



Synthesis of novel 2-(1-adamantanylcaboxamido)thiophene derivatives. Selective cannabinoid type 2 (CB2) receptor agonists as potential agents for the treatment of skin inflammatory disease

This is the peer reviewed version of the following article:

Original:

Mugnaini, C., Rabbito, A., Brizzi, A., Palombi, N., Petrosino, S., Verde, R., et al. (2019). Synthesis of novel 2-(1-adamantanylcaboxamido)thiophene derivatives. Selective cannabinoid type 2 (CB2) receptor agonists as potential agents for the treatment of skin inflammatory disease. EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, 161, 239-251 [10.1016/j.ejmech.2018.09.070].

Availability:

This version is available <http://hdl.handle.net/11365/1063401> since 2018-11-21T18:02:23Z

Published:

DOI:10.1016/j.ejmech.2018.09.070

Terms of use:

Open Access

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. Works made available under a Creative Commons license can be used according to the terms and conditions of said license.

For all terms of use and more information see the publisher's website.

(Article begins on next page)

Synthesis of Novel 2-(1-Adamantanylcarboxamido)thiophene Derivatives. Selective Cannabinoid Type 2 (CB2) Receptor Agonists as Potential Agents for the Treatment of Skin Inflammatory Disease

Claudia Mugnaini^{a,*}, Alessandro Rabbito^b, Antonella Brizzi^a, Nastasja Palombi^a, Stefania Petrosino^b, Roberta Verde^b, Vincenzo Di Marzo^b, Alessia Ligresti^{b,**}, Federico Corelli^a

^a*Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via Aldo Moro 2, 53100 Siena, Italy*

^b*Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via dei Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy*

Abbreviations: CB1R, cannabinoid type-1 receptor; CB2R, cannabinoid type-2 receptor; ClogP, calculated logarithm of the partition coefficient (P); CNS, central nervous system; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's Medium; ECS, endocannabinoid system; DNFB, 2,4-dinitrofluorobenzene; FCS, fetal calf serum; MCP-2, human monocyte chemotactic protein-2; PET, positron emission tomography; SAR, structure-activity relationship; SI, selectivity index; TPSA, topological polar surface area.

*Corresponding author. Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via Aldo Moro 2, 53100 Siena, Italy.

**Corresponding author. Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via dei Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy

E-mail addresses: claudia.mugnaini@unisi.it (C. Mugnaini), aligresti@icb.cnr.it (A. Ligresti).

ABSTRACT

A set of CB2R ligands, based on the thiophene scaffold, was synthesized and evaluated in in vitro assays. Compounds **8c-i**, **k**, **l**, bearing the 3-carboxylate and 2-(adamantan-1-yl)carboxamido groups together with apolar alkyl/aryl substituents at 5-position or at 4- and 5-positions of the thiophene ring possess high CB2R affinity at low nanomolar concentration, good receptor selectivity, and agonistic functional activity. The full agonist **8g**, showing the best balance between receptor affinity and selectivity, was tested in vitro in an experimental model of allergic contact dermatitis and proved to be able to block the release of MCP-2 in HaCaT cells at 10 μ M concentration.

Keywords:

CBR ligands

CB2R agonists

Skin inflammation

Chemokine release

1. Introduction

In 1988, twenty-four years after the isolation of Δ^9 -THC [1], the main active ingredient of *Cannabis*, type-1 cannabinoid receptor (CB1R) was discovered in the brain where it regulates the release of a number of neurotransmitters controlling emotions, pain, movement, memory, food intake [2]. In 1993, a second receptor was identified, predominantly expressed at a peripheral level, in immune cells, gastrointestinal tract, liver, heart, kidney, bones [3]. More recently, it was demonstrated that type-2 cannabinoid receptor (CB2R) is also expressed in the CNS during inflammatory processes [4]. Both CBRs share an overall homology of 44%, but the similarity in the 7-transmembrane region, where the ligand-binding domain has been identified, reaches 68%.

In the same years, the first endogenous ligands of CBRs, namely anandamide [5] and 2-arachidonoylglycerol (2-AG) [6] were also discovered. Since then, new ligands have been identified able to modulate CBRs together with the enzymes responsible for their synthesis and degradation, making the complex picture of endocannabinoid system (ECS) increasingly exhaustive [7]. Due to their wide distribution, CBRs are implicated in a number of physiological and pathological conditions both centrally and peripherally [8], and hence they are considered interesting targets for the design of drug candidates potentially useful for the treatment of disorders such as chronic inflammation [9], pain [10], multiple sclerosis [11], cancer [12], and addiction [13].

Despite the initial controversy on the presence of functional cannabinoid CB2R in the CNS, it is now well accepted that this receptor is present in limited amounts and distinct locations in the brain of several animal species, including humans [14]. The inducible nature of these receptors under neuroinflammatory conditions and the lack of the undesired psychoactive effects make them attractive targets for the development of novel therapeutic approaches for the treatment of various pathological conditions, such as neurodegenerative disorders, chronic and inflammatory pain, ischemic stroke, diabetic neuropathy and nephropathy, liver cirrhosis, pruritus, osteoporosis, and cancers [15]. Of current interest are also skin inflammatory diseases, such as allergic contact dermatitis, that affect a significant percentage of population, and mainly women, in industrialized

countries, where are among the main causes of occupational diseases. Notably, the CB2R agonist S-777469 has been advanced into a phase II clinical trial to assess its efficacy in subjects with mild to moderate atopic dermatitis [16].

Because of the high homology between the ligand-binding domains of the two receptors and the overall higher tissue expression of CB1R, the development of selective CB2R ligands, devoid of the centrally CB1R-mediated psychotropic effects, is a challenging task [17]. Despite the plethora of structurally diverse, selective CB2R agonists and inverse agonists unveiled so far and the progress of some of them into clinical trials [18], none of these bioactive compounds has been further developed into a successful clinical drug. Although it is unclear why some of the candidates have failed, generally the main reasons for failure can be found in low receptor selectivity, poor in vitro DMPK and pharmacokinetics, off-target activity, deficiencies in the predictive utility of the preclinical models as well as in the understanding of the molecular mode of action of CB2R agonists [17].

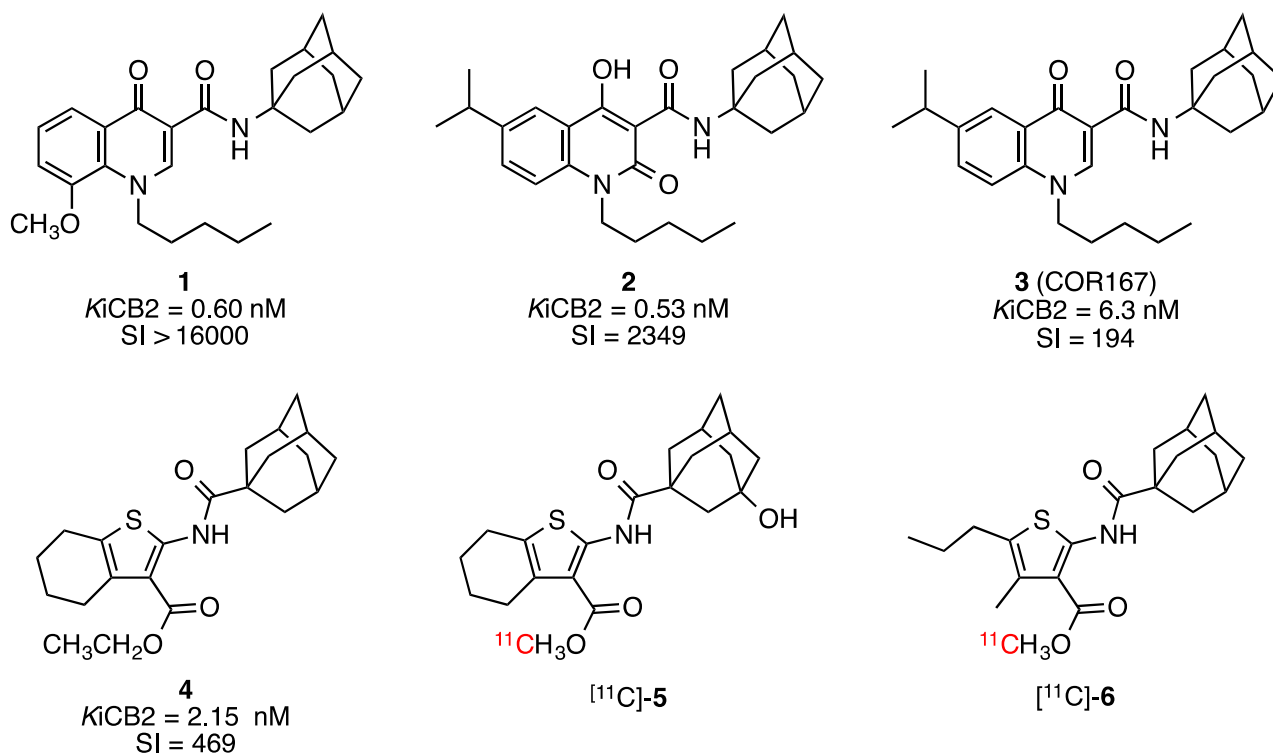


Fig. 1. Representative examples of quinolones and thiophene derivatives previously developed

Intensive research efforts have been devoted in our laboratories to the design and synthesis of new compounds showing high CB2R affinity and selectivity over the CB1R subtype. In particular, within a significantly large set of 4-quinolone-3-carboxamides [19a,b] and 4-hydroxy-2-quinolone-3-carboxamides [19c] described in the last years, a number of compounds proved to possess a remarkable in vitro profile, with binding constant (K_i) values at CB2R usually in the low nanomolar range and selectivity indexes ($SI = K_{iCB1}/K_{iCB2}$) even $>2,000$, as exemplified by compounds **1** [19b] and **2** [19c] (Fig. 1). The 6-isopropyl analog **3** (usually referred to as COR167) [19a] was further characterized in in vitro, ex vivo, and in vivo assays, where it elicited: a) potent immunomodulatory activity on immune cells from healthy subjects and patients with multiple sclerosis [20]; b) protective effects on rat brain tissues toward ischemia and reperfusion-induced injury [21]; c) anti-nociceptive properties [22], thereby validating the CB2R as a therapeutic target and becoming a useful pharmacological tool for further investigations.

More recently, we engaged also in the evaluation of alternative scaffolds to develop new CB2R selective ligands. Among the diverse building blocks that are of more prospective use in medicinal chemistry, the 2-aminothiophene scaffolds, widely represented in several classes of compounds possessing various biological activities [23a-c], including CB2R agonism [23d,e], have kindled our attention [24-26]. In 2016, Oman et al. reported the synthesis of pyrrole- and tetrahydrobenzo[b]thiophene-carboxamides as CB2R ligands [27]. While arylamides and sulfonamides showed modest or no CB2R affinity, the amides and retroamides bearing the adamantan-1-yl moiety elicited high affinity and selectivity. In particular, compound **4** (Fig. 1) exhibited the highest CB2R affinity ($K_i = 2.15$ nM), as well as the highest CB2R/CB1R subtype selectivity ($SI = 469$). In addition, due to the current interest in CB2R as potential indicator of neurodegeneration [28], recently two thiophene-based radioligands [^{11}C]-**5** and [^{11}C]-**6** were developed as potential positron emission tomography (PET) tracers to visualize CB2R in the brain [29].

