Accepted Manuscript

Synthesis and Structure Activity Relationship Investigation of Triazolo[1,5-*a*]pyrimidines as CB_2 Cannabinoid Receptor Inverse Agonists

Mojgan Aghazadeh Tabriz, Pier Giovanni Baraldi, Emanuela Ruggiero, Giulia Saponaro, Stefania Baraldi, Giulio Poli, Tiziano Tuccinardi, Annalisa Ravani, Fabrizio Vincenzi, Pier Andrea Borea, Katia Varani

PII: S0223-5234(16)30112-X

DOI: 10.1016/j.ejmech.2016.02.032

Reference: EJMECH 8383

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 17 November 2015

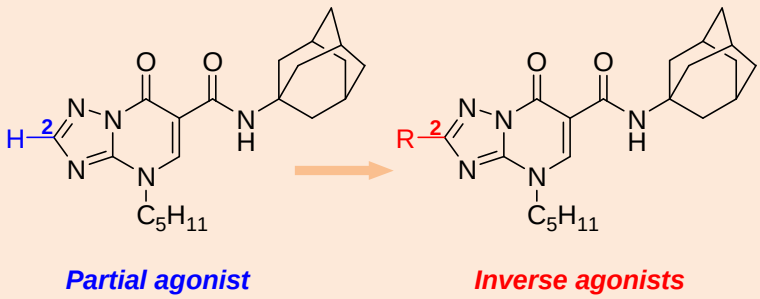
Revised Date: 26 January 2016

Accepted Date: 11 February 2016

Please cite this article as: M.A. Tabriz, P.G. Baraldi, E. Ruggiero, G. Saponaro, S. Baraldi, G. Poli, T. Tuccinardi, A. Ravani, F. Vincenzi, P.A. Borea, K. Varani, Synthesis and Structure Activity Relationship Investigation of Triazolo[1,5-*a*]pyrimidines as CB_2 Cannabinoid Receptor Inverse Agonists, *European Journal of Medicinal Chemistry* (2016), doi: 10.1016/j.ejmech.2016.02.032.

Abstract graphic

Synthesis and Structure Activity Relationship Investigation of Triazolo[1,5-*a*]pyrimidines as CB_2 Cannabinoid Receptor Inverse Agonists



Synthesis and Structure Activity Relationship Investigation of Triazolo[1,5-*a*]pyrimidines as CB_2 Cannabinoid Receptor Inverse Agonists

Mojgan Aghazadeh Tabriz[†*], Pier Giovanni Baraldi[†*], Emanuela Ruggiero[†], Giulia Saponaro[†], Stefania Baraldi[†], Giulio Poli[‡], Tiziano Tuccinardi[‡], Annalisa Ravani[⊥], Fabrizio Vincenzi[⊥], Pier Andrea Borea[⊥], Katia Varani[⊥]

[†]Dipartimento di Scienze Chimiche e Farmaceutiche, Università di Ferrara, Via Fossato di Mortara 17-19, 44121 Ferrara, Italy

[‡]Dipartimento di Farmacia, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy

[⊥]Dipartimento di Scienze Mediche, Sezione di Farmacologia, Università di Ferrara, Via Fossato di Mortara 17-19, 44121, Ferrara, Italy

[*]Dr. Mojgan Aghazadeh Tabrizi, Dipartimento di Scienze Chimiche e Farmaceutiche, Università di Ferrara, 44121, Ferrara, Italy, Via Fossato di Mortara 17-19, 44121, Ferrara Italy, Tel: +39-0532-455926, Fax: +39-0532-455921, E-mail: tbj@unife.it

[*]Prof. Pier Giovanni Baraldi, Dipartimento di Scienze Chimiche e Farmaceutiche, Università di Ferrara, 44121, Ferrara, Italy, Via Fossato di Mortara 17-19, 44121, Ferrara Italy, Tel: +39-0532-455921, Fax: +39-0532-455921, E-mail: baraldi@unife.it

Abstract

CB_2 cannabinoid receptor ligands are known to be therapeutically important for the treatment of numerous diseases. Recently, we have identified the heteroaryl-4-oxopyridine/7-oxopyrimidine derivatives as highly potent and selective CB_2 receptor ligands, showing that the pharmakodynamics of the new compounds was controlled by the nature of the heterocycle core. In this paper we describe the synthesis and biological evaluation of 7-oxo-4-pentyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide derivatives that led to the identification of novel CB_2 receptor inverse agonists. Cyclic AMP experiments on CB_2 receptors expressed in CHO cells revealed that introduction of structural modifications at position 2 of triazolopyrimidine template changes the functional activity from partial to inverse agonism. The molecular docking analysis of the novel structures is reported.

Keywords

Cannabinoid; CB_2 receptor; inverse agonists; triazolopyrimidine; molecular docking.

Introduction

The endocannabinoid system includes a group of neuromodulatory lipids and their receptors that are involved in a diversity of physiological and pathological conditions. The main endocannabinoids, or endogenous cannabis-like substances, are small molecules resulting from arachidonic acid, N-arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoyl-glycerol (2-AG) [1]. Two diverse types of G protein-coupled receptors (GPCRs) have been discovered and named as cannabinoid CB_1 and cannabinoid CB_2 receptors. The CB_1 and CB_2 receptors are negatively coupled to adenylyl cyclase, exert an inhibitory effect on cyclic AMP (cAMP) production, alter the mitogen-activated protein kinase (MAPK) pathway, and regulate voltage-gated L-, N- and P- or Q-type Ca2+ channels [2].

CB_2 receptors were originally supposed to be present in periphery, primarily to immune cells, but at present it has been reported that these receptors are localized neuronally in different species. They are expressed in hematopoietic cells, natural killer cells, macrophages and neutrophils, and represent an attractive target for the treatment of the inflammatory status [3]. These receptors also mediate a significant protection in brain microglia against neurotoxicity by modulating the release of anti- or pro-inflammatory cytokines [4]. CB_2 receptors appear to be implicated in allergic dermatitis where their modulation mediates an inflammatory response obtained from antigen-specific effecter T cells upon frequent allergen contact [5]. Contrasting preclinical studies are reported regarding the involvement of CB_2 receptors and allergic dermatitis, supposing the potential use of both CB_2 agonists or inverse agonists/antagonists in the pharmacological therapy [6].

In recent years, pharmaceutical companies and academic research laboratories have challenged to identify novel CB_2 agonists without CB_1 central effects by focusing on the discovery of high affinity and selective agonists for CB_2 receptors with a therapeutic potential in the treatment of different pathologies such as neurodegenerative diseases, pain transduction and perception, ischemic stroke, severe inflammation, autoimmune diseases, osteoporosis and cancers [4,7].

From the pharmacological point of view, CB_2 agonists demonstrate various ways of interacting with the receptor site, suggesting different binding and functional properties well represented by pharmacodynamic concepts of full, partial and inverse agonism [8–11].

The therapeutic potential of the cannabinoids has encouraged the development of several CB_2 receptor selective inverse agonists (Chart 1). In particular, the immunomodulatory activities associated with cannabinoid CB_2 receptor inverse agonists have been demonstrated by oral administration of the inverse agonists **1** (JTE-907) and **2** (SR144528) that inhibited carrageenin-induced paw oedema in mice [12]. The efficacy of triaryl bissulphone **3** (Sch414319)**,** a CB_2 receptor inverse agonist, was investigated in vivo in diverse disease models, showing its ability to modulate immune cell mobility and bone damage in antigen-induced mono-articular arthritis in the rat [13]. Additional reports suggest that compound **4** (MH), an inverse agonists of CB_2 receptor, showed anti-inflammatory and antiosteoclastogenic properties [14]. Moreover, N,N′-((4-(dimethylamino)phenyl)methylene)bis(2-phenylacetamide) derivatives such as **5** were found to be CB_2 inverse agonists and potential therapeutic agents for the treatment of antiosteoporosis [15].

The 4-oxo-1,8-naphthyridine-3-carboxamide and 4-oxo-1,4-dihydroquinoline-3-carboxamide cannabinoid based ligands are reported with high affinity and selectivity toward CB_2 receptors. Some of these analogues were demonstrated to act as agonists or inverse agonists in functional activity assays, depending on the nature of the substituents on the different positions of the heterocyclic scaffold. Within this series, the 8-methoxy derivative **6 (**hCB_2 K_i = 0.6 nM, hCB_1 K_i > 10000 nM) (Chart 1) behaved as an inverse agonist and showed antihyperalgesic effects [16].

Chart 1 should be inserted here

Our group previously reported the synthesis and cannabinoid receptor binding of 4-oxopyrazolopyridines as CB_2 partial agonists [10]. In this family, compound **7** (Chart 2) displayed high affinity and selectivity for CB_2 receptor. Changing the orientation of the pyrazole ring led to the 7-oxopyrazolopyrimidines, such as compound **8** that exhibited selective inverse agonist activity at CB_2 receptor [11]. Replacement of the pyrazolo ring of the parent nucleus with differently substi-

tuted 5-membered heterocycles allowed to the isoxazolo/thienopyridines that were found to be CB_2 full agonists. In this context, the triazolopyrimidines **9**, **10** (Chart 2) were found to be potent CB_2 receptor ligands and in functional assays compound **9** behaved as partial agonist.

In light of these results, we sought to explore the influence of substituent arrangement at the C-2 position of triazolo heterocycle on the activity versus CB_2 receptor. In this attempt, we have synthesized a new series of 2-substituted-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide derivatives based on the previously well-known pharmacophores in the series of heteroaryl pyridine/pyrimidine compounds. Consequently, the *n*-pentyl chain was retained at the N-4 position of heterocycle and the cyclohexyl, cycloheptyl, and adamantyl moieties were introduced on the carboxamide moiety (Chart 2). All the newly synthesized compounds were tested on membranes prepared from Chinese hamster ovary (CHO) cells expressing human CB_1 (hCB_1) and CB_2 (hCB_2) receptors, in order to estimate their affinity and selectivity for CB_2 receptor. The cyclic AMP assays on hCB_2 CHO cells to test the functionality of these ligands is reported. Moreover, a molecular docking analysis was carried out into the three-dimensional model of CB receptors recently constructed, in order to clarify the structure activity relationships (SARs) experimentally observed.

Chart 2 should be inserted here

Chemistry

Synthesis of derivatives **15-41** was carried out by the general methodology shown in Scheme 1. Cyclocondensation of aminotriazoles **11a-m** with diethyl ethoxymethylenmalonate (DEEM) in glacial acetic acid gave the ethyl 4,7-dihydro-2-substituted-7-oxo-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylates **12a-m** in good yields. Alkylation of the bicyclic nucleus with 1-pentylbromide in the presence of K_2CO_3 led to the formation of **13a-m**. The subsequent hydrolysis with 10% aqueous NaOH yielded the related carboxylic acids **14a-m,** which were converted to the corresponding carboxamide derivatives **15-41** by coupling reaction with appropriate amines using 1-ethyl- 3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCl) or ***N,N,N′,N′***-tetramethyl-*O*-(1*H*-

benzotriazol-1-yl)uronium hexafluorophosphate in dimethylformamide. The starting 1,2,4-triazol-3-amines **11a-b** were commercially available, while derivatives **11c-m** were prepared following two different procedures. According to method A via cyclocondensation of amidoguanidines, a suitable hydrazide was reacted with S-methylisothiourea hemisulfate upon heating in water to give the amidoguanidine intermediate. The subsequent cyclization in 10% aqueous NaOH at 100 °C yielded aminotriazoles **11c-h** [17,18]. The aminotriazoles **11i-m** were prepared according to method B, by the reaction of N-cyanocarbonimidodithioic acid dimethyl ester with one molar equivalent of an appropriate amine followed by the reaction with hydrazine monohydrate in refluxing acetonitrile (as except for **11i** that was prepared without adding hydrazine) [19].

Scheme 1 should be inserted here

Biological results and discussion

All of the newly synthesized compounds were examined in [^{3}H]CP-55,940 competition binding experiments for their affinity and selectivity toward the human recombinant CB_1 and CB_2 receptors. The results are reported in Table 1 together with K_i values of previously published triazolopyrimidines **9**, **10** and the CB_2 agonist WIN55,212-2. According to our previous pharmacophore investigations, compounds bearing cyclohexyl, cycloheptyl, or adamantanyl moieties on the carboxamide function had shown the highest affinity for CB_2 receptor. We had also confirmed that the presence of the 1-pentyl chain on the pyridine/pyrimidine nucleus was required for CB_2 receptor affinity. Consequently, we introduced structural modifications at position 2 of the 7-oxo-triazolo[1,5-*a*]pyrimidine-6-carboxamide scaffold, in order to analyze the SARs of the new series. Examination of the binding data for the novel compounds reveals some potent and highly selective ligands for the CB_2 receptor, e.g. compounds **23, 26-28, 32** and **39** (Table 1). It is worth noting that the new derivatives although showed decreased in affinity for CB_2 receptor in comparison to the reference compound (**10**), highlighted a higher selectivity profile versus CB_1 receptor. Introduction of cycloheptyl and 3,5-dimethyladamantan-1-yl groups ((**15** and **16**, respectively) produced drastic decrease

in affinity and selectivity at CB_2 receptor compared with the parent compound **9** bearing the adamantyl moiety. The same results were obtained by the 2-phenyl derivatives **19-21**, when the adamantly carboxamide was replaced, showing lower affinity and selectivity in comparison with **10.** The best affinity and selectivity profile was observed by compound **21** (K_iCB_2 = 8.4 nM, K_iCB_1 = 653 nM, K_iCB_1/ K_iCB_2 = 77). Introduction of a methyl chain at position 2, such as in compounds **17** and **18**, resulted in a substantial reduction of affinity and a total loss of selectivity for both derivatives. Intriguingly, these results are in contrast with those of the previously pyrazolo[3,4-*b*]pyridone series [10], where the most active compounds bore a methyl group or an hydrogen atom, indicating a probable different binding mode of this new series to the receptor. Subsequently, analogues by introduction of 4-substituted-phenyl at the C-2 of the triazolopyrimidine nucleus were prepared, while the cyclohexyl, cycloheptyl, and adamantyl moieties on the carboxamide were maintained (compounds **22-28,** Table 1). The 4-chlorophenyl group at C-2 of the triazolopyrimidine nucleus (compounds **22** and **23**) resulted in a decreased of affinity and selectivity for the cyclohexylcarboxamide **22** compared to the parent phenyl analogue **19.** The adamantylcarboxamide derivative **23** resulted in a 10-fold decrease in affinity for the CB_1 receptor (**23**: K_iCB_1 = 5216 nM versus **10**: K_iCB_1 = 523 nM), showing higher selectivity and comparable affinity for CB_2 receptor relative to that of analogue compound **10** (**23**: K_iCB_2 = 11.3 nM, **10**: K_iCB_2 = 3.5 nM).

