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Discovery of novel Tetrahydrobenzo[*b*]thiophene and pyrrole based scaffolds as potent and selective CB2 receptor ligands: The structural elements controlling binding affinity, selectivity and functionality

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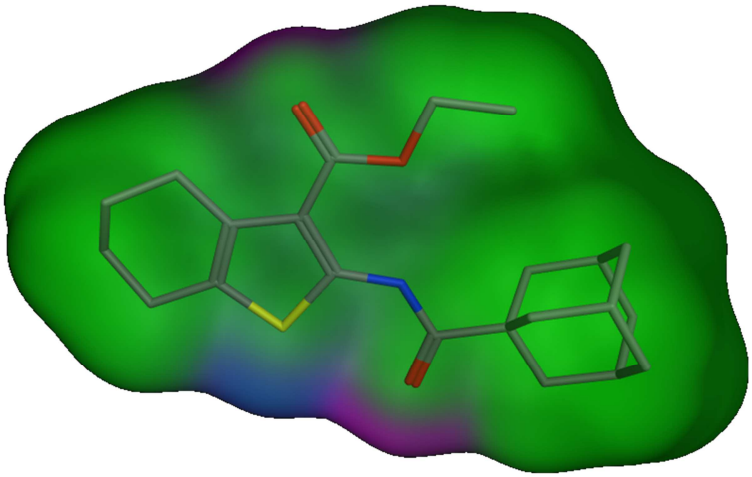
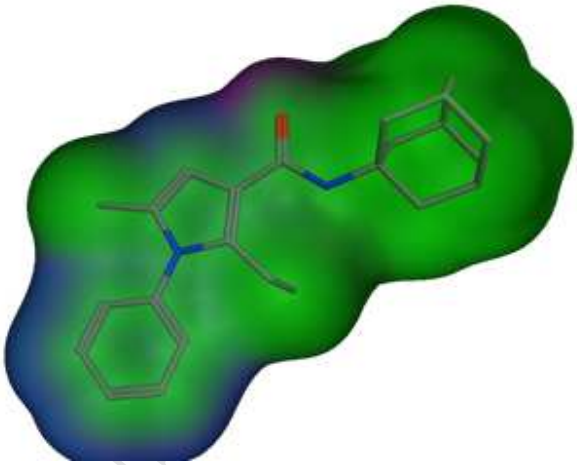
GRAPHICAL ABSTRACT



The molecular surface of **6b**
CB2 selective agonist
hCB2 K_i = 2.15 nM,
hCB1 K_i/hCB2 K_i **SI 195**



The molecular surface of **19b**
CB2 inverse agonist
hCB2 K_i = 6.15 nM,
hCB1 K_i/hCB2 K_i **SI 469**

Discovery of Novel Tetrahydrobenzo[b]thiophene and Pyrrole Based Scaffolds as Potent and Selective CB2 Receptor Ligands: The Structural Elements Controlling Binding Affinity, Selectivity and Functionality.

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ABSTRACT: CB2-based therapeutics show strong potential in the treatment of diverse diseases such as inflammation, multiple sclerosis, pain, immune-related disorders, osteoporosis and cancer, without eliciting the typical neurobehavioral side effects of CB1 ligands. For this reason, research activities are currently directed towards the development of CB2 selective ligands. Herein, the synthesis of novel heterocyclic-based CB2 selective compounds is reported. A set of 2,5-dialkyl-1-phenyl-1H-pyrrole-3-carboxamides, 5-subtituted-2-(acylamino)/(2-sulphonylamino)-thiophene-3-carboxylates and 2-(acylamino)/(2-sulphonylamino)-tetrahydrobenzo[b]thiophene-3-carboxylates were synthesized. Biological results revealed compounds with remarkably high CB2 binding affinity and CB2/CB1 subtype selectivity. Compound **19a** and **19b** from the pyrrole series exhibited the highest CB2 receptor affinity (K_i = 7.59 and 6.15 nM, respectively), as well as the highest CB2/CB1 subtype selectivity (~70 and ~200-fold, respectively). In addition, compound **6b** from the tetrahydrobenzo[b]thiophene series presented the most potent and selective CB2 ligand in this series (K_i = 2.15 nM and CB2 subtype selectivity of almost 500-fold over CB1). Compound **6b** showed a full agonism, while compounds **19a** and **19b** acted as inverse agonists when tested in an adenylate cyclase assay. The present findings thus pave the way to the design and optimization of heterocyclic-based scaffolds with lipophilic carboxamide and/or retroamide substituent that can be exploited as potential CB2 receptor activity modulators.

KEYWORDS: CB2 selective ligands; CB2 agonist; CB2 inverse agonist; cannabinoids

ABBREVIATIONS

CB1: Cannabinoid receptor 1; CB2: Cannabinoid receptor 2;; DIPEA: *N*,*N*-Diisopropylethylamine; EDC: 1-Ethyl-3-(3'-dimethylamino)carbodiimide HCl salt; HATU: 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3 triazolo[4,5-*b*]pyridinium 3-oxid

hexafluorophosphate; HBTU: O-(1H-benzotriazol-1-yl)-N,N,N',N' tetramethyluronium hexafluorophosphate; HEK-293: Human embryonic kidney 293 cells; Hz: Hertz; IBD: Inflammatory Bowel Disease; IC_{50}: Half maximal (50%) inhibitory concentration; K_i: Inhibition constant; m.p.: Melting point; M.wt: Molecular weight; nM: Nanomolar; SI: Selectivity index; TLC: thin layer chromatography

INTRODUCTION

Cannabinoid receptors, endogenous cannabinoid receptor ligands "endocannabinoids", and enzymes catalyzing their formation and degradation collectively constitute the endocannabinoid system. The potential modulation of this ubiquitous system for therapeutic gain has become a central focus of research during the last decade. Two cannabinoid receptor subtypes have been identified, to date: CB1 and CB2. Both are G protein-coupled receptors which have variable tissue distribution. Although the CB1 receptor is present in various peripheral tissues, its highest expression is in the CNS where it mediates the psychotropic effects of Δ^9-THC. Such psychotropic effects include euphoria, drowsiness, memory lapses, disruption of motor skills, lack of concentration and disorientation[1-5]. Conversely, the CB2 subtype is predominantly, but not exclusively, expressed in the periphery, primarily in cells of the immune system. It has also been found to be expressed in osteoclasts, and osteoblasts, as well as in various tumors and the tumor cell microenviroment[6]. Interestingly, in the case of colorectal and endometrial carcinoma, the CB2 receptor is over-expressed and its levels correlate with tumor malignancy[7, 8]. This cellular distribution has increased the popularity of CB2 receptors for their immunomodulatory[9], anti-inflammatory[10], analgesic[11], bone remodeling[12] and anti-tumor effects[13]. In addition, studies have demonstrated that CB2 receptors are overexpressed in chronically activated microglial cells, which are thought to play an important role in neurodegenerative disorders[14]. Hence,

targeting this receptor also holds promise for the treatment of neuro-inflammatory disorders such as dementia, multiple sclerosis and Alzheimer's[15, 16].

We are thus left with the conclusion that the CB2 receptor represents an attractive therapeutic target for the treatment of many conditions with important unmet medical needs. More importantly, because this receptor is significantly found outside the brain, compounds selective for the CB2 receptor do not exhibit the same psychotropic side effects that have plagued CB1 receptor-based therapeutics.[5] This has, consequently, prompted the development of several chemical classes of CB2 receptor selective ligands.

Over the past few years, a diverse number of CB2 ligands have been developed, either as agonists, partial agonists, or antagonists/inverse agonists (Figure 1). Among the well-known CB2 agonists are the classical cannabinoids (CC); (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran (**1**, JWH-133) in this class has shown to suppress colitis in several experimental models of IBD in rodents. {4-[4-(1,1-dimethylheptyl)-2,6-dimethoxy-phenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-en-2-yl}-methanol (**2**, HU-308) is a bicyclic CC analogue that exhibits a 400 fold higher selectivity for the CB2 receptor subtype over CB1.[17] The aminoalkylindoles (AAI) is also one of the most extensively studied classes represented by R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4 benzoxazin-6-yl]-1-naphthalenylmethanonemesylate (**3**, R-(+)-WIN55212) which exhibits a slightly higher affinity towards CB2 versus CB1.[18] Second-generation CB2 agonists based on AAI structure-activity relationship (SAR) studies is exemplified by novel compounds such as (1-(2-morpholin-4-yl-ethyl)-1H-indol-3-yl)-(2,2,3,3-tetramethylcyclopropyl) methanone (**4**, A-796260). It has been found to display analgesic activity in inflammatory, osteoarthritic, neuropathic and postoperative rodent pain models.[19] With respect to selective CB2 antagonists/

inverse agonists, fewer classes have been reported. Among these are the diarylpyrazole carboxamides represented by the first to be discovered and one of the most potent members: 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,4R,6S)-1,5,5-trimethyl-6-bicyclo[2.2.1]-heptanyl]pyrazole-3-carboxamide (**5**, SR144528).[20] More recently, other series have been disclosed which include the quinolone amide derivatives. Compound **6** is a representative of this series endowed with a high affinity (K_i = 0.6 nM and selectivity (>16,666-fold) for the CB2 receptor over CB1.[21] Also, the 1,8-Naphthyridin-2(1H)-one-3-carboxamide series has been recently identified to act as potent and selective CB2 ligands, where it has been shown that their functional activity is controlled by the presence of the substituents at certain positions of the naphthyridine scaffold. Thus, it is pretty clear that the functional activity can be modulated by changing the nature of substituents around the heterocyclic scaffold.[22]

In the light of these findings and as an extension of our research project aimed at the identification of novel CB2-selective chemotypes, we decided to design and synthesise a novel series of pyrrole-3-carboxamide derivatives, introducing structural modifications, which have been previously reported to increase CB2 receptor subtype affinity and selectivity (Figure 2).[23] Moreover, it was noted that both 1,3-thiazole- and indole-based chemical scaffolds presented potent and selective CB2 ligands. What is common among these scaffolds is the presence of either a single or fused heterocycle with an amide substituent at the heterocyclic 2-position (Figure 2)[23]. Such observations encouraged us to embark an isosterism approach and to synthesize another novel series of amide derivatives in which a thiophene or a tetrahydrobenzo[b]thiophene ring represents their heterocyclic cores. A thiophene ring replaced the 1,3-thiazole ring while a tetrahydrobenzo[b]thiophene ring was chosen to replace the indole ring. It is worth to note that, in the latter case, the disruption of planarity was due to the fusion of

the heterocycle with a cyclohexane ring in the tetrahydrobenzo[b]thiophene scaffold, versus an aromatic benzene ring in the original indole scaffold. Additionally, in order to obtain greater chemical diversity and based on recent findings that indicated the presence of sulphonamide functionalities in promising CB2 ligands[24-26], we also synthesized a series of thiophene and tetrahydrobenzo[b]thiophene-based scaffolds that displayed a substituted sulphonamide in place of the classical amide functionality. The general structures of the novel chemical scaffolds are shown in Figure 2.