Given the promising findings obtained with compound **4** and bearing in mind the remarkably

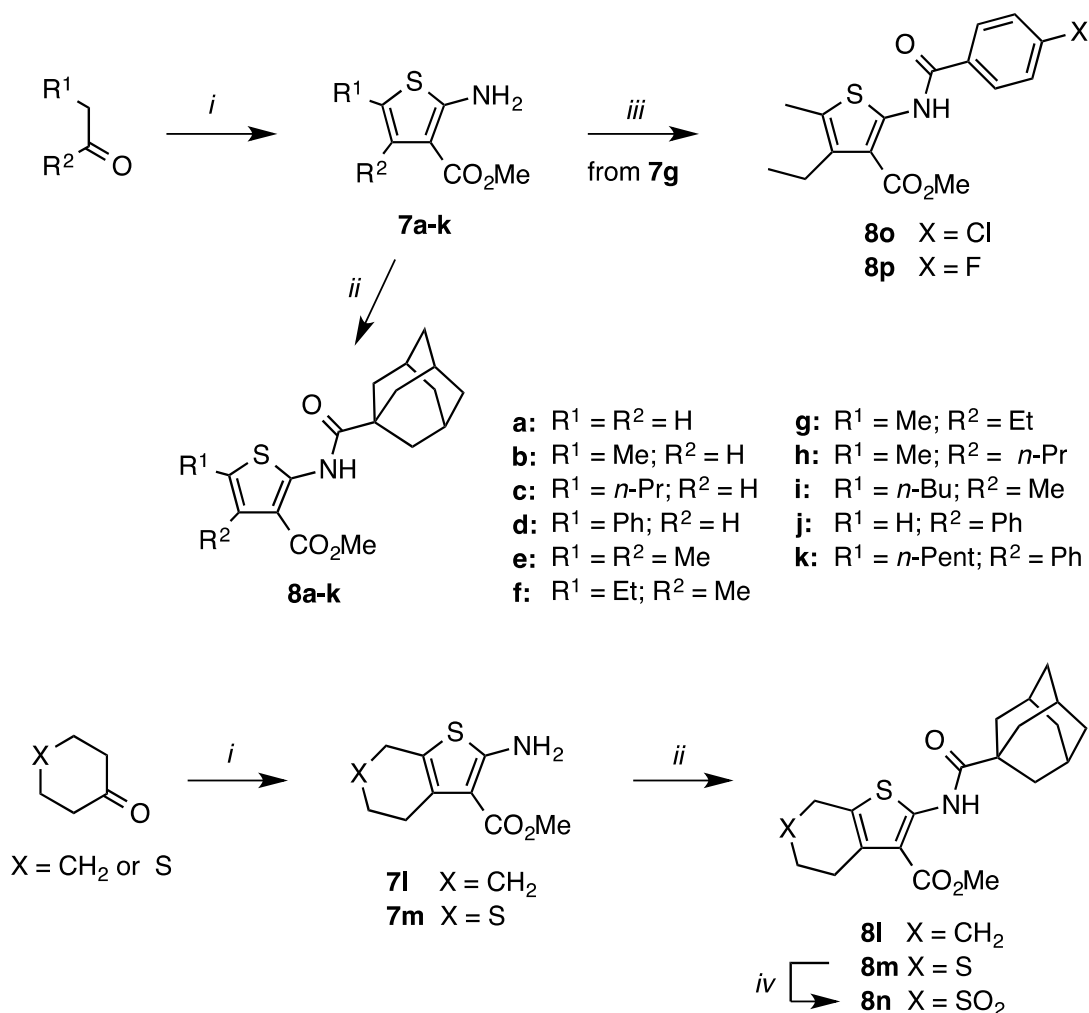
positive effect that the adamantyl group can exert in terms of both CBRs affinity and selectivity [19,23d,23e,27], we embarked on the synthesis and biological evaluation of a family of amidothiophene derivatives characterized by the adamantan-1-carbonyl moiety. Furthermore, also the methyl ester group was retained in all of the compounds as it had previously proven to be stable toward esterases [26] and to confer comparable CB2R affinity as well as lower lipophilicity than corresponding amides, such as the *n*-propylamide [23d]. Major aim was to evaluate in vitro the biological profile of the new compounds and possibly to establish their structure-activity relationship (SAR). In addition, we decided also to test the effects on biological activity of the isosteric replacement of the thiophene ring with other heterocyclic scaffolds as well as the modification of the amide functionality into a urea or carbamate group to modulate lipophilicity and introduce further H-bond donor/acceptor groups.

2. Results and discussion

2.1. Chemistry

Among the aminothiophene derivatives **7** (Scheme 1) used as starting substrates, compound **7a** was commercially available, while the analogs **7b-k** were prepared in-house according to the Gewald procedure, as previously reported for the synthesis of **7g** [26]. Condensation of methyl cyanoacetate in the presence of sulfur and morpholine with the appropriate aldehydes or ketones yielded intermediates **7b-d** and **7e-k**, respectively. Similarly, Gewald reaction between methyl cyanoacetate and cyclohexanone or tetrahydro-4*H*-thiopyran-4-one afforded the bicyclic aminothiophene intermediates **7l** [26] and **7m**, respectively. Subsequent acylation of **7a-m** with adamantan-1-carbonyl chloride was best performed in refluxing 1,4-dioxane as solvent in the absence of any added base. Under these conditions, the final amidothiophene derivatives **8a-m** were obtained in 13-95% yield by avoiding a possible bis-acylation of the amino group of **7**, as observed previously when this reaction was carried out in the presence of a base at room temperature [25,26]. Compound **8m** was converted into the corresponding sulfone derivative **8n** by oxidation with

Oxone®. In addition, two more compounds **8o** [26] and **8p** were prepared by acylation of **7g** with 4-chlorobenzoyl chloride or 4-fluorobenzoyl chloride, respectively, to compare their biological activity to that of **8g**.

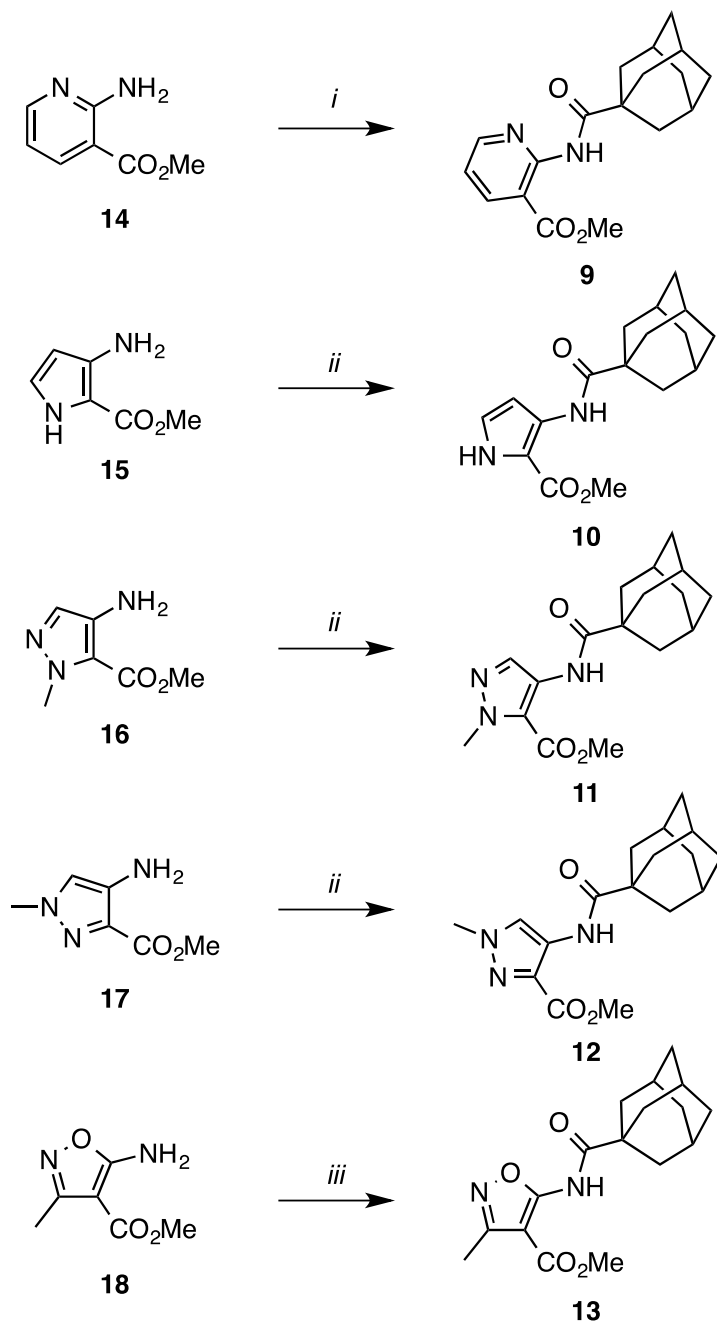


Scheme 1. Synthesis of 2-(1-adamantylcarboxamido)thiophene derivatives.^a

^aReagents and conditions: (i) methyl cyanoacetate, S_8 , morpholine, MeOH, reflux, overnight; (ii) adamantane-1-carbonyl chloride, 1,4-dioxane, 100 °C, 1-2 h; (iii) 4-chlorobenzoyl chloride or 4-fluorobenzoyl chloride, 1,4-dioxane, 100 °C, 1-2 h; (iv) Oxone®, MeOH, H_2O , rt, overnight.

According to the scaffold hopping approach [30], although not exhaustively applied, the thiophene nucleus was replaced with some other heterocycles able to provide final compounds with different electronic properties but basically similar steric characteristics. As starting substrates for the preparation of compounds **9-13** (Scheme 2), we used heterocyclic amino esters commercially available, such as methyl 2-aminopyridine-3-carboxylate (**14**), or synthesized following published

procedures, as for methyl 3-amino-1*H*-pyrrole-2-carboxylate (**15**) [31], methyl 4-amino-1-methyl-1*H*-pyrazole-5-carboxylate (**16**), and the isomeric methyl 4-amino-1-methyl-1*H*-pyrazole-3-carboxylate (**17**) [32].



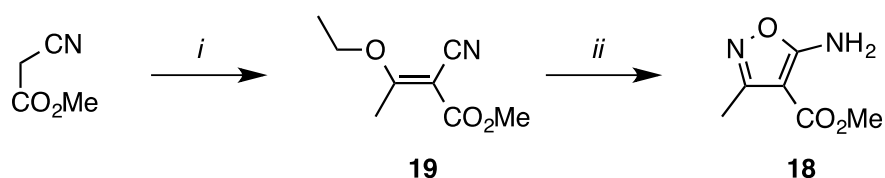
Scheme 2. Synthesis of isosteric compounds **9-13**.^a

^aReagents and conditions: (i) adamantane-1-carbonyl chloride, TEA, DCM, 0 °C to rt, 18 h; (ii) adamantane-1-carbonyl chloride, 1,4-dioxane, 100 °C, 18 h; (iii) adamantane-1-carbonyl chloride, NaH, dry THF, rt to reflux, 2 h.