Changing of the chlorine atom with an electron donating group, such as methoxy, resulted in derivatives **24** and **25**, which showed lower affinity for the CB_2 receptors (K_iCB_2 = 96 and 47 nM, respectively) in comparison with the phenyl analogue (**10**). Introduction of a 4-methylphenyl group at C-2, as with compounds **26-28**, increased selectivity in comparison with **10**, and the best results were obtained with adamantylamide derivative **28** that showed high affinity and selectivity at CB_2 receptor (K_iCB_2 = 6.8 nM, K_iCB_1/ K_iCB_2 = 223). These results seem to indicate that electronic effects on the aromatic moiety do not significantly affect the binding for CB_2 receptors confirming our previously results found for the pyrazolopyrimidine series [11]. Replacement of the 2-phenyl ring of **10** with a bioisosteric heterocycle such as 2-furyl (**29**) resulted in a decrease of both affinity

and selectivity at the CB receptors (Table 1, K_iCB_2 = 45 nM, K_iCB_1/ K_iCB_2 = 26). Substitution of the 5-membered heterocycle with a 6-membered aromatic ring such as 3-pyridyl moiety (**30**) determined a dramatic loss of affinities at both CB receptors (K_iCB_2 = 228 nM, K_iCB_1 > 10000 nM).

Replacement of the aromatic ring of the C-2 position by additional substitution on the heterocyclic nucleus had a profound effect on affinity versus CB_1 receptor as shown by compounds **31-44** with a K_iCB_1 > 10000 nM (Table 1). The introduction of a thiomethyl at the C-2 position (compounds **31, 32**) significantly alters affinity at the CB_2 receptor relative to that of parent compounds **19** and **10**, respectively (K_i = 716 and 47 versus 43 and 3.5 nM), although the selectivity increased about 1.5-fold for **32** (K_iCB_1/K_iCB_2 > 213 versus K_iCB_1/ K_iCB_2 = 146). Among the 2-morpholino derivatives, the highest affinity and selectivity was observed with the 6-adamantylcarboxamide **35** (K_iCB_2 = 53 nM, K_iCB_1/K_iCB_2 > 188), while neither the 2-piperazine compounds (**36**, **37)** nor the diallylamine derivatives (**40**, **41**) showed affinity against the CB_2 receptor.

The enhancement in affinity and selectivity was obtained with the 2-N-methylbenzyl chain and adamantyl moiety on the 6-carboxamide group (**39**, K_iCB_2 = 21 nM, K_iCB_1/K_iCB_2 = 476). This compound was the most selective of whole series probably because of different binding mode into CB_2 receptor due to the shape and volume of the C-2 substituent as shown by molecular modeling studies (Figure 3).

Human CB_2 receptor functional assay

The most active compounds of this series and previously derivatives **9** and **10** were analyzed in cAMP assays for evaluation of their effect on cAMP production. The new ligands, as well as compound 10, revealed an inverse agonist behavior, as they were able to significantly increase cAMP production, with the exception of compound **16** which acted as partial agonist (Figure 1). The best values of Emax have been obtained by compounds **21**, **23** and **28**, that increased the second messenger production by 243%, 223% and 256% at 10 μM, respectively. It is noteworthy that the same compounds were also identified as the most active derivatives in binding assays, revealing a good

correlation between affinity and efficacy of the developed series. The remaining compounds provided a comparable trend, since cAMP production increase was obtained within 53% and 135% at 1μM, and from 132% to 185% at 10 μM, respectively. Interestingly, the new 2-unsubstituted triazolopyrimidine **16** acted as partial agonist as same as its parent compound **9** with values of efficacies (E_{max}) = 57 and 60%, respectively. Moreover, the potency value of **16** measured in functional assays is in the nanomolar range (EC_{50} = 88 nM) and strictly correlated with the binding affinity value. The difference in the functional activity between the 2-substituted derivatives and the compounds lacking this substituent indicates that introduction of structural modifications at C-2 position plays a crucial role in establishing the functionality of the novel cannabinoid ligands switching their activity to inverse agonism. Furthermore, the antagonism of the new compounds was investigated, evaluating their capability to counteract WIN55,212-2 inhibition of forskolin-stimulated cAMP production. The IC_{50} values obtained with the concentration-response curves of the new cannabinoids ligands were strictly correlated with their affinity values (Figure 2).

Table 1 should be inserted here

Figure 1 should be inserted here

Figure 2 should be inserted here

Molecular modeling studies

The synthesized compounds were docked into the inactive CB_2 and CB_1 receptor models by using AUTODOCK4.2 [20], and for each ligand, the top-scored pose belonging to the cluster of solutions with the best average of estimated free energy was considered. The resulting ligand-protein complexes were then energy minimized with AMBER11 [21] in a lipid bilayer and water environment, since molecular dynamic simulation studies carried out on the potent AMG3 CB ligand suggested that the lipid bilayer environment can affect the binding of ligands to CB receptors [22,23]. Figure 3A shows the minimized structures of the receptor-ligand complexes obtained by docking com-

pound **28**, presenting the highest CB_2 affinity, into both CB_2 and CB_1 receptor models. In the CB_2 receptor model the triazolopyrimidine core of the ligand is placed between the α-helixes belonging to the third and the sixth transmembrane domains (TM3 and TM6), predominantly interacting with M6.55(265) and L6.59(269) through hydrophobic interactions and forming an H-bond with T3.33(114). The *n*-pentyl chain interacts into a sort of small lipophilic channel mainly delimited by L3.26(107), L3.27(108) and L6.59(269), and is directed towards the TM4 α-helix, taking contacts with P4.60(168) and L4.61(169). The adamantyl ring interacts with V4.56(164), P4.60(168), Y5.39(190), W5.43(194) and feels the effects of a strong van der Waals interaction with F5.46(197), which is a nonconserved residue (V5.46 in the CB_1 receptor) and could thus contribute to the selectivity of these ligands for CB_2 versus CB_1 receptor, consistently with site-directed mutagenesis studies. [24,25]. Finally, the 4-CH_3-phenyl moiety directed toward the α-helixes of the second and the seventh transmembrane domains (TM2 and TM7) is located in a small lipophilic cavity mainly delimited by F2.57(87), I3.29(110), L6.54(264) and F7.35(281). In the CB_1 receptor model, compound **28** shows a binding pose in which it is orthogonally oriented with respect to its disposition into the CB_2 receptor (Figure 3B), thus interacting only with four transmembrane domains (TM3-TM6). More in details, the 4-CH_3-phenyl moiety of the ligand is placed between TM3 and TM4 α-helixes, surrounded by L3.26(190), F3.27(191) and L4.61(252) with which it establishes van der Waals contacts, whereas the *n*-pentyl chain points toward TM6 interacting mainly with V6.59(367). The central scaffold and the adamantyl ring of the ligand are placed in the hydrophobic pocket primarily constituted by L3.29(193), T3.33(197), I4.56(247), A4.57(248), P4.60(251), and W5.43(279), thus forming lipophilic interactions with these residues. Due to its disposition into CB_1 receptor the ligand not only lacks the strong van der Waals interactions with residue 5.46 (which is V282 instead of F197, as in CB_2) but it is not even able to form any H-bond with T3.33(197) through its triazolopyrimidine core. These two differences, together with the fewer hydrophobic contacts established within the CB_1 receptor, could be the main responsible for the selectivity for CB_2 versus CB_1 receptor shown by compound **28** and, in general, for this series of ligands.

Figure 3 should be inserted here

Figure 4 should be inserted here

Ligands such as compound **18**, bearing no substituents or only a methyl group in position 2 of the central scaffold, show a general decreased CB_2 affinity with respect to ligands presenting phenyl or aryl groups in the same positions. The energy minimized complex of compound **18** into CB_2 receptor (Figure 4A) shows a very similar binding mode for this ligand with respect to the one predicted for compound **28**. The two ligands share an almost identical disposition of their *n*-pentyl chains and adamantyl ring, which thus take van der Waals contacts with the same residues. Anyway, compound **18** is not able to fill the small lipophilic cavity mainly delimited by F2.57(87), I3.29(110), L6.54(264) and F7.35(281), showing only limited contacts with I3.29(110) and L6.64(264). This could be at the basis of the reduced affinity for CB_2 receptor showed by this ligand and its analogues lacking of larger substituents in C-2 position. Compound **18** shares with compound **28** also a similar binding pose into CB_1 receptor (Figure 4B). Anyway, the smaller C-2 substituent of this ligand allows it to adopt a slightly different orientation where the methyl group points toward the side chain of L4.61(252) and the triazole ring of the central scaffold is closer to P4.60(251). In this disposition, the ligand is able to form an H-bond with T3.33(197) through the carbonyl oxygen of its central scaffold; this interaction could constitute the reason of the highest affinity of this compound for CB_1 receptor with respect to what observed for compound **28** and generally for all those ligands bearing bulkier C-2 substituents that wouldn't allow such disposition due to steric bumps with F3.27(191) and L4.61(252) side chains. Compound **39**, bearing a *N*-benzyl-*N*-methyl group in 2 position of the central scaffold showed a strong affinity for CB_2 receptor and the highest selectivity versus CB_1 receptor among this series of compounds. As shown in Figure 5A, the ligand adopts a different binding mode into CB_2 receptor with respect to the previously analyzed ligands, due to the shape and volume of the C-2 substituent that cannot be placed into the small hydrophobic pocket formed by F2.57(87), I3.29(110), L6.54(264) and F7.35(281). Nevertheless, the compound is still

able to establish the strong van der Waals interactions with F5.46(197) through its adamantyl ring, which shows the same location within the receptor as for compounds **18** and **28**. Moreover, the ligand forms an H-bond with T3.33(114) through the carbonyl oxygen of its amide moiety, thus maintaining both the interactions that are thought to be responsible of the CB_2 versus CB_1 selectivity of these ligands. Finally, the C-2 substituent of the compound is efficiently positioned into the hydrophobic channel mainly delimited by L3.26(107), L3.27(108), L4.61(169) and Y5.39(190), forming lipophilic interactions with all these residues and, in particular, a strong π-π stacking with Y5.39(190). The energy minimized complex of compound **39** docked into CB_1 receptor model (Figure 5B) shows that the ligand does not interact as in the CB_2 receptor model. The compound shows a binding mode similar to that observed for **28** and **18** into the CB_1 receptor model, with a slight rotation of the central core in order to place the bulky benzyl moiety between TM3 and TM4 α-helixes. Adopting this disposition the ligand is not able to form any H-bond with T3.33(197) and although it maintains a good interaction with F3.27(191) through the benzyl group, it shows reduced contacts with L3.26(190) and V6.59(367), as well as weaker hydrophobic interactions with T3.33(197), A3.34(198) and I4.65(247) through the adamantyl ring, with respect to compounds **28** and **18**. This could explain the low CB_1 affinity of compound **39** and thus its strong CB_2 versus CB_1 selectivity.

Figure 5 should be inserted here

Conclusions

In order to progress the CB_2 receptor affinity and selectivity and to develop structure activity relationship for the series of triazolo[1,5-*a*]pyrimidine-6-carboxamide, we synthesized and evaluated twenty-seven new derivatives belonging to this chemical class. Structural modifications around the central scaffold, especially at C-2 and C-6 positions, led us to achieve greater selectivity, with respect to the parent compounds **9** and **10**. The best affinity values associated with high selectivity were obtained with compounds **23** (K_iCB_2 = 11.3 nM; K_iCB_1/K_iCB_2 = 462) and **39** (K_iCB_2 = 21

nM; $K_iCB_1/K_iCB_2 > 476$) bearing an adamantan-1-yl moiety on the carboxamide function and a 4-Cl-phenyl or N-benzyl-N-methylamine residues at C-2, respectively. cAMP experiments on CB_2 receptors expressed in CHO cells surprisingly revealed a different behavior, basing on the substitutions at C-2. Compound **16** as its parent **9** lacking the substituent in position C-2 of the triazolepyrimidine nucleus showed partial agonist activities. 2-Substituted derivatives provided an inverse agonism behavior, with high efficacy levels from 152% to 246% of increase in cAMP production. Thus, this novel series of compounds proposes an attractive starting point for more optimization, representing novel pharmacological tools to evaluate the therapeutic potential of CB_2 inverse agonists in diverse disease settings.