RESULTS AND DISCUSSION

Chemistry

Synthesis of 2-(Acylamino/Sulphonylamino)-thiophene derivatives and 2-(Acylamino/Sulphonylamino)-tetrahydrobenzo[b]thiophene derivatives was afforded via a two-step synthetic route, outlined in **Scheme 1.** The first step involved the synthesis of the 2-aminothiophene intermediates **2a,b** adapting the famous one –pot Gewald reaction. This multi-component reaction involves 3 components: aldehydes or ketones, α-activated acetonitriles and sulphur in the presence of a base such as morpholine or diethylamine. Reaction takes place in solvents like methanol, ethanol or DMF, usually at 50-60 °C, in two subsequent steps – Knoevenagel-Cope condensation and intramolecular ring closure of formed sulfanyl substituted α,β-unsaturated nitrile. The reaction generally produces polysubstituted 2-aminothiophenes in yields varying between 35-90%.[27, 28]

Thus, condensation of phenylacetaldehyde or cyclohexanone with ethyl cyanoacetate in the presence of sulfur and a base such as morpholine or diethyl amine yielded the ethyl 2-aminothiophene-3-carboxylate derivatives **2a** and **2b**, respectively. Several variations of the reaction were tried in order to optimise reaction yield and shorten reaction time. In an attempt to

prepare compound **2a**, the respective reactants were all added in one pot using ethanol as a solvent and left to stir overnight at room temperature. TLC monitoring showed that no new product was formed, even after it was left to stir over another night. The same reaction was repeated by overnight heating at 60-65°C in an oil bath. TLC monitoring revealed that reaction completion occurred after 24 h. By pouring the reaction mixture onto ice-water, a significant amount of precipitate was noticed, producing the target compound in 90% yield. To further optimise reaction conditions, the microwave-assisted Gewald synthesis was attempted. Here, the multi-reactants vial was submitted to microwave irradiation for 30 min at 77 °C (Pmax = 80 W), while monitoring reaction by TLC. It was obvious that nearly all reactants disappeared after 20 minutes of microwave irradiation. Reaction workup was done in the same way but this time giving the target products in 96% yield. Therefore, this shows that the microwave-accelerated method provides a straightforward and a very efficient preparation of the target compounds in high yields.

Secondly, the 2-aminothiophene derivatives (**2a,b**) were then reacted with different carboxylic acids/acyl chlorides and sulphonyl chlorides yielding carboxamides (**3a,b-6b**) and sulphonamides (**7a,b-12a**), respectively. Carboxamide derivatives were produced either by coupling with carboxylic acids using a coupling agent (HATU, HBTU or EDCI/HOBt in the presence of DIPEA) or by direct reaction with acyl chlorides in the presence of TEA. Generally, reaction yields did not exceed 30% using the former method (irrespective of the coupling agent type). In contrast to this, the latter method gave yields that reached up to 90%. The reason for this is that the 2-amino group is of poor nucleophilicity due to resonance with the aromatic thiophene system and thus needed the more reactive acyl chlorides for the reaction to happen in a good yield. Sulphonamides (**7a,b-11b**) were produced by reaction with sulphonyl chlorides in

the presence pyridine, while stirring at room temperature overnight. In a few cases, the *N*,*N*-disulphonylamino product (**12a**) was isolated instead of the monosulphonylamino product. The increased acidity of the monosulphonylamino derivative promoted further deprotonation of the sulphonamide and subsequent formation of the *N*,*N*-disulphonylamino product.

Synthesis of the desired 2,5-Dialkyl-1-phenyl-1*H*-pyrrole-3-carboxamide derivatives was carried using a four-step procedure that is depicted in **Scheme 2**. Ethylacetoacetate or ethylpropionylacetate (β-keto-ester) was first alkylated with chloroacetone (haloketone) by refluxing in dry acetone in the presence of potassium carbonate and potassium fluoride. Reaction was monitored by TLC and required refluxing for 48-72 h to yield the corresponding 1,4-diketoesters (**14a,b**). The intermediate compounds **14a,b** were then allowed to undergo Paal-Knorr reaction, by refluxing with aniline in acetic acid for ≈ 5 h, to afford the corresponding ethyl 2,5-dialkyl-1-phenyl-1*H*-pyrrole-3-carboxylates (**15a,b**) in an average yield of 55-65%. Successful reaction was evident from ^{1}H-NMR spectra of **15a** and **15b**. ^{1}H-NMR of **15a** showed a singlet peak at 2.27 ppm that corresponds to the methyl protons at the pyrrole-2-position. On the other hand, ^{1}H-NMR of **15b** showed a quartet signal at 2.70 ppm, J = 7.5 Hz and a triplet signal at 0.97 ppm, J = 7.5 Hz, corresponding to the methylene and methyl protons of the ethyl group at the pyrrole-2-position. Ester hydrolysis of **15a,b** was accomplished, in a nearly 100% yield, through refluxing with 10% aqueous NaOH, followed by neutralization with 10% HCl to afford the corresponding carboxylic acids (**16a,b**). Coupling **16a,b** with the appropriate amines in the presence of HBTU and DIPEA, while stirring in DMF at room temperature, successfully afforded the corresponding amide derivatives (**17a,b-22a,b**) in an average yield of 80%.

In Vitro CB1 and CB2 receptor binding affinity and structure–activity relationships. All newly synthesized compounds were evaluated in radioligand binding assays for their ability to

displace [^{3}H]-CP-55,940 (a high affinity radioligand; 0.14 nM / K_d = 0.18 nM and 0.084nM / K_d = 0.31 nM, respectively for CB1 and CB2 receptor) from human recombinant CB1 and CB2 receptors. Preliminary screening assays for hit discovery were run at 1 and 10 µM. Compounds showing greater than 50% displacement at the screening doses were tested in a dose response curve to determine their IC_{50} values. K_i values were calculated by applying the Cheng-Prusoff equation to the IC_{50} values. Selectivity indices (K_i CB1/K_i CB2) were also calculated. The binding affinities are reported in Table 1.

Regarding Scheme 1 compounds, binding data showed that most compounds with a 5-phenyl thiophene scaffold (**4a**, **7a**-**10a**) did not show any binding affinity to the CB2 receptor. In fact, only compound **3a**, with a 1-naphthylamide substituent at position 2, displayed a K_i on CB2 = 59.81 nM and > 167-fold selectivity for CB2 over CB1. It was also evident from the binding data (Table 1) that replacing the 5-phenyl-thiophene substituent with a tetramethylene chain linking the 4- and 5- positions of the thiophene ring yielded the tetrahydrobenzo[b]thiophene derivatives (**3b**-**11b**), which was accompanied in most cases (**3b**-**6b**, **8b** and **10b**) with a noticeable increase in the CB2 receptor affinity, irrespective of the functional group at position 2. To explore the effect of having variable functionalities at position 2 of the thiophene/ tetrahydrobenzo[b]thiophene scaffold, both amide and sulphonamide derivatives were synthesized. It was clear that having a sulphonamide functionality at position 2, generally, provided compounds with a lower (**8b** and **10b**) or a nearly abolished affinity to the CB2 receptor (**7b**, **9b**, **11b**, **7a**-**10a** and **12a**). Comparing **8b** and **10b**, we can find that K_i values dropped from 1993.94 nM to 800.00 nM when the 1-phenylsulphonamide substituent in **8b** was replaced by the bulkier 1-naphthylsulphonamide substituent in **10b**.

For compounds having an amide functionality at position 2 (**3b**-**6b** and **3a**), a marked enhancement in the CB2 receptor affinity and selectivity was noted. Interestingly, compound **6b** was the most potent and selective compound of the current series, showing a remarkably high affinity at the CB2 receptor (K_i = 2.15 nM) and a CB2 receptor subtype selectivity that is almost 500-fold over CB1. Such an affinity is seemingly owed to the 1-admantyl substituent of the amide functionality. Pharmacomodulations of the substituent on the carboxamide function was also carried out. Replacing the 1-adamantyl group with a 1-naphthyl (aromatic) group yielded compound, **3b**, the second most potent and selective compound of the current class (K_i = 16.80 nM and 80-fold selectivity for CB2 over CB1. Substituting the 1-naphthyl group with its positional isomer, 2-naphthyl (**4b**), led to a 100-fold lower CB2 affinity, which reflects that the 1-naphthyl moiety is more favourable compared to its 2-naphthyl isomer. Substitution with other lipophilic groups such as 2-phenylethyl (**5b**) in the same position led to relatively lower CB2 binding affinity (K_i = 111.87 nM), however displaying a CB2 selectivity profile that is almost identical to that of **3b**. It can be, therefore, concluded that the type of the heteroayl nucleus together with its substitution pattern, type of functionality at the 2-position and the substituents attached to such functionalities are all crucial modulators of the CB2 receptor biniding affinity and selectivity profiles.

Regarding the second set of compounds synthesized, Scheme 2 was carried out to afford the 2,5-dialkyl-1-phenyl-1*H*-pyrrole-3-carboxamide derivatives. Two series of these derivatives were synthesized that were characterized by either having a 2-methyl pyrrole substituent, represented as "**series a**" or a 2-ethyl pyrrole substituent represented as "**series b**". It is worth to note that all compounds in both series showed quite impressive CB2 receptor binding affinities, all being in the nanomolar range. Some CB2 subtype selectivities reached almost as high as 200

fold over CB1. Biological results in Table 1 revealed that a bulky, lipophilic 1-adamantly amide gives a very potent CB2 ligand when either an ethyl (**19b**) or a methyl group (**19a)** was used at the 2-position of the pyrrole ring. In particular, compound **19b** (K_i = 6.15 nM, CB2 selectivity of almost 200-fold over CB1) showed a slightly higher CB2 receptor affinity and a much higher CB2 receptor subtype selectivity, when compared to **19a** (K_i = 7.59 nM, 68-fold selectivity for CB2 over CB1). In an effort to test the effect of less bulkier aliphatic amides, cyclohexyl amide analogues (**22a** and **22b**) were prepared. As evident from Table 1, reduced CB2 receptor affinity and selectivity were observed. In an attempt to explore the affinity of aromatic carboxamide substituents, 2-naphthylamide derivatives were prepared; yielding compounds **18a** and **18b** with a CB2 receptor affinity and selectivity that is higher when compared to their cyclohexyl counterparts while lower than their 1-adamantyl counterparts. Replacing the 2-naphthyl substituent of **18b** (K_i = 57 nM) with its 1-naphthyl positional isomer (**17b**, K_i = 20.02 nM) demonstrated an enhancement of the CB2 receptor affinity. However, an opposite effect was observed in **series a** when **18a** (K_i = 58.28 nM) was compared to **17a** (K_i = 84.22 nM). In order to further evaluate the importance of the hydrophobic character of the amide substituent, we also synthesized 1-phenyl ethyl (**21a** and **21b**) and 2-phenyl ethyl amide derivatives (**20a** and **20b**). K_i values revealed deterioration in the CB2 affinity of both derivatives when compared to their naphthyl analogues. This effect was less marked with the 1-phenyl ethyl derivatives (**21a**, K_i = 546.44 nM and **21b**, K_i = 540.10 nM) than their 2-phenylethyl counterparts (**20a**, K_i = 584.17 nM and **20b**, K_i = 718.20 nM). Therefore, we can say that increasing spacer length from 1- to 2-C atoms detrimentally affects the CB2 receptor affinity.