For the synthesis of methyl 5-amino-3-methyl-4-isoxazolecarboxylate (**18**), we adopted a reaction

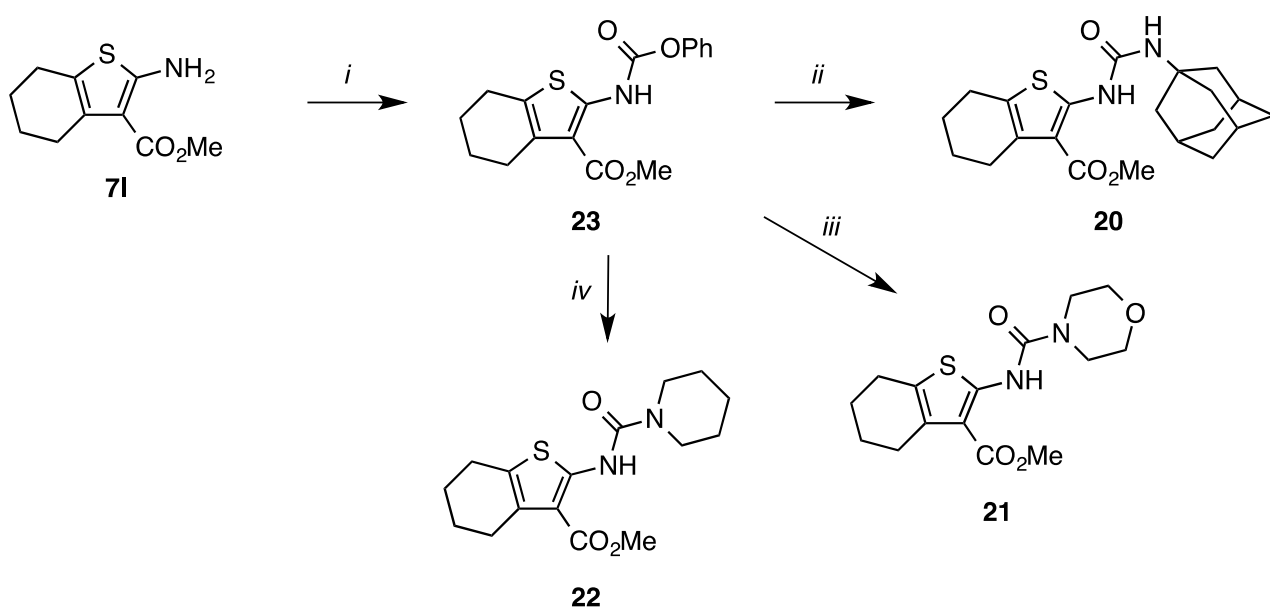
sequence (Scheme 3) slightly modified compared to those described in the only two literature reports concerning its preparation [33,34], and entailing the reaction of methyl cyanoacetate with triethyl orthoacetate to give methyl 2-cyano-3-ethoxy-2-butenoate (**19**) followed by cyclocondensation with hydroxylamine. Precursors **14-18** were then converted into the corresponding adamantanecarboxamides **9-13** under appropriate acylation conditions.

Finally, compounds **20-22** (Scheme 4), where the amide functionality is replaced by urea moieties, were also synthesized in 50-87% yield by reaction of 1-aminoadamantane, morpholine, and piperidine with phenyl carbamate **23**, in turn obtained by acylation of **71** with phenyl chloroformate.



Scheme 3. Synthesis of methyl 5-amino-3-methylisoxazole-4-carboxylate.^a

^aReagents and conditions: (i) triethyl orthoacetate, Ac₂O, reflux, 3 h; (ii) hydroxylamine hydrochloride, dry pyridine, reflux, 4 h.



Scheme 4. Synthesis of ureido derivatives **20-22**.^a

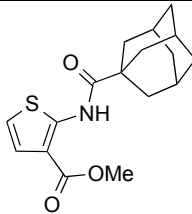
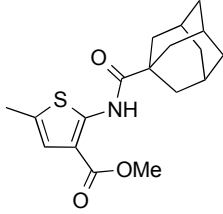
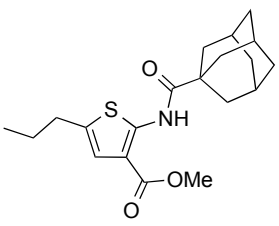
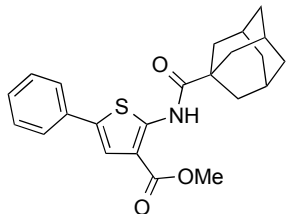
^aReagents and conditions: (i) phenyl chloroformate, 1,4-dioxane, 100 °C, overnight; (ii) 1-aminoadamantane, 1,4-dioxane, 100 °C, overnight; (iii) morpholine, 1,4-dioxane, 100 °C, 5 h; (iv)

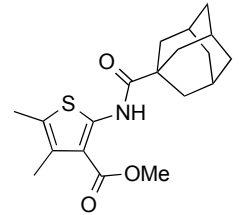
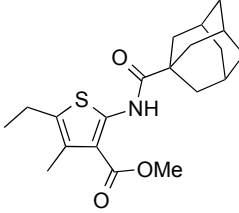
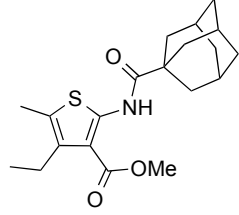
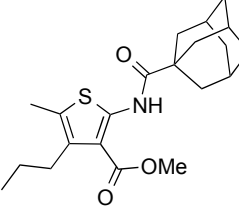
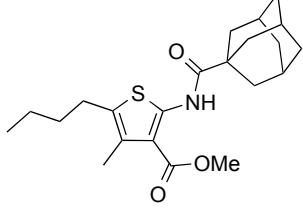
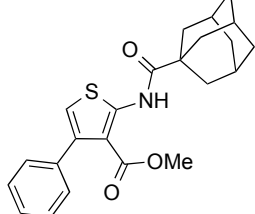
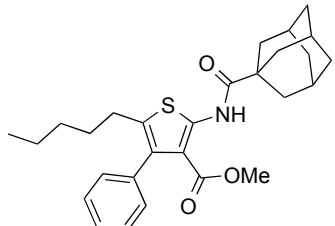
piperidine, 1,4-dioxane, 100 °C, 1 h.

2.2. Binding affinity for cannabinoid receptors and SAR

The binding affinities (K_i values) of compounds **8a-p**, **9-13**, and **20-24** for human recombinant CB1R and CB2R are reported in Table 1. The tested compounds were evaluated in parallel with **24** (SR144528) [35a] and **25** (AM630) [35b] as reference CB2R ligands.

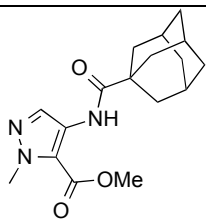
Table 1.
CB1R and CB2R affinity values for compounds **8a-p**, **9-13**, and **20-24**.^{a,b}

Compd	Structure	CB1R ^c	CB2R ^d	SI ^e
		K_i^f (nM)	K_i^f (nM)	
8a		>10,000	1324.12 ± 283.34	>68
8b		>10,000	23.09 ± 2.93	>433
8c		1339.29 ± 230.43	7.21 ± 0.77	186
8d		>10,000	6.48 ± 1.88	>1540

8e		>10,000	9.24 ± 1.45	>1080
8f		2120 ± 110	5.92 ± 0.21	358
8g		185.14 ± 31.49	2.46 ± 2.08	75
8h		1100 ± 120	4.28 ± 0.65	257
8i		58.89 ± 6.03	1.72 ± 0.14	34
8j		>10,000	35.00 ± 1.95	>285
8k		518.57 ± 165.43	8.03 ± 0.05	65

8l		>10,000	5.08 ± 0.03	>1968
8m		>10,000	50.70 ± 13.62	>197
8n		>10,000	1673.74 ± 312.44	>6
8o		>10,000	621.00 ± 150.85	>16
8p		>10,000	495.77 ± 208.59	>20
9		>10,000	1220.19 ± 281.21	>8
10		>10,000	2141.79 ± 167.09	>4

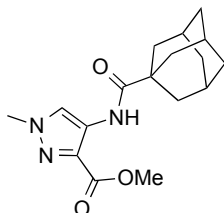
11



>10,000

 1081.88 ± 232.16 >9

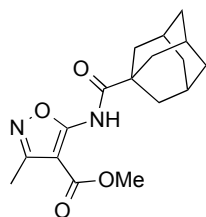
12



>10,000

 845.42 ± 34.25 >11

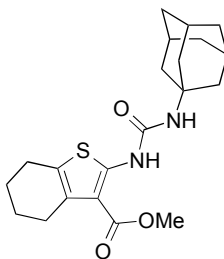
13



>10,000

 64.72 ± 3.93 >154

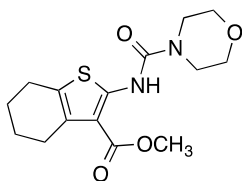
20



>10,000

 20.97 ± 1.81 >477

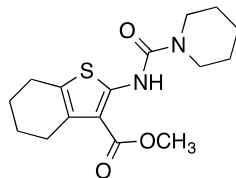
21



>10,000

 2494.08 ± 32.50 >4

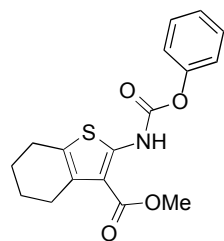
22



>10,000

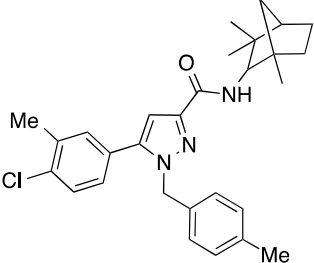
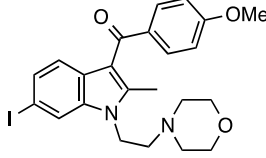
 195.70 ± 43.70 >51

23



>10,000

 244.06 ± 49.88 >41

24 (SR144528) ^{g,h}		116.8	1.8	64.8
25 (AM630) ^{g,h}		5152	31.2	165

^aData represent mean values for at least three separate experiments performed in duplicate and are expressed as K_i (nM). ^bFor both receptor binding assays, the new compounds were tested using membranes from HEK cells transfected with either the CB1R or CB2R and [³H]-(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol ([³H]CP-55,940). ^cCB1R: human cannabinoid type 1 receptor. ^dCB2R: human cannabinoid type 2 receptor. ^eSI: selectivity index for CB2R, calculated as $K_i(\text{CB1R})/K_i(\text{CB2R})$ ratio. ^f K_i : “Equilibrium dissociation constant”, that is, the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors. ^gCB2 reference compound. ^hThe binding affinities of reference compound were evaluated in parallel with test compounds under the same conditions.

As to the thiophene derivatives **8a-p**, they showed higher affinity for CB2R than CB1R, with $K_i(\text{CB2})$ values spanning three orders of magnitude (from 1.72 to 1673 nM), while $K_i(\text{CB1})$ is mostly >1,000 nM, excepted compounds **8g**, **8i**, and **8k** which exhibited affinity binding to CB1R of 185.14, 58.89, and 518.57 nM, respectively. In particular, nine compounds (**8c-i** and **8k,l**) out of sixteen are the most potent CB2R ligands [$K_i(\text{CB2}) < 10$ nM] showing comparable affinity to the reference compound SR144528, and three compounds (**8b**, **8j**, and **8m**) show noticeable CB2R affinity ranging between 23.09 and 50.70 nM. As a result of the capacity of compounds **8a-p** to

bind preferentially to CB2R, SI values range from 34 to approximately 2,000, with SI >1,000 for compounds **8d**, **8e**, and **8i**, which therefore can be considered the most selective CB2R ligands.

