (m, 2H), 2.33 (s, 6H), 3.90–4.10 (m, 1H), 5.03 (s, 2H), 5.41 (d, 1H, J = 9.2 Hz, NH, exch. with con D_2O), 6.38 (s, 1H), 6.92 (d, 2H, J = 7.4 Hz), 7.00–7.22 (m, 4H), 7.30 (d, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 16.25 (CH_3), 20.03 (CH_2), 21.18 (CH_3 ×2), 22.15 (CH_2), 23.86 (CH_2), 26.84 (CH), 31.84 (CH_3), 34.55 (CH_2), 43.35 (CH), 48.37 (CH_2), 49.49 (CH), 50.92 (CH), 106.96 (CH), 118.02 (C), 119.84 (CH), 125.33 (CH), 126.67 (CH), 127.51 (CH ×2), 129.01 (C), 129.43 (CH ×2), 130.66 (C), 131.53 (CH), 134.27 (C), 134.96 (C), 136.12 (C), 137.47 (C), 163.63 (C=O); MS (ESI): $C_{30}H_{37}ClN_2O$ requires *m/z* 477, found 478 $[M + 1]^+$; Anal. calcd for $C_{30}H_{37}ClN_2O$: C, 75.53; H, 7.82; Cl 7.43; N, 5.87. Found: C, 75.59; H, 7.90; Cl, 7.47; N, 5.90.

5.1.13. N-(R)-(−)-Cyclohexylethyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**14**)

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(R)-(−)-cyclohexylethylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 7:3) to afford **13** (41.7 mg, 32%) as a brown solid. $R_f = 0.25$ (petroleum ether/EtOAc 8:2); mp 211–214 °C; IR 1640 (C=O), 3350 (NH); ^{1}H NMR (DMSO) δ 1.16 (d, 3H, J = 6.8 Hz), 1.19–1.24 (m, 3H), 1.30–1.45 (m, 2H), 1.64–1.82 (m, 6H), 2.33 (s, 3H), 2.38 (s, 3H), 4.03–4.10 (m, 1H), 5.03 (s, 2H), 5.57 (d, 1H, J = 9.2 Hz, NH, exch. with D_2O), 6.38 (d, 1H, J = 1.6 Hz), 6.90 (d, 2H, J = 8.0 Hz), 7.04 (d, 1H, J = 8.4 Hz), 7.10 (d, 2H, J = 8.0 Hz), 7.15 (d, 1H, J = 1.6 Hz), 7.30 (d, 2H, J = 8.0 Hz); ^{13}C NMR (DMSO) δ 17.84 (CH_3), 19.51 (CH_3), 20.59 (CH_3), 25.75 (CH_2 ×2), 26.02 (CH_2), 28.90 (CH_2), 29.25 (CH_2), 42.54 (CH), 48.20 (CH), 50.22 (CH_2), 108.71 (CH), 119.92 (C), 125.98 (CH), 126.08 (CH ×2), 127.16 (CH), 128.93 (CH), 129.13 (CH ×2), 130.94 (CH), 131.19 (C), 132.15 (C), 132.75 (C), 135.11 (C), 135.63 (C), 136.56 (C), 162.52 (C=O); MS (ESI): $C_{28}H_{33}ClN_2O$ requires *m/z* 449, found 450 $[M + 1]^+$; Anal. calcd for $C_{28}H_{33}ClN_2O$: C, 74.90; H, 7.41; Cl, 7.90; N, 6.24. Found: C, 74.72; H, 7.40; Cl, 7.88; N, 6.23.

5.1.14. N-(S)-(+)-Cyclohexylethyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**15**)

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(S)-(+)-cyclohexylethylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 7:3) to afford **15** (100 mg, 77%) as a brown solid. $R_f = 0.19$ (petroleum ether/EtOAc 8:2); mp 205–207 °C; IR 1645 (C=O), 3340 (NH); ^{1}H NMR (DMSO) δ 1.05–1.23 (m, 8H), 1.64–1.86 (m, 6H), 2.33 (s, 6H), 4.03–4.10 (m, 1H), 5.03 (s, 2H), 5.57 (d, 1H, J = 9.2 Hz, NH, exch. with D_2O), 6.38 (d, 1H, J = 1.6 Hz), 6.91 (d, 2H, J = 8.0 Hz), 6.98–7.20 (m, 4H), 7.21–7.33 (m, 2H); ^{13}C NMR (DMSO) δ 17.84 (CH_3), 19.51 (CH_3), 20.59 (CH_3), 25.75 (CH_2 ×2), 26.02 (CH_2), 28.90 (CH_2), 29.25 (CH_2), 42.54 (CH), 48.20 (CH), 50.22 (CH_2), 108.71 (CH), 119.92 (C), 125.98 (CH), 126.08 (CH ×2), 127.16 (CH), 128.93 (CH), 129.13 (CH ×2), 130.94 (CH), 131.19 (C), 132.30 (C), 132.75 (C), 135.10 (C), 135.63 (C), 136.56 (C), 162.52 (C=O); MS (ESI): $C_{28}H_{33}ClN_2O$ requires *m/z* 449, found 450 $[M + 1]^+$; Anal. calcd for $C_{28}H_{33}ClN_2O$: C, 74.90; H, 7.41; Cl, 7.90; N, 6.24. Found: C, 74.75; H, 7.40; Cl, 7.88; N, 6.23.

5.1.15. N-Cyclohexyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**16**)

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-cyclohexylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 7:3) to afford **16** (79.3 mg, 65%) as a beige solid. $R_f = 0.35$ (petroleum ether/EtOAc 7:3); mp 100–104 °C; IR 1640 (C=O), 3360 (NH); ^{1}H NMR ($CDCl_3$) δ 1.07–1.78 (m, 8H), 1.87–1.98 (m, 2H), 2.32 (s, 6H), 3.79–4.01 (m, 1H), 5.03 (s, 2H), 5.60 (d, 1H, NH, exch. with D_2O, J = 8.0 Hz), 6.38 (d, 1H, J = 2.0 Hz), 6.90 (d, 2H, J = 7.8 Hz), 6.97–7.17 (m, 4H), 7.19–7.37 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 19.79 (CH_2), 20.05 (CH_3), 21.07 (CH_3), 25.00 (CH_2), 25.65 (CH_2), 30.92 (CH), 33.45 (CH_2), 47.93 (CH_2), 50.97 (CH_2), 107.22 (CH), 120.03 (CH), 125.31 (CH), 126.66 (CH), 126.72 (C), 127.13 (CH), 129.08 (CH), 129.50 (CH), 130.77 (C), 130.91 (C), 131.60 (CH), 134.27 (C), 134.39 (CH), 135.45 (C), 136.19 (C), 137.52 (C), 163.63 (C=O); MS (ESI): $C_{26}H_{29}ClN_2O$ requires *m/z* 420, found 421 $[M + 1]^+$; Anal. calcd for $C_{26}H_{29}ClN_2O$: C, 74.18; H, 6.94; Cl, 8.42; N, 6.65. Found: C, 74.24; H, 7.01; Cl, 8.45; N, 6.69.