In summary, it is evident that the combination of a 1-aryl substituent with small alkyl substituents on positions 2 and 5 of the pyrrole-3-carboxamide nucleus led to very potent and

selective CB2 ligands. Lipophilic, aliphatic and aromatic, carboxamide substituents both yielded potent CB2 ligands. The best results in this series being demonstrated by compounds displaying the 1-adamantyl susbtituent (**19a** and **19b**) at the pyrrole-3-carboxamide position. In addition, the abolished CB2 affinity exhibited by the pyrrole-3-carboxylic acid intermediates (**16a** and **16b**) (K_i > 10000 nM) highlights the importance of the amide substitution at this position. It is also worth noting that, in general, **series b** compounds displayed either a very similar or a higher CB2 binding affinity and selectivity compared to **series a**. However, exceptions to this were only noted with the 2-phenyl ethyl amide (**20a** and **20b**) and the cyclohexyl amide (**22a** and **22b**) derivatives. In the former, compound **20a** gave a 1.2-fold higher CB2 receptor affinity than **20b** while in the latter, compound **22a** showed more than double the CB2 receptor selectivity of **22b**.

In vitro CB2 functional activity. Compounds **6b**, **19a** and **19b**, taken as representatives of relatively potent and selective CB2 ligands from Scheme 1 and 2 respectively, were subject to further in vitro pharmacological evaluation. We performed the Hunter™ eXpress GPCR assay to measure the modulation of intracellular cAMP levels induced by the water-soluble analog of forskolin, NKH-477. Opposite to compound **6b**, which showed a typical agonist behavior by reducing the NKH-477 induced cAMP (IC_{50} of 474.9 nM); compound **19a** and **19b** showed a similarity with the reference compound SR144528 (in terms of both potency and functionality), as indicated by the stimulation of cAMP production over the NKH-477 stimulus (IC_{50} of 108.6 and 398.3 nM, respectively). With the aim of investigating whether these compounds were able to antagonize CB2 receptor activation, we tested compounds also in the presence of an EC_{80} concentration of a selective CB2 agonist (JWH-133) as indicated in the Methods section. As shown in Figure 3 (A-B) compounds **19a** and **19b** increased cAMP levels far beyond that induced by NKH-477 both in absence and in presence of the EC_{80} ligand challenge suggesting

that they act orthosterically as inverse agonists. Conversely, compound **6b** reduced the cAMP levels induced by the NKH-477 stimulus as expected for an orthosteric Gi agonist. Consistently, this compound showed no activity in presence of the EC_{80} ligand challenge (Figure 3, C-D). Thus, it is obvious that the functional activity of these ligands is dictated by the nature of their heterocyclic cores as well as the substituents around these cores. Ligands with a pyrrole core that has a pendant benzene ring at the 1-position were shown to be inverse agonists. It is interesting to note that the latter ligands confer structural similarity to the well-known potent CB2 inverse agonist/antagonist; **SR144528**[20] (Figure 1). In the light of these findings, it can be deduced that an N-containing 5-membered heteroaryl central scaffold with a 1-phenyl/benzyl substituent are crucial elements that can be utilised to direct the functionality of the designed CB2 ligands towards an inverse agonist/ antagonist activity. Conversely, replacing the pyrrole ring with its isostere "thiophene", fusing the latter with a cyclohexane ring while abolishing the pendant phenyl substituent from the central heterocyclic scaffold has led to a full agonist functionality. Taken together, the present study confirms that these novel chemotypes are attractive leads that can be further optimised to produce selective CB2 ligands, acting either as full or inverse agonists.

CONCLUSION

2-(acylamino)tetrahydrobenzo[b]thiophene and pyrrole-3-carboxamide series were designed and synthesised as CB2 receptor ligands, tested in radioligand binding studies, and functionally characterized in adenylate cyclase assays. Both heterocyclic-based scaffolds presented novel chemical classes of potent and selective CB2 ligands, displaying K_i values in the nanomolar range and CB2 selectivities reaching up to 500-fold over CB1. In both series, best results were

demonstrated by compounds having the bulky 1-adamantyl substituent (**6b**, **19a** and **19b**) attached to the heterocyclic-amide functionality. The latter two compounds behaved as potent inverse agonists in functional assay, whereas the former one behaved as an agonist. Along with these data, we also confirmed specific structural elements that lead to agonism/inverse agonism activity. These findings pave the way to the design and/or optimisation of heterocyclic-based scaffolds with lipophilic carboxamide and/or retroamide substituents that can be exploited as potential CB2 receptor activity modulators and hence evaluating the therapeutic utility of such modulators in various disease settings.

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EXPERIMENTAL

Chemistry. All starting materials, reactants and solvents were obtained from Sigma–Aldrich and were used without further purification. Melting points were determined on Buchi B-540 Melting Point apparatus and are uncorrected. ^{1}H NMR and ^{13}C-NMR spectra were recorded on Varian 300 MHz spectrometer or 500 MHz spectrometer using $CDCl_3$ as a solvent; chemical shifts (δ) were reported in parts per million (ppm) downfield from TMS; multiplicities are abbreviated as: s: singlet; bs: broad singlet; d: doublet; q: quartet; p: pintet; m: multiplet; dd: doublet of doublet; ddd: doublet of doublet of doublet, dt: doublet of triplet and all coupling constants (*J*) are given in Hz. HR-ESI mass spectra were recorded on Bruker micrOTOF-Q II instrument and were acquired either in positive or in negative mode and data are reported as m/z. All masses were reported either as $(M+H)^+$, $(M+Na)^+$ or $(M+K)^+$ in case of positive mode or as $(M-H)^-$ in case of

negative mode. Flash chromatography was performed using Biotage Isolera One purification system using silica gel (0.06-0.2 mm) cartridges (KP-SIL) and UV monitoring at 254-280 nm. Reaction progress was monitored by TLC performed on Merck silica gel plates 60 and detection of the components was made by UV light (254 nm). Microwave irradiations were conducted using a Monowave 300 synthesis reactor with IR temperature sensor from Anton Paar. All reactions were carried out under nitrogen when inert atmosphere was needed.

General procedure for the synthesis of 2-Amino-4/5-substituted-thiophene-3-carboxylic acid ethyl ester (2a,b)[29]. A mixture of the respective aldehyde or ketone (4 mmol), ethylcyanoacetate (4 mmol), S8 (0.14 g, 4.4 mmol), and morpholine or diethylamine (5 mL) in EtOH (7 mL) was put in a vial (G30 size) and submitted to microwave irradiation for 20 minutes at 77 °C (Pmax = 80 W). After cooling, the solution was poured onto 50 mL ice water to yield a precipitate which was filtered, washed with water and dried under vacuum. The solid was used without further purification.

2-Amino-5-phenyl-thiophene-3-carboxylic acid ethyl ester (2a).[30] Brown powder, 95%; MS (HR-ESI) m/z calcd for $C_{13}H_{13}NNaO_2S$ $(M+Na)^+$: 270.0559, found: 270.0562.

2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (2b).[30] Yellow powder, 96%; MS (HR-ESI) m/z calcd for $C_{11}H_{15}NNaO_2S$ $(M+Na)^+$: 248.0716, found: 248.0709.

General procedure for the synthesis of 2-(Acylamino)-4/5-substituted-thiophene-3-carboxylic acid ethyl ester (3b, 6b). The appropriate acyl chloride (1.5 mmol) was slowly added under a nitrogen atmosphere to a cooled (0 °C) solution of aminothiophene derivative **21a** or **21b** (1 mmol) and TEA (1.5 mmol) in dry DCM (10 mL). After being stirred at room temperature for 18 h, the solution was washed with 1 N HCl, saturated solution of $NaHCO_3$ and

brine, then dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude product was purified by flash column chromatography using the reported eluent system.

2-[(Naphthalene-1-carbonyl)-amino]-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (3b). Yellow solid, 90%; m.p.: 126-128 ^{o}C; ^{1}H-NMR (300 MHz) δ 11.88 (s, 1H), 8.54 (d, *J* = 7.7 Hz, 1H), 7.96 (d, *J* = 8.2 Hz, 1H), 7.89 – 7.79 (m, 2H), 7.60 – 7.44 (m, 3H), 4.27 (q, *J* = 7.1 Hz, 2H), 2.79 – 2.76 (m, 2H), 2.69-2.66 (m, 2H), 1.86 – 1.71 (m, 4H), 1.31 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (75 MHz) δ 166.61, 165.57, 147.58, 133.86, 131.99, 131.93, 131.09, 130.50, 128.38, 127.51, 127.09, 126.61, 126.02, 125.50, 124.77, 112.16, 60.52, 26.42, 24.43, 23.02, 22.88, 14.28; MS (HR-ESI) m/z calcd for $C_{22}H_{21}NNaO_3S$ $(M+Na)^+$: 402.1134, found: 402.1132.

2-[(Adamantane-1-carbonyl)-amino]-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (6b). White solid, 91%; m.p.: 133-135 ^{o}C; ^{1}H-NMR (300 MHz) δ 11.54 (s, 1H), 4.34 (q, J = 7.1 Hz, 2H), 2.78-2.75 (m, 2H), 2.65-2.67 (m, 2H), 2.10 (s, 3H), 1.98 (d, J = 2.7 Hz, 6H), 1.91 (dd, J = 15.8, 2.7 Hz, 1H), 1.76 (bs, 9H), 1.38 (t, J = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{22}H_{29}NNaO_3S$ $(M+Na)^+$: 410.1754, found: 410.1760.