Chemistry

Materials and methods

Positive ion electrospray ionization (ESI) mass spectra were performed with an Agilent 1100 Series LC/MSD model. All melting points are uncorrected and were assigned by a Buchi-Tottoli apparatus. The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on either a Bruker AC 200 (200MHz) or a Varian Mercury Plus 400 (400MHz) spectrometer. Chemical shifts are reported in δ values (parts per million), using appropriate deuterated solvents (DMSO-d_6 and $CDCl_3$). The purity of tested compounds was determined by combustion elemental analyses performed by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara with a Yanagimoto MT-5 CHN recorder elemental analyzer. All tested compounds obtained data consistent with a purity of at least 95% as compared with the theoretical values.

Reactions were monitored by TLC (Thin Layer Chromatography) on silica gel (precoated Merck F254 plates), and compounds visualization was performed by UV light. Flash chromatography was accomplished using 230-400 mesh silica gel and a mixture of ethyl acetate and petroleum ether or ethyl acetate and methanol in different ratios as eluting phase. Organic extracts were dried over an-

hydrous sodium sulfate (Na_2SO_4). All starting materials, reagents and solvents were obtained from Sigma-Aldrich or Alfa Aesar.

*General procedure A for the synthesis of compounds **11c-h***

A solution of *S*-methylisothiourea hemisulfate salt (22 mmol), appropriate hydrazide (22 mmol), and water (35 mL) was refluxed for 6 h. The solvent was concentrated under reduced pressure and the residue was washed with ethanol (3 x 10 mL). The resulting solid was poured into 10 mL of 10% NaOH and heated to reflux for additional 6 h. The reaction was cooled to room temperature and adjusted to pH 5 using 1N HCl. The precipitate was filtered, washed with cold water and dried.

*5-Phenyl-2H-1,2,4-triazol-3-amine (**11c**).* Following general procedure A, compound **11c** was isolated as a white solid. Yield 82%, mp = 233-234 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.58 (br s, 1H); 7.97-7.85 (m, 2H); 7.62-7.35 (m, 3H); 5.97 (br s, 2H).

*5-(4-Chlorophenyl)-2H-1,2,4-triazol-3-amine (**11d**).* Following general procedure A, compound **11d** was isolated as a white solid. Yield 90%, mp = 229-230 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.75 (br s, 1H); 7.95-7.89 (d, *J* = 8.6 Hz, 2H); 7.58-7.52 (d, *J* = 8.6 Hz, 2H); 5.11 (br s, 2H).

*5-(4-Methoxyphenyl)-2H-1,2,4-triazol-3-amine (**11e**).* Following general procedure A, compound **11e** was isolated as pale yellow solid. Yield 87%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.85 (br s, 1H); 7.80-7.78 (d, *J* = 9 Hz, 2H); 6.96-6.94 (d, *J* = 8.4 Hz, 2H); 5.98 (br s, 2H); 3.77 (s, 3H).

*5-p-Tolyl-2H-1,2,4-triazol-3-amine (**11f**).* Following general procedure A, compound 11f was isolated as a white solid. Yield 78%, mp = 190-193 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.21 (br s, 1H); 7.78-7.73 (d, *J* = 8.2 Hz, 2H); 7.21-7.17 (d, *J* = 8 Hz , 2H); 5.91 (br s. 2H); 2.31 (s, 3H).

*5-(Furan-2-yl)-2H-1,2,4-triazol-3-amine (**11g**).* Following general procedure A, compound **11g** was isolated as a white solid. Yield 82%, mp = 225-226 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.11 (br s, 1H); 7.60 (d, *J* = 1 Hz, 1H); 6.67 (d, *J* = 3.4 Hz, 1H); 6.53 (dd, *J* = 3.4 and 1.5 Hz, 1H); 6.07 (br s, 2H).

*5-(Pyridin-3-yl)-2H-1,2,4-triazol-3-amine (**11h**).* Following general procedure A, compound **11h** was isolated as a white solid. Yield 75%, mp = 203-204 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.23 (br s, 1H); 9.04-9.03 (d, *J* = 1.6 Hz, 1H); 8.54-8.52 (dd, *J* = 5.4 and 1.5 Hz, 1H); 8.18-8.15 (dt, *J* = 8 and 1.6 Hz, 1H); 7.44-7.41 (dd, *J* = 7.8 and 4.9 Hz, 1H); 6.17 (br s, 2H).

*General procedure B for the synthesis of compounds **11i-m***

A solution of *N*-cyanocarbonimidodithioic acid dimethyl ester (17.4 mmol), acetonitrile (10 mL), and appropriate amine (17.4 mmol) was refluxed for 2 h. After cooling to room temperature, hydrazine monohydrate (25.6 mmol) was added and the reaction mixture was further refluxed for additional 5 h. The solvent was evaporated under reduced pressure and the resulting product recrystallized from ethyl acetate.

*5-(Methylthio)-2H-1,2,4-triazol-3-amine (**11i**).* Following general procedure B, compound **11i** was isolated as a white solid. Yield 90%, mp = 134 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 11.78 (br s, 1H); 5.95 (br s, 2H); 2.39 (s, 3H).

*5-Morpholino-2H-1,2,4-triazol-3-amine (**11j**).* Following general procedure B, compound **11j** was isolated as a white solid. Yield 82%, mp = 161-162 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 10.98 (br s, 1H); 5.71 (br s, 2H); 3.64-3.59 (m, 4H); 3.13-3.08 (m, 4H).

*5-(4-Methylpiperazin-1-yl)-2H-1,2,4-triazol-3-amine (**11k**).* Following general procedure B, compound **11k** was isolated as a pale yellow solid. Yield 80%, mp = 103-104 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 10.95 (br s, 1H); 5.72 (br s, 2H); 3.16-3.11 (m, 4H); 2.34-2.29 (m, 4H); 2.17 (s, 3H).

*N-benzyl-N-methyl-1H-1,2,4-triazole-3,5-diamine (**11l**).* Following general procedure B, compound **11l** was isolated as a white solid. Yield 86%, mp = 163-164 °C. 1H NMR (200 MHz, DMSO-d6) δ: 10.85 (br s, 1H); 7.27-7.23 (m, 5H); 5.75 (br s, 2H); 4.42 (s, 2H); 2.70 (s, 3H).

*N,N-Diallyl-1H-1,2,4-triazole-3,5-diamine (**11m**).* Following general procedure B, compound 11m was isolated as a white solid. Yield 95%, mp = 244-245 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ:

10.75 (br s, 1H); 5.82-5.69 (m, 2H); 5.54 (br s, 2H); 5.16-5.02 (m, 4H); 3.81-3.78 (d, *J* = 5.6 Hz, 4H).

General procedure C for the synthesis of ethyl 4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylates ***12a-m***

A solution of aminotriazole **11a-m** (3.1 mmol), glacial acetic acid (5 mL), and diethyl ethoxymethylenemalonate (4.6 mmol) was refluxed for 3 h. After cooling to room temperature, the resulting precipitate was collected by filtration, washed with cold water and dried. The residue was stirred with cold ethyl ether (20 mL) and filtered to afford the desired product.

*Ethyl 4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12a**).* Following general procedure C, compound **12a** was isolated as a white solid. Yield 76%, mp > 300 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 14.30 (br s, 1H); 8.95 (s, 1H); 8.84 (s, 1H); 4.20 (q, *J* = 6.8 Hz, 2H); 1.29 (t, *J* = 6.8 Hz, 3H).

*Ethyl 4,7-dihydro-2-methyl-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12b**).* Following general procedure C, compound **12b** was isolated as a pale yellow solid 70%, mp = 237 °C dec. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.9 (br s, 1H); 8.58 (s, 1H); 4.28-4.17 (q, *J* = 7.4 Hz, 2H); 2.38 (s, 3H); 1.31-1.27 (t, *J* = 7 Hz, 3H).

*Ethyl 4,7-dihydro-2-phenyl-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12c**).* Following general procedure C, compound **12c** was isolated as a white solid. Yield 67%, mp > 300 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 14.00 (br s, 1H); 8.64 (s, 1H); 8.14–8.10 (m, 2H); 7.55 (m, 3H); 4.26 (q, *J* = 7.0 Hz, 2H); 1.28 (t, *J* = 6.8 Hz, 3H).

*Ethyl 2-(4-chlorophenyl)-4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12d**).* Following general procedure C, compound **12d** was isolated as a pale yellow solid. Yield 60%, mp > 300 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 13.41 (br s, 1H); 8.65 (s, 1H); 8.14-8.10 (d, *J* = 8.4 Hz, 2H); 7.63-7.59 (d, *J* = 8.6 Hz, 1H); 4.29-4.22 (q, *J* = 7 Hz, 2H); 1.33-1.25 (t, *J* = 7.4 Hz, 3H).

*Ethyl 2-(4-methoxyphenyl)-4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12e**).* Following general procedure C, compound **12e** was isolated as a pale yellow solid. Yield

65%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.25 (br s, 1H); 8.61 (s, 1H); 8.06-8.04 (d, *J* = 8.8 Hz, 2H); 7.10-7.08 (d, *J* = 8.8 Hz, 1H); 4.25-4.23 (q, *J* = 7.2 Hz, 2H); 3.83 (s, 3H); 1.31-1.27 (t, *J* = 7.2 Hz, 3H).

*Ethyl 4,7-dihydro-7-oxo-2-p-tolyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12f**).* Following general procedure C, compound **12f** was isolated as a pale yellow solid. Yield 70%, mp = 268 °C dec. ^{1}H NMR (200 MHz, DMSO-d_6) δ. 13.91 (br s, 1H); 8.63 (s, 1H); 8.03-7.99 (d, *J* = 8 Hz, 2H); 7.37-7.32 (d, *J* = 8.2 Hz, 2H);4.27-4.23 (q, *J* = 7 Hz, 2H); 2.38 (s, 3H); 1.33-1.26 (t, *J* = 7.4 Hz, 3H).

*Ethyl 2-(furan-2-yl)-4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12g**).* Following general procedure C, compound **12g** was isolated as a white solid. Yield 75%, mp > 300 °C. ^{1}H NMR (200 MHZ-DMSO-d_6) δ: 13.24 (br s, 1H); 8.59 (s, 1H); 7.88-7.87 (d, *J* = 1 Hz, 1H); 7.11-7.09 (d, *J* = 3.4 Hz, 1H); 6.67-6.65(d, *J* = 3.4 Hz, 1H); 4.23-4.20 (q, *J* = 7.2 Hz, 2H); 1.31-1.24 (t, *J* = 7 Hz, 3H).

*Ethyl 4,7-dihydro-7-oxo-2-(pyridin-3-yl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12h**).* Following general procedure C, compound **12h** was isolated as a white solid. Yield 65%, mp = 250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (br s, 1H); 9.27 (d, *J* = 1.6 Hz, 1H); 8.74-8.72 (dd, *J* = 6.4 and 1.6 Hz, 1H); 8.67 (s, 1H); 8.48-8.46 (dt *J* = 6.9 and 1.7 Hz,1H); 7.62-7.60 (dd, *J* = 7 and 5.1 Hz, 1H); 4.28-4.23 (q, *J* = 7.2 Hz, 2H); 1.31-1.27 (t, *J* = 7.2 Hz, 3H).

*Ethyl 4,7-dihydro-2-(methylthio)-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12i**).* Following general procedure C, compound **12i** was isolated as a white solid. Yield 70%, mp = 277 °C dec. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.41 (br s, 1H); 8.56 (s, 1H); 4.27-4.20 (q, *J* = 7 Hz, 2H); 2.60 (s, 3H); 1.31-1.24 (t, *J* = 7.2 Hz, 3H).

*Ethyl 4,7-dihydro-2-morpholino-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12j**).* Following general procedure C, compound **12j** was isolated as a white solid. Yield 75%, mp = 283-284 °C dec. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.5 (br s, 1H); 8.45 (s, 1H); 4.23-4.20 (q, *J* = 7.2 Hz, 2H); 3.70-3.66 (m, 4H); 3.41-3.37 (m, 4H); 1.30-1.23 (t, *J* = 7.2 Hz, 3H).

*Ethyl 4,7-dihydro-2-(4-methylpiperazin-1-yl)-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12k**).* Following general procedure C, compound **12k** was isolated as a white solid. Yield 78%, mp = 220 °C dec. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 9.95 (br s, 1H); 8.41 (s, 1H); 4.17-4.07 (q, *J* = 7 Hz, 2H); 3.78-3.45 (m, 4H); 2.76 (s, 3H); 2.52-2.49 (m, 4H); 1.26-1.19 (t, *J* = 7.2 Hz, 3H).

*Ethyl 2-(N-benzyl-N-methylamino)-4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylates (**12l**).* Following general procedure C, compound **12l** was isolated as a white solid. Yield 69%, mp = 284 °C. ^{1}H NMR (200 MHz, DMSO-d6) δ: 13.37 (br s, 1H); 8.42 (s, 1H); 7.30-7.28 (m, 5H); 4.64 (s, 2H); 4.25-4.17 (q, J = 7 Hz, 2H); 2.95 (s, 3H); 1.30-1.23 (t, J = 7 Hz, 3H).