Compound **8a**, the prototypical element in the series **8a-p**, was only marginally active, but its conversion into the corresponding 5-methyl analog **8b** resulted in a 50-fold boost of potency, an outcome that traces back to the so-called “magic methyl” effect [36]. Although the important role played by methyl groups in biologically active small molecules is well documented [37], a survey of the medicinal chemistry literature performed by Jorgensen and co-workers has suggested that the introduction of a methyl group in a lead molecule could give rise to a 10-fold increase in activity in 8% of the time, while 100-fold gain in potency is far less likely, occurring with a 0.4% frequency [38]. Thus, the transition from **8a** to **8b** features an extreme example of boosting effect of the methyl substituent, that cannot be ascribed merely to the slight increase in lipophilicity or to favourable conformational changes, but might be sooner attributed to the ability of **8b** to place its methyl nicely into the receptor binding site, so as to establish profitable hydrophobic interactions. This hypothesis seems to be corroborated by replacing the methyl group of **8b** with the *n*-propyl chain or the phenyl ring to produce compounds **8c** and **8d**, respectively, which gained a further 3-fold increase in potency. The progression from **8a** to **8b**, to **8c**, and to **8d** maintained receptor selectivity, probably because the increasingly larger and more hydrophobic substituents at position 5 of the thiophene ring well sit within the CB2R pocket, but are unfavourable or uninfluential for interaction with CB1R. The shift of the phenyl substituent from the 5 (compound **8d**) to the 4 position (compound **8j**) on thiophene did not invert receptor selectivity, despite CB2R affinity was decreased by five times. 4,5-Disubstituted thiophene derivatives generally displayed better CB2R affinity compared to the monosubstituted thiophene derivatives, although with some loss of receptor selectivity for compounds **8g**, **8i**, and **8k**. Although the CB1R affinity of these three compounds cannot be definitely rationalized, we can envisage that the lengthening of the 5-chain to four/five carbon atoms (**8i** and **8k**) or the homologation of the methyl into an ethyl group at 4 position (**8g**) have an influence on selectivity by conferring higher CB1R affinity. On the other hand, further

homologation of the 4-ethyl group of **8g** into a 4-*n*-propyl chain (**8h**) led to a more selective CB2R ligand, though with a modest decrease in potency. Compound **8l**, featuring the annulation product of **8a** with cyclohexane, showed a boost in potency of 260 times with respect to the prototype. Interestingly, the binding profile of **8l** is much more similar to that of **8f** than **8g**, with the complete loss of affinity for CB1R ($K_i > 10,000$ nM). It is not easy to explain why the replacement of a methylene group with a sulfur atom (from **8l** to **8m**), that resulted in a slight decrease of ClogP (from 4.77 to 4.30) and no change in TPSA [39], was able to cause a 10-fold decrease in CB2R affinity. The same result was obtained by Nelson *et al.* when in a compound similar to **8l** a methylene group was replaced by an oxygen atom [23d], which however cause a more pronounced decrease of ClogP and a slight increase of TPSA compared to a sulfur atom. It is worth mentioning that **8l** showed an increase in selectivity, accompanied by reduced affinity, compared to compound **4**, differing only in the ester functionality. As expected, the oxidation of **8m** to the corresponding sulfone **8n** produced a basically inactive compound, probably because it possesses a polar group that would project inside a very hydrophobic pocket of the receptor. Finally, the biological evaluation of compounds **8o** and **8p**, bearing a 4-substituted phenyl ring instead of the adamantyl group, once more confirmed the positive impact of the adamantylamide on CB2R binding [19].

The isosteric analogs **9-12** elicited no affinity for CB1R, but also low binding ability to CB2R with K_i approximately around 1,000 nM. This finding seems to be due to the polarity of these compounds, that show TPSA values higher than that of thiophene derivatives. However, the isoxazole derivative **13**, though even more polar compared to the other isosteres **9-12**, is a selective CB2R ligand with significant affinity of 64.72 nM. At moment, we are not able to make any plausible conjecture to explain the discrepant behaviour of **13** with respect to compounds **9-12**.

Within the other subset of carbamate and ureido analogs **20-23**, while compounds **21-23** only elicited moderate affinity for CB2R, compound **20** still represents a potent and selective CB2R ligand, with an SI value > 477 and only 4-fold decreased CB2R affinity compared to **8l**.

It is interesting to compare the biological profile of the new thiophene compounds with that of

quinolones **1-3** that we have previously investigated. Thus, while quinolones **1** and **2** appear to be superior in terms of both affinity and selectivity, most of the new compounds compare well with quinolone **3** and exhibit in some cases better affinity or selectivity. Finally, thiophene derivatives have the advantage over quinolones of being attainable through a simple two-step synthesis rather than a more demanding preparation.

In vitro CB2R functional activity

The most potent compounds **8e**, **8f**, **8g**, **8i** and **8l** were subjected to further in vitro pharmacological evaluation in order to assess their capability of activating CB2R. We performed the cAMP Hunter™ assay enzyme fragment complementation chemiluminescent detection kit to measure whether compounds modulate intracellular cAMP levels in NKH-477-stimulated CHO cells overexpressing CB2R.

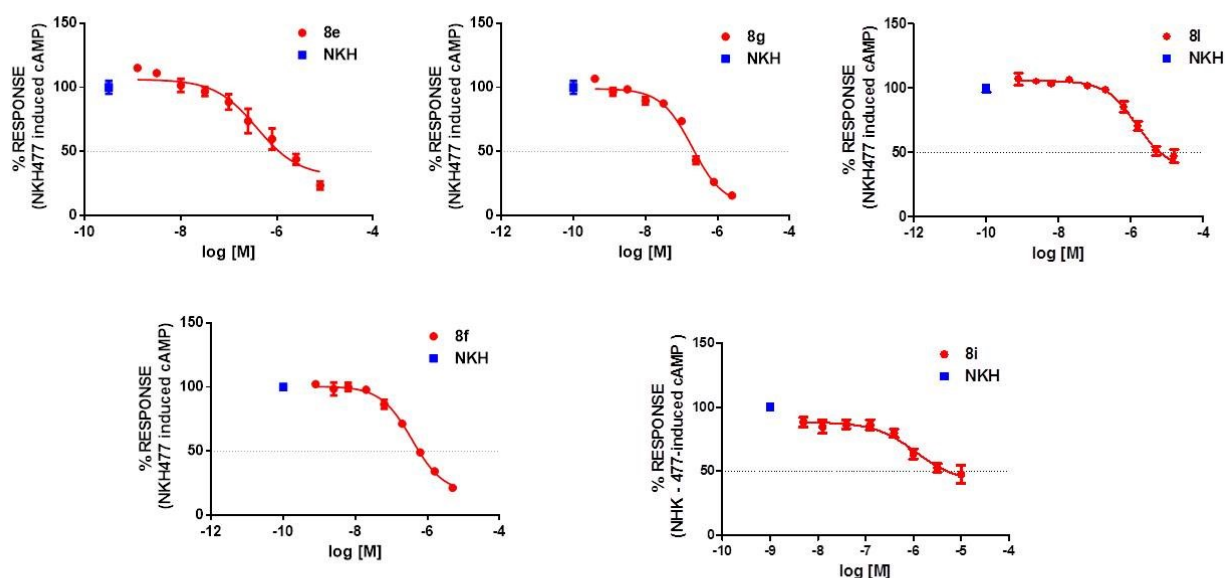


Fig. 2. Concentration-response curves of compounds in cAMP Hunter™ assay enzyme fragment complementation chemiluminescent detection kit.

As shown in Fig. 2, all compounds displayed a typical agonist behavior by reducing cAMP levels induced by the NKH-477 (a water-soluble analog of forskolin) as expected for an orthosteric G_i agonist. However, we noticed that the nature of substituent on the position 4 and 5 of the thiophene ring influenced the compounds activity, being the compound **8g** the most active full agonist (EC₅₀ = 197 nM) followed by compounds **8e** and **8f** (EC₅₀ 318 and 371 nM, respectively). The elongation of the acyl chain in position 5 or the insertion of the cyclohexyl moiety were instead detrimental for functional activity, as compound **8i** and **8l** acted as partial agonists (EC₅₀ 1135 and 1531 nM, respectively) since they were not able to decrease cAMP content under basal conditions not even at the highest concentrations tested (Fig. 2). Consistently, all compounds showed no activity in presence of an EC₈₀ CB2-ligand challenge (3 μM of JWH-133, not shown).

The curves show the effect of increasing concentrations of compounds on NKH-477-induced cAMP levels in stable CHO cells expressing the human CB2R. Data were normalized to the maximal and minimal response observed. 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 as indicated in the experimental section.

Effect on MCP-2 chemokine release in polyinosinic polycytidylic acid [poly-(i:c)]-stimulated human keratinocyte (HaCaT) cells

Endocannabinoids and their receptors constitute part of an adaptive system that also regulates cutaneous inflammation. Cannabinoids exert their anti-inflammatory effects in skin via their actions on keratinocyte cytokine production or their capability of modulating immune cells [40]. Pharmacological inhibition of CBR augments 2,4-dinitrofluorobenzene (DNFB)-induced dermatitis in mice [41], suggesting a potential therapeutic effect for CB2R agonists in skin inflammation disease. For this reason we tested the effect of the most active full agonist compound of the series on the Poly-(I:C)-induced release of human monocyte chemotactic protein-2 (MCP-2) in HaCaT cells, a widely used in vitro model of allergic contact dermatitis that highly expresses CB2R. Upon

a 100 $\mu\text{g}/\text{mL}$ stimulus of Poly-(I:C), we found a significant increase of MCP-2 release ($p < 0.001$) that was completely abolished ($p < 0.001$) in presence of 10 μM of compound **8g** (Fig. 3). Co-incubation with the CB2R antagonist AM630 (0.1 μM) did not affect per se the release of pro-inflammatory cytokine MCP-2, but was able to significantly revert ($p < 0.001$) the anti-inflammatory effect of the full agonist **8g**.

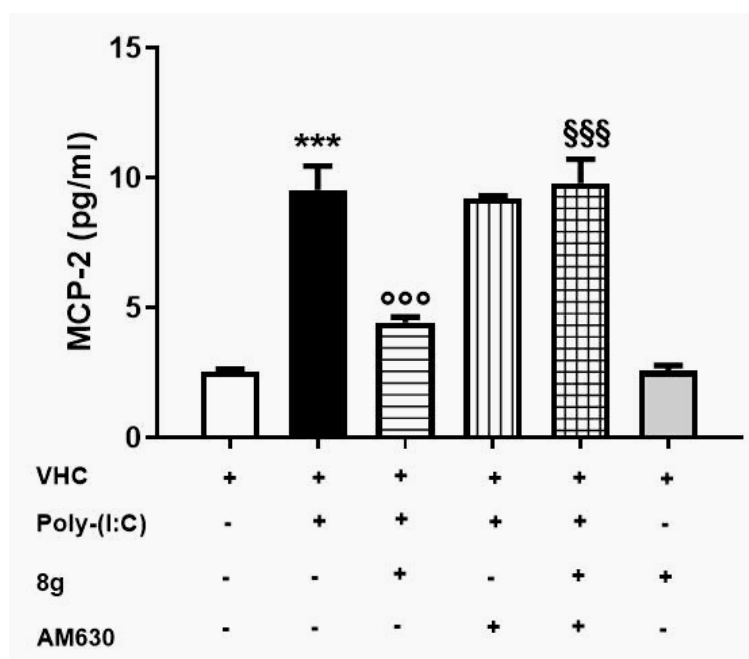


Fig. 3. Effect of the full agonist **8g** (10 μM) on Poly-(I:C)-induced release of MCP-2 in HaCaT cells in presence and absence of *per se* inactive concentration (0.1 μM) of the CBR2 antagonist AM630.