General procedure for the synthesis of 2-(Acylamino)-4/5-substituted-thiophene-3-carboxylic acid ethyl ester (3a, 4a-b, 5b). To a magnetically stirred solution of the respective aminothiophene derivative (1.20 mmol) in anhydrous DMF (15 mL) were successively added *N*,*N*-diisopropylethylamine (Hunig's base) (3.60 mmol), HBTU (1.2 mmol) in case of **5b**/ HATU (1.2 mmol) in case of **4b**/ EDCI and HOBt (1.2 mmol each) in case of **3a** and **4a** , and the acid derivative (1 mmol). After stirring overnight, the resulting mixture was concentrated in vacuo. The residue was dissolved in EtOAc, successively washed with aqueous $NaHCO_3$ solution,

water, and brine, dried over $MgSO_4$, filtered, and concentrated to give a crude solid. This solid was further purified by flash chromatography using the reported eluent system.

2-[(Naphthalene-1-carbonyl)-amino]-5-phenyl-thiophene-3-carboxylic acid ethyl ester (3a). Orange solid, 30%; m.p.: 150-152 ^{o}C; ^{1}H-NMR (300 MHz) δ 11.71 (s, 1H), 8.65 (d, *J* = 8.2 Hz, 1H), 8.05 (d, *J* = 8.2 Hz, 1H), 7.94 (dd, *J* = 7.3, 1.1 Hz, 2H), 7.70–7.75 (m, 5H), 7.52 (s, 1H), 7.46 – 7.38 (m, 2H), 7.35 – 7.29 (m, 1H), 4.38 (q, *J* = 7.1 Hz, 2H), 1.42 (t, *J* = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{24}H_{19}NNaO_3S$ $(M+Na)^+$: 424.0978, found: 424.0974.

2-[(Naphthalene-2-carbonyl)-amino]-5-phenyl-thiophene-3-carboxylic acid ethyl ester (4a). Brown solid, 12%; m.p.: 218-220 ^{o}C; ^{1}H-NMR (500 MHz) δ 12.18 (s, 1H), 8.59 (s, 1H), 8.09 – 8.03 (m, 2H), 7.99 (d, *J* = 8.6 Hz, 1H), 7.92 (d, *J* = 8.0 Hz, 1H), 7.66 – 7.64 (m, 2H), 7.63 – 7.57 (m, 2H), 7.50 (s, 1H), 7.44 – 7.38 (m, 2H), 7.32 – 7.28 (m, 1H), 4.45 (q, *J* = 7.1 Hz, 2H), 1.47 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (126 MHz) δ 165.99, 163.64, 148.58, 135.30, 134.03, 133.79, 132.67, 129.43, 129.19, 128.99, 128.97, 128.84, 128.38, 127.83, 127.47, 127.03, 125.51, 123.27, 119.34, 114.14, 61.00, 14.45; MS (HR-ESI) m/z calcd for $C_{24}H_{19}NNaO_3S$ $(M+Na)^+$: 424.0978, found: 424.0962.

2-[(Naphthalene-2-carbonyl)-amino]-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (4b). Brown powder, 13%; m.p.: 174-176 ^{o}C; ^{1}H-NMR (300 MHz) δ 12.47 (s, 1H), 8.56 (s, 1H), 8.08 – 7.88 (m, 4H), 7.63-7.57 (m, 2H), 4.41 (q, *J* = 7.1 Hz, 2H), 2.89 – 2.79 (m, 2H), 2.74-2.69 (m, 2H), 1.87-1.79 (m, 4H), 1.43 (t, *J* = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{22}H_{21}NNaO_3S$ $(M+Na)^+$: 402.1134, found: 402.1127.

2-(3-Phenyl-propionylamino)-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (5b). Yellow resin, 15%; ^{1}H-NMR (300 MHz) δ 11.27 (s, 1H), 7.33 – 7.16 (m, 5H), 4.30

(q, *J* = 7.1 Hz, 2H), 3.11 – 3.02 (m, 2H), 2.82 – 2.70 (m, 4H), 2.69 – 2.59 (m, 2H), 1.82 – 1.74 (m, 4H), 1.36 (t, *J* = 7.1, 3H); MS (HR-ESI) m/z calcd for $C_{20}H_{23}NNaO_3S$ $(M+Na)^+$: 380.1296, found: 380.1291.

General procedure for the synthesis of 2-(Sulphonylamino)-4/5-substituted-thiophene-3-carboxylic acid ethyl ester (7a,b-10a,b; 11b and 12a)[31]. The appropriate sulphonyl chloride (1.5 mmol equiv) was slowly added to a 0 °C solution of thiophene-2-amine derivative (1 mmol) in pyridine (10 mL). The reaction mixture was stirred at room temperature under inert atmosphere overnight. After addition of EtOAc, the solution was washed with 1 N HCl and brine. The organic phase was dried over $MgSO_4$ and evaporated under vacuum. The residue was purified by flash chromatography using the reported eluent system.

2-Methanesulphonylamino-5-phenyl-thiophene-3-carboxylic acid ethyl ester (7a). Brown solid, 60%; m.p.: 155-157 ^oC; ^1H-NMR (300 MHz) δ 7.64 (s, 1H), 7.61 – 7.55 (m, 2H), 7.46 – 7.35 (m, 3H), 4.39 (q, J = 7.1 Hz, 2H), 3.52 (s, 3H), 1.40 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (75MHz) δ 161.50, 145.06, 133.81, 132.59, 129.31, 129.21, 126.15, 123.77, 61.57, 43.26, 14.43; MS (HR-ESI) m/z calcd for $C_{14}H_{14}NO_4S_2$ $(M-H)^-$: 324.0370, found: 324.0369.

2-Methanesulphonylamino-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (7b). Yellow crystalline solid, 30%; m.p.: 74-76 ^oC; ^1H-NMR (300 MHz) δ 10.16 (s, 1H), 4.30 (q, *J* = 7.1 Hz, 2H), 3.05 (s, 3H), 2.77 – 2.68 (m, 2H), 2.64 – 2.55 (m, 2H), 1.83 – 1.69 (m, 4H), 1.35 (t, *J* = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{12}H_{17}NNaO_4S_2$ $(M+Na)^+$: 326.0491, found: 326.0493.

2-Benzenesulphonylamino-5-phenyl-thiophene-3-carboxylic acid ethyl ester (8a). Red sticky powder, 22%; m.p.: 72-74 ^oC; ^1H-NMR (500 MHz) δ 7.98 – 7.94 (m, 1H), 7.83 – 7.80 (m, 1H),

7.62 – 7.46 (m, 4H), 7.42 – 7.27 (m, 4H), 7.24 (s, 1H), 4.29 (q, *J* = 7.1 Hz, 2H), 1.35 (t, *J* = 7.1 Hz, 3H); MS (ESI) m/z calcd for $C_{19}H_{16}NO_4S_2$ $(M-H)^-$: 386.1, found: 386.0.

2-Benzenesulphonylamino-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (8b). White solid, 48%; m.p.: 116-118 ^oC; ^1H-NMR (300 MHz) δ 10.48 (s, 1H), 7.96 – 7.89 (m, 2H), 7.62 – 7.53 (m, 1H), 7.53 – 7.45 (m, 2H), 4.25 (q, *J* = 7.1 Hz, 2H), 2.70 –2.56 (m, 4H), 1.83 – 1.66 (m, 4H), 1.32 (t, *J* = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{17}H_{19}NNaO_4S_2$ $(M+Na)^+$: 388.0648, found: 388.0646.

2-(4-Chloro-benzenesulphonylamino)-5-phenyl-thiophene-3-carboxylic acid ethyl ester (9a). Red powder, 22%; m.p.: 92-94 ^oC; ^1H-NMR (500 MHz) δ 7.91 – 7.86 (m, 1H), 7.62 – 7.50 (m, 1H), 7.47 – 7.41 (m, 2H), 7.40 – 7.27 (m, 4H), 7.24 (s, 1H), 7.20 (t, *J* = 7.4 Hz, 1H) 4.30 (q, *J* = 7.1 Hz, 2H), 1.37 (t, *J* = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{19}H_{15}ClNO_4S_2$ $(M-H)^-$: 420.0137, found: 420.0143.

2-(4-Chloro-benzenesulphonylamino)-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (9b). Orange solid, 28%; m.p.: 95-97 ^oC; ^1H-NMR (500 MHz) δ 10.50 (s, 1H), 7.86 – 7.82 (m, 2H), 7.46 – 7.42 (m, 2H), 4.24 (q, *J* = 7.1 Hz, 2H), 2.68 – 2.63 (m, 2H), 2.61 – 2.56 (m, 2H), 1.78 – 1.70 (m, 4H), 1.31 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (126 MHz) δ 166.04, 147.38, 140.00, 137.52, 132.30, 129.56, 128.90, 127.23, 114.06, 60.91, 26.53, 24.64, 22.94, 22.66, 14.31; MS (HR-ESI) m/z calcd for $C_{17}H_{17}ClNO_4S_2$ $(M-H)^-$: 398.0293, found: 398.0291.

2-(Naphthalene-1-sulphonylamino)-5-phenyl-thiophene-3-carboxylic acid ethyl ester (10a). Yellow solid, 30%; m.p.: 163-165 ^oC; ^1H-NMR (300 MHz) δ 10.68 (s, 1H), 8.69 (d, J = 9.3 Hz, 1H), 8.40 (dd, J = 7.4, 1.2 Hz, 1H), 8.07 (d, J = 8.3 Hz, 1H), 7.96 – 7.89 (m, 1H), 7.79 – 7.70 (m,

1H), 7.66 – 7.27 (m, 7H), 7.19 (s, 1H), 4.23 (q, J = 7.1 Hz, 2H), 1.28 (t, J = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{23}H_{18}NO_4S_2$ $(M-H)^-$: 436.0683, found: 436.0672.

2-(Naphthalene-1-sulphonylamino)-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (10b). Yellow solid, 31%; m.p.: 175-177 ^oC; ^1H-NMR (500 MHz) δ 10.95 (s, 1H), 8.68 – 8.64 (m, 1H), 8.35 (dd, *J* = 7.4, 1.2 Hz, 1H), 8.05 (d, *J* = 8.2 Hz, 1H), 7.91 (d, *J* = 8.2 Hz, 1H), 7.74 – 7.69 (m, 1H), 7.61 – 7.57 (m, 1H), 7.54 – 7.50 (m, 1H), 4.18 (q, *J* = 7.1 Hz, 2H), 2.61 – 2.48 (m, 4H), 1.72 – 1.63 (m, 4H), 1.25 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (126 MHz) δ 165.91, 147.82, 135.10, 134.34, 133.52, 132.04, 130.94, 129.19, 128.74, 128.09, 127.10, 126.34, 124.41, 124.11, 113.02, 60.74, 26.45, 24.55, 22.96, 22.66, 14.29; MS (HR-ESI) m/z calcd for $C_{21}H_{21}NNaO_4S_2$ $(M+Na)^+$: 438.0804, found: 438.0802.