*Ethyl 2-(diallylamino)-4,7-dihydro-7-oxo-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**12m**).* Following general procedure C, compound **12m** was isolated as a white solid. Yield 73%, mp = 240-241 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 13.91 (br s, 1H); 8.41 (s, 1H); 5.93-5.76 (m, 2H); 5.21-5.13 (m, 4H); 4.26-4.16 (q, *J* = 7.2 Hz, 2H); 4.02-3.99 (d, *J* = 6.2 Hz, 4H); 1.30-1.23 (t, *J* = 7.2 Hz, 3H).

*General procedure D for the synthesis of ethyl 4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylates **13a-m***

To a solution of appropriate carboxylic acid ethyl ester **12a-m** (0.54 mmol) in anhydrous *N*,*N*-dimethylformamide was added K_2CO_3 (1.62 mmol), and the mixture was stirred at 70 °C for 1 h. 1-Pentyl bromide (1.62 mmol) was added, and the mixture was heated at 100 °C for 16 h. The reaction mixture was cooled to room temperature, the solvent was evaporated under reduced pressure and the residue partitioned between water (20 mL) and ethyl acetate (60 mL). The organic phase was dried was dried (Na_2SO_4), and after removal of the solvent, the residue was purified by flash column chromatography, eluting with ethyl acetate/petroleum ether or ethyl acetate/methanol.

*Ethyl 4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13a**).* Following general procedure D, compound **13a** was isolated as a white solid. Eluent: petroleum ether-ethyl ac-

etate 3/2. Yield 75%, mp = 85-86 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.88 (s, 1H), 8.33 (s, 1H), 4.32-4.22 (m, 4H), 1.95-1.79 (m, 2H), 1.33-1.26 (m, 7H), 0.89-0.82 (t, *J* = 6.8 Hz, 3H).

*Ethyl 4,7-dihydro-2-methyl-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13b**).* Following general procedure D, compound **13b** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield 78%, mp = 151-152 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.82 (s, 1H), 4.30-4.21 (m, 4H), 2.39 (s, 3H), 1.91-1.77 (m, 2H), 1.32-1.25 (m, 7H), 0.89-0.84 (t, *J* = 6.8 Hz, 3H).

*Ethyl 4,7-dihydro-7-oxo-4-pentyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13c**).* Following general procedure D, compound **13c** was isolated as a pale yellow solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield 75%, mp = 112-113 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.87 (s, 1H), 8.16-8.11 (m, 2H), 7.56-7.53 (m, 3H), 4.37-4.23 (m, 4H), 1.93-1.81 (m, 2H), 1.37-1.26 (m, 7H), 0.91-0.85 (t, *J* = 6.6 Hz, 3H).

*Ethyl 2-(4-chlorophenyl)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13d**).* Following general procedure D, compound **13d** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 78%, mp = 155-156 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.88 (s, 1H); 8.16-8.11 (d, *J* = 8.6 Hz, 2H); 7.64-7.60 (d, *J* = 8.4 Hz, 2H); 4.32-4.23 (m, 4H); 1.96-1.83 (m, 2H); 1.36-1.27 (m, 7H); 0.91-0.84 (t, *J* = 6.8 Hz, 3H).

*Ethyl 4,7-dihydro-2-(4-methoxyphenyl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13e**).* Following general procedure D, compound **13e** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 80%, mp = 147-148 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.85 (s, 1H); 8.07-8.05 (d, *J* = 8.8 Hz, 2H); 7.10-7.08 (d, *J* = 8.8 Hz, 2H); 4.33-4.25 (m, 4H); 3.83 (s, 1H); 1.94-1.81 (m, 2H); 1.34-1.29 (m, 7H); 0.90-0.86 (t, *J* = 7.2 Hz, 3H).

*Ethyl 4,7-dihydro-7-oxo-4-pentyl-2-p-tolyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13f**).* Following general procedure D, compound **13f** was isolated as a pale yellow solid. Eluent: petroleum ether-ethyl acetate 1/4. Yield 82%, mp = 216-217 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.86

(s, 1H), 8.03-8.01 (d, *J* = 8 Hz, 2H), 7.36-7.34 (d, *J* = 7.6 Hz, 2H), 4.32-4.25 (m, 4H), 2.38 (s, 3H), 1.91-1.72 (m, 2H), 1.35-1.29 (m, 7H), 0.89-0.86 (t, *J* = 6.8 Hz, 3H).

Ethyl 2-(furan-2-yl)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate ***(13g)***. Following general procedure D, compound **13g** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield **77**%, mp = 151-152 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.86 (s, 1H); 7.94-7.93 (d, *J* = 1.6 Hz, 1H); 7.20-7.18 (d, *J* = 3.4 Hz, 1H); 6.72-6.69 (dd, *J* = 3.4 and 1.6 Hz, 1H); 4.33-4.23 (m, 4H); 1.93-1.79 (m, 2H); 1.33-1.26 (m, 7H); 0.90-0.84 (t, *J* = 6.6 Hz, 3H).

Ethyl 4,7-dihydro-7-oxo-4-pentyl-2-(pyridin-3-yl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate ***(13h).*** Following general procedure D, compound **13h** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield **77**%, mp = 104-105 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.46 (d, *J* = 1.6 Hz, 1H); 8.72-8.70 (dd, *J* = 6.6 and 1.6 Hz, 1H); 8.57-8.55 (dt, *J* = 7.8 and 1.6 Hz, 1H); 8.47 (s, 1H); 7.42-7.39 (dd, *J* = 8 and 5 Hz, 1H); 4.45-4.39 (q, *J* = 7.2 Hz, 2H); 4.34-4.30 (t, *J* = 7.6 Hz, 2H); 2.01-1.97 (m, 2H); 1.43-1.39 (m, 7H); 0.95-0.92 (t, *J* = 7.2 Hz, 3H).

Ethyl 4,7-dihydro-2-(methylthio)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate ***(13i)***. Following general procedure D, compound **13i** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 81%, mp = 120-121 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.79 (s, 1H); 4.30-4.20 (m, 4H); 2.61 (s, 3H); 1.91-1.72 (m, 2H); 1.32-1.25 (m, 7H); 0.89-0.84 (t, *J* = 6.8 Hz, 3H).

Ethyl 4,7-dihydro-2-morpholino-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate ***(13j).*** Following general procedure D, compound **13j** was isolated as a white solid. Eluent: ethyl acetate-methanol 9.5/0.5. Yield 82%, mp = 113-114 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.68 (s, 1H); 4.30-4.13 (m, 4H); 3.71-3.66 (m, 4H); 3.43-3.38 (m, 4H); 1.95-1.72 (m, 2H); 1.31-1.24 (m, 7H); 0.89-0.83 (t, *J* = 6.6 Hz, 3H).

Ethyl 4,7-dihydro-2-(4-methylpiperazin-1-yl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate ***(13k)***. Following general procedure D, compound **13k** was isolated as a white solid. Eluent: ethyl acetate-methanol 9/1. Yield 70%, mp = 183-184 °C. ^{1}H NMR (200 MHz, DMSO-d_6)

δ: 8.67 (s, 1H); 4.26-4.16 (m, 4H); 3.45-3.42 (m, 4H); 2.41-2.37 (m, 4H); 2.20 (s, 3H); 1.96-1.79 (m, 2H); 1.31-1.24 (m, 7H); 0.89-0.82 (t, *J* = 6.8 Hz, 3H).

*Ethyl 2-(N-benzyl-N-methylamino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13l**)*. Following general procedure D, compound **13l** was isolated as a white solid. petroleum ether-ethyl acetate 1/4. Yield 82%, mp = 77-78 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.66 (s, 1H); 7.34-7.29 (m, 5H); 4.65 (s, 2H); 4.29-4.15 (m, 4H); 2.95 (s, 3H); 1.91-1.78 (m, 2H); 1.31-1.24 (m, 7H); 0.88-0.81 (t, J = 6.8 Hz, 3H).

*Ethyl 2-(diallylamino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**13m**)*. Following general procedure D, compound **13m** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/4. Yield 81%, mp = 70-72 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.65 (s, 1H); 5.87-5.76 (m, 2H); 5.23-5.14 (m, 4H); 4.25-4.16 (m, 4H); 4.03-4.00 (d, *J* = 5.8 Hz, 2H); 1.91-1.75 (m, 2H); 1.32-1.24 (m, 7H); 0.89-0.82 (t, *J* = 7 Hz, 3H).

*General procedure E for the synthesis of carboxylic acid derivatives **14a-m***

A solution of appropriate carboxylic acid ethyl ester **13a-m** (1.27 mmol), methanol (20 mL), and NaOH 10% (10 mL) was stirred at room temperature for 6 h. The suspension was acidified with 10% HCl, the precipitate was collected by filtration and washed with cold water to afford the desired product.

*4,7-Dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14a**)*. Following general procedure E, compound **14a** was isolated as a white solid. Yield 72%, mp = 215 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.12 (br s, 1H); 8.73 (s, 1H); 8.28 (s, 1H); 4.27-4.20 (t, *J* = 7 Hz, 2H); 1.84-1.77 (m, 2H); 1.32-1.25 (m, 4H); 0.88-0.81 (t, *J* = 6.6 Hz, 3H).

*4,7-Dihydro-2-methyl-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14b**)*. Following general procedure E, compound **14b** was isolated as a white solid. Yield 85%, mp = 164-165 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.37 (br s, 1H); 8.68 (s, 1H); 4.23-4.16 (t, *J* = 7.2 Hz, 2H); 2.38 (s, 3H); 1.81-1.73 (m, 2H); 1.30-1.25 (m, 4H); 0.88-0.81 (t, *J* = 6.8 Hz, 3H).

*4,7-Dihydro-7-oxo-4-pentyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14c**).* Following general procedure E, compound **14c** was isolated as a white solid. Yield 91%, mp >300 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ:12.24 (br s, 1H); 8.63 (s, 1H); 8.14-8.10 (m, 2H); 7.54-7.51 (m, 3H); 4.30-4.26 (t, *J* = 7 Hz, 2H); 1.98-1.81 (m, 2H); 1.39-1.27 (m, 4H); 0.89-0.82 (t, *J* = 6.6 Hz, 3H).

*2-(4-Chlorophenyl)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14d**).* Following general procedure E, compound **14d** was isolated as a white solid. Yield (87%), mp = 204-205 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.81 (br s, 1H); 8.92 (s, 1H); 8.16-8.12 (d, *J* = 8.6 Hz, 2H); 7.64-7.60 (d, *J* = 8.6 Hz, 2H); 4.37-4.30 (t, *J* = 7.2 Hz, 2H); 1.81-1.75 (m, 2H); 1.36-1.28 (m, 4H); 0.90-0.84 (t, *J* = 6.6 Hz, 3H).

*4,7-Dihydro-2-(4-methoxyphenyl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14e**).* Following general procedure E, compound **14e** was isolated as a white solid. Yield 86%, mp = 200-201 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.79 (br s, 1H); 8.90 (s, 1H); 8.08-8.06 (d, *J* = 8.8 Hz, 2H); 7.11-7.08 (d, *J* = 8.4 Hz, 2H); 4.35-4.31 (t, *J* = 7.2 Hz, 2H); 1.89-1.86 (m, 2H); 1.34-1.32 (m, 4H); 0.89-0.86 (t, *J* = 6.4 Hz, 3H).

*4,7-Dihydro-7-oxo-4-pentyl-2-p-tolyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14f**).* Following general procedure E, compound **14f** was isolated as a pale yellow solid. Yield 87%, mp = 207-208 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.65 (br s, 1H); 8.87 (s, 1H); 8.05-8.01 (d, *J* = 8.4 Hz, 2H); 7.37-7.33 (d, *J* = 8 Hz, 2H); 4.34-4.31 (t, *J* = 6.8 Hz, 2H); 2.38 (s, 3H); 1.96-1.79 (m, 2H); 1.35-1.30 (m, 4H); 0.90-0.84 (t, *J* = 6.6 Hz, 3H).

*2-(Furan-2-yl)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14g**).* Following general procedure E, compound **14g** was isolated as a pale yellow solid. Yield 87%, mp = 187-188 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.81 (br s, 1H); 8.90 (s, 1H); 7.95-7.94 (d, *J* = 1.8 Hz, 1H); 7.21-7.19 (d, *J* = 3.4 Hz, 1H); 6.72-6.70 (dd, *J* = 3.4 and 1.6 Hz, 1H); 4.32-4.26 (t, *J* = 6.8 Hz, 2H); 1.87-1.81 (m, 2H); 1.34-1.27 (m, 4H); 0.90-0.83 (t, *J* = 6.6 Hz, 3H).

*4,7-Dihydro-7-oxo-4-pentyl-2-(pyridin-3-yl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14h**).* Following general procedure E, compound **14h** was isolated as a white solid. Yield 86%, mp = 158-159 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.84 (br s, 1H); 9.28 (d, *J* = 1.6 Hz, 1H); 8.94 (s, 1H); 8.74-8.73 (dd, *J* = 6.4 and 1.8 Hz, 1H); 8.48-8.45 (dt, *J* = 8 and 1,6 Hz, 1H); 7.61-7.58 (dd, *J* = 8 and 4.8 Hz, 1H); 4.37-4.33 (t, *J* = 7.2 Hz, 2H); 1.90-1.87 (m, 2H); 1.37-1.32 (m, 4H); 0.89-0.86 (t, *J* = 6.8 Hz, 3H).