*5.1.16. N-Piperidinyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**17**)*

General procedure for the synthesis of carboxamides was used to convert **5** and piperidine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 8:2) to afford **17** (73.4 mg, 60%) as a brown solid. $R_f = 0.375$ (petroleum ether/EtOAc 6:4); mp 104–105 °C; IR 1635 (C=O), 1703 (C=O), 3350 (NH); ^{1}H NMR (DMSO) δ 1.48–1.55 (m, 4H), 1.58–1.64 (m, 2H), 2.24 (s, 3H), 2.30 (s, 3H), 3.55–3.60 (m, 4H), 5.18 (s, 2H), 6.36 (d, 1H, J = 2.0 Hz), 6.85 (d, 2H, J = 7.6 Hz), 7.09 (d, 2H, J = 7.6 Hz), 7.19 (d, 2H, J = 8.0 Hz), 7.21–7.25 (m, 1H), 7.28 (s, 1H), 7.35–7.40 (m, 2H); ^{13}C NMR (DMSO) δ 19.51 (CH_3), 20.58 (CH_3), 24.24 (CH_2 ×2), 25.83 (CH_2), 50.11 (CH_2 ×3), 109.81 (CH), 118.17 (C), 126.21 (CH), 126.28 (CH ×2), 126.46 (CH), 128.89 (CH), 129.10 (CH ×2), 130.97 (C), 131.09 (CH), 133.33 (C), 134.36 (C), 135.14 (C), 135.61 (C), 136.52 (C), 164.53 (C=O); MS (ESI): $C_{30}H_{35}ClN_2O$ requires *m/z* 406, found 407 $[M + 1]^+$; Anal. calcd for $C_{25}H_{27}ClN_2O$: C, 73.79; H, 6.69; Cl, 8.71; N, 6.88. Found: C, 73.77; H, 6.68; Cl, 8.69; N, 6.86.

*5.1.17. N-(4-(2-Chlorophenyl)piperazin-1-yl)-(5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**18**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-(4-(2-chlorophenyl)piperazine) into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 7:3) to afford **18** (40 mg, 25%) as a brown solid. $R_f = 0.16$ (petroleum ether/EtOAc 7:3); mp 67–71 °C; IR 1635 (C=O), 3350 (NH); ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H), 2.33 (s, 3H), 3.06 (t, 4H, J = 4.8 Hz), 3.95 (t, 4H, J = 4.8 Hz), 5.03 (s, 2H), 6.39 (s, 1H), 6.91 (d, 2H, J = 8.0 Hz), 7.01 (d, 2H, J = 8.0 Hz), 7.05–7.11 (m, 4H), 7.17–7.22 (m, 2H), 7.25 (d, 1H, J = 8.0 Hz), 7.30 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 20.08 (CH_3), 21.09 (CH_3), 50.89 (CH_2 ×4), 51.58 (CH_2), 109.53 (CH), 118.32 (C), 120.03 (CH), 124.16 (CH), 125.72 (CH), 126.72 (CH ×2), 126.79 (CH), 128.94 (C), 129.10 (CH), 130.75 (CH ×3), 131.64 (CH), 132.06 (CH), 133.88 (C ×2), 133.90 (C), 136.23 (C), 137.55 (C), 148.87 (C), 166.32 (C=O); MS (ESI): $C_{30}H_{29}Cl_2N_3O$ requires *m/z* 517, found 518 $[M + 1]^+$; Anal. calcd for $C_{30}H_{29}Cl_2N_3O$: C, 69.50; H, 5.64; Cl, 13.68; N, 8.10. Found: C, 69.48; H, 5.62; Cl, 13.65; N, 8.08.

*5.1.18. N-Pyrrolidinyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carbohydrazide (**19**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-aminopyrrolidine hydrochloride into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 3:7) to afford **19** (22 mg, 18%) as a brown solid. $R_f = 0.375$ (petroleum ether/EtOAc 3:7); mp 141–143 °C; IR 1640 (C=O), 3330 (NH); ^{1}H NMR ($CDCl_3$) δ 1.69–1.95 (m, 4H), 2.33 (s, 6H), 2.88–3.00 (m, 4H), 5.02 (s, 2H), 6.30–6.40 (br s 1H, NH, exch. with D_2O), 6.89–6.92 (m, 2H) 7.03–7.15 (m, 4H), 7.29–7.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 20.20 (CH_3), 21.32 (CH_3), 23.12 (CH_2 ×2), 46.30 (CH_2), 58.24 (CH_2), 108.40 (CH), 112.29 (C), 123.45 (CH), 126.15 (CH), 127.32 (CH ×2), 129.15 (CH ×2), 129.67 (CH), 131.04 (C), 131.62 (C), 134.48 (C ×2), 135.27 (C), 136.40 (C), 139.42 (C), 165.00 (C=O); MS (ESI): $C_{24}H_{26}ClN_3O$ requires *m/z* 407, found 408 $[M + 1]^+$; Anal. calcd for $C_{24}H_{26}ClN_3O$: C, 70.66; H, 6.42; Cl, 8.69; N, 10.30. Found: C, 70.71; H, 6.49; Cl, 8.72; N, 10.25.

*5.1.19. N-Piperidinyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carbohydrazide (**20**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-aminopiperidine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 6:4–4:6) to afford **20** (50.2 mg, 41%) as a beige solid. $R_f = 0.15$ (petroleum ether/EtOAc 6:4); mp 82–84 °C; IR 1649 (C=O), 3214 (NH); ^{1}H NMR ($CDCl_3$) δ 1.36–1.48 (m, 2H), 1.65–1.73 (m, 4H), 2.34 (s, 6H), 2.67–2.90 (m, 4H), 5.03 (s, 2H), 6.11 (br s 1H, NH, exch. with D_2O), 6.37–6.42 (br s, 1H), 6.85–6.95 (m, 2H), 7.01–7.27 (m, 4H), 7.50–7.67 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 20.04 (CH_3), 21.04 (CH_3), 23.29 (CH_2), 25.34 (CH_2), 25.66 (CH_2), 51.01 (CH_2), 57.33 (CH_2), 57.92 (CH_2), 107.71 (CH), 118.17 (C), 126.68 (CH), 127.16 (CH), 127.59 (CH), 129.05 (CH), 129.47 (CH ×2), 130.88 (CH), 130.97 (C), 131.59 (CH), 133.33 (C), 134.05 (C), 135.19 (C), 136.15 (C), 137.56 (C), 164.53 (C=O); MS (ESI): $C_{25}H_{28}ClN_3O$ requires *m/z* 421, found 422 $[M + 1]^+$; Anal. calcd for $C_{25}H_{28}ClN_3O$: C, 71.16; H, 6.69; Cl, 8.40; N, 9.96. Found: C, 71.20; H, 6.73; Cl, 8.43; N, 9.94.