2-(Naphthalene-2-sulphonylamino)-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester (11b). White powder, 63%; m.p.: 173-175 ^oC; ^1H-NMR (300 MHz) δ 10.60 (s, 1H), 8.51 (s, 1H), 8.02 – 7.83 (m, 4H), 7.69 – 7.56 (m, 2H), 4.22 (q, *J* = 7.1 Hz, 2H), 2.69 – 2.48 (m, 4H), 1.80 – 1.61 (m, 4H), 1.29 (t, *J* = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{21}H_{20}NO_4S_2$ $(M-H)^-$: 414.0839, found: 414.0815.

2-(Bis-naphthalene-1-sulphonylamino)-5-phenyl-thiophene-3-carboxylic acid ethyl ester (12a). Brown solid, 57%; m.p.: 113-115 ^oC; ^1H-NMR (300 MHz) δ 8.56 (d, J = 11.5 Hz, 2H), 8.06 – 7.90 (m, 7H), 7.75 – 7.55 (m, 7H), 7.53 – 7.35 (m, 4H), 3.47 (q, J = 7.1 Hz, 2H), 0.87 (t, J = 7.1 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{33}H_{25}NNaO_6S_3$ $(M+Na)^+$: 650.0736, found: 650.0722.

General procedure for the synthesis of the diketoesters (14a,b)[32]. A mixture of Ethylacetoacetate (for **14a**) or Ethylpropionylacetate (for **14b**) (1.0 mmol), anhydrous potassium

carbonate (2.0 mmol), and potassium fluoride (1.0 mmol) in dry acetone was refluxed under nitrogen atmosphere and continuously stirred for half an hour, and then a solution of chloroacetone (1.1 mmol) in dry acetone was added, refluxing for another 48-72 h and monitoring the reaction by TLC. The reaction mixture was filtered, the residue was washed with acetone, and the combined filtrate and washings were evaporated. The residue was dissolved in ethylacetate, and the solution was washed with water, dried and evaporated under reduced pressure.

2-Acetyl-4-oxo-pentanoic acid ethyl ester (14a)[32]. Brown resin, 78%; MS (ESI) m/z calcd for $C_9H_{14}NaO_4$ $(M+Na)^+$: 209.0, found: 209.1.

3-Oxo-2-(2-oxo-propyl)-pentanoic acid ethyl ester (14b)[33]. Brown resin, 96%; MS (ESI) m/z calcd for $C_{10}H_{16}NaO_4$ $(M+Na)^+$: 223.0, found: 223.1.

General procedure for the synthesis of 2,5-Dialkyl-1-phenyl-1H-pyrrole-3-carboxylic acid ethyl ester (15a,b).[34] To a solution of **14a,b** (11 mmol) in acetic acid (40 mL), aniline (11 mmol) was added. The mixture was heated under reflux for 5-6 h until disappearance of reactants (TLC monitorage). Water was then added to the resulting solution and the solution extracted with ethylacetate (3 times). The combined organic layer was then dried using $MgSO_4$, evaporated under reduced pressure and the residue purified by flash chromatography according to the reported eluent.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid ethyl ester (15a).[35] Yellow liquid, 55%; MS (ESI) m/z calcd for $C_{15}H_{18}NO_2$ $(M+H)^+$: 244.1, found: 244.1.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid ethyl ester (15b). Brown resin, 64%; ^{1}H-NMR (500 MHz) δ 7.50 – 7.42 (m, 3H), 7.21 – 7.16 (m, 2H), 6.37 (d, J = 0.9 Hz, 1H),

4.28 (q, J = 7.1 Hz, 2H), 2.70 (q, *J* = 7.5 Hz, 2H), 1.91 (d, *J* = 0.9 Hz, 3H), 1.35 (t, J = 7.1 Hz, 3H), 0.97 (t, *J* = 7.4 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{16}H_{20}NO_2$ $(M+H)^+$: 258.1489, found: 258.1475.

General procedure for the synthesis of 2,5-Dialkyl-1-phenyl-1H-pyrrole-3-carboxylic acid (16a,b). The appropriate ethyl ester (5.8 mmol) was refluxed for 3-4 h in a mixture of aqueous 10% sodium hydroxide (20 mL) and ethanol (20 mL). After cooling, the solution was adjusted to pH 3 with aqueous 10% hydrochloric acid. The resulting precipitate was collected by filtration, washed with H_2O, dried under vacuum and used without further purification.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid (16a).[36] White powder, 95%; m.p.: 216-218 ^oC; ^1H-NMR (300 MHz) δ 7.56 – 7.44 (m, 3H), 7.23 – 7.18 (m, 2H), 6.44 (d, *J* = 0.9 Hz, 1H), 2.32 (s, 3H), 1.99 (d, *J* = 0.9 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{13}H_{12}NO_2$ $(M-H)^-$: 214.0874, found: 214.0866.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid (16b). Brown powder, 100%; m.p.: 197-199 ^oC; ^1H-NMR (500 MHz) δ 7.52 – 7.44 (m, 3H), 7.23 – 7.18 (m, 2H), 6.39 (d, *J* = 0.9 Hz, 1H), 2.72 (q, *J* = 7.5 Hz, 2H), 1.93 (d, *J* = 0.9 Hz, 3H), 0.99 (t, *J* = 7.4 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{14}H_{15}NNaO_2$ $(M+Na)^+$: 252.0995, found: 252.0989.

General procedure for the synthesis of 2,5-Dialkyl-1-phenyl-1H-pyrrole-3-carboxamides (17a,b; 18a,b; 19a,b; 20a,b; 21a,b and 22a, b).[37] To a magnetically stirred suspension of the carboxylic acid (1.6 mmol) in anhydrous DMF were successively added *N,N*-diisopropylethylamine (Hunig's base) (6 eq) and *O*-benzotriazol-1-yl-*N,N,N¢,N¢*-tetramethyluronium hexafluorophosphate (HBTU) (1.2 eq) and the mixture was allowed to stir at room temperature for 30 miuntes. The appropriate amine (1.2 equiv) was then added, and the

solution was stirred at room temperature overnight. The mixture was diluted with EtOAc, washed with aqueous $NaHCO_3$ solution, 1N HCl and brine, dried over $MgSO_4$ and concentrated to give a crude resin. The crude product was further purified by flash chromatography according to the reported eluent.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid naphthalen-1-ylamide (17a). Grey solid, 81%; m.p.: 145-147 ^{o}C; ^{1}H-NMR (300 MHz) δ 8.08 (d, *J* = 8.4 Hz, 1H), 7.61 – 7.48 (m, 5H), 7.45 – 7.38 (m, 1H), 7.30 – 7.19 (m, 5H), 6.67 (s, 1H), 2.34 (s, 3H), 2.05 (s, 3H); MS (HR-ESI) m/z calcd for $C_{23}H_{20}N_2NaO$ $(M+Na)^+$: 363.1468, found: 363.1474.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid naphthalen-1-ylamide (17b). Beige solid, 40%; m.p.: 106-108 ^{o}C; ^{1}H-NMR (300 MHz) δ 8.08 (d, *J* = 8.4 Hz, 1H), 7.99 – 7.86 (m, 1H), 7.61 – 7.48 (m, 5H), 7.45 – 7.38 (m, 1H), 7.30 – 7.26 (m, 1H), 7.26 – 7.18 (m, 3H), 6.67 (d, *J* = 0.9 Hz, 1H), 2.73 (q, *J* = 7.4 Hz, 2H), 2.02 (d, *J* = 0.9 Hz, 3H), 1.02 (t, *J* = 7.4 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{24}H_{22}N_2NaO$ $(M+Na)^+$: 377.1624, found: 377.1614.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid naphthalen-2-ylamide (18a). Beige solid, 83%; m.p.: 142-144 ^{o}C; ^{1}H-NMR (300 MHz) δ 8.08 (d, *J* = 8.4 Hz, 1H), 7.61 – 7.37 (m, 7H), 7.27 – 7.19 (m, 4H), 6.67 (d, *J* = 0.9 Hz, 1H), 2.34 (s, 3H), 2.05 (d, *J* = 0.9 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{23}H_{20}N_2NaO$ $(M+Na)^+$: 363.1468, found: 363.1461.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid naphthalen-2-ylamide (18b). Brown resin, 35%; ^{1}H-NMR (300 MHz) δ 8.08 (d, *J* = 8.4, 1H), 7.87 – 7.19 (m, 11H), 6.67 (d, *J* = 1.0 Hz, 1H), 2.74 (q, *J* = 7.4 Hz, 2H), 2.02 (d, *J* = 1.0 Hz, 3H), 1.02 (t, *J* = 7.4 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{24}H_{23}N_2O$ $(M+H)^+$: 355.1805, found: 355.1795.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid adamantan-1-ylamide (19a). Yellow resin, 81%; ^{1}H-NMR (300 MHz) δ 7.52 – 7.41 (m, 3H), 7.20 – 7.12 (m, 2H), 6.02 (d, *J* = 0.9 Hz, 1H), 2.29 (s, 3H), 2.13 (bs, 9H), 1.96 (bs, 3H), 1.72 (bs, 6H); ^{13}C NMR (75 MHz) δ 165.66, 133.79, 129.45, 128.52, 128.48, 128.40, 115.32, 104.58, 51.80, 42.23, 36.66, 29.73, 27.07, 12.83, 12.41; MS (HR-ESI) m/z calcd for $C_{23}H_{29}N_2O$ $(M+H)^+$: 349.2274, found: 349.2272.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid adamantan-1-ylamide (19b). White solid, 40%; m.p.: 143-144 ^{o}C; ^{1}H-NMR (300 MHz) δ 7.66 – 7.55 (m, 3H), 7.35 – 7.29 (m, 2H), 6.13 (d, *J* = 0.8 Hz, 1H), 2.87 (q, *J* = 7.4 Hz, 2H), 2.25 (bs, 9H), 2.07 (d, *J* = 0.7 Hz, 3H), 1.82 (bs, 6H), 1.11 (t, *J* = 7.4 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{24}H_{31}N_2O$ $(M+H)^+$: 363.2431, found: 363.2430.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid phenethyl-amide (20a). Yellow oil, 100%; ^{1}H NMR (300 MHz) δ 7.53 – 7.40 (m, 3H), 7.37 – 7.29 (m, 2H), 7.29 – 7.20 (m, 3H), 7.20 – 7.13 (m, 2H), 5.95 (s, 1H), 3.67 (t, *J* = 6.9 Hz, 2H), 2.92 (t, *J* = 6.9 Hz, 2H), 2.29 (s, 3H), 1.96 (s, 3H); MS (HR-ESI) m/z calcd for $C_{21}H_{22}KN_2O$ $(M+K)^+$: 357.1364, found: 357.1363.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid phenethyl-amide (20b). Beige solid, 42%; m.p.: 139-141 ^{o}C; ^{1}H NMR (300 MHz) δ 7.55 – 7.43 (m, 3H), 7.38 – 7.15 (m, 7H), 5.93 (s, 1H), 3.67 (t, *J* = 6.8 Hz, 2H), 2.91 (t, *J* = 6.8 Hz, 2H), 2.75 (q, *J* = 7.4 Hz, 2H), 1.92 (s, 3H), 0.97 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz) δ 165.78, 140.29, 139.57, 137.91, 129.38, 129.01, 128.73, 128.67, 128.58, 126.50, 113.53, 104.38, 40.51, 36.30, 19.16, 14.86, 12.78; MS (HR-ESI) m/z calcd for $C_{22}H_{24}N_2NaO$ $(M+Na)^+$: 355.1781, found: 355.1776.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid (1-phenyl-ethyl)-amide (21a). Beige crystalline solid, 50%; m.p.: 149-151 ^{o}C; ^{1}H NMR (300 MHz) δ 7.55 – 7.26 (m, 8H), 7.20 – 7.12