*4,7-Dihydro-2-(methylthio)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14i**).* Following general procedure E, compound **14i** was isolated as a white solid. Yield 98%, mp = 165-166 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.51 (br s, 1H); 8.78 (s, 1H); 4.22-4.19 (t, *J* = 6.8 Hz, 2H); 2.61 (s, 3H); 1.84-1.77 (m, 2H); 1.35-1.26 (m, 4H); 0.88-0.83 (t, *J* = 6.4 Hz, 3H).

*4,7-Dihydro-2-morpholino-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14j**).* Following general procedure E, compound **14j** was isolated as a white solid. Yield 98%, mp = 276-277 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.69 (br s, 1H); 8.38 (s, 1H); 4.13-4.09 (t, *J* = 6.8 Hz, 2H); 3.70-3.66 (m, 4H); 3.41-3.36 (m, 4H); 1.78-1.73 (m, 2H); 1.30-1.25 (m, 4H); 0.88-0.81 (t, *J* = 6.6 Hz, 3H).

*4,7-Dihydro-2-(4-methylpiperazin-1-yl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14k**).* Following general procedure E, compound **14k** was isolated as a white solid. Yield 81%, mp = 225 °C dec. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.72 (br s, 1H); 8.73 (s, 1H); 4.21-4.17 (t, *J* = 6.6 Hz, 2H); 3.50-3.47 (m, 4H); 3.38-3.33 (m, 4H); 2.28 (s, 3H); 1.81-1.77 (m, 2H); 1.29-1.26 (m, 4H); 0.88-0.82 (t, *J* = 6.6 Hz, 3H).

*2-(N-Benzyl-N-methylamino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14l**).* Following general procedure E, compound **14l** was isolated as a white solid. Yield 94%, mp = 130 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.71 (br s, 1H); 8.76 (s, 1H); 7.33-7.24 (m, 5H); 4.64 (s, 2H); 4.22-4.19 (t, J = 7.2 Hz, 2H); 2.95 (s, 3H); 1.81-1.78 (m, 2H); 1.29-1.24 (m, 4H); 0.84-0.81 (t, J = 6.8 Hz, 3H).

*2-(Diallylamino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid (**14m**).* Following general procedure E, compound **14m** was isolated as a white solid. Yield 95%, mp = 142-143 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.95 (br s, 1H); 8.77 (s, 1H); 5.92-5.78 (m, 2H); 5.23-5.15 (m, 4H); 4.24-4.17 (t, *J* = 6.8 Hz, 2H); 4.05-4.02 (d, *J* = 5.6 Hz, 4H); 1.84-1.79 (m, 2H); 1.30-1.26 (m, 4H); 0.88-0.82 (t, *J* = 6.6 Hz, 3H).

*General procedure F for the synthesis of adamantan-1-yl-carboxamide derivatives **16**, **18**, **21**, **23**, **25**, **28-30**, **32**, **35**, **39**, **41***

To a stirred solution of the appropriate carboxylic acid (0.72 mmol) in anhydrous dimethylformamide (10 mL) was added diisopropylethylamine (3.24 mmol). The resulting solution was stirred at room temperature for 10 min before addition of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU, 1.08 mmol). The solution was stirred for another 3 h at room temperature. 1-Adamantylamine or 3,5-dimethyl-1-adamantylamine (1.08 mmol) was added and stirring was continued for 16 h. The solvent was removed under reduce pressure, the residue was dissolved in ethyl acetate (50 mL), then washed with saturated aqueous sodium bicarbonate (10 mL), water (10 mL), and brine (10 mL). The organic extract was dried (Na_2SO_4), filtered, and evaporated to dryness, and the residue was purified by flash chromatography on silica gel, eluting with ethyl acetate-petroleum ether.

*4,7-Dihydro-N-(3,5-dimethyladamantan-1-yl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**16**).* Following general procedure F, compound **16** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 60%, mp = 182 °C. MS (ESI): m/z 412.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.82 (s, 1H); 8.67 (br s, 1H); 8.38 (s, 1H); 4.33-4.30 (t, *J* = 7 Hz, 2H); 2.18-2.04 (m, 1H); 1.90-1.65 (m, 9H); 1.38-1.24 (m, 9H); 0.89-0.82 (m, 9H). Anal. for $C_{23}H_{33}N_5O_2$. Calcd: C, 67.12; H, 8.08; N, 17.02. Found: C, 67.22; H, 8.18; N, 17.051.

*N-Adamantan-yl-4,7-dihydro-2-methyl-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**18**).* Following general procedure F, compound **18** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 3/2. Yield 56%, mp = 189-190 °C. MS (ESI): m/z 398.2 (M+H).

^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.78 (s, 1H); 8.68 (br s, 1H); 4.30-4.22 (t, J = 7 Hz, 2H); 2.41 (s, 3H); 2.05 (s, 9H); 1.93-1.81 (m, 2H); 1.66 (s, 6H); 1.34-1.24 (m, 4H); 0.89-0.82 (t, J = 6.4 Hz, 3H). Anal. for $C_{22}H_{31}N_5O_2$. Calcd: C, 66.47; H, 7.86; N, 17.62. Found: C, 66.22; H, 7.73; N, 17.37.

*4,7-Dihydro-N-(3,5-dimethyladamantan-1-yl)-7-oxo-4-pentyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**21**).* Following general procedure F, compound **21** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 55%, mp = 96 °C. MS (ESI): m/z 488.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.81 (s, 1H); 8.72 (br s, 1H); 8.16-8.13 (m, 2H); 7.57-7.54 (m, 3H); 4.39-4.35 (t, J = 7 Hz, 2H); 2.19-2.07 (m, 1H); 1.93-1.68 (m, 9H); 1.39-1.18 (m, 9H); 0.91-0.84 (m, 9H). Anal. for $C_{29}H_{37}N_5O_2$. Calcd: C, 71.43; H, 7.65; N, 14.36. Found: C, 71.34; H, 7.60; N, 14.29.

*N-Adamantan-1-yl-2-(4-chlorophenyl)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**23**).* Following general procedure F, compound **23** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 53%, mp = 213 °C. MS (ESI): m/z 494.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.85 (s, 1H); 8.69 (br s, 1H); 8.16-8.12 (d, J = 8.6 Hz, 2H); 7.65-7.61 (d, J = 8.4 Hz, 2H); 4.38-4.34 (t, J = 7.2 Hz, 2H); 2.06 (s, 9H); 1.97-1.84 (m, 2H); 1.68 (s, 6H); 1.36-1.31 (m, 4H); 0.90-0.84 (t, J = 6.2 Hz, 3H). Anal. for $C_{27}H_{32}ClN_5O_2$. Calcd: C, 65.64; H, 6.53; N, 14.18. Found: C, 65.59; H, 6.42; N, 14.29.

*N-Adamantan-1-yl-4,7-dihydro-2-(4-methoxyphenyl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**25**).* Following general procedure F, compound **25** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 46%, mp = 176 °C. MS (ESI): m/z 490.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.81 (s, 1H); 8.73 (br s, 1H); 8.09-8.05 (d, J = 8.8 Hz, 2H); 7.12-7.08 (d, J = 9 Hz, 2H); 4.37-4.34 (t, J = 7.2 Hz, 2H); 3.83 (s, 3H); 2.06 (s, 9H); 1.96-1.81 (m, 2H); 1.67 (s, 6H); 1.36-1.32 (m, 4H); 0.91-0.85 (t, J = 6.6 Hz, 3H). Anal. for $C_{28}H_{35}N_5O_3$. Calcd: C, 68.69; H, 7.21; N, 14.30. Found: C, 68.75; H, 7.28; N, 14.39.

*N-Adamantan-1-yl-4,7-dihydro-7-oxo-4-pentyl-2-p-tolyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**28**).* Following general procedure F, compound **28** was isolated as a white solid. Elu-

ent: petroleum ether-ethyl acetate 4/1. Yield 44%, mp = 229 °C. MS (ESI): m/z 474.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.82 (s, 1H); 8.72 (br s, 1H); 8.05-8.01 (d, *J* = 8 Hz, 2H); 7.38-7.34 (d, *J* = 8.2 Hz, 2H); 4.38-4.33 (t, *J* = 7 Hz, 2H); 2.38 (s, 3H); 2.06 (s, 9H); 1.90-1.83 (m, 2H); 1.68 (s, 6H); 1.36-1.30 (m, 4H); 0.90-0.84(t, *J* = 6.4 Hz, 3H). Anal. for $C_{28}H_{35}N_5O_2$. Calcd: C, 71.01; H, 7.45; N, 14.79. Found: C, 71.15; H, 7.52; N, 15.75.

*N-Adamantan-1-yl-2-(furan-2-yl)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**29**).* Following general procedure F, compound **29** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 54%, mp = 175 °C. MS (ESI): m/z 450.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.82 (s, 1H); 8.67 (br s, 1H); 7.96-7.94 (d, *J* = 1.6 Hz, 1H); 7.22-7.19 (d, *J* = 3.4 Hz, 1H); 6.73-6.70 (dd, *J* = 3.4 and 1.8 Hz, 1H); 4.37-4.29 (t, *J* = 6.8 Hz, 2H); 2.06 (s, 9H); 1.96-1.82 (m, 2H); 1.67 (s, 6H); 1.37-1.30 (m, 4H); 0.90-0.84 (t, *J* = 6.2 Hz, 3H). Anal. for $C_{25}H_{31}N_5O_3$. Calcd: C, 66.79; H, 6.95; N, 15.58. Found: C, 66.75; H, 6.96; N, 15.61.

*N-Adamantan-1-yl-4,7-dihydro-7-oxo-4-pentyl-2-(pyridin-3-yl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**30**).* Following general procedure F, compound **30** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 45%, mp = 160 °C. MS (ESI): m/z 461.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 9.29 (d, *J* = 1.6 Hz, 1H); 8.87 (s, 1H); 8.76-8.72 (dd, *J* = 7.4 and 1.8 Hz, 1H); 8.68 (br s, 1H); 8.49-8.43 (dt, *J* = 7.8 and 1.6 Hz, 1H); 7.63-7.57 (dd, *J* = 7.8 and 4.2 Hz, 1H); 4.41-4.36 (t, *J* = 6.8 Hz, 2H); 2.07 (s, 9H); 1.99-1.85 (m, 2H); 1.74-1.59 (m, 8H); 1.36-1.32 (m, 2H); 0.91-0.84 (t, *J* = 6 Hz, 3H). Anal. for $C_{26}H_{32}N_6O_2$. Calcd: C, 67.80; H, 7.00; N, 18.25. Found: C, 67.74; H, 7.23; N, 18.33.

*N-Adamantan-1-yl-4,7-dihydro-2-(methylthio)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**32**).* Following general procedure F, compound **32** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 55%, mp = 161 °C. MS (ESI): m/z 430.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.75 (s, 1H); 8.67 (br s, 1H); 4.27-4.22 (t, *J* = 7 Hz, 2H); 2.61 (s, 3H); 2.05 (s, 9H); 1.91-1.74 (m, 2H); 1.67 (s, 6H); 1.33-1.25 (m, 4H); 0.89-0.83 (t, *J* = 6.4 Hz, 3H). Anal. for $C_{22}H_{31}N_5O_2S$. Calcd: C, 61.51; H, 7.27; N, 16.30. Found: C, 61.34; H, 7.29; N, 16.45.

*N-Adamantan-1-yl-4,7-dihydro-2-morpholino-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**35**).* Following general procedure F, compound **35** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield 40%, mp = 148 °C. MS (ESI): m/z 469.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.81 (br s, 1H); 8.64 (s, 1H); 4.08-3.99 (t, *J* = 6.8 Hz, 2H); 3.71-3.67 (m, 4H); 3.44-3.40 (m, 4H); 2.03 (s, 9H); 1.85-1.70 (m, 2H); 1.66 (s, 6H); 1.33-1.26 (m, 4H); 0.89-0.83 (t, *J* = 6.6 Hz, 3H). Anal. for $C_{25}H_{36}N_6O_3$. Calcd: C, 64.08; H, 7.74; N, 17.93. Found: C, 64.19; H, 7.79; N, 17.90.

*N-Adamantan-1-yl-2-(N-benzyl-N-methyl-amino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**39**).* Following general procedure F, compound **39** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield 60%, mp = 139 °C. MS (ESI): m/z 519.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.85 (br s, 1H); 8.61 (s, 1H); 7.34-7.29 (m, 5H); 4.65 (s, 2H); 4.24-4.19 (t, J = 7 Hz, 2H); 2.97 (s, 3H); 2.03 (s, 9H); 1.87-1.75 (m, 2H); 1.66 (s, 6H); 1.30-1.26 (m, 4H); 0.88-0.82 (t, J = 6.2 Hz, 3H). Anal. for $C_{29}H_{38}N_6O_2$. Calcd: C, 69.29; H, 7.62; N, 16.72. Found: C, 69.32; H, 7.77; N, 16.68.

*N-Adamantan-1-yl-2-(diallylamino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**41**).* Following general procedure F, compound **41** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 45%, mp = 110 °C. MS (ESI): m/z 479.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.84 (br s, 1H); 8.60 (s, 1H); 5.87-5.78 (m, 2H); 5.24-5.14 (m, 4H); 4.22-4.16 (t, *J* = 6.8 Hz, 2H); 4.04-4.01 (d, *J* = 5.6 Hz, 4H); 2.03 (s, 9H); 1.90-1.72 (m, 2H); 1.66 (s, 6H); 1.32-1.25 (m, 4H); 0.88-0.82 (t, *J* = 6.6 Hz, 3H). Anal. for $C_{27}H_{38}N_6O_2$. Calcd: C, 67.75; H, 8.00; N, 17.56. Found: C, 67.71; H, 7.98; N, 17.49.