Student's t test: *** $p < 0.001$ versus Vehicle; °°° $p < 0.001$ versus Poly-(I:C) and §§§ $p < 0.001$ versus compound **8g** under Poly-(I:C) stimulus.

3. Conclusions

Thiophene-2-carboxamide derivatives were designed, synthesized, and evaluated in vitro for their ability to bind selectively at CB2R over CB1R. In the whole set of compounds both 3-carboxylate and 2-(adamantan-1-yl)carboxamide groups were kept unchanged, whereas different substituents were introduced at positions 4 and 5 of the heterocyclic ring to get some insight into SAR. Thus,

compound **8a**, having no substituents at positions 4 and 5, showed only weak affinity for CB2R, demonstrating that the 3-carboxylate and 2-carboxamido moieties are not decisive for receptor binding. On the other hand, the insertion of substituents at position 5 seems to be much more fruitful, as a progressive increase of CB2R affinity, often accompanied by higher selectivity, can be noticed in compounds **8b**, **8c**, **8d** bearing an increasingly larger substituent. A further gain in affinity/selectivity was achieved by substituting also the position 4 of the ring, as in compounds **8e-i** and **8k,l**. However, polar groups, such as the sulfone group of **8n**, or the isosteric replacement of thiophene with more polar heterocycles led to far less active compounds. All the most potent CB2R ligands showed a functional profile as agonists, being capable of activating this receptor. In conclusion, a set of potent and selective CB2R agonists, based on a novel chemotype for cannabimimetic agents, was obtained. In an experimental model of allergic contact dermatitis, the potent full agonist **8g** was able to completely abolish the MCP-2 release in HaCaT cells at 10 μ M concentration, demonstrating to have a potential for the treatment of skin inflammatory disease.

4. Experimental

4.1. Chemistry

Reagents were purchased from commercial suppliers and used without further purification. Anhydrous reactions were run under a positive pressure of dry N₂. Merck silica gel 60 was used for flash chromatography (23-400 mesh). ¹H NMR and ¹³C NMR were recorded at 400 and 100 MHz on a Bruker Advance DPX400. Chemical shifts are reported relative to tetramethylsilane at 0.00 ppm. Mass spectral (MS) data were obtained using Agilent 1100 LC/MSD VL system (G1946C) with a 0.4 mL/min flow rate using a binary solvent system of 95:5 methanol/water. UV detection was monitored at 254 nm. Mass spectra were acquired either in positive or in negative mode scanning over the mass range of 105-1500. High resolution molecular ion determinations (HRMS) were performed using a Dionex Ultimate 3000 RS UHPLC system coupled to an Orbitrap™ Q-Exactive high-resolution mass spectrometer (Thermo Scientific) operating in ESI positive full scan

(m/z range 100-800 at resolution 70,000 FWHM at 200 m/z). Melting points were determined on a Gallenkamp apparatus and are uncorrected. Elemental analyses were performed on a Perkin-Elmer PE 2004 elemental analyzer and the data for C, H, and N are within 0.4% of the theoretical values. The chemical purity of the target compounds was determined using the following conditions: an Agilent 1100 series LC/MSD with a Lichrocart 125-4 Lichrospher 100 RP-18 (4.6 mm x 100 mm, 5 μ m) reversed phase column; method: 86% (v/v) of MeOH in H₂O, isocratic, flow rate of 1 mL/min, UV detector, 254 nm. The purity of each compound was \geq 95% in either analysis.

4.1.1. General procedure for the synthesis of aminothiophene precursors **7a-m**

Most of these compounds were commercially available or were prepared according to known procedures [42]. As an example, the preparation of the new compound **7h** is reported below.

4.1.1.1. *Methyl 2-Amino-5-methyl-4-(n-propyl)thiophene-3-carboxylate (7h)*. A mixture of 3-hexanone (1.0 g, 10 mmol), methyl cyanoacetate (882 μ L, 10 mmol), sulfur (321 mg, 10 mmol), and morpholine (872 μ L, 10 mmol) in MeOH (4 mL) was refluxed overnight. After cooling, the reaction mixture was poured into ice (50 g) and the precipitate was filtered and dried under vacuum. The crude product was purified by silica gel chromatography (PE/AcOEt 9:1 as eluent) to give the title compound (230 mg, 10%) as a white solid. ¹H NMR (400 MHz, CDCl₃): δ 5.87 (bs, 2H), 3.79 (s, 3H), 2.59 (t, *J* = 8.0 Hz, 2H), 2.14 (s, 3H), 1.45 (m, 2H), 0.91 (t, *J* = 8.0 Hz, 3H).

4.1.2. General procedure for the synthesis of methyl 2-(acylamino)thiophene-3-carboxylate **8a-m**, **8o**, **8p** and related isosteres **10-12**.

A solution of the appropriate acyl chloride (adamantane-2-carbonyl chloride or 4-substituted benzoyl chloride, 1.3 mmol) in dry dioxane (3 mL) was added dropwise to a solution of **7a-m**, **15**, **16**, **17** (1.0 mmol) in 10 mL of the same solvent, maintained at 70 °C. After the addition was complete, the mixture was refluxed until the formation of hydrogen chloride stopped (1-18 h). The

solution was concentrated in vacuo and the residue was dissolved in DCM (30 mL), washed with NaHCO₃ solution (10 mL), dried over anhydrous Na₂SO₄, and evaporated to dryness. The crude product was purified by recrystallization from MeOH or flash column chromatography on silica gel using the reported eluent system.

4.1.2.1. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)thiophene-3-carboxylate (8a)*. Yield: 50%. Mp 207-208 °C. ¹H NMR (400 MHz, CDCl₃): δ 7.17 (d, *J* = 5.6 Hz, 1H), 6.70 (d, *J* = 5.6 Hz, 1H), 3.88 (s, 3H), 2.10-1.99 (m, 9H), 1.79-1.72 (m, 6H). MS (ESI): *m/z* 342 [M + Na]⁺.

4.1.2.2. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-methylthiophene-3-carboxylate (8b)*. Eluent: DCM. Yield: 56%. Mp 133-134 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.10 (br s, 1H), 6.76 (s, 1H), 3.80 (s, 3H), 2.30 (s, 3H), 2.20 (m, 7H), 2.04 (m, 2H), 1.93 (m, 6H). MS (ESI): *m/z* 334 [M + H]⁺, 356 [M + Na]⁺, 372 [M + K]⁺.

4.1.2.3. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-(*n*-propyl)-thiophene-3-carboxylate (8c)*. Eluent: DCM. Yield: 56%. Mp 124-127 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.12 (br s, 1H), 6.78 (s, 1H), 3.80 (s, 3H), 2.61-2.58 (t, *J* = 7.4 Hz, 2H), 2.03 (m, 2H), 1.93 (m, 7H), 1.69 (m, 6H), 1.62-1.56 (q, *J* = 7.3 Hz, 2H), 0.88 (t, *J* = 7.3 Hz, 3H). MS (ESI): *m/z* 362 [M + H]⁺, 384 [M + Na]⁺, 400 [M+K]⁺.

4.1.2.4. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-phenylthiophene-3-carboxylate (8d)*. Yield: 69%. Mp 167-168 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.23 (br s, 1H), 7.52-7.50 (m, 2H), 7.32-7.28 (m, 2H), 7.19 (m, 2H), 3.85 (s, 3H), 2.06 (m, 2H), 1.96 (m, 6H), 1.72 (m, 7H). MS (ESI): *m/z* 418 [M + Na]⁺.

4.1.2.5. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-4,5-dimethylthiophene-3-carboxylate (8e)*.

Yield: 84%. Mp 155-156 °C. ¹H NMR (400 MHz, CDCl₃): δ 3.87 (s, 3H), 2.23 (s, 3H), 2.21 (s, 3H), 2.12-1.96 (m, 9H), 1.78-1.72 (m, 6H). MS (ESI): *m/z* 348 [M + H]⁺, 370 [M + Na]⁺.

4.1.2.6. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-ethyl-4-methylthiophene-3-carboxylate (8f)*.

Yield: 13%. Mp 99-100 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.54 (s, 1H), 3.88 (s, 3H), 2.67 (q, *J* = 7.6 Hz, 2H), 2.23 (s, 3H), 2.09 (br s, 3H), 1.97 (br s, 6H), 1.75 (br s, 6H), 1.19 (t, *J* = 7.6 Hz, 3H). MS (ESI): *m/z* 362 [M + H]⁺, 384 [M + Na]⁺, 400 [M + K]⁺.

4.1.2.7. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-4-ethyl-5-methylthiophene-3-carboxylate (8g)*.

Yield: 98%. Mp 112-116 °C. ¹H-NMR (400 MHz, CDCl₃) δ 11.49 (s, 1H), 3.83 (s, 3H), 2.65 (q, *J* = 8.0 Hz, 2H), 2.18 (s, 3H), 2.03 (br s, 3H), 1.91 (br s, 6H), 1.69 (br s, 6H), 0.98 (t, *J* = 8.0 Hz, 3H). MS (ESI): *m/z* 362 [M + H]⁺.

4.1.2.8. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-methyl-4-(n-propyl)-thiophene-3-carboxylate (8h)*.

Yield: 95%. Mp 75-76 °C. ¹H NMR (400 MHz, CDCl₃): δ 3.88 (s, 3H), 2.67 (t, *J* = 8.1 Hz, 2H), 2.24 (s, 3H), 2.09 (m, 3H), 1.98 (m, 6H), 1.75 (m, 6H), 1.46 (m, 2H), 0.90 (t, *J* = 8.0 Hz, 3H). MS (ESI): *m/z* 376 [M + H]⁺, 398 [M + Na]⁺, 414 [M + K]⁺.

4.1.2.9. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-(n-butyl)-4-methylthiophene-3-carboxylate (8i)*.

Eluent: DCM. Yield: 51%. Mp 80-82 °C. ¹H NMR (400 MHz, CDCl₃): δ 3.82 (s, 3H), 2.60-2.56 (t, *J* = 7.5 Hz, 3H), 2.17 (s, 3H), 2.03 (m, 2H), 1.92 (m, 7H), 1.69 (m, 6H), 1.53-1.46 (m, 2H), 1.33-1.24 (m, 2H), 0.86-0.82 (t, *J* = 7.2 Hz, 2H). MS (ESI): *m/z* 391 [M + H]⁺, 413 [M + Na]⁺, 429 [M + K]⁺.

4.1.2.10. Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-4-phenylthiophene-3-carboxylate (**8j**).

Yield: 58%. Mp 194-196 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.23 (br s, 1H), 7.29-7.19 (m, 5H), 6.51 (s, 1H), 3.63 (s, 3H), 2.06 (m, 2H), 1.96 (m, 7H), 1.72 (m, 6H). MS (ESI): *m/z* 418 [M + Na]⁺.