(m, 2H), 6.07 (s, 1H), 5.31 (m, 1H), 2.31 (s, 3H), 1.97 (s, 3H), 1.58 (d, *J* = 6.8 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{21}H_{23}N_2O$ $(M+H)^+$: 319.1805, found: 319.1795.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid (1-phenyl-ethyl)-amide (21b). Beige crystalline solid, 32%; m.p.: 168-170 ^oC; ^1H NMR (300 MHz) δ 7.54 – 7.44 (m, 3H), 7.43 – 7.30 (m, 4H), 7.29 – 7.16 (m, 3H), 6.07 (s, 1H), 5.38 – 5.26 (m, 1H), 2.85 – 2.68 (m, 2H), 1.94 (s, 3H), 1.57 (d, *J* = 6.9 Hz, 3H), 0.98 (t, *J* = 7.4 Hz, 3H); MS (HR-ESI) m/z calcd for $C_{22}H_{24}N_2NaO$ $(M+Na)^+$: 355.1781, found: 355.1785.

2,5-Dimethyl-1-phenyl-1H-pyrrole-3-carboxylic acid cyclohexylamide (22a). White solid, 87%; m.p.: 115-117 ^oC; ^1H NMR (300 MHz) δ 7.51 – 7.37 (m, 3H), 7.19 – 7.10 (m, 2H), 6.06 (d, J = 0.9 Hz, 1H), 4.01 – 3.86 (m, 1H), 2.30 (s, 3H), 2.07 – 1.96 (m, 2H), 1.96 (d, J = 0.9 Hz, 3H), 1.79 – 1.57 (m, 3H), 1.49 – 1.32 (m, 2H), 1.27 – 1.10 (m, 3H); ^{13}C NMR (75 MHz) δ 165.17, 137.88, 133.78, 129.38, 128.56, 128.45, 128.28, 114.49, 104.35, 47.77, 33.62, 26.97, 25.77, 25.11, 12.76, 12.26; MS (HR-ESI) m/z calcd for $C_{19}H_{24}N_2NaO$ $(M+Na)^+$: 319.1781, found: 319.1782.

2-Ethyl-5-methyl-1-phenyl-1H-pyrrole-3-carboxylic acid cyclohexylamide (22b). White crystalline solid, 45%; m.p.: 129-131 ^oC; ^1H NMR (300 MHz) δ 7.52 – 7.40 (m, 3H), 7.22 – 7.15 (m, 2H), 6.04 (d, *J* = 0.8 Hz, 1H), 5.63 (d, *J* = 6.9 Hz, 1H), 4.02 – 3.86 (m, 1H), 2.75 (q, *J* = 7.4 Hz, 2H), 2.07 – 1.96 (m, 2H), 1.94 (d, *J* = 0.8 Hz, 3H), 1.79 – 1.57 (m, 3H), 1.49 – 1.32 (m, 2H), 1.27 – 1.10 (m, 3H), 0.98 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz) δ 164.90, 140.17, 137.92, 129.35, 128.62, 128.57, 113.80, 104.32, 47.82, 33.69, 25.82, 25.17, 19.17, 14.82, 12.73; MS (HR-ESI) m/z calcd for $C_{20}H_{26}N_2NaO$ $(M+Na)^+$: 333.1937, found: 333.1934.

Competition Binding Assay. Membranes from HEK-293 cells over-expressing the respective human recombinant CB1 receptor (B_{max}= 2.5pmol/mg protein) and human recombinant CB2 receptor (B_{max}= 4.7pmol/mg protein) were incubated with [^{3}H]-CP-55,940 (0.14nM/K_d=0.18nM and 0.084nM/K_d=0.31nM, respectively for CB1 and CB2 receptor) as the high affinity ligand. Competition curves were performed by displacing [^{3}H]-CP-55,940 with increasing concentration of the newly synthesized compounds (0.1nM–10μM). Nonspecific binding was defined by 10μM of WIN55,212-2 as the heterologous competitor (Ki values 9.2nM and 2.1nM respectively for CB_1 and CB2 receptor). All compounds were tested following the procedure described by the manufacturer (Perkin Elmer, Italy). Displacement curves were generated by incubating drugs with [^{3}H]-CP-55,940 for 90 minutes at 30ºC. K_i values were calculated by applying the Cheng-Prusoff equation to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compound. Data represent mean values for at least three separate experiments performed in duplicate and are expressed as K_i (nM), average SEM < 10%.

Functional Activity at CB2 Receptors in Vitro. The cAMP Hunter™ assay enzyme fragment complementation chemiluminescent detection kit was used to characterize the functional activity in CB2 receptor-expressing cell lines. Gi-coupled cAMP modulation was measured following the manufacturer's protocol (DiscoveRx, Fremont, CA). Briefly, CHO-K1 cells overexpressing the human CB2 receptor were plated into a 96 well plate (30,000 cells/well), and incubated overnight at 37 °C, 5% CO2. Media was aspirated and replaced with 30μl of assay buffer. Cells were incubated 30 min at 37°C with 15μl of 3x dose-response solutions of samples prepared in presence of cell assay buffer containing a 3x of 25μM NKH-477 solution (a water soluble analogue of Forskolin) to stimulate adenylate cyclase and enhance basal cAMP levels. We

further investigated the effect upon receptor activation by testing compounds in the presence of JWH-133 selective agonist. Cells were pre-incubated with samples (15 min at 37°C at 6x the final desired concentration) followed by 30 min incubation with JWH-133 agonist challenge at the EC80 concentration (EC80=4µM, previously determined in separate experiments) in presence of NKH-477 to stimulate adenylate cyclase and enhance cAMP levels. For all protocols, following stimulation, cell lysis and cAMP detection were performed as per the manufacturer's protocol. Luminescence measurements were measured using a GloMax Multi Detection System (Promega, Italy). Data are reported as mean ± SEM of three independent experiments conducted in triplicate and were normalized considering the NKH-477 stimulus alone as 100% of the response. The percentage of response was calculated using the following formula: % RESPONSE = 100% x (1- (RLU of test sample - RLU of NKH-477 positive control) / (RLU of vehicle - RLU of NKH-477 positive control). When tested in presence of JWH-133, the percentage of response was calculated using the following formula: % RESPONSE = 100% x (RLU test sample - RLU of EC_{80} control) / (mean RLU NKH-477 positive control - RLU EC_{80} control). The data were analyzed using PRISM software (GraphPad Software Inc, San Diego, CA).

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version.

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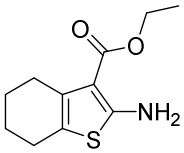
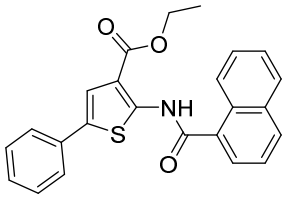
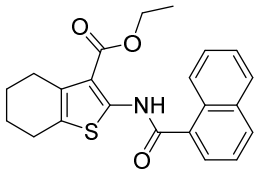
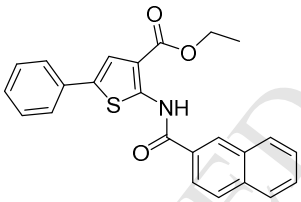
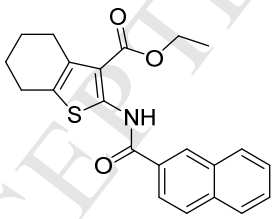
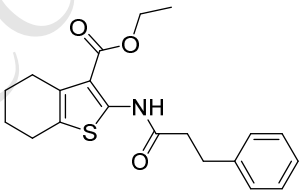
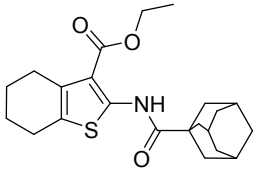
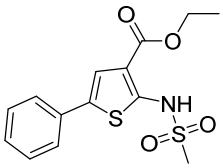
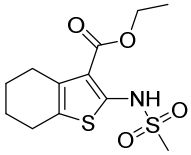
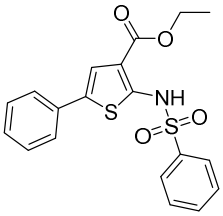
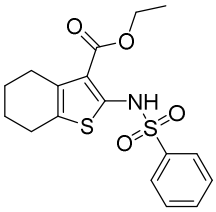
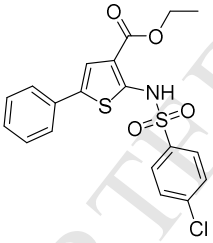
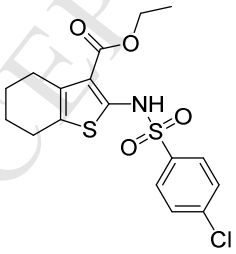
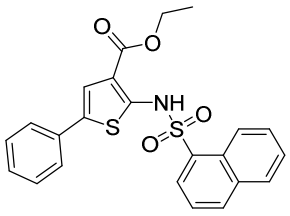
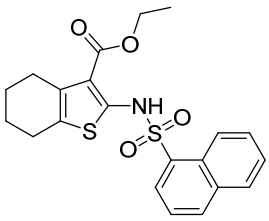
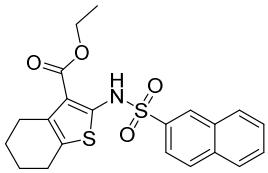
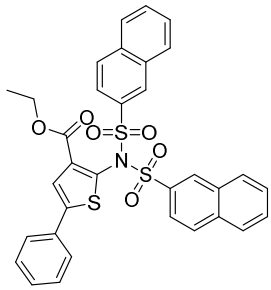
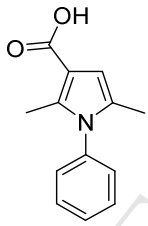
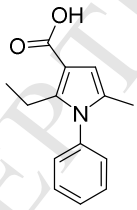
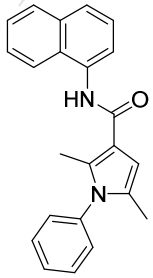
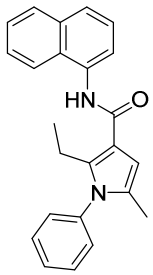
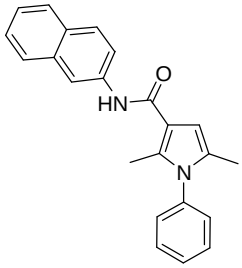
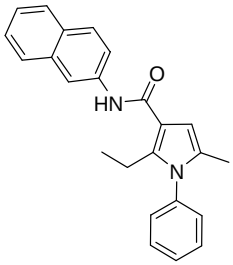
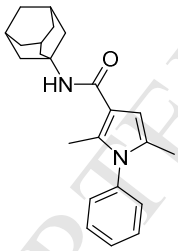
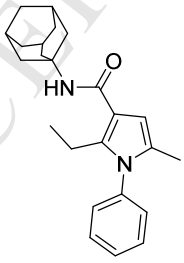
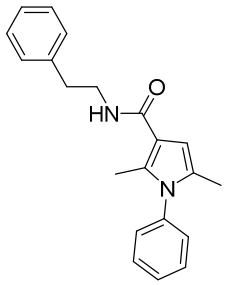
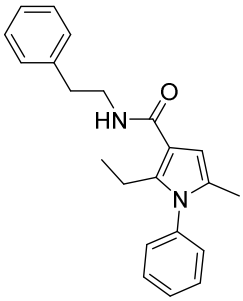
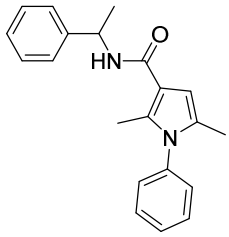
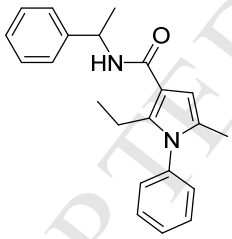
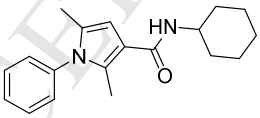
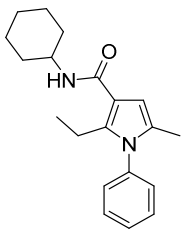
Table 1: CB1 and CB2 Receptor Affinity Values for the reference and synthesized compounds.