*5.1.20. N-Homopiperidinyl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carbohydrazide (**21**)*

General procedure for the synthesis of carboxamides was used to convert **5** and *N*-aminohomopiperidine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 65:35) to afford **21** (50.6 mg, 40%) as a brown solid. $R_f = 0.175$ (petroleum ether/EtOAc 7:3); mp 220–224 °C; IR 1669 (C=O), 3210 (NH); ^{1}H NMR ($CDCl_3$) δ 1.42–1.85 (m, 10H), 2.34 (s, 6H), 3.02–3.22 (m, 2H), 5.03 (s, 2H), 6.38 (s 1H, NH, exch. with D_2O), 6.53 (s, 1H), 6.88–6.95 (m, 2H), 7.01–7.17 (m, 4H), 7.24–7.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 19.64 (CH_3), 20.66 (CH_2 ×2), 25.73 (CH_3), 26.73 (CH_2 ×2), 50.47 (CH_2), 57.80 (CH_2 ×2), 107.71 (CH), 118.17 (C), 126.27 (C), 126.35 (CH), 127.14 (CH), 128.65 (CH ×2), 129.02 (CH ×2), 131.05 (CH), 133.89 (CH), 134.07 (C), 135.62 (C ×2), 136.13 (C), 137.54 (C), 166.36 (C=O); MS (ESI): $C_{26}H_{30}ClN_3O$ requires *m/z* 435, found 436 $[M + 1]^+$; Anal. calcd for $C_{26}H_{30}ClN_3O$: C, 71.63; H, 6.94; Cl, 8.13; N, 9.64. Found: C, 71.60; H, 6.93; Cl, 8.13; N, 9.63.

*5.1.21. N-(Naphthalen-1-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carboxamide (**22**)*

General procedure for the synthesis of carboxamides was used to convert **5** and 1-naphtylamine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 7:3) to afford **22** (93.8 mg, 70%) as a beige solid. $R_f = 0.54$ (petroleum ether/EtOAc 7:3); mp 170–172 °C; IR 1640 (C=O), 3345 (NH); ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H), 2.35 (s, 3H), 5.03 (s, 2H), 6.62 (s, 1H), 6.91 (d, 1H, J = 8.0 Hz), 7.10–7.12 (m, 3H), 7.19 (s, 1H), 7.32 (d, 1H, J = 8.0 Hz), 7.42–7.49 (m, 4H), 7.67 (d, 1H, J = 8.0 Hz), 7.85 (d, 1H, J = 8.0 Hz), 7.91 (d, 1H, J = 8.0 Hz), 7.95 (s, 1H), 7.99 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 20.11 (CH_3), 21.11 (CH_3), 51.16 (CH_2), 107.43 (CH), 119.72 (C), 120.86 (CH), 125.44 (CH), 125.87 (CH ×2), 126.15 (CH), 126.20 (CH), 126.75 (CH ×2), 126.82 (CH), 127.44 (CH), 127.74 (C), 128.74 (CH), 129.18 (CH), 129.58 (CH ×2), 130.54 (C), 131.73 (CH), 132.74 (C), 134.13 (C), 134.15 (C), 134.17 (C), 134.97 (C), 136.33 (C), 137.68 (C), 163.14 (C=O); MS (ESI): $C_{30}H_{25}ClN_2O$ requires *m/z* 464, found 465 $[M + 1]^+$; Anal. calcd for $C_{30}H_{25}ClN_2O$: C, 77.49; H, 5.42; Cl, 7.62; N, 6.02. Found: C, 74.24; H, 7.01; Cl, 8.45; N, 6.69.

*5.1.22. Synthesis of 1-(5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-yl)methyl)-fenchylamine (**7**)*

To a solution of carboxamide **6** (100 mg, 0.21 mmol, 1 eq) in THF_{an} (3 1 L) under N_2 at 0 °C, was portionwise added a solution of 2 M $LiAlH_4$ in THF_{an} (0.17 mL). The resulting solution was refluxed for 12 h, then cooled to room temperature and added of 10% NaOH (few drops). The resulting precipitate was removed under vacuum and the filtrate extracted with EtOAc which was dried (Na_2SO_4) and concentrated under reduced pressure to give derivative **7** as a yellow solid (40 mg, 41%). $R_f = 0.18$ (petroleum ether/EtOAc 7:3); mp 108–111 °C; IR 3390 (NH); ^{1}H NMR ($CDCl_3$) δ 0.96 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), 1.28–1.48 (m, 2H), 1.46–1.68 (m, 5H), 2.25–2.30 (br s, 1H, NH, exch. with D_2O), 2.32 (s, 6H), 3.56 (d, 1H, J = 13.2 Hz), 3.72 (d, 1H, J = 12.8 Hz), 5.02 (d, 2H, J = 8.8 Hz), 6.20 (s, 1H), 6.65 (s,

1H), 6.91 (d, 2H, J = 8.0 Hz), 7.02–7.12 (m, 3H), 7.15 (d, 2H, J = 8.0); ^{13}C NMR ($CDCl_3$) δ 19.63 (CH_3), 19.71 (CH_3), 21.02 (CH_3), 25.98 (CH_2), 26.65 (CH_2), 31.91 (CH_3), 31.98 (CH_3), 39.12 (CH_2), 43.01 (CH_2), 45.66 (CH_2), 48.71 (C), 48.87 (CH), 50.57 (C), 70.95 (CH), 109.90 (CH), 123.13 (CH), 126.44 (CH ×2), 127.03 (CH), 128.83 (CH), 128.98 (CH ×2), 129.21 (CH), 133.33 (C), 134.41 (C), 135.35 (C ×2), 135.68 (C), 137.16 (C ×2); MS (ESI): $C_{30}H_{37}ClN_2$ requires *m/z* 461, found 462 $[M + 1]^+$; Anal. calcd for $C_{30}H_{37}ClN_2$: C, 78.15; H, 8.09; Cl 7.69; N, 6.08. Found: C, 78.07; H, 8.08; Cl 7.68; N, 6.07.