4.1.2.11. Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-5-(*n*-pentyl)-4-phenylthiophene-3-

carboxylate (**8k**). Eluent: DCM. Yield: 69%. Mp 103-104 °C. ¹H NMR (400 MHz, CDCl₃): δ 7.29-7.22 (m, 3H), 7.07-7.05 (d, *J* = 6.9 Hz, 2H), 3.40 (s, 3H), 2.41-2.38 (t, *J* = 7.8 Hz, 3H), 2.05 (m, 2H), 1.95-1.94 (m, 7H), 1.71 (m, 6H), 1.50-1.42 (m, 2H), 1.13-1.11 (m, 4H), 0.75-0.72 (m, 2H). MS (ESI): *m/z* 466 [M + H]⁺, 488 [M + Na]⁺, 504 [M + K]⁺.

4.1.2.12. Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-

carboxylate (**8l**). Yield: 56%. Mp 90-91 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.51 (s, 1H), 3.86 (s, 3H), 2.74-2.72 (m, 2H), 2.63-2.60 (m, 2H), 2.09-1.97 (m, 9H), 1.77-1.72 (m, 10H). MS (ESI): *m/z* 396 [M + Na]⁺.

4.1.2.13. Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-4,7-dihydro-5*H*-thieno[2,3-*c*]thiopyran-3-

carboxylate (**8m**). Yield: 75%. Mp 149-150 °C. ¹H NMR (400 MHz, CDCl₃): δ 3.80 (s, 3H), 3.00 (m, 2H), 2.83 (m, 2H), 2.14 (m, 3H), 1.92 (m, 6H), 1.69 (m, 6H). MS (ESI): *m/z* 414 [M + Na]⁺.

4.1.2.14. Methyl 2-(((4-Chlorophenyl)carbonyl)amino)-4-ethyl-5-methylthiophene-3-carboxylate

(**8o**). Its physicochemical properties have been described previously [26].

4.1.2.15. Methyl 4-Ethyl-2-(((4-fluorophenyl)carbonyl)amino)-5-methylthiophene-3-carboxylate

(**8p**). Yield: 90%. Mp 126-128 °C. ¹H NMR (400 MHz, CDCl₃): δ 8.02-7.98 (m, 2H), 7.19-7.15 (m, 2H), 3.92 (s, 3H), 2.29 (s, 3H), 2.74 (q, *J* = 7.4 Hz, 2H), 1.07 (t, *J* = 7.5 Hz, 3H). MS (ESI): *m/z* 322 [M + H]⁺, 344 [M + Na]⁺.

4.1.2.16. *Methyl 3-(((Adamantan-1-yl)carbonyl)amino)-1H-pyrrole-2-carboxylate (10)*. Eluent: PE/AcOEt (2:1). Yield: 46%. Mp 221-224 °C. ¹H NMR (200 MHz, CDCl₃): δ 9.52 (s, 1H), 8.89 (s, 1H), 7.06-7.03 (m, 1H), 6.81-6.78 (m, 1H), 3.84 (s, 3H), 2.05-1.96 (m, 9H), 1.71-1.65 (m, 6H). MS (ESI): *m/z* 303 [M + H]⁺.

4.1.2.17. *Methyl 4-(((Adamantan-1-yl)carbonyl)amino)-1-methyl-1H-pyrazole-5-carboxylate (11)*. Yield: 64%. Mp 205-206 °C. ¹H NMR (400 MHz, CDCl₃): δ 9.06 (s, 1H), 8.19 (s, 1H), 4.00 (s, 3H), 3.90 (s, 3H), 2.00 (s, 3H), 1.87 (m, 6H), 1.70-1.63 (m, 6H). MS (ESI): *m/z* 319 [M + H]⁺.

4.1.2.18. *Methyl 4-(((Adamantan-1-yl)carbonyl)amino)-1-methyl-1H-pyrazole-3-carboxylate (12)*. Mp 214-216 °C. ¹H NMR (400 MHz, CDCl₃): δ 9.26 (s, 1H), 8.13 (s, 1H), 3.90 (s, 3H), 3.85 (s, 3H), 2.02 (s, 3H), 1.90 (m, 6H), 1.69 (m, 6H). MS (ESI): *m/z* 319 [M + H]⁺.

4.1.3. *Methyl 2-(((Adamantan-1-yl)carbonyl)amino)-4,7-dihydro-5H-thieno[2,3-*c*]thiopyran-3-carboxylate 6,6-dioxide (8n)*

A solution of Oxone (466 mg, 0.75 mmol) in water (3 mL) was slowly added to a solution of **8m** (100 mg, 0.25 mmol) in MeOH (3 mL) and the mixture was maintained under stirring at room temperature overnight. After evaporation of MeOH, the aqueous phase was extracted with DCM (3x10 mL) and the organic layer was washed with water (10 mL) and dried over anhydrous sodium sulfate. Evaporation of the solvent left a residue which was purified by flash chromatography on silica gel with DCM/MeOH (95:5) as eluent. Fractions containing the higher spot were pooled and solvent was removed under reduced pressure. The solid residue was further purified by trituration with PE to give 76 mg (70% yield) of the sulfone **8n**. Mp 263-264 °C. ¹H NMR (400 MHz, CDCl₃): δ 7.20 (s, 1H), 4.14 (s, 2H), 3.85 (s, 3H), 3.42 (m, 2H), 3.16 (m, 2H), 2.07 (m, 3H), 1.92 (m, 6H), 1.71 (m, 6H). MS (ESI): *m/z* 446 [M + Na]⁺, 462 [M + K]⁺.

4.1.4. Methyl 2-[(Adamantan-1-yl)carbonyl]amino]pyridine-3-carboxylate (**9**)

A solution of 1-adamantanecarbonyl chloride (198 mg, 1 mmol) in dry DCM (1 mL) was slowly added to a cooled (0-5 °C) solution of methyl 2-aminopyridine-3-carboxylate (**14**) (152 mg, 1 mmol) and TEA (210 µL, 1.5 mmol) in dry DCM (3 mL). After stirring for 18 h, the solution was diluted with DCM (5 mL), washed with saturated solution of NaHCO₃ (5 mL) and brine (5 mL), then dried over anhydrous sodium sulfate, and evaporated to dryness. The crude residue was purified by recrystallization from MeOH to afford the title compound in 40% yield. Mp 183-184 °C. ¹H NMR (200 MHz, CDCl₃): δ 10.88 (s, 1H), 8.64 (m, 1H), 8.30-8.27 (m, 1H), 7.04-7.01 (m, 1H), 3.94 (s, 3H), 2.10-2.02 (m, 9H), 1.76-1.74 (m, 6H). MS (ESI): *m/z* 337 [M+Na]⁺.

4.1.5. Methyl 5-[(Adamantan-1-yl)carbonyl]amino]-3-methylisoxazole-4-carboxylate (**13**)

Sodium hydride powder (31 mg, 1.29 mmol) was added to a solution of **18** (100 mg, 0.64 mmol) in dry THF (4 mL) and the mixture was stirred at rt under nitrogen for 15 min. Then a solution of 1-adamantanecarbonyl chloride (140 mg, 0.7 mmol) in dry THF (2 mL) was added dropwise and the reaction mixture was stirred at rt for 30 min and then was refluxed for 2 h. After removal of most of solvent, the residue was diluted with water (15 mL), neutralized by adding 1 N HCl, and extracted with DCM (3x10 mL). The organic phase was washed with water (10 mL), dried over anhydrous sodium sulfate, and evaporated to give a crude solid which was purified by flash column chromatography on silica gel (PE/AcOEt 1:1 as eluent) to obtain pure **13** in 41% yield. Mp 168-169 °C. ¹H NMR (400 MHz, CDCl₃): δ 10.06 (s, 1H), 3.82 (s, 3H), 2.31 (s, 3H), 1.96 (br s, 3H), 1.90 (br s, 6H), 1.70-1.68 (m, 6H). MS (ESI): *m/z* 320 [M + H]⁺.

4.1.6. Methyl 5-Amino-3-methylisoxazole-4-carboxylate (**18**)

A solution of methyl cyanoacetate (2 mL, 20 mmol) and triethyl orthoacetate (4 mL, 22 mmol) in acetic anhydride (12 mL) was refluxed for 3 h. After cooling, the solution was poured into ice (150

g) and the precipitated solid was filtered and washed with water (100 mL). The crude product was purified by trituration with diethyl ether to give 616 mg (20% yield) of methyl 2-cyano-3-ethoxy-2-butenate (**19**). Mp 87-88 °C. A solution of **19** (600 mg, 3.6 mmol) and hydroxylamine hydrochloride (380 mg, 5.4 mmol) in pyridine (6 mL) was refluxed for 4 h, then poured into ice (50 g) and extracted with AcOEt (3x20 mL). The organic layer was separated, washed with brine (20 mL), dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel eluting with PE/AcOEt 1:1 to afford 214 mg (38% yield) of **18** as a colorless solid, mp 137-138 °C (lit. [33]: 140-141 °C).

4.1.7. Methyl 2-[(Phenoxyacetyl)amino]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (**23**)

To a solution of **7I** (300 mg, 1.42 mmol) in dry dioxane (6 mL) warmed at 70 °C were added DIPEA (495 µL, 2.84 mmol) and then phenyl chloroformate (267 µL, 2.13 mmol). After heating at 100 °C overnight, volatiles were removed under vacuum and the residue was purified by flash column chromatography on silica gel (PE/DCM 2:1 as eluent) to provide the title compound **23** in 99% yield (465 mg) as a white solid, mp 124-125 °C. ¹H NMR (400 MHz, CDCl₃): δ 10.75 (br s, 1H), 7.34-7.30 (m, 2H), 7.19-7.14 (m, 3H), 3.81 (s, 3H), 2.70- 2.68 (m, 2H), 2.58-2.55 (m, 2H), 1.73-1.72 (m, 4H). MS (ESI): *m/z* 354 [M+Na]⁺.

4.1.8. General Procedure for the preparation of ureido derivatives **20-22**

1-Aminoadamantane, morpholine, or piperidine (1.3 mmol) was added to a solution of **23** (331 mg, 1 mmol) in dioxane (4 mL) and the reaction mixture was refluxed for the appropriate time (18 h, 5 h, and 1 h, respectively). After cooling to rt, the solution was diluted with DCM (10 mL) and washed with 2 N NaOH (5 mL), 2 N HCl (5 mL), and brine (5 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness to leave a residue which was purified as described below.

4.1.8.1. *Methyl 2-[(1-Adamantylamino)carbonyl]amino]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (20)*. Crystallized from MeOH. Yield: 87%. White solid, mp 125-129 °C. ¹H NMR (400 MHz, CDCl₃): δ 10.31 (br s, 1H), 3.63 (s, 3H), 2.64-2.62 (m, 2H), 2.52-2.49 (m, 2H), 2.02-1.94 (m, 9H), 1.72-1.61 (m, 11H). MS (ESI): *m/z* 389 [M+H]⁺, 411 [M+Na]⁺.

4.1.8.2. *Methyl 2-[(4-Morpholinylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (21)*. Crystallized from MeOH. Yield: 43%. White solid, mp 173-174 °C. ¹H NMR (400 MHz, CDCl₃): δ 11.10 (br s, 1H), 3.77 (s, 3H), 3.69-3.67 (m, 4H), 3.48-3.46 (m, 4H), 2.66 (m, 2H), 2.55 (m, 2H), 1.71 (m, 4H). MS (ESI): *m/z* 325 [M+H]⁺, 347 [M+Na]⁺.