*General procedure G for the synthesis of N-cyclohexyl/cycloheptyl carboxamide derivatives **15**, **17**, **19**, **20**, **22**, **24**, **26**, **27**, **31**, **33**, **34**, **36-38**, **40***

To a solution of the appropriate carboxylic acid (0.57 mmol) in DMF, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide (EDCI, 0.85 mmol) and 1-hydroxybenzotriazole (HOBt, 0.85 mmol) were added and the reaction was stirred at room temperature for 10 min before adding the suitable

amine (0.85 mmol) and the stirring continued at the same temperature for 16h. The solvent was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (30 mL), washed with aqueous bicarbonate (10 mL), water (10 mL) and brine (10 mL). The organic extract was dried, filtered and concentrated and resulting products were purified by flash chromatography on silica gel, eluting with ethyl acetate and petroleum ether or ethyl acetate and methanol.

N-Cycloheptyl-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide ***(15).*** Following general procedure G, compound **15** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield 46%, mp = 149 °C. MS (ESI): m/z 346.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.89 (s, 1H); 8.85-8.83 (br d, *J* = 7.8 Hz, 1H); 8.39 (s, 1H); 4.34-4.30 (t, *J* = 7 Hz, 2H); 4.17-4.01 (m, 1H); 1.96-1.75 (m, 4H); 1.70-1.42 (m, 10H); 1.35-1.24 (m, 4H); 0.88-0.83 (t, *J* = 6.4 Hz, 3H). Anal. for $C_{18}H_{27}N_5O_2$. Calcd: C, 62.58; H, 7.88; N, 20.27. Found: C, 62.51; H, 7.82; N, 20.39.

N-Cyclohexyl-4,7-dihydro-2-methyl-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide ***(17).*** Following general procedure G, compound **17** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 2/3. Yield 41%, mp = 120 °C. MS (ESI): m/z 346.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.83 (s, 1H); 8.81-8.77 (br d, *J* = 7.8 Hz, 1H); 4.31-4.24 (t, *J* = 7 Hz, 2H); 3.91-3.78 (m, 1H); 2.41 (s, 3H); 1.94-1.54 (m, 6H); 1.43-1.24 (m, 10H); 0.89-0.82 (t, *J* = 6.6 Hz, 3H). Anal. for $C_{18}H_{27}N_5O_2$. Calcd: C, 62.58; H, 7.88; N, 20.27. Found: C, 62.61; H, 7.59; N, 20.32.

N-Cyclohexyl-4,7-dihydro-7-oxo-4-pentyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide ***(19).*** Following general procedure G, compound **19** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 55%, mp = 161 °C. MS (ESI): m/z 408.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.90 (s, 1H); 8.85-8.81 (br d, *J* = 8 Hz, 1H); 8.17-8.12 (m, 2H); 7.57-7.54 (m, 3H); 4.40-4.35 (t, *J* = 7 Hz, 2H); 3.94-3.81 (m, 1H); 1.91-1.58 (m, 6H); 1.39-1.28 (m, 10H); 0.90-0.84 (t, *J* = 6.4 Hz, 3H). Anal. for $C_{23}H_{29}N_5O_2$. Calcd: C, 67.79; H, 7.17; N, 17.19. Found: C, 67.68; H, 6.94; N, 17.25.

*N-Cycloheptyl-4,7-dihydro-7-oxo-4-pentyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**20**).* Following general procedure G, compound **20** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 48%, mp = 155 °C. MS (ESI): m/z 422.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ; 8.90-8.98 (m, 2H); 8.15-8.12 (m, 2H); 7.57-7.54 (m, 3H); 4.40-4.36 (t, *J* = 7 Hz, 2H); 4.15-4.03 (m, 1H); 1.93-1.82 (m, 4H); 1.71-1.44 (m, 10H); 1.36-1.30 (m, 4H); 0.91-0.85(t, *J* = 6.4 Hz, 3H). Anal. for $C_{24}H_{31}N_5O_2$. Calcd: C, 68.38; H, 7.41; N, 16.61. Found: C, 68.22; H, 7.55; N, 16.55.

*2-(4-Chlorophenyl)-N-cyclohexyl-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**22**).* Following general procedure G, compound **22** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 4/1. Yield 53%, mp = 212 °C. MS (ESI): m/z 442.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.90 (s, 1H); 8.83-8.79 (br d, *J* = 8.2 Hz, 1H); 8.16-8.12 (d, *J* = 8.4 Hz, 2H); 7.65-7.61 (d, *J* = 8.4 Hz, 2H); 4.41-4.35 (t, *J* = 7.2 Hz, 2H); 3.97-3.82 (m, 1H); 2.41 (s, 3H); 1.90-1.57 (m, 6H); 1.39-1.23 (m, 10H); 0.90-0.84 (t, *J* = 6.4 Hz, 3H). Anal. for $C_{23}H_{28}ClN_5O_2$. Calcd: C, 62.51; H, 6.39; N, 15.85. Found: C, 62.59; H, 6.20; N, 15.72.

*N-Cyclohexyl-4,7-dihydro-2-(4-methoxyphenyl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**24**).* Following general procedure G, compound **24** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 53%, mp = 190 °C. MS (ESI): m/z 438.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.88 (s, 1H); 8.86 (br s, 1H); 8.10-8.05 (d, *J* = 9.2 Hz, 2H); 7.13-7.08 (d, *J* = 8.6 Hz, 2H); 4.39-4.35 (t, *J* = 7.2 Hz, 2H); 3.42-3.84 (m, 1H); 3.83 (s, 3H); 1.98-1.61 (m, 6H); 1.38-1.30 (m, 10H); 0.90-0.85 (t, *J* = 6.6 Hz, 3H). Anal. for $C_{24}H_{31}N_5O_3$. Calcd: C, 65.88; H, 7.14; N, 16.01. Found: C, 65.68; H, 7.22; N, 16.12.

*N-Cyclohexyl-4,7-dihydro-7-oxo-4-pentyl-2-p-tolyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**26**).* Following general procedure G, compound **26** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 50%, mp = 200 °C. MS (ESI): m/z 422.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.88 (s, 1H); 8.87-8.86 (br d, *J* = 7.8 Hz, 1H); 8.05-8.01 (d, *J* = 8.4 Hz, 2H); 7.38-7.34 (d, *J* = 8 Hz, 2H); 4.39-4.36 (t, *J* = 7 Hz, 2H); 3.93-3.81 (m, 1H); 2.38 (s, 3H);

1.89-1.58 (m, 6H); 1.36-1.25 (m, 10H); 0.90-0.84(t, *J* = 6.4 Hz, 3H). Anal. for $C_{24}H_{31}N_5O_2$. Calcd: C, 68.38; H, 7.41; N, 16.61. Found: C, 68.42; H, 7.45; N, 16.69.

*N-Cycloheptyl-4,7-dihydro-7-oxo-4-pentyl-2-p-tolyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**27**).* Following general procedure G, compound **27** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 48%, mp = 195 °C. MS (ESI): m/z 436.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.91-8.90 (br d, *J* = 7.8 Hz, 1H); 8.87 (s, 1H); 8.05-8.01 (d, *J* = 8 Hz, 2H); 7.38-7.34 (d, *J* = 7.8 Hz, 2H); 4.39-4.36 (t, *J* = 7 Hz, 2H); 4.17-4.02 (m, 1H); 2.38 (s, 3H); 1.97-1.81 (m, 4H); 1.74-1.49 (m, 10H); 1.36-1.28 (m, 4H); 0.90-0.84(t, *J* = 6.4 Hz, 3H). Anal. for $C_{25}H_{33}N_5O_2$. Calcd: C, 68.94; H, 7.64; N, 16.08. Found: C, 68.74; H, 7.68; N, 16.12.

*N-Cyclohexyl-4,7-dihydro-2-(methylthio)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**31**).* Following general procedure G, compound **31** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 7/3. Yield 50%, mp = 192 °C. MS (ESI): m/z 378.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.80-8.77 (m, 2H); 4.27-4.21 (t, *J* = 6.8 Hz, 2H); 3.87-3.75 (m, 1H); 2.60 (s, 3H); 1.91-1.59 (m, 6H); 1.42-1.21 (m, 10H); 0.88-0.81 (t, *J* = 6.4 Hz, 3H). Anal. for $C_{18}H_{27}N_5O_2S$. Calcd: C, 57.27; H, 7.21; N, 18.55. Found: C, 57.21; H, 7.25; N, 18.61.

*N-Cyclohexyl-4,7-dihydro-2-morpholino-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**33**).* Following general procedure G, compound **33** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 44%, mp = 143 °C. MS (ESI): m/z 417.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.95-8.91 (d, *J* = 7.8 Hz, 1H); 8.70 (s, 1H); 4.24-4.20 (t, *J* = 7 Hz, 2H); 3.89-3.72 (m, 1H); 3.71-3.67 (m, 4H); 3.45-3.40 (m, 4H); 1.81-1.47 (m, 6H); 1.39-1.20 (m, 10H); 0.88-0.82 (t, *J* = 6.4 Hz, 3H). Anal. for $C_{21}H_{32}N_6O_3$. Calcd: C, 60.56; H, 7.74; N, 20.18. Found: C, 60.61; H, 7.69; N, 20.59.

*N-Cycloheptyl-4,7-dihydro-2-morpholino-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**34**).* Following general procedure G, compound **34** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 50%, mp = 172 °C. MS (ESI): m/z 431.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 9.00-8.96 (br d, *J* = 7.8 Hz, 1H); 8.69 (s, 1H); 4.25-4.18 (t, *J* = 6.8

Hz, 2H); 4.15-4.02 (m, 1H); 3.71-3.67 (m, 4H); 3.45-3.40 (m, 4H); 1.89-1.73 (m, 4H); 1.66-1.48 (m, 10H); 1.32-1.24 (m, 4H); 0.88-0.82 (t, *J* = 6.6 Hz, 3H). Anal. for $C_{22}H_{34}N_6O_3$. Calcd: C, 61.37; H, 7.96; N, 19.52. Found: C, 61.46; H, 7.86; N, 19.58.

*N-Cyclohexyl-4,7-dihydro-2-(4-methylpiperazin-1-yl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**36**).* Following general procedure G, compound **36** was isolated as a white solid. Eluent: ethyl acetate-methanol 4/1. Yield 40%, mp = 168 °C. MS (ESI): m/z 430.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.96-8.92 (br d, *J* = 8 Hz, 1H); 8.68 (s, 1H); 4.24-4.18 (t, *J* = 6.6 Hz, 2H); 3.89-3.78 (m, 1H); 3.47-3.42 (m, 4H); 2.40-2.36 (m, 4H); 2.21 (s, 3H); 1.81-1.49 (m, 6H); 1.41-1.22 (m, 10H); 0.89-0.82 (t, *J* = 6.8 Hz, 3H). Anal. for $C_{22}H_{35}N_7O_2$. Calcd: C, 61.51; H, 8.21; N, 22.83. Found: C, 61.54; H, 8.24; N, 22.69.

*N-Cycloheptyl-4,7-dihydro-2-(4-methylpiperazin-1-yl)-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**37**).* Following general procedure G, compound **37** was isolated as a white solid. Eluent: ethyl acetate-methanol 4/1. Yield 40%, mp = 183 °C. MS (ESI): m/z 444.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 9.00-8.97 (br d, *J* = 7.8 Hz, 1H); 8.67 (s, 1H); 4.24-4.17 (t, *J* = 7 Hz, 2H); 4.12-3.99 (m, 1H); 3.47-3.42 (m, 4H); 2.40-2.36 (m, 4H); 2.21 (s, 3H); 1.87-1.72 (m, 4H); 1.64-1.46 (m, 10H); 1.35-1.24 (m, 4H); 0.88-0.82 (t, *J* = 6.6 Hz, 3H). Anal. for $C_{23}H_{37}N_7O_2$. Calcd: C, 62.28; H, 8.41; N, 22.10. Found: C, 62.39; H, 8.49; N, 22.35.

*2-(N-Benzyl-N-methylamino)-N-cyclohexyl-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**38**).* Following general procedure G, compound **38** was isolated as a white solid. Eluent: petroleum ether-ethyl acetate 1/1. Yield 62%, mp = 157 °C. MS (ESI): m/z 451.2 (M+H). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.17-9.15 (br d, *J* = 7.6 Hz, 1H); 8.47 (s, 1H); 7.33-7.24 (m, 5H); 4.73 (s, 2H); 4.18-4.14 (t, *J* = 7.2 Hz, 2H); 3.06 (s, 3H); 1.98-1.73 (m, 5H); 1.56-1.30 (m, 11H); 0.91-0.87 (t, *J* = 6.8 Hz, 3H). Anal. for $C_{25}H_{34}N_6O_2$. Calcd: C, 66.64; H, 7.61; N, 18.65. Found: C, 66.51; H, 7.68; N, 18.76.