5.1.23. Synthesis of [5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-yl]methanol (**23**)

To a solution of acid **5** (510 mg, 1.5 mmol, 1 eq) in anhydrous THF (3 mL) under N_2 at 0 °C, was portionwise added a solution of 2 M $LiAlH_4$ in anhydrous THF (1.3 mL). The resulting solution was stirred at room temperature for 4 h, then 10% NaOH was added. The precipitate was removed under vacuum and the filtrate extracted with EtOAc, which dried (Na_2SO_4) and concentrated under reduced pressure gave derivative **23** as yellow oil (400 mg, 82%). R_f = 0.12 (petroleum ether/EtOAc 8:2); ^{1}H NMR ($CDCl_3$) δ 1.50–1.65 (br s, 1H, OH exch. with D_2O), 2.32 (s, 6H), 4.56 (s, 2H), 5.01 (s, 2H), 6.25 (s, 1H), 6.71 (s, 1H), 6.91 (d, 2H, J = 8.2 Hz), 7.0–7.16 (m, 4H), 7.28 (d, 1H, J = 8.0 Hz).

5.1.24. Synthesis of 5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrole-3-carbaldehyde (**24**)

To a solution of alcohol **23** (400 mg, 1.22 mmol, 1eq) in CH_2Cl_2 (7 mL) was added MnO_2 (10 eq) in small portions and the resulting mixture was refluxed for 12 h. Then the solution was cooled to room temperature and the catalyst removed by filtration on a bed of celite®. The organic solution concentrate under reduced pressure gave derivative **24** as yellow oil (310 mg, 82%). R_f = 0.21 (petroleum ether/EtOAc 8:2); ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 6H), 5.06 (s, 2H), 6.66 (d, 1H, J = 1.8 Hz), 6.92 (d, 2H, J = 7.8 Hz), 7.04–7.21 (m, 4H), 7.33 (d, 1H, J = 8.4 Hz), 9.75 (s, 1H).

5.1.25. General procedure for the synthesis of amines **25–26**

To a stirred solution of carbaldehyde **24** (1000 mg, 0.31 mmol, 1 eq) and the appropriate arylpiperazine (2 eq) in MeOH (5 mL), AcOH was added until pH = 5–6. The mixture was added of $NaCNBH_4$ (2 eq) at 0 °C and the whole stirred at room temperature for 5 (for **25**) or 12 h (for **26**). The solvent was removed under reduce pressure and the resulting yellow oil dissolved in Et_2O. The organic solution was washed (H_2O), dried (Na_2SO_4) and concentrated under reduced pressure. The analytically pure product was isolated by flash chromatography purification.

5.1.26. 1-{[5-(4-Chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrol-3-yl]methyl}-4-(4-chlorophenyl)piperazine (**25**)

General procedure for the synthesis of amines was used to convert **24** and 1-(4-chlorophenyl)piperazine into the title product. The mixture was purified by flash chromatography ($CHCl_3$//MeOH 98:2) to afford **25** (60 mg, 38%) as a white solid. R_f = 0.15 (petroleum ether/EtOAc 1:1); mp 99–102 °C; ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 6H), 2.60–2.67 (m, 4H), 3.17 (t, 4H, J = 9.6 Hz), 3.48 (s, 2H), 5.04 (s, 2H), 6.21 (s, 1H), 6.63 (s, 1H), 6.82 (d, 2H, 9.2 Hz), 7.02–7.13 (m, 3H), 7.15–7.23 (m, 3H), 7.24–7.25 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 20.09 (CH_3), 21.09 (CH_3), 49.12 (CH_2 ×2), 50.46 (CH_2), 52.70 (CH_2 ×2), 55.39 (CH_2), 110.45 (CH), 117.15 (CH ×3), 119.49 (C), 124.31 (CH), 126.41 (C), 126.56 (CH), 128.25 (CH), 129.14 (CH ×3), 129.33 (CH ×2), 129.40 (CH ×2), 131.24 (C), 133.08 (C), 133.94 (C), 135.62 (C), 135.98 (C), 137.12 (C), 150.05 (C); MS (ESI): $C_{30}H_{31}Cl_2N_3$ requires *m/z* 503, found 504 $[M + 1]^+$; Anal. calcd for $C_{30}H_{31}Cl_2N_3$: C, 71.42; H, 6.19; Cl, 14.05; N, 8.33. Found: C, 71.44; H, 6.20; Cl, 14.03; N, 8.32.

5.1.27. 1-{[5-(4-Chloro-3-methylphenyl)-1-(4-methylbenzyl)-1H-pyrrol-3-yl]methyl}-4-(3,4-dichlorophenyl)piperazine (**26**)

General procedure for the synthesis of amines was used to convert **24** and 1-(3,4-dichlorophenyl)piperazine into the title product. The mixture was purified by flash chromatography (petroleum ether/EtOAc 7:3) to afford **26** (80 mg, 47%) as a yellow sticky solid. R_f = 0.20 (petroleum ether/EtOAc 7:3); mp 170–172 °C (triturated with petroleum ether); IR 1640 (C═O), 3345 (NH); ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 6H), 2.60–2.65 (m, 4H), 3.19 (t, 4H, J = 9.6 Hz), 3.48 (s, 2H), 5.04 (s, 2H), 6.21 (s, 1H), 6.64 (s, 1H), 6.73 (dd, 1H, J_m = 8.8 Hz, J_o = 2.8 Hz), 6.90 (d, 1H, J = 8.0 Hz), 6.94 (s, 1H), 7.06–7.15 (m, 3H), 7.18–7.28 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 20.07 (CH_3), 21.07 (CH_3), 48.61 (CH_2 ×2), 50.46 (CH_2), 52.48 (CH_2 ×2), 55.28 (CH_2), 110.38 (CH), 115.19 (CH), 117.10 (CH), 119.49 (C), 122.74 (CH), 126.39 (CH), 126.41 (C), 126.55 (CH), 127.20 (CH ×2), 128.97 (CH), 129.39 (CH ×2), 130.38 (CH), 131.23 (CH), 131.34 (C), 133.10 (C), 133.95 (C), 135.60 (C), 135.97 (C), 137.15 (C), 149.07 (C); MS (ESI): $C_{30}H_{30}Cl_3N_3$ requires *m/z* 537, found 538 $[M + 1]^+$; Anal. calcd for $C_{30}H_{30}Cl_3N_3$: C, 66.86; H, 5.61; Cl, 19.73; N, 7.80. Found: C, 66.85; H, 5.60; Cl, 19.70; N, 7.79.