4.1.8.3. *Methyl 2-[(1-Piperidinylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (22)*. The reaction crude was purified by flash column chromatography on silica gel (DCM as eluent) to give compound **22** in 26% yield. ¹H NMR (400 MHz, CDCl₃): δ 11.10 (br s, 1H), 8.13-8.11 (d, *J* = 8.2 Hz, 1H), 7.64-7.62 (d, *J* = 7.9 Hz, 1H), 7.32-7.28 (t, *J* = 7.6 Hz, 1H), 7.19-7.15 (m, 1H), 3.95 (s, 3H), 3.51 (m, 4H), 1.61 (m, 6H). MS (ESI): *m/z* 342 [M+K]⁺.

4.2. Competition binding assay

Membranes from HEK-293 cells over-expressing the respective human recombinant CB1R (B_{max} = 2.5 pmol/mg protein) and human recombinant CB2R (B_{max} = 4.7 pmol/mg protein) were incubated with [³H]-CP-55,940 (0.14 nM/*K*_d = 0.18 nM and 0.084 nM/*K*_d = 0.31 nM, respectively, for CB1R and CB2R) as the high affinity ligand. Competition curves were performed by displacing [³H]-CP-55,940 with increasing concentration of compounds (0.1 nM – 10 μM). Nonspecific binding was defined by 10 μM of WIN55,212-2 as the heterologous competitor (*K*_i values 9.2 nM and 2.1 nM, respectively, for CB1R and CB2R). IC₅₀ values were determined for compounds showing >50% displacement at 10 μM. All compounds were tested following the procedure

described by the manufacturer (Perkin Elmer, Italy). Displacement curves were generated by incubating drugs with [³H]-CP-55,940 for 90 minutes at 30 °C. *K_i* values were calculated by applying the Cheng-Prusoff equation to the IC₅₀ 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 independent experiments performed in duplicate and are expressed as the average of *K_i* (nM) ± standard deviation.

4.3. Functional activity at CB2R 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 CB2R 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 30 μL of assay buffer. Cells were incubated 30 min at 37 °C with 15 μL of 3x dose-response solutions of samples prepared in presence of cell assay buffer containing a 3x of 25 μM NKH-477 solution (a water soluble analogue of Forskolin) to stimulate adenylate cyclase and enhance basal cAMP levels. For those compounds showing an increase of cAMP levels, we further investigated their 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 = 4 μM, 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)). The data were analyzed using PRISM software (GraphPad Software Inc, San Diego, CA).

4.4. Poly-(I:C)-induced allergic contact dermatitis in HaCaT cells

Polyinosinic-polycytidylic acid [poly-(I:C)] was purchased from InvivoGen (Aurogene Srl, Roma, Italy). Human Keratinocyte (HaCaT) cell line was purchased from CLS Cell Lines Service (Eppelheim, Germany). The human monocyte chemotactic protein-2 (MCP-2) ELISA kit was purchased from RayBiotech, Inc (Tebu-Bio Srl, Milano, Italy). HaCaT cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/mL), streptomycin (50 mg/mL) and 10% Fetal Bovine Serum (FBS) at 37 °C in humidified 5% CO₂. HaCaT cells were plated into twenty-four-well culture plates at a cell density of 2 x 10⁵ cells per well, and after 1 day were stimulated with poly-(I:C) (100 µg/mL) [43] or vehicle (water) and incubated for 6 h at 37 °C in 5% CO₂. Poly-(I:C)-stimulated HaCaT cells were treated with vehicle (dimethyl sulfoxide, DMSO) or compound 8g (10 µM) in the presence or absence of a per se inactive concentration (0.1 µM) of CB2 antagonist AM630. After 6 h, the supernatants were collected and used for MCP-2 ELISA assay according to the manufacturer's instructions (RayBiotech), and by using a reader GENios Pro (Tecan). Data are expressed as pg/mL of released MCP-2. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). The data are expressed as means ± SEM of three independent experiments. Student's t-test was used for analysis. P values < 0.05 were considered statistically significant.

4.5. Interference compounds screening

All tested compounds were also subjected to Pan-assay interference compounds (PAINS) filter [44], and none of them was filtered out as problematic structure.

Author contribution

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Declarations of interest

None.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors from Dipartimento di Biotecnologie, Chimica e Farmacia acknowledge the partial support by MIUR Progetto *Dipartimenti di Eccellenza 2018-2022*.

Acknowledgments

The authors thank Dr. Marco Allarà for his technical assistance in binding experiments and Dr. Simone Esposito, Department of DMPK, IRBM, Pomezia (Rome), Italy, for HRMS determinations.

Appendix A. Supplementary Data.

Supplementary data related to this article can be found at <https://>

These data include ¹³C NMR, HRMS, and elemental analysis data as well as molecular formula strings (CSV) for the most important compounds described in this article.

References

1. Gaoni, Y.; Mechoulam, R. Isolation, structure and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* **1964**, *86*, 1646-1647.
2. Devane, W.A.; Dysarz, F. A. 3rd; Johnson, M. R.; Melvin, L. S.; Howlett, A. C. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol.* **1988**, *34*, 605-613.

3. Munro, S.; Thomas, K. L.; Abu-Shaar, M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, **1993**, *365*, 61-65.
4. Cabral, G. A.; Marciano-Cabral, F. Cannabinoid receptors in microglia of the central nervous system: immune functional relevance. *J. Leukoc. Biol.* **2005**, *78*, 1192-1197.
6. Mechoulam, R.; Ben-Shabat, S.; Hanus, L.; Ligumsky, M.; Kaminski, N. E.; Schatz, A. R.; Gopher, A.; Almog, S.; Martin, B. R.; Compton, D. R. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol.* **1995**, *50*, 83-90.
7. Barna, I.; Zelena, D. The biochemical complexity of the endocannabinoid system with some remarks on stress and related disorders: A minireview. *Endocr. Regul.* **2012**, *46*, 107-124.
8. Maccarrone, M.; Bab, I.; Biró, T.; Cabral, G. A.; Dey, S. K.; Di Marzo, V.; Konje, J. C.; Kunos, G.; Mechoulam, R.; Pacher, P.; Sharkey, K. A.; Zimmer, A. Endocannabinoid signaling at the periphery: 50 years after THC. *Trends Pharmacol. Sci.* **2015**, *36*, 277-296.
9. Pini, A.; Mannaioni, G.; Pellegrini, G. D.; Passani, M. B.; Mastroianni, R.; Bani, D.; Masini, E. The role of cannabinoids in inflammatory modulation of allergic respiratory disorders, inflammatory pain and ischemic stroke. *Curr. Drug Targ.* **2012**, *13*, 984-993.
10. (a) Malek, N.; Mrugala, M.; Makuch, W.; Kolosowska, N.; Przewlocka, B.; Binkowski, M.; Czaja, M.; Morera, E.; Di Marzo, V.; Starowicz, K. A multi-target approach for pain treatment: dual inhibition of fatty acid amide hydrolase and TRPV1 in a rat model of osteoarthritis. *Pain* **2015**, *156*, 890–903. (b) Starowicz, K.; Malek, N.; Przewlocka, B. Cannabinoid receptors and pain. *WIREs Membr. Transp. Signal.* **2013**, *2*, 121-132.
11. Zaiicek, J. P.; Apostu, V. I. Role of cannabinoids in multiple sclerosis. *CNS Drugs.* **2011**, *25*, 187-201.
12. Hermanson, D. J.; Marnett, L. J. Cannabinoids, Endocannabinoids and Cancer. *Cancer Metastasis Rev.* **2011**, *30*, 599-612.
13. Parsons, L. H.; Hurd, Y. L. Endocannabinoid signaling in reward and addiction. *Nat. Rev.*

Neurosci. **2015**, *16*, 579-594.

14. Benito, C.; Tolón, R. M.; Pazos, M. R.; Casillo, A. I.; Romero, J. Cannabinoid CB₂ receptors in human brain inflammation. *Br. J. Pharmacol.* **2008**, *153*, 277–285.

15. Tabrizi, M. A.; Baraldi, P. G.; Borea, P. A.; Varani, K. Medicinal chemistry, pharmacology, and therapeutic benefits of cannabinoid CB₂ receptor agonists. *Chem. Rev.* **2016**, *116*, 519–560.

16. Haruna, T.; Soga, M.; Morioka, Y.; Imura, K.; Furue, Y.; Yamamoto, M.; Hayakawa, J.; Deguchi, M.; Arimura, A.; Yasui, K. The inhibitory effect of S-777469, a cannabinoid type 2 receptor agonist, on skin inflammation in mice. *Pharmacology* **2017**, *99*, 259–267.

17. Soethoudt, M.; Grether, U.; Fingerle, J.; Grim, T. W.; Fezza, F.; de Petrocellis, L.; Ullmer, C.; Rothenhäusler, B.; Perret, C.; van Gils, N.; Finlay, D.; MacDonald, C.; Chicca, A.; Dalghi Gens, M.; Stuart, J.; de Vries, H.; Mastrangelo, N.; Xia, L.; Alachouzos, G.; Baggelaar, M. P.; Martella, A.; Mock, E. D.; Deng, H.; Heitman, L. H.; Connor, M.; Di Marzo, V.; Gertsch, J.; Lichtman, A. H.; Maccarrone, M.; Pacher, P.; Glass, M.; van der Stelt, M. Cannabinoid CB₂ receptor ligand profiling reveals signalling and off-target activity. *Nat. Commun.* **2017**, *8*, 13958.

18. Morales, P.; Hernandez-Folgado, L.; Goya, P.; Jagerovic, N. Cannabinoid receptor 2 (CB₂) agonists and antagonists: a patent update. *Expert Opin. Ther. Pat.* **2016**, *26*, 843–856.

19. (a) Pasquini, S.; Botta, L.; Semeraro, T.; Mugnaini, C.; Ligresti, A.; Palazzo, E.; Maione, S.; Di Marzo, V.; Corelli, F. Investigations on the 4-quinolone-3-carboxylic acid motif. 2. Synthesis and structure-activity relationship of potent and selective cannabinoid-2 receptor agonists endowed with analgesic activity in vivo. *J. Med. Chem.* **2008**, *51*, 5075–5084. (b) Pasquini, S.; De Rosa, M.; Pedani, V.; Mugnaini, C.; Guida, F.; Luongo, L.; De Chiaro, M.; Maione, S.; Dragoni, S.; Frosini, M.; Ligresti, A.; Di Marzo, V.; Corelli, F. Investigations on the 4-quinolone-3-carboxylic acid motif. 4. Identification of new potent and selective ligands for the cannabinoid type 2 receptor with diverse substitution patterns and anti-hyperalgesic effects in mice. *J. Med. Chem.* **2011**, *54*, 5444–5453. (c) Mugnaini, C.; Brizzi, A.; Ligresti, A.; Allarà, M.; Lamponi, S.; Vacondio, F.; Silva, C.; Mor, M.; Di Marzo, V.; Corelli, F. Investigations on the 4-quinolone-3-carboxylic acid motif. 7.