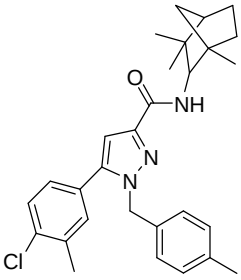
Cpd no	Structure	K_i on *h*CB1 (nM)	K_i on *h*CB_2 (nM)	Selectivity Index (K_i *h*CB_1/K_i *h*CB_2)
2b		>10000	>10000	–
3a		>10000	59.81	>167.20
3b		1373.03	16.80	81.73
4a		>10000	>10000	–
4b		>10000	1785.22	>5.60
5b		>10000	111.87	>89.39
6b		1008.48	2.15	469

7a		>10000	>10000	_
7b		>10000	>10000	_
8a		>10000	>10000	_
8b		>10000	1993.94	>5.02
9a		>10000	>10000	_
9b		>10000	>10000	_
10a		>10000	>10000	_

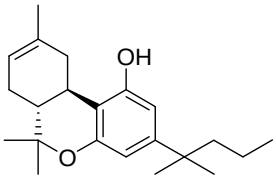
10b		>10000	800.00	>12.5
11b		>10000	>10000	–
12a		>10000	>10000	–
16a		>10000	>10000	–
16b		>10000	>10000	–
17a		317.03	84.22	3.76

17b		505.03	20.02	25
18a		281.06	58.28	4.82
18b		633.03	57.00	11.11
19a		518.20	7.59	68.27
19b		1196.70	6.15	194.59

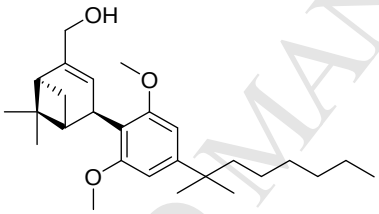
20a		5926.70	584.17	10.15
20b		>10000	718.20	>13.92
21a		2621.82	546.44	4.80
21b		3736.70	540.10	6.92
22a		>10000	167.81	>59.59
22b		4571.51	170.81	26.76

SR144528		116.79	1.82	64.17

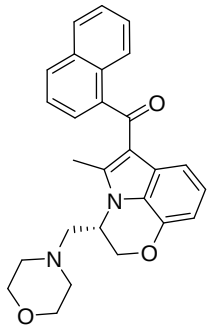
FIGURES



1, JWH-133



2, HU-308



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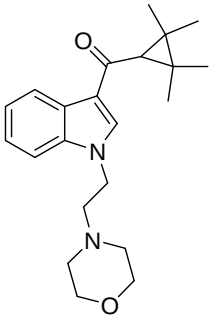

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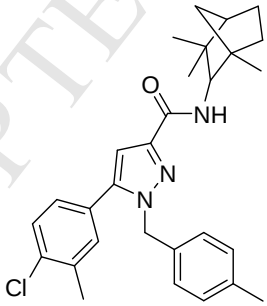

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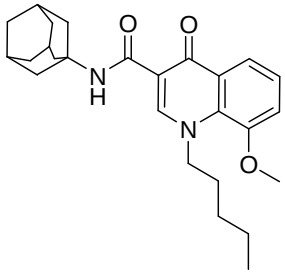


6

Figure 1: Structures of representative CB2 selective ligands.

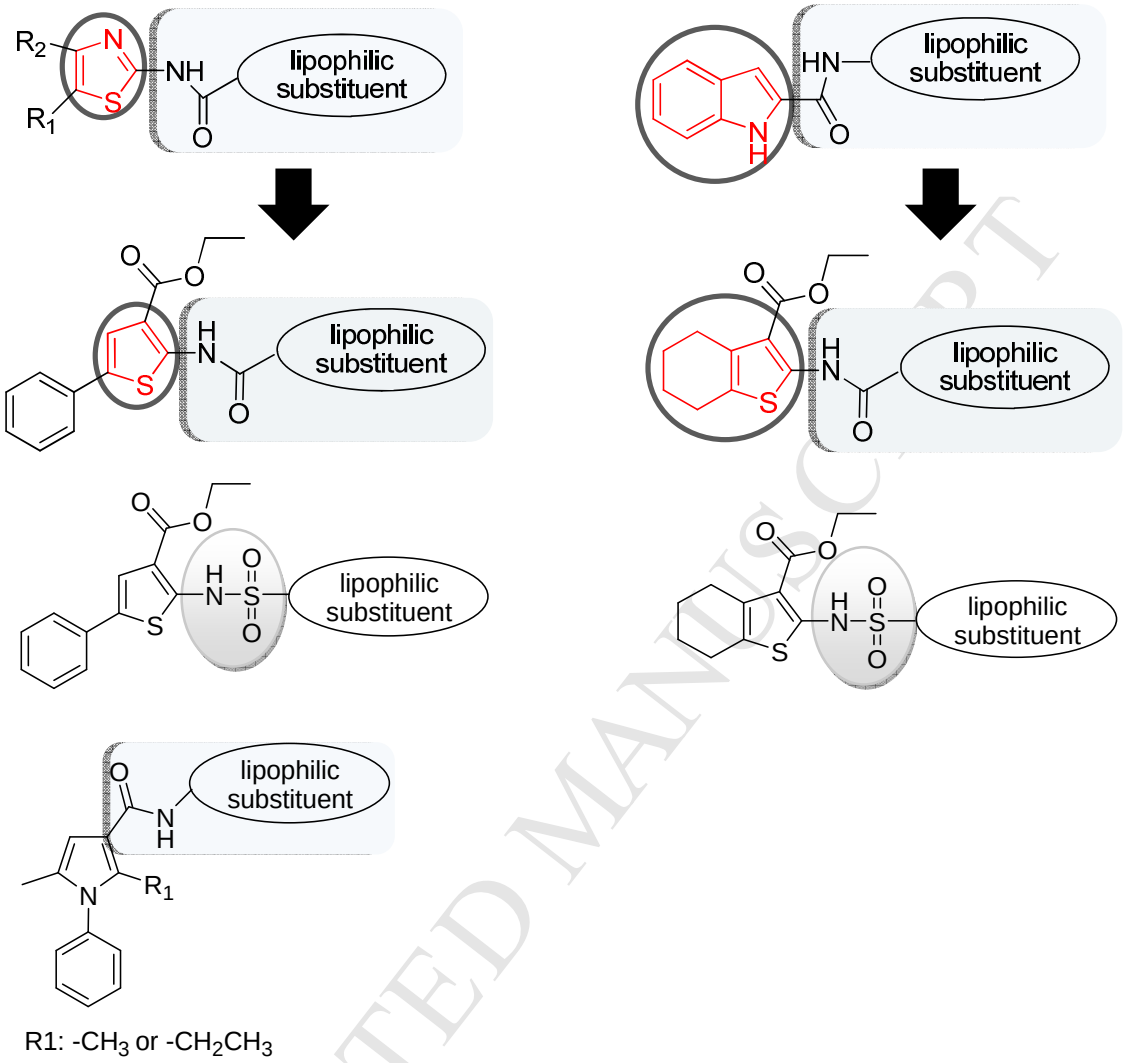


Figure 2: Design of thiophene and tetrahydrobenzo[b]thiophene cores (bottom) from 1,3-thiazole and indole scaffolds (top) and general structure of the compounds with a pyrrole-based scaffold.