*N-Cyclohexyl-2-(diallylamino)-4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxamide (**40**).* Following general procedure G, compound **40** was isolated as a white solid. Elu-

ent: petroleum ether-ethyl acetate 7/3. Yield 42%, mp = 78 °C. MS (ESI): m/z 427.2 (M+H). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 8.99-8.95 (br d, J = 7.8 Hz, 1H); 8.66 (s, 1H); 5.87-5.79 (m, 2H); 5.23-5.14 (m, 4H); 4.25-4.18 (t, J = 7.2 Hz, 2H); 4.04-4.01 (d, J = 5.6 Hz, 4H); 3.96-3.82 (m, 1H); 1.89-1.55 (m, 6H); 1.43-1.24 (m, 10H); 0.88-0.81 (t, J = 6.2 Hz, 3H). Anal. for $C_{23}H_{34}N_6O_2$. Calcd: C, 64.76; H, 8.03; N, 19.70. Found: C, 64.49; H, 7.96; N, 19.44.

Pharmacology

The affinity and the functionality of the novel compounds were studied by using human CB_1 and CB_2 receptors expressed in CHO cells (Perkin-Elmer Life and Analytical Sciences, U.S.). To calculate the affinity values, competition binding assays were performed by using [^{3}H]CP-55,940 as radioligand (specific activity, 180 Ci/mmol; Perkin-Elmer Life and Analytical Sciences, U.S.). The effect of these compounds was also evaluated in cyclic AMP experiments that used [^{3}H]-cAMP as radioligand (specific activity, 58 Ci/mmol; Perkin-Elmer Life and Analytical Sciences U.S.). All other reagents were of analytical grade and obtained from commercial sources.

Competition Binding Experiments on CB_1 and CB_2 Receptors. CHO cell lines expressing human CB_1 and CB_2 receptors were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 U/mL), and Geneticin (G418, 0.4 mg/ml) in 5% CO_2/95% air at 37 °C [8,10]. For membrane preparations, the culture medium was removed and the cells were washed with PBS, then scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and centrifuged at 1000*g* for 10 min, and the supernatant was then centrifuged at 100000*g* for 30 min. The membrane pellet was suspended in 50 mM Tris-HCl buffer, 0.5% BSA (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA or 2.5 mM EDTA for *h*CB_1 or *h*CB_2 receptor, respectively.

Competition binding experiments were performed using 0.5 nM [^{3}H]CP-55,940 along with different concentrations (1 nM to 10 μM) of the examined compounds or a reference agonist (WIN 55,212-2) for an incubation time of 90 or 60 min at 30 °C for CB_1 or CB_2 receptors, respectively.

Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/C glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted on a Perkin-Elmer 2810 TR scintillation counter (Perkin-Elmer Life and Analytical Sciences, U.S.).

Cyclic AMP Assay for Human CB_2 Receptors. CHO cells transfected with human CB_2 receptors were washed with phosphate-buffered saline, diluted trypsin and centrifuged at 200*g* for 10 min. The pellet containing CHO cells (1×10^6 cells/assay) was suspended in 0.5 mL of incubation mixture: KCl 2.7 mM, NaCl 150 mM, $MgSO_4$ 1 mM, NaH_2PO_4 0.37 mM, $CaCl_2$ 1 mM, $MgCl_2$ 10 mM, HEPES 5 mM, glucose 5 mM, pH 7.4 at 37 °C. Then 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as a phosphodiesterase inhibitor was added and the mixture preincubated for 10 min in a shaking bath at 37 °C [11]. The effect of the novel CB compounds was studied in the presence of forskolin, 1 μM, in comparison with the well-known CB agonist WIN 55,212-2. The antagonism of the new CB ligands was also investigated evaluating their inhibition of the agonist WIN 55,212-2 (20 nM) response. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged for 10 min at 2000*g* at 4 °C, and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay. Samples of cyclic AMP standard (0-10 pmol) were added to each test tube containing the incubation buffer (Trizma base 0.1 M, aminophylline 8.0 mM, 2-mercaptoethanol 6.0 mM, pH 7.4) and [^{3}H]cyclic AMP in a total volume of 0.5 mL. The binding protein previously prepared from beef adrenals was added to the samples previously incubated at 4 °C for 150 min, and after the addition of charcoal, they were centrifuged for 10 min at 2000*g*. The clear supernatant was counted by using a Perkin-Elmer 2810 TR scintillation counter (Perkin-Elmer Life and Analytical Sciences, U.S.).

Data Analysis. The protein concentration was determined following a Bio-Rad method (Bradford, 1976) with bovine albumin as reference standard [26]. Inhibitory binding constants, K_i, were calculated from the IC_{50} values according to the Cheng and Prusoff equation:[27] $K_i = IC_{50}/(1 + [C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant. A weighted nonlinear least-squares curve fitting program, LIGAND [28] ,was used for computer analysis of the inhibition experiments. All the data are expressed as the mean ± SEM of $n = 4$ independent experiments. Statistical analysis of the data was performed using unpaired two-sided Student's *t* test.

Docking studies

The ligands were built with Maestro [29] and subjected to minimization into a water environment (employing the generalized Born/surface area model) by using Macromodel [30]. The minimizations were carried out by means of the conjugate gradient, the MMFFs force field and a distance-dependent dielectric constant of 1.0, until a convergence value of 0.05 kcal/(Å•mol) was attained. The inactive CB_1 and CB_2 receptor models employed for docking studies were generated by applying a previously reported procedure [31] and then further optimized based on the recently deposited PDB structures of adenosine (PDB code 3EML) [31] and rhodopsine (PDB code 1U19) [32] receptors. The ligands were docked using AUTODOCK4.2 [20] AUTODOCK TOOLS [33] was employed to define the torsion angles in the ligands, to add the solvent model and to assign partial atomic charges (Gasteiger for the ligands and Kollman for the receptors). The docking sites were defined according to the previously published WIN 55212-2 docked into the CB1 and CB2 receptor models [34]. The new CB_1 and CB_2 models were aligned to the corresponding protein structures complexed with WIN 55212-2 and the ligand was considered as the central group of a grid of 54, 50, and 52 points in the x, y, and z directions. The energetic map calculations were carried out by using a grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant. The ligands were able to form an intramolecular H-bond and previous quantum mechanical studies highlighted that it was a strong interaction [34]. On this basis, this interaction was supposed to be pre-

served in the ligands binding pose within the receptor sites and the torsions involved in this intramolecular H-bond were kept fixed during AUTODOCK calculations, so that to avoid its loss in the docking process. The ligands were subjected to 100 runs of the AUTODOCK search using the Lamarckian genetic algorithm with 5 000 000 steps of energy evaluation; an rms tolerance of 1.0 Å was used to carry out the cluster analysis of the docking solutions and all other settings were left as their defaults. For each docked ligand, the top-scored pose belonging from the cluster with the best average of estimated free energy was taken into account for energy minimization studies.

Energy Minimizations. In order to further validate the docking results and also in order to partially take into account the receptor flexibility and the possibility of small adjustments in the ligand-receptor interaction, for each docked ligand the top-scored pose was energy minimized. The energy minimizations of the receptor-ligand complexes obtained as a result of the docking protocol were carried out by using AMBER11 [21].The complexes were embedded into a phospholipid bilayer made up of POPC molecules, which was created by means of VMD [35]. This software was also used to place the ligand-protein complexes inside the phospholipid bilayer. The systems were set in a rectangular parallelepiped water-box (TIP3P explicit solvent model) and solvated on both the "extracellular" and "intracellular" side with a 12 Å water cap. Chlorine ions were then added for the neutralization of the systems. Three steps of energy minimization were performed, each one carried out through 1000 steps of steepest descent followed by 9000 steps of conjugate gradient but with the application of different position restraints. In the first step the phospholipids and the protein were kept fixed with a restraint of 100 kcal/(mol•Å2), so that only the position of the water molecules was energy minimized. During the second step the position restraint of 100 kcal/(mol•Å2) was applied just to the receptor, thus energy minimizing the surrounding phospholipid–water environment. Finally, a third step was performed by applying no restraints and thus leaving the whole system free. All the minimized ligand-receptor complexes were very similar to that obtained after docking calculations and the minimized ligand dispositions never differed more than 1.5 Å (RMSD) with respect to the docked poses.

Acknowledgments

The authors gratefully acknowledged Alberto Casolari for excellent technical assistance.

References

[1] V. Di Marzo, M. Bifulco, L. De Petrocellis, The endocannabinoid system and its therapeutic exploitation., Nat. Rev. Drug Discov. 3 (2004) 771–84. doi:10.1038/nrd1495.

[2] A.C. Howlett, F. Barth, T.I. Bonner, G. Cabral, P. Casellas, W.A. Devane, et al., International Union of Pharmacology. XXVII. Classification of cannabinoid receptors, Pharmacol. Rev. 54 (2002) 161–202.

[3] G.T. Whiteside, G.P. Lee, K.J. Valenzano, The role of the cannabinoid CB2 receptor in pain transmission and therapeutic potential of small molecule CB2 receptor agonists., Curr. Med. Chem. 14 (2007) 917–36.

[4] A.M. Malfitano, S. Basu, K. Maresz, M. Bifulco, B.N. Dittel, What we know and do not know about the cannabinoid receptor 2 (CB2)., Semin. Immunol. 26 (2014) 369–79.

[5] E. Gaffal, M. Cron, N. Glodde, T. Tüting, Anti-inflammatory activity of topical THC in DNFB-mediated mouse allergic contact dermatitis independent of CB1 and CB2 receptors., Allergy. 68 (2013) 994–1000.

[6] S. Han, J. Thatte, D.J. Buzard, R.M. Jones, Therapeutic utility of cannabinoid receptor type 2 (CB(2)) selective agonists, J. Med. Chem. 56 (2013) 8224–8256.

[7] R.P. Picone, D.A. Kendall, Minireview: from the bench, toward the clinic: therapeutic opportunities for cannabinoid receptor modulation., Mol. Endocrinol. 29 (2015) 801–13.

[8] P.G. Baraldi, G. Saponaro, A.R. Moorman, R. Romagnoli, D. Preti, S. Baraldi, et al., 7-Oxo-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxamides as selective CB(2) cannabinoid receptor ligands: structural investigations around a novel class of full agonists, J. Med. Chem. 55 (2012) 6608–6623.

[9] V. Lucchesi, D.P. Hurst, D.M. Shore, S. Bertini, B.M. Ehrmann, M. Allarà, et al., CB2-selective cannabinoid receptor ligands: synthesis, pharmacological evaluation, and molecular modeling investigation of 1,8-Naphthyridin-2(1H)-one-3-carboxamides., J. Med. Chem. 57 (2014) 8777–91.

[10] M. Aghazadeh Tabrizi, P.G. Baraldi, G. Saponaro, A.R. Moorman, R. Romagnoli, D. Preti, et al., Design, synthesis, and pharmacological properties of new heteroarylpyridine/heteroarylpyrimidine derivatives as CB(2) cannabinoid receptor partial agonists, J. Med. Chem. 56 (2012) 1098–1112.

[11] M. Aghazadeh Tabrizi, P.G. Baraldi, G. Saponaro, A.R. Moorman, R. Romagnoli, D. Preti, et al., Discovery of 7-oxopyrazolo[1,5-a]pyrimidine-6-carboxamides as potent and selective CB(2) cannabinoid receptor inverse agonists, J. Med. Chem. 56 (2013) 4482–4496.

[12] H. Iwamura, H. Suzuki, Y. Ueda, T. Kaya, T. Inaba, In Vitro and in Vivo Pharmacological Characterization of JTE-907, a Novel Selective Ligand for Cannabinoid CB2 Receptor, J. Pharmacol. Exp. Ther. 296 (2001) 420–425.

[13] C.A. Lunn, E.-P. Reich, J.S. Fine, B. Lavey, J.A. Kozlowski, R.W. Hipkin, et al., Biology and therapeutic potential of cannabinoid CB 2 receptor inverse agonists, Br. J. Pharmacol. 153 (2008) 226–239.

[14] W. Schuehly, J.M.V. Paredes, J. Kleyer, A. Huefner, S. Anavi-Goffer, S. Raduner, et al., Mechanisms of osteoclastogenesis inhibition by a novel class of biphenyl-type cannabinoid CB(2) receptor inverse agonists., Chem. Biol. 18 (2011) 1053–64.

[15] P. Yang, K.-Z. Myint, Q. Tong, R. Feng, H. Cao, A.A. Almehizia, et al., Lead Discovery, Chemistry Optimization, and Biological Evaluation Studies of Novel Biamide Derivatives as CB 2 Receptor Inverse Agonists and Osteoclast Inhibitors, J. Med. Chem. 55 (2012) 9973–9987.

[16] S. Pasquini, M. De Rosa, V. Pedani, C. Mugnaini, F. Guida, L. Luongo, et al., 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 antihyperalgesic effects in mice., J. Med. Chem. 54 (2011) 5444–53.

[17] D. Thomae, E. Perspicace, S. Hesse, G. Kirsch, P. Seck, Synthesis of substituted [1,3]thiazolo[4,5-b]pyridines and [1,3]thiazolo[4,5-d][1,2,3]triazines, Tetrahedron. 64 (2008) 9309–9314.

[18] Y. Huang, X.-Q. Hu, D.-P. Shen, Y.-F. Chen, P.-F. Xu, Synthesis of 1H-imidazo[1,2-b]-1,2,4-triazol-6-amines via multicomponent reaction, Mol. Divers. 11 (2007) 73–80.