Synthesis and pharmacological evaluation of 4-quinolone-3-carboxamides and 4-hydroxy-2-quinolone-3-carboxamides as high affinity cannabinoid receptor 2 (CB2R) ligands with improved aqueous solubility. *J. Med. Chem.* **2016**, *59*, 1052–1067.

20. Annunziata, P.; Cioni, C.; Mugnaini, C.; Corelli, F. Potent immunomodulatory activity of a highly selective cannabinoid CB2 agonist on immune cells from healthy subjects and patients with multiple sclerosis. *J. Neuroimmunol.* **2017**, *303*, 66–74.

21. Contartese, A.; Valoti, M.; Corelli, F.; Pasquini, S.; Mugnaini, C.; Sgaragli, G. P.; Frosini, M. A novel substituted 4-quinolone-3-carboxylic acid derivative CB2 agonist, COR167, potently protects rat brain cortical slices against OGD and reperfusion injury. *Pharmacol. Res.* **2012**, *66*, 555–563.

22. Cascio, M. G.; Bolognini, D.; Pertwee, R. G.; Palazzo, E.; Corelli, F.; Pasquini, S.; Di Marzo, V.; Maione, S. In vitro and in vivo pharmacological characterization of two novel selective cannabinoid CB2 receptor inverse agonists. *Pharmacol. Res.* **2010**, *61*, 349354.

23. (a) Bozorov, K.; Zhao, J.-Y.; Nie, L. F.; Ma, H.-R.; Bobakulov, K.; Hu, R.; Rustamova, N.; Huang, G.; Efferth, T.; Aisa, H. A. Synthesis and in vitro biological evaluation of novel diaminothiophene scaffolds as antitumor and anti-influenza virus agents. Part 2. *RSC Adv.* **2017**, *7*, 31417–31427. (b) Hrast, M.; Anderluh, M.; Knez, D.; Randall, C. P.; Barreteau, H.; O'Neill, A. J.; Blanot, D.; Gobec, S. Design, synthesis and evaluation of second generation MurF inhibitors based on a cyanothiophene scaffold. *Eur. J. Med. Chem.* **2014**, *73*, 83–96. (c) Massari, S.; Nannetti, G.; Goracci, L.; Sancineto, L.; Muratore, G.; Sabatini, S.; Manfroni, G.; Mercorelli, B.; Cecchetti, V.; Facchini, M.; Palù, G.; Cruciani, G.; Loregian, A.; Tabarrini, O. Structural investigation of cycloheptathiophene-3-carboxamide derivatives targeting influenza virus polymerase assembly. *J. Med. Chem.* **2013**, *56*, 10118–10131. (d) Nelson, D. W.; Frost, J. M.; Tietje, K. R.; Florjancic, A. S.; Ryther, K.; Carroll, W. A.; Dart, M. J.; Daza, A. V.; Hooker, B. A.; Grayson, G. K.; Fan, Y.; Garrison, T. R.; El-Kouhen, O. F.; Yao, B.; Pai, M.; Chandran, P.; Zhu, C.; Hsieh, G. C.; Meyer, M. D. Synthesis and evaluation of 2-amido-3-carboxamide thiophene CB₂ receptor agonists for pain management. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2604–2608. (e) Thur, Y.; Bhalerao, A.; Munshi,

Z.; Pansare, N.; Mann, K.; Hanauer, G.; Kley, H.-P.; Nappe, S.; Weiss-Haljiti, C.; Ostermann, C.; Zitt, K.; Schaefer, M.; Mondal, D.; Siddiki, A. A.; Armugam, V.; Gudaghe, V.; Gupta, M.; Rayudu, P.; Dautzenberg, F. M.; Das Sarma, K. Structure-activity relationships of 2-arylamido-5,7-dihydro-4*H*-thieno[2,3-*c*]pyran-3-carboxamide derivatives as cannabinoid receptor agonists and their analgesic action. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 7314–7321.

24. Castelli, M. P.; Casu, A.; Casti, P.; Lobina, C.; Carai, M. A. M.; Colombo, G.; Solinas, M.; Giunta, D.; Mugnaini, C.; Pasquini, S.; Tafi, A.; Brogi, S.; Gessa, G. L.; Corelli, F. Characterization of methyl 2-(adamantan-1-yl-carboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627) and methyl 2-(cyclohexylcarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628), two novel positive allosteric modulators of the GABA_B receptor. *J. Pharmacol. Exp. Ther.* **2012**, *340*, 529–538.

25. Mugnaini, C.; Pedani, V.; Giunta, D.; Sechi, B.; Solinas, M.; Casti, A.; Castelli, M. P.; Giorgi, G.; Corelli, F. Synthesis, structural properties, and pharmacological evaluation of 2-(acylamino)thiophene-3-carboxamides and analogues thereof. *RSC Adv.* **2014**, *4*, 1782–1793.

26. Mugnaini, C.; Pedani, V.; Casu, A.; Lobina, C.; Casti, A.; Maccioni, P.; Porcu, A.; Giunta, D.; Lamponi, S.; Solinas, M.; Dragoni, S.; Valoti, M.; Colombo, G.; Castelli, M. P.; Gessa, G. L.; Corelli, F. Synthesis and pharmacological characterization of 2-(acylamino)thiophene derivatives as metabolically stable, orally effective, positive allosteric modulators of the GABA_B receptor. *J. Med. Chem.* **2013**, *56*, 3620–3635.

27. Osman, N. A.; Ligresti, A.; Klein, C. D.; Allarà, M.; Rabbito, A.; Di Marzo, V.; Abouzeid, K. A.; Abadi, A. H. 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. *Eur. J. Med. Chem.* **2016**, *122*, 619–634.

28. Aso, E.; Ferrer, I. CB₂ cannabinoid receptors as potential target against Alzheimer's disease. *Front. Neurosci.* **2016**, *10*, article 243.

29. (a) Haider, A.; Müller Herde, A.; Slavik, R.; Weber, M.; Mugnaini, C.; Ligresti, A.; Schibli, R.;

Mu, L.; Ametamey, S. M. Synthesis and biological evaluation of thiophene-based cannabinoid receptor type 2 radiotracers for PET imaging. *Front. Neurosci.* **2016**, *10*, article 350. (b) Moldovan, R.-P.; Teodoro, R.; Gao, Y.; Deuther-Conrad, W.; Kranz, M.; Wang, Y.; Kuwabara, H.; Nakano, M.; Valentine, H.; Fischer, S.; Pomper, M. G.; Wong, D. F.; Dannals, R. F.; Brust, P.; Horti, A. G. Development of a high-affinity PET radioligand for imaging cannabinoid subtype 2 receptor. *J. Med. Chem.* **2016**, *59*, 7840–7855. (c) Slavik, R.; Grether, U.; Müller Herde, A.; Gobbi, L.; Fingerle, J.; Ullmer, C.; Krämer, S.; Schibli, R.; Mu, L.; Ametamey, S. M. Discovery of a high affinity and selective pyridine analog as a potential positron emission tomography imaging agent for cannabinoid type 2 receptor. *J. Med. Chem.* **2015**, *58*, 4266–4277.

30. Hu, Y.; Stumpfe, D.; Bajorath, J. Recent advances in scaffold hopping. *J. Med. Chem.* **2017**, *60*, 1238–1246.

31. Furneaux, R. H.; Tyler, P. C. Improved syntheses of 3*H*,5*H*-pyrrolo[3,2-*d*]pyrimidines. *J. Org. Chem.* **1999**, *64*, 8411–8412.

32. Regiec, A.; Mastalarz, H.; Mastalarz, A.; Kochel, A. Methylation of 4-nitro-3(5)-pyrazolecarboxylic acid. *Tetrahedron Lett.* **2009**, *50*, 2624–2627.

33. Baba, H.; Hori, I.; Hayashi, T.; Midorikawa, H. Reactions of α -cyano- β -methoxy- β -alkylacrylic esters with hydrazine and hydroxylamine. *Bull. Chem. Soc. Jpn.* **1969**, *42*, 1653–1659.

34. Regiec, A.; Gadzinski, P.; Ploszaj, P. New methods for preparing of esters of 5-amino-3-methyl-4-isoxazolecarboxylic acid. PL 216764 B1 20140530.

35. (a) Rinaldi-Carmona, M.; Barth, F.; Millan, J.; Derocq, J. M.; Casellas, P.; Congy, C.; Oustric, D.; Sarran, M.; Bouaboula, M.; Calandra, B.; Portier, M.; Shire, D.; Breliere, J. C.; Le Fur, G. L. SR144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 644–650. (b) Ross, R. A.; Brockie, H. C.; Stevenson, L. A.; Murphy, V. L.; Templeton, F.; Makriyannis, A.; Pertwee, R. G. Agonist-inverse agonist characterization at CB1 and CB2 cannabinoid receptors of L759633, L759656 and AM630. *Br. J. Pharmacol.* **1999**, *126*, 665–672.

36. Kuntz, K. W.; Campbell, J. E.; Keilhack, H.; Pollock, R. M.; Knutson, S. K.; Porter-Scott, M.; Richon, V. M.; Sneeringer, C. J.; Wigle, T. J.; Allain, C. J.; Majer, C. R.; Moyer, M. P.; Copeland, R. A.; Chesworth, R. The importance of being Me: magic methyls, methyltransferase inhibitors, and the discovery of tazemetostat. *J. Med. Chem.* **2016**, *59*, 1556–1564.
37. Barreiro, E. J.; Kümmerle, A. E.; Fraga, C. A. M. The methylation effect in medicinal chemistry. *Chem. Rev.* **2011**, *111*, 5215–5246.
38. Leung, C. S.; Leung, S. S. F.; Tirado-Rives, J.; Jorgensen, W. L. Methyl effects on protein-ligand binding. *J. Med. Chem.* **2012**, *55*, 4489–4500.
39. Calculated with Molinspiration Cheminformatics Software (www.molinspiration.com).
40. Klein, T. W.; Newton, C. A. Therapeutic potential of cannabinoid-based drugs. *Adv. Exp. Med. Biol.* **2007**, *601*, 395–413.
41. Karsak, M.; Gaffal, E.; Date, R.; Wang-Eckhardt, L.; Rehnelt, J.; Petrosino, S.; Starowicz, K.; Steuder, R.; Schlicker, E.; Cravatt, B.; Mechoulam, R.; Buettner, R.; Werner, S.; Di Marzo, V.; Tüting, T.; Zimmer, A. Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science* **2007**, *316*, 1494–1497.
42. Reviews: (a) Sabnis, R. W. The Gewald Synthesis. *Sulfur Rep.* **1994**, *16*, 1–17. (b) Sabnis, R. W.; Rangnekar, D. W.; Sonawane, N. D. 2-Aminothiophenes by the Gewald Reaction. *J. Heterocyclic Chem.* **1999**, *36*, 333–345. (c) Huang, Y.; Dömling, A. The Gewald multicomponent reaction. *Mol. Divers.* **2011**, *15*, 3–33.
43. Petrosino, S.; Cristino, L.; Karsak, M.; Gaffal, E.; Ueda, N.; Tüting, T.; Bisogno, T.; De Filippis, D.; D'Amico, A.; Saturnino, C.; Orlando, P.; Zimmer, A.; Iuvone, T.; Di Marzo, V. Protective role of palmitoylethanolamide in contact allergic dermatitis. *Allergy* **2010**, *65*, 698–711.
44. Baell, J. B.; Holloway, G. A. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* **2010**, *53*, 2719–2740.