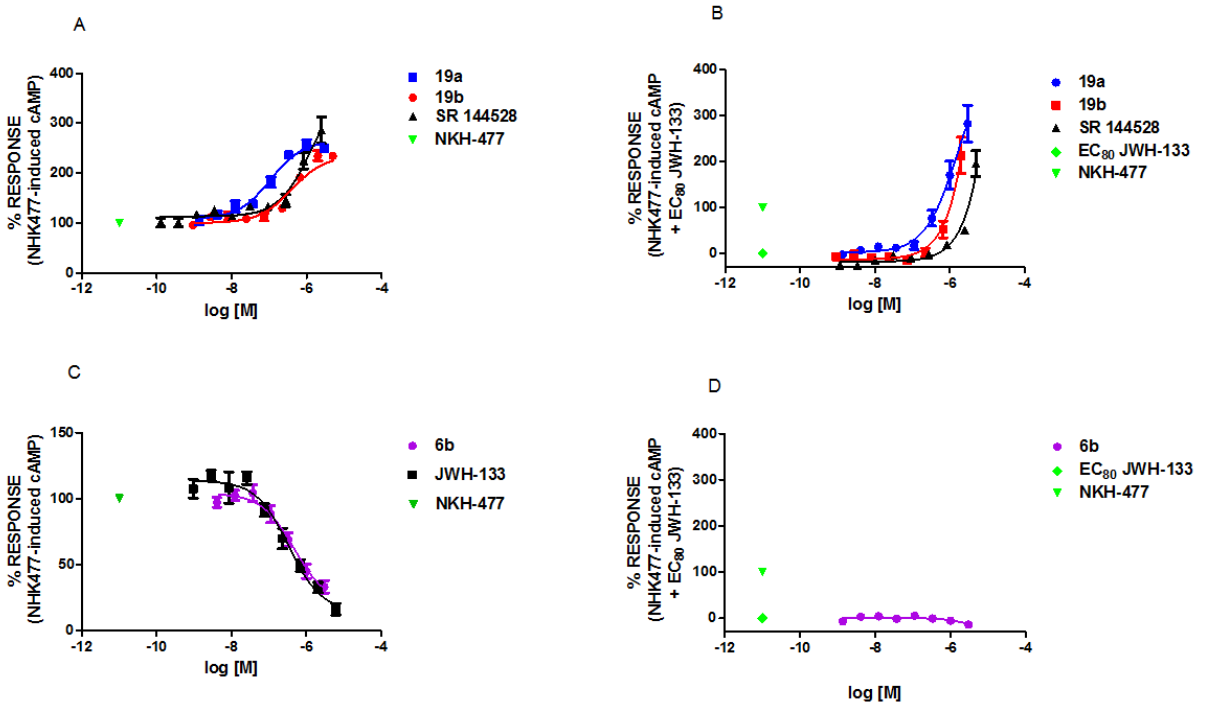
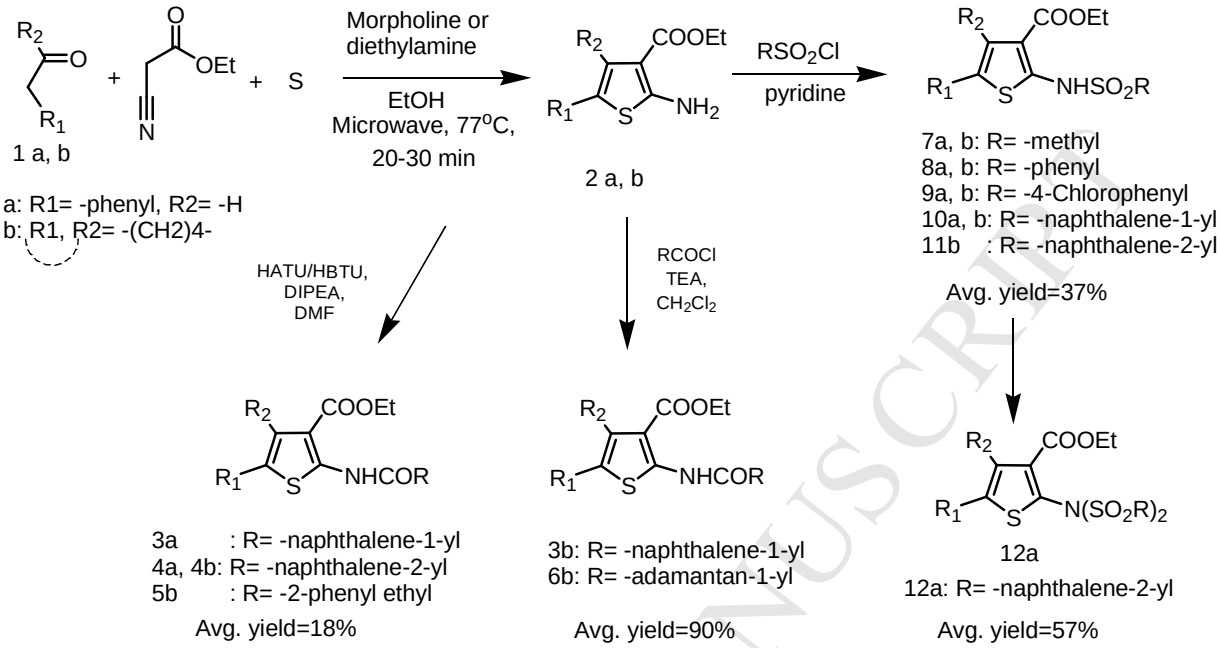


Figure 3: Concentration-response curves of compounds in cAMP-based functional assay. (A-C) The curves show the effect of increasing concentrations of compounds on NKH-477-induced cAMP levels in stable CHO cells expressing the human CB2 receptor. (B-D) Effect of compounds following the incubation with the JWH-133 agonist challenge at the EC80 concentration. Data were normalized to the maximal and minimal response observed respectively with NKH-477 alone and in presence of the EC80 concentration of ligand.

Scheme 1



1 a, b

a: R1= -phenyl, R2= -H
b: R1, R2= -(CH2)4-

2 a, b

7a, b: R= -methyl
8a, b: R= -phenyl
9a, b: R= -4-Chlorophenyl
10a, b: R= -naphthalene-1-yl
11b : R= -naphthalene-2-yl

Avg. yield=37%

3a : R= -naphthalene-1-yl
4a, 4b: R= -naphthalene-2-yl
5b : R= -2-phenyl ethyl

Avg. yield=18%

3b: R= -naphthalene-1-yl
6b: R= -adamantan-1-yl

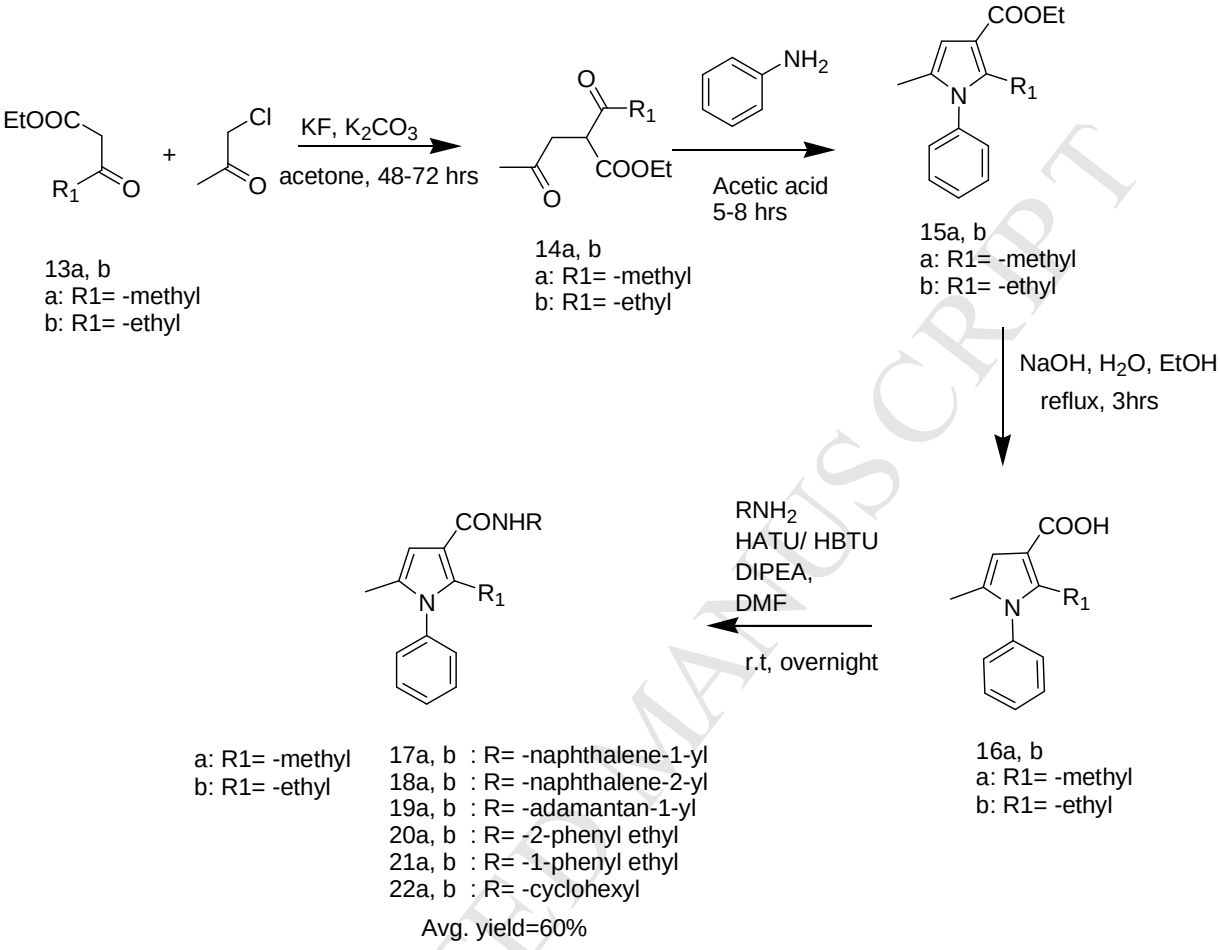
Avg. yield=90%

12a

12a: R= -naphthalene-2-yl

Avg. yield=57%

Scheme 2



13a, b
a: R1= -methyl
b: R1= -ethyl

14a, b
a: R1= -methyl
b: R1= -ethyl

15a, b
a: R1= -methyl
b: R1= -ethyl

16a, b
a: R1= -methyl
b: R1= -ethyl

a: R1= -methyl
b: R1= -ethyl

17a, b : R= -naphthalene-1-yl
18a, b : R= -naphthalene-2-yl
19a, b : R= -adamantan-1-yl
20a, b : R= -2-phenyl ethyl
21a, b : R= -1-phenyl ethyl
22a, b : R= -cyclohexyl

Avg. yield=60%

Highlights

- Novel Pyrrole and Tetrahydrobenzo[b]thiophene based scaffolds as CB2 selective ligands.
- Both series exhibited high CB2 binding affinity and CB2 subtype selectivity.
- Pyrrole based chemotypes act as inverse agonists.
- Tetrahydrobenzo[b]thiophene chemotypes act as full agonists.
- Structural elements controlling CB2 affinity, selectivity and functionality are provided.