5.1.28. Synthesis of 2-methyl-3-nitrobenzaldehyde (**29**) [20]

The compound was synthesized starting from a solution of alcohol **28** [13] (1.10 g, 6.58 mmol, 1eq) in CH_2Cl_2 as reported in US Patent 73850 [20]. The brown solid residue obtained after work up was purified by flash chromatography (petroleum ether/EtOAc 8:2) to obtain derivative **29** as a yellow solid (900 mg, 83.3%). R_f = 0.28 (petroleum ether/EtOAc 8:2); mp 54–57 °C; ^{1}H NMR ($CDCl_3$) δ 2.78 (s, 3H), 7.53 (t, 1H, J = 8.0 Hz), 7.98 (d, 1H, J = 8.0 Hz), 8.06 (d, 1H, J = 8.0 Hz), 10.39 (s, 1H).

5.1.29. Synthesis of (E)-3-(2-methyl-3-nitrophenyl)propenoic acid (**30**)

To a mixture of aldehyde **29** (500 mg, 3 mmol, 1 eq) and malonic acid (2.2 eq) in dry pyridine (11.5 mL) was added piperidine (0.1 mL). The mixture was refluxed for 18 h, then was cooled to room temperature and poured onto concd. HCl (8 mL) and ice. The resulting precipitate was filtered, washed (5% aqueous HCl) and air dried to give derivative **30** as a yellow solid (490 mg, 79%). R_f = 0.04 (petroleum ether/EtOAc 7:3); mp 190–192 °C; ^{1}H NMR ($CDCl_3$) δ 2.54 (s, 3H), 6.40 (d, 1H, J = 16.0 Hz), 7.38 (t, 2H, J = 8.0 Hz), 7.77 (t, 1H, J = 8.8 Hz), 8.08 (d, 1H, J = 16.0 Hz).

5.1.30. Synthesis of 3-(3-amino-2-methylphenyl)propanoic acid (**31**)

To a suspension of acid **30** (800 mg, 3.86 mmol, 1 eq) in $EtOH_{abs}$ (7.2 mL) was added Pd/C-10% (0.1 eq) and the mixture was hydrogenated at 30 psi for 12 h at room temperature. Then the catalyst was removed by filtration on bed of celite® and the filtrate concentrated under reduce pressure to yield **31** as a brown solid (635 mg, 93%). R_f = 0.13 (petroleum ether/EtOAc 1:1); mp 158–161 °C; ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 3H), 2.59 (t, 2H, J = 8.8 Hz), 2.70–2.85 (br s, 2H, NH_2, exch. with D_2O), 2.91 (t, 2H, J = 8.8 Hz), 6.60 (d, 1H, J = 8.0 Hz), 6.97 (s, 1H), 7.28 (s, 1H), 11.10 (s, 1H, OH, exch. with D_2O).

5.1.31. Synthesis of 3-(3-chloro-2-methylphenyl)propanoic acid (**32**)

To a solution of acid **31** (500 mg, 2.79 mmol, 1eq) in conc. HCl (5.3 mL) cooled at −5 °C was dropwise added an aqueous solution (5 mL) of $NaNO_2$ (2.6 eq) and CuCl (1.25 eq). The mixture was stirred at room temperature for 12 h, then slowly added of 10% NaOH until pH 5–6. The precipitate was removed under vacuum and the filtrate extracted with Et_2O. The organic solution dried (Na_2SO_4) and concentrated under reduced pressure to furnish a brown

residue, whose flash chromatography purification (petroleum ether/EtOAc 4:6) gave derivative **32** as a yellow solid (349 mg, 63%). R_f = 0.20 (petroleum ether/EtOAc 4:6); mp 121–124 °C; ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H), 2.65 (t, 2H, J = 7.8 Hz), 3.02 (t, 2H, J = 8.0 Hz), 6.81 (d, 1H, J = 9.0 Hz), 7.06 (d, 1H, J = 4.6 Hz), 7.91 (d, 1H, J = 8.8 Hz), 11.09 (s, 1H, OH, exch. with D_2O).

*5.1.32. Synthesis of 4-methy-5-chloro-indan-1-one (**33**)*

A solution of acid **32** (2 g, 10 mmol) in CF_3SO_3H (10 mL) was stirred at room temperature for 5 h. Then crushed ice was slowly added and the solution extracted with Et_2O. The organic layer washed with 10% $NaHCO_3$, H_2O and brine, dried (Na_2SO_4) and concentrated under reduced pressure to give a yellow solid, whose flash chromatography purification (petroleum ether/EtOAc 8:2) afforded derivative **33** as a pallid yellow solid (720 mg, 40%). R_f = 0.23 (petroleum ether/EtOAc 8/2); mp 100–102 °C; ^{1}H NMR ($CDCl_3$) δ 2.38 (s, 3H), 2.72 (t, 2H, J = 5.2 Hz), 3.06 (t, 2H, J = 5.2 Hz), 7.38 (d, 1H, J = 8.2 Hz), 7.53 (d, 1H, J = 8.4 Hz).

*5.1.33. Synthesis of 2-bromo-4-methy-5-chloro-indan-1-one (**34**)*

To a solution of indanone **33** (1 g, 5.5 mmol, 1 eq) in AcOH (4 mL), Br_2 (1 eq) was dropwise added and the mixture stirred at room temperature for 4 h. Then H_2O (3 mL) was poured into reaction flask, and the resulting precipitate filtered under vacuum, washed (H_2O) and air dried to furnish a yellow solid whose purification by flash chromatography (petroleum ether/EtOAc 95:5) gave derivative **34** as a white solid (850 mg, 60%). R_f = 0.12 (petroleum ether/EtOAc 95:5); mp 131–134 °C; δ 2.37 (s, 3H), 3.33 (d, 1H, J = 15.6 Hz), 3.76 (dd, 1H, J_m = 18 Hz, Jo = 7.2 Hz), 6.66 (d, 1H, J = 7.6 Hz), 7.45 (d, 1H, J = 8.4 Hz), 7.62 (d, 1H, J = 8.0 Hz).

*5.1.34. Synthesis of ethyl 2-(5-chloro-4-methyl-1-oxo-1H-indan-2-yl)-3-oxobutanoate (**35**)*

A solution of ethyl-acetoacetate (1.2 eq) in anhydrous THF (1 mL) cooled to 0 °C was slowly added 60% NaH in mineral oil (3 eq) and the suspension was stirred at room temperature for 20 min, under N_2. Then, a solution of bromo-indanone **34** (850 mg, 3.27 mmol, 1 eq) in anhydrous THF (4 mL) was dropwise added and the mixture was stirred at room temperature for 24 h H_2O was added to the solution and extracted with Et_2O. The organic phase dried (Na_2SO_4) and concentrated at reduced pressure to give an oily residue, which was purified by gradient flash chromatography (petroleum ether/EtOAc 9:1–8:2) to give derivative **35** as a yellow oil (650 mg, 65%); R_f = 0.21 (petroleum ether/EtOAc 8:2); ^{1}H NMR ($CDCl_3$) 1.34 (t, 3H, J = 6.8 Hz), 2.30 (s, 3H), 2.42 (s, 3H), 2.88–3.00 (m, 1H), 3.15–3.40 (8m, 2H), 4.00–4.15 (m, 2H), 4.1 (q, 2H, J = 6.8 Hz), 7.38 (d, 1H, J = 8.0 Hz), 7.54 (d, 1H, J = 8.0 Hz).

*5.1.35. Synthesis of ethyl 6-chloro-2,5-dimethyl-1,4-dihydroindeno [1,2-b]pyrrole-3-carboxylate (**36**)*

To a solution of keto-ester **35** (700 mg, 2.27 mmol, 1 eq) in toluene (4.5 mL) were added NH_4OAc (2.5 eq) and SiO_2 (0.2 eq) and the mixture of reaction was subjected to mW irradiation at 110 °C for 2.5 h. Then the suspension was filtered under vacuum, and the residue washed with EtOAc. The filtrate was concentrated under reduced pressure to furnish a dark solid whose purification by flash chromatography (petroleum ether/EtOAc 8:2) gave derivative **36** as a brown solid (400 mg, 61%); R_f = 0.15 (petroleum ether/AcOEt 8:2); mp = 152–155 °C; ^{1}H NMR ($CDCl_3$) δ 1.40 (t, 3H, J = 7.2 Hz), 2.42 (s, 3H), 2.65 (s, 3H), 3.58 (s, 2H), 4.33 (d, 2H, J = 7.2 Hz), 7.02 (d, 1H, J = 7.6 Hz), 7.25–7.28 (m, 1H), 8.32 (br s, 1H, NH, exch. with D_2O).

*5.1.36. Synthesis of ethyl 6-chloro-2,5-dimethyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxylate (**37**)*

To a solution of tricyclic-ester **36** (405 mg, 1.40 mmol, 1 eq) in anhydrous DMF (4.8 mL) under N_2, was portionwise added 60% NaH in mineral oil (1.2 eq). The solution was stirred at room temperature for 15–20 min, then a solution of 4-methyl-benzyl chloride (1 eq) in anhydrous THF (1.5 mL) was added dropwise: the resulting mixture was stirred at room temperature for 12 h. The solution was poured in H_2O (8.5 mL) and extracted with CH_2Cl_2, which was washed (H_2O), dried (Na_2SO_4) and concentrated under reduced pressure to furnish an oily dark residue, whose flash chromatography purification (petroleum ether/EtOAc 95:5) gave derivative **37** as a purple solid (480 mg, 87%). R_f = 0.21 (petroleum ether/EtOAc 8:2); ^{1}H NMR ($CDCl_3$) δ 1.40 (t, 3H, J = 7.2 Hz), 2.32 (s, 3H), 2.47 (s, 3H), 2.61 (s, 3H), 3.64 (s, 2H), 4.36 (d, 2H, J = 7.2 Hz), 5.18 (s, 2H), 6.97 (d, 2H, J = 8.4 Hz), 7.02 (d, 1H, J = 7.6 Hz), 7.15 (d, 2H, J = 8.0 Hz), 7.25–7.28 (m, 1H).

*5.1.37. Synthesis of 6-chloro-2,5-dimethyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxylic acid (**38**)*

A solution of indenopyrrole-ester **37** (470 mg, 1.2 mmol) in a 10% hydro-alcoholic NaOH solution (11.5 mL, 60% EtOH) was refluxed 6 h. The solution was cooled at room temperature and acidified with 37% HCl. The resulting precipitate was filtered, washed (H_2O) and air-dried to give the acid **38** as an orange-red solid (270 mg, 65%). R_f = 0.10 (petroleum ether/EtOAc 7:3); mp 193–196 °C; ^{1}H NMR ($CDCl_3$) δ 2.10 (br s, 1H, OH, exch. with D_2O), 2.32 (s, 3H), 2.46 (s, 3H), 2.61 (s, 3H), 3.63 (s, 2H), 5.20 (s, 2H), 6.96 (d, 2H, J = 8.2 Hz), 7.03 (d, 1H, J = 7.6 Hz), 7.15 (d, 2H, J = 8.0 Hz), 7.25–7.28 (m, 1H).

*5.1.38. Synthesis of N-(1)-(S)-fenchyl-6-chloro-2,5-dimethyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-b]pyrrole-3-carboxamide (**39**)*

General procedure for the synthesis of carboxamides was used to convert acid **38** and *N*-(1)-(*S*)-fenchylamine into the title product. The mixture was refluxed for 4 h and purification by flash chromatography (petroleum ether/EtOAc 85:15) afforded **39** (51 mg, 35%) as a red solid. R_f = 0.10 (petroleum ether/EtOAc 85:15); mp 192–193 °C; IR 1633 (C=O), 3354 (NH); ^{1}H NMR ($CDCl_3$) δ 0.89 (s, 3H), 1.09 (s, 3H), 1.16 (s, 3H), 1.20–1.28 (m, 3H), 1.65–2.00 (m, 6H), 2.33 (s, 3H). 2.57 (s, 3H), 2.61 (s, 3H), 3.88 (d, 1H, J = 7.8 Hz), 5.15 (s, 2H), 6.55 (d, 1H, J = 7.8 Hz), 6.99 (d, 2H, J = 7.8 Hz), 7.07 (d, 1H, J = 7.8 Hz), 7.16 (d, 2H, J = 7.8 Hz), 8.17 (d, 1H, NH, exch. with D_2O); ^{13}C NMR ($CDCl_3$) δ 10.88 (CH_3), 13.52 (CH_3), 19.86 (CH_3), 21.07 (CH_3), 21.55 (CH_3), 26.19 (CH_2), 27.29 (CH_2), 31.18 (CH_3), 39.38 (C), 42.93 (CH_2), 48.39 (CH_2), 48.46 (CH), 48.60 (C ×2), 63.79 (CH), 113.57 (C), 115.39 (CH), 119.99 (C), 125.85 (CH ×2), 129.92 (CH ×2), 131.93 (C), 132.00 (CH), 134.40 (C), 135.85 (C), 136.29 (C), 137.84 (C), 138.05 (C), 142.11 (C), 148.49 (C); MS (ESI): $C_{32}H_{37}ClN_2O$ requires *m/z* 501, found 502 $[M + 1]^+$; Anal. calcd for $C_{32}H_{37}ClN_2O$: C, 76.70; H, 7.44; Cl 7.08; N, 5.59. Found: C, 76.68; H, 7.43; Cl 7.07; N, 5.57.

5.2. Radioligand binding assays for CB_1 and CB_2 receptors

CB_1R/CB_2R binding studies were performed using membrane fractions of human CB_1R/CB_2R transfected cells purchased from Perkin–Elmer Life and Analytical Sciences (Boston, MA). HEK293EBNA membranes were resuspended in Tris buffer (50 mM Tris–HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL BSA fatty acid free, pH 7.4). Fractions of the final membrane suspension (about 0.415 mg/mL of protein for CB_1 and about 0.18 mg/mL of protein for CB_2) were incubated at 30 °C for 90 min with 0.54 nM [^{3}H]-CP55940 (139.6 Ci/mmol) for CB_1 and 0.33 nM [^{3}H]-CP55940 (139.6 Ci/mmol) for CB_2, in the presence or absence of several concentrations of the competing drug, in a final volume of 0.2 mL

for CB_1 and 0.6 mL for CB_2 of assay buffer (50 mM Tris–HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL BSA fatty acid free, pH 7.4). Nonspecific binding was determined in the presence of 10 μM WIN55,212-2. Silanized tubes were used throughout the experiment to minimize receptor binding loss due to tube adsorption. The reaction was terminated by rapid vacuum filtration with a filter mate Harvester apparatus (Perkin–Elmer) through Filtermat A GF/C filters presoaked in 0.05% polyethylenimine (PEI). The filters were washed nine times with ice-cold buffer for CB1 (50 mM Tris–HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL BSA fatty acid free, pH 7.4) for CB_1 and CB_2 (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 1 mg/mL BSA fatty acid free, pH 7.5), and bound radioactivity was measured with a 1450 LSC & Luminiscence counter Wallac MicroBeta TriLux (Perkin–Elmer). The binding assay showed the appropriate sensitivity to CB_1 and CB_2 ligands. Thus, WIN55,212-2 inhibited the binding with a *K*i value of 36.2 nM (CB_1R) and WIN55,212-2 and HU-308 inhibited the binding with K_i values of 3.7 and 11.2 nM (CB_2R), respectively. For all binding experiments, competition binding curves were analysed by using an iterative curve-fitting procedure GraphPad Prism version 5.02 (GraphPad Software Inc., San Diego, CA, USA) and K_i values are expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

5.3. [^{35}S]-GTPγS binding analysis

[^{35}S]-GTPγS binding analyses were carried out for compounds **6** and **10** using CB_2R-containing membranes (HTS020M2, Eurofins Discovery Services). To this end, membranes (5 μg/well) were permeabilized by addition of saponin (Sigma–Aldrich), then mixed with 0.3 nM [^{35}S]-GTPγS (Perkin–Elmer) and 10 μM GDP (Sigma–Aldrich) in 20 mM HEPES (Sigma–Aldrich) buffer containing 100 mM NaCl (Merck) and 10 mM $MgCl_2$ (Merck), at pH 7.4. 30 nM CP55,940 (Sigma–Aldrich) and increasing concentrations of compound **6** or **10** (from 10^{-11} to 10^{-5} M) were added in a final volume of 100 μl and incubated for 30 min at 30 °C. The non-specific signal was measured with 10 μM GTPγS (Sigma–Aldrich). All 96-well plates and the tubes necessary for the experiment were previously silanized with Sigmacote (Sigma–Aldrich). The reaction was terminated by rapid vacuum filtration with a filter mate Harvester apparatus (Perkin–Elmer) through Filtermat A GF/C filters. The filters were washed nine times with ice-cold filtration buffer (10 mM sodium phosphate, pH 7.4), and bound radioactivity was measured with a 1450 LSC & Luminiscence counter Wallac MicroBeta TriLux (Perkin–Elmer). [^{35}S]-GTPγS binding data were analysed to determine the IC50 values by using an iterative curve-fitting procedure with the GraphPad Prism version 5.02 (GraphPad Software Inc.). IC50 values are expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

5.4. Determination OF CB_2 receptor-mediated functional activity in a cultured cell-based bioassay

The functional activity of the new compounds for CB_2R was also evaluated in cultured BV-2 cells, a mouse microglial cell line. Cells were plated at a density of 5×10^5 cells per well in 12-well culture plates previously coated with 15 μg/ml Poly-L-ornithine (Sigma), and incubated overnight in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) supplemented with 10% foetal bovine serum (FBS, Lonza), 2 mM Ultraglutamine and antibiotics (Lonza) in a humidified atmosphere of 5% CO_2 at 37 °C. One hour before treatment, medium was replaced with DMEM supplemented with 1% FBS, 2 mM Ultraglutamine and antibiotics. Cells were treated for 16 h with 1 μg/ml Lipopolysaccharides (LPS from *Escherichia coli* 055:B5, Sigma), alone or in combination with the investigated compound, used at a concentration 10-fold the K_i obtained in binding studies. 10 μM WIN55,212-2 (Sigma) and 10 μM SR144528 (Santa Cruz Biotechnology) were used as reference compounds because of their capability to either activate or block the CB_2R, respectively. Media were then removed and used for the determination of PG-E2 release using the ELISA kit DetectX® Prostaglandin E2 (Arbor Assays).

5.5. Molecular modelling

5.5.1. Conformational analysis

Complete conformational analyses of compounds listed in Table 1 were performed by first using the semi-empirical RM1 forcefield to conduct conformational search calculations and then optimizing resulting unique conformers with *ab initio* Hartree–Fock calculations at the 6-31G* level as encoded in Spartan '08 (Wavefunction, Inc., Irvine, CA). In each conformer search, local energy minima were identified by rotation of a subject torsion angle through 360° in 45° increments (8-fold search), followed by semi-empirical RM1 energy minimization of each rotamer generated. Duplicate conformations were removed and HF 6-31G* energy minimizations of each unique conformer were performed. To calculate the energy difference between the global minimal energy conformer of each compound and its final docked conformation, the single point energy of each was calculated in OPLS 2005 and difference was calculated.

5.5.2. Unique volume map calculation

To probe the steric limits of the CB_2 binding pocket, we used a modification of the Active Analogue Approach [17]. Here, we calculated that volume of space occupied by poor affinity ($K_i > 5000$ nM) analogues in Table 1 that was not occupied by those analogues in Table 1 with higher affinities ($K_i < 5000$ nM). All conformers within 5.00 kcal/mol of the global minimum conformer were considered to be accessible conformers. These conformers were then superimposed at their central five membered ring. Using the Surface facilities within Maestro 9.8 (Schrödinger Inc.), a density of 3.33 points per Å, and a probe radius of 1.4 Å, the UNION of Van der Waals (VdW's) volume maps of each of the conformers identified belonging to the binding group was calculated. The UNION of the VdW's volume maps of the non-binding group was separately calculated. Using a logical NOT operation, the region of space that the conformers of the non-binding group did not share with that of the binding group was then calculated.

5.5.3. Docking study in CB_2R

Compound **10** was docked into the SR144528 CB_2R binding site previously identified [16] by Glide docking studies. This CB_2 inactive state receptor model was pre-equilibrated in a stearoyldocosahexaenoylphosphatidylcholine (SDPC) bilayer for 300 ns to allow it to adjust to a lipid environment [16]. The selected conformer was docked using Glide 6.6 and the dock with the best Glide score modified by the strain energy (relative to the global min) was chosen. Glide was used to generate a grid based on the centroid of select residues in the binding pocket. The box size was set to the default value of 14 × 14 × 14 Å, with the inner box size was set to 10 × 10 × 10 Å. This default box size encompasses the entire CB_2R binding pocket both in width and depth. Standard precision (SP) and flexible docking with ring sampling were selected for the docking setup. Only trans amides were allowed. After docking with ring sampling, a 500 step conjugate gradient minimization was performed by Glide (dielectric = 1).

Conflict of interest

None of the authors have a conflict of interest to declare.

Acknowledgements

GM acknowledges Regione Autonoma della Sardegna for economic support (grant n. CRP-26417, LR n. 7/2007 and INNOVA.RE-POR FESR 2007-2013). PM is recipient of a CSIC fellowship JAE-Pre-2010-01119 from Junta para la Ampliación de Estudios co-financed by FSE. MGA was recipient of a postdoctoral fellowship from the PICATA Program, CEI-Moncloa. NJ thanks the Spanish Ministry of Economy and Competitivity for the grant SAF2012-40075, and with JFR the "Programa de Biomedicina, Comunidad de Madrid" (S2011/BMD-2308).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.06.057.

References

[1] P. Pacher, G. Kunos, Modulating the endocannabinoid system in human health and disease-successes and failures, FEBS J. 280 (2013) 1918–1943.
[2] R.G. Pertwee, Targeting the endocannabinoid system with cannabinoid receptor agonists: pharmacological strategies and therapeutic possibilities, Philos. Trans. R. Soc. Lond. B Biol. Sci. 367 (2012) 3353–3363.
[3] V. Di Marzo, M. Bifulco, L. De Petrocellis, The endocannabinoid system and its therapeutic exploitation, Nat. Rev. Drug Discov. 3 (2004) 771–784.
[4] A.C. Howlett, F. Barth, T.I. Bonner, G. Cabral, P. Casellas, W.A. Devane, C.C. Felder, M. Herkenham, K. Mackie, B.R. Martin, R. Mechoulam, R.G. Pertwee, International Union of Pharmacology. XXVII. Classification of cannabinoid receptors, Pharmacol. Rev. 54 (2002) 161–202.
[5] I. Svízenská, P. Dubový, A. Sulcová, Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures – a short review, Pharmacol. Biochem. Behav. 90 (2008) 501–511.
[6] J. Fernández-Ruiz, The endocannabinoid system as a target for the treatment of motor dysfunction, Br. J. Pharmacol. 156 (2009) 1029–1040.
[7] A. Quartilho, H. Mata, M. Ibrahim, T. Vanderah, F. Porreca, A. Makriyannis, T. Malan, Inhibition of inflammatory hyperalgesia by activation of peripheral CB2 cannabinoid receptors, Anesthesiology 99 (2003) 955–960.
[8] N. Clayton, F.H. Marshall, C. Bountra, C.T. O'Shaughnessy, CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain, Pain 96 (2002) 253–260.
[9] 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.
[10] J. Flygare, K. Gustafsson, E. Kimby, B. Christensson, B. Sander, Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma, FEBS Lett. 579 (2005) 6885–6889.
[11] C. Sánchez, M.L. De Ceballos, T. Gómez, C. Sa, M. Guzma, Inhibition of glioma growth in vivo by selective activation of the CB 2 cannabinoid receptor, Cancer Res. (2001) 5784–5789.
[12] T.W. Klein, C. Newton, K. Larsen, L. Lu, I. Perkins, L. Nong, H. Friedman, The cannabinoid system and immune modulation, J. Leukoc. Biol. 74 (2003) 486–496.
[13] P. Pacher, R. Mechoulam, Is lipid signaling through cannabinoid 2 receptors part of a protective system? Prog. Lipid Res. 50 (2011) 193–211.
[14] A.I. Idris, S.H. Ralston, Role of cannabinoids in the regulation of bone remodeling, Front Endocrinol. (Lausanne) 3 (2012) 136.
[15] M. Rinaldi-carmona, F. Barth, J. Millan, J.-M. Derocq, P. Casellas, C. Congy, D. Oustric, M. Sarran, M. Bouaboula, B. Calandra, M. Portier, D. Shire, J. Breliére, G. Le Fur, SR 144528, the first potent and selective antagonist of the CB2, Cannabinoid Recept. 284 (1998) 644–650.
[16] E. Kotsikorou, F. Navas, M.J. Roche, A.F. Gilliam, B.F. Thomas, H.H. Seltzman, P. Kumar, Z.-H. Song, D.P. Hurst, D.L. Lynch, P.H. Reggio, The importance of hydrogen bonding and aromatic stacking to the affinity and efficacy of cannabinoid receptor CB2 antagonist, 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide, J. Med. Chem. 56 (2013) 6593–6612.
[17] J.R. Sufrin, D.A. Dunn, G.R. Marshall, Steric mapping of the L-methionine binding site of ATP: L-methionine S-adenosyltransferase, Mol. Pharmacol. 19 (1981) 307–313.
[18] J.A. Suchocki, E.L. May, T.J. Martin, C. George, B.R. Martin, Synthesis of 2-exo- and 2-endo-mecamylamine analogues. Structure–activity relationships for nicotinic antagonism in the central nervous system, J. Med. Chem. (1991) 1003–1010.
[19] J.M. Kraus, C.L.M.J. Verlinde, M. Karimi, G.I. Lepesheva, M.H. Gelb, F.S. Buckner, Rational modification of a candidate cancer drug for use against Chagas disease, J. Med. Chem. 52 (2009) 1639–1647.
[20] R.J. Altenbach, M.D. Meyer, J.F. Kerwin Jr., A. Khilevich, T. Kolasa, J. Rohde, W.A. Carroll, X. Searle, F. Yang, 4-Imidazole Derivatives of Benzyl and Restricted Benzyl Sulfonamides, Sulfamides, Ureas, Carbamates, and Amides and their Use, 2003. US Patent 73850.