[19] S.A. El Bialy, M.M. Nagy, H.M. Abdel-Rahman, Efficient regioselective three-component domino synthesis of 3-(1,2,4-Triazol-5-yl)-1,3-thiazolidin-4-ones as potent antifungal and antituberculosis agents., Arch. Pharm. (Weinheim). 344 (2011) 821–9.

[20] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D. Goodsell, et al., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility., J. Comput. Chem. 30 (2009) 2785–91.

[21] D.A. Case, T.A. Darden, T.E. Cheatham, C.L. Simmerling, J. Wang, R.E. Duke, et al., AMBER version 11, (2010) University of California, San Francisco, CA.

[22] S. Durdagi, H. Reis, M.G. Papadopoulos, T. Mavromoustakos, Comparative molecular dynamics simulations of the potent synthetic classical cannabinoid ligand AMG3 in solution and at binding site of the CB1 and CB2 receptors., Bioorg. Med. Chem. 16 (2008) 7377–87.

[23] S. Durdagi, M.G. Papadopoulos, P.G. Zoumpoulakis, C. Koukoulitsa, T. Mavromoustakos, A computational study on cannabinoid receptors and potent bioactive cannabinoid ligands: homology modeling, docking, de novo drug design and molecular dynamics analysis., Mol. Divers. 14 (2010) 257–76.

[24] Z.H. Song, C.-A.A. Slowey, D.P. Hurst, P.H. Reggio, The difference between the CB1 and CB2 cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN55212-2 for CB2, Mol. Pharmacol. 56 (1999) 834–840.

[25] C. Manera, T. Tuccinardi, A. Martinelli, Indoles and related compounds as cannabinoid ligands., Mini Rev. Med. Chem. 8 (2008) 370–387.

[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[27] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction., Biochem. Pharmacol. 22 (1973) 3099–3108.

[28] P.J. Munson, D. Rodbard, Ligand: a versatile computerized approach for characterization of ligand-binding systems., Anal. Biochem. . 107 (1980) 220–239.

[29] Maestro, version 9.0. Schrödinger Inc: Portland, OR, 2009., (2009).

[30] Macromodel, Version 9.7. Schrödinger Inc: Portland, OR, 2009, (2009).

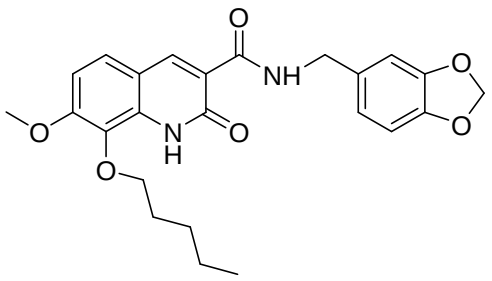
[31] V.-P. Jaakola, M.T. Griffith, M.A. Hanson, V. Cherezov, E.Y.T. Chien, J.R. Lane, et al., The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist., Science. 322 (2008) 1211–7.

[32] T. Okada, M. Sugihara, A.-N. Bondar, M. Elstner, P. Entel, V. Buss, The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure., J. Mol. Biol. 342 (2004) 571–83.

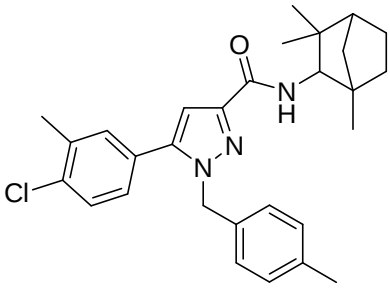
[33] M.F. Sanner, Python: a programming language for software integration and development, J. Mol. Graph. Model. 17 (1999) 57–61.

[34] T. Tuccinardi, P.L. Ferrarini, C. Manera, G. Ortore, G. Saccomanni, A. Martinelli, Cannabinoid CB2/CB1 selectivity. Receptor modeling and automated docking analysis., J. Med. Chem. 49 (2006) 984–994.

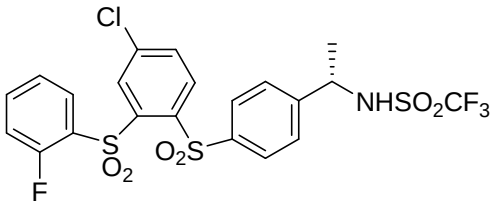
[35] W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics, J. Mol. Graph. 14 (1996) 33–38.



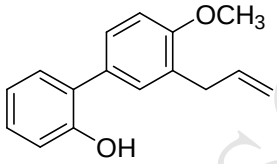
1, JTE-907



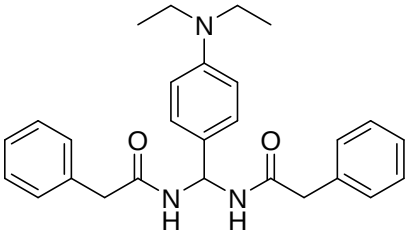
2, SR144528



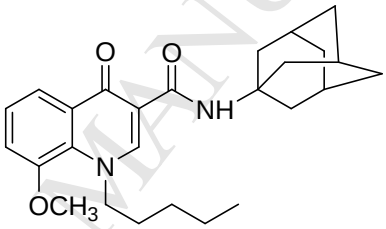
3, Sch414319



4, MH



5



6

Chart 1. Chemical structures of representative CB_2 inverse agonists

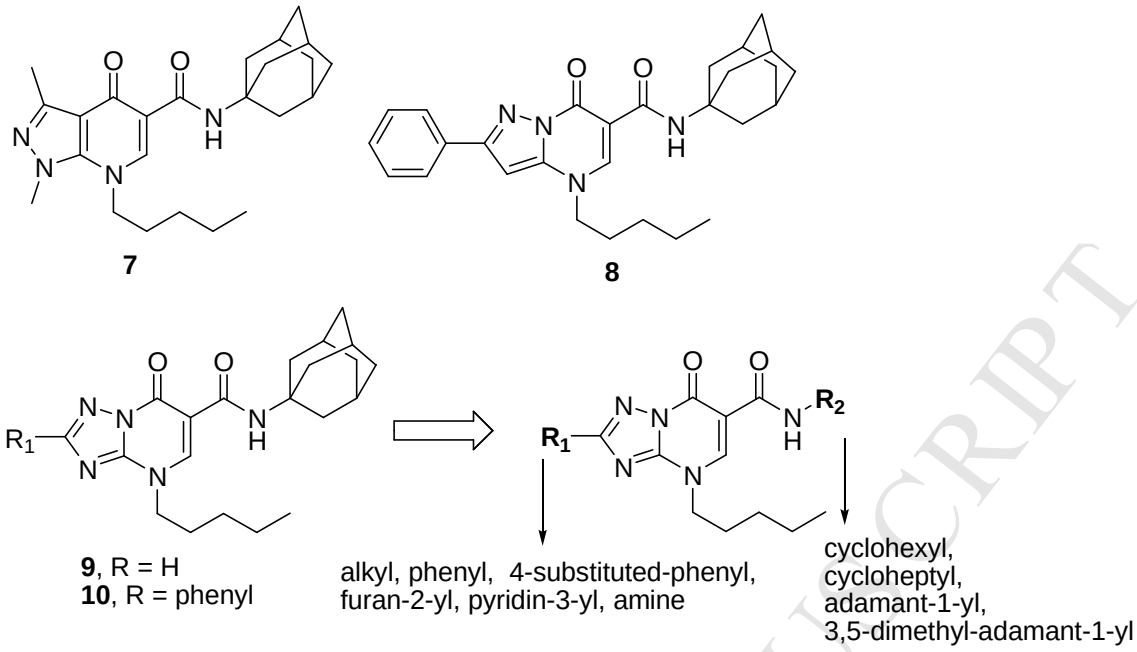
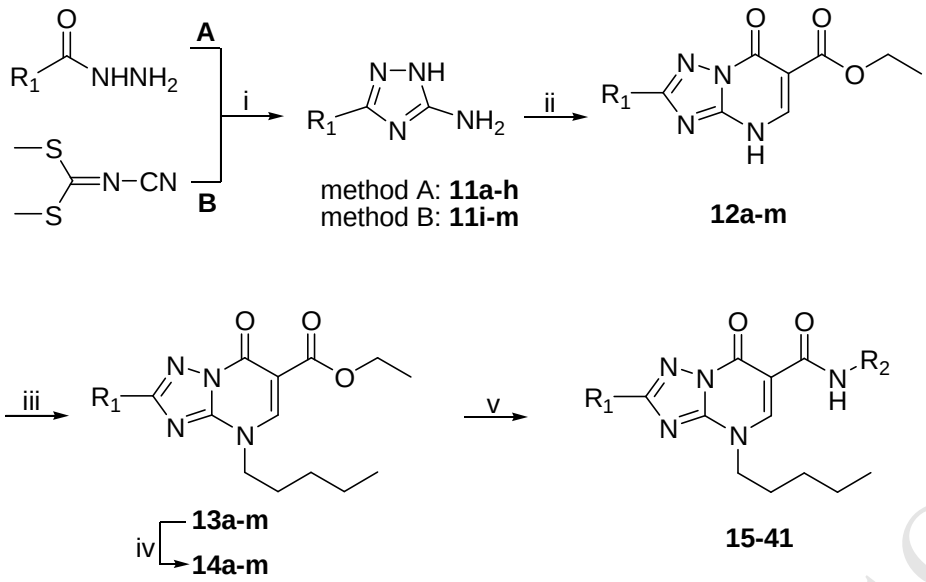


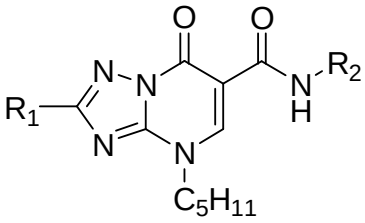
Chart 2. Structural investigations around the 4,7-dihydro-7-oxo-4-pentyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide scaffold



R^1 = a: H; b: CH_3; c: phenyl; d: 4-Cl-phenyl; e: 4-OCH_3-phenyl; f: 4-CH_3-phenyl; g: furan-2-yl; h: pyridin-3-yl; i: S-CH_3; j: morpholine; k: N-methylpiperazine; l: N-benzyl-N-methyl; m: N,N-diallylamine.

Scheme 1. Reagents and conditions: i) **A**: S-methylisothiourea hemisulfate, water, 100 °C, 6 h then NaOH 10%, 100 °C, 6 h; **B**: R_1NH_2, hydrazine monohydrate, CH_3CN, 70 °C, 7 h; ii) DEEM, CH_3COOH, 3 h, 120 °C; iii) K_2CO_3, DMF, 1-pentylbromide, 100 °C, 6 h; iv) 10% NaOH, MeOH, rt, 3 h; v) EDC, HOBt, DMF, amine, rt, 16 h or HBTU, DIEA, DMF, amine, rt, 16 h.

Table 1. Affinity (K_i, nM) and selectivity on human CB_1 and CB_2 receptors[a]



9-41

Compd	*R_1*	*R_2*	*K_i hCB_1*[b] (nM)	*K_i hCB_2*[c] (nM)	*CB_1/CB_2*
	WIN55,212-2		*12.5 ±1.6*	*4.6 ±0.4*	*2.7*
9	*H*	*adamantan-1-yl*	*712 ±64*	*20 ±2*	*36*
10	*Ph*	*adamantan-1-yl*	*523±47*	*3.6 ±0.3*	*146*
15	H	cycloheptyl	717 ± 66	514 ± 49	1
16	H	3,5-dimethyladamantan-1-yl	622 ± 53	25 ± 3	25
17	CH_3	cyclohexyl	488 ± 46	674 ± 62	1
18	CH_3	adamantan-1-yl	471 ± 42	383 ± 35	1
19	Ph	cyclohexyl	3214 ± 28	43 ± 4	75
20	Ph	cycloheptyl	2483 ± 237	48 ± 5	52
21	Ph	3,5-dimethyladamantan-1-yl	653 ± 61	8.4 ± 0.9	77
22	4-Cl-Ph	cyclohexyl	4718 ± 413	117 ± 9	40
23	4-Cl-Ph	adamantan-1-yl	5216 ± 483	11.3 ± 1.9	462
24	4-OCH_3-Ph	cyclohexyl	5849 ± 544	96 ± 8	61
25	4-OCH_3-Ph	adamantan-1-yl	3723 ± 318	47 ± 4	79
26	4-CH_3-Ph	cyclohexyl	>10000	31 ± 3	323
27	4-CH_3-Ph	cycloheptyl	6352 ± 594	22 ± 2	289
28	4-CH_3-Ph	adamantan-1-yl	1519 ± 141	6.8 ± 0.6	223
29	furan-2-yl	adamantan-1-yl	1172 ± 106	45 ± 4	26
30	pyridin-3-yl	adamantan-1-yl	>10000	228 ± 19	>44
31	S-CH_3	cyclohexyl	>10000	716 ± 62	>14
32	S-CH_3	adamantan-1-yl	>10000	47 ± 3	>213

33	morpholine	cyclohexyl	>10000	>10000	
34	morpholine	cycloheptyl	>10000	566 ± 52	>18
35	morpholine	adamantan-1-yl	>10000	53 ± 5	140
36	*N*-CH_3-piperazine	cyclohexyl	>10000	>10000	>188
37	*N*-CH_3-piperazine	cycloheptyl	>10000	982 ± 87	>10
38	*N*-benzyl-*N*-methyl	cyclohexyl	>10000	1332 ± 128	>7
39	*N*-benzyl-*N*-methyl	adamantan-1-yl	>10000	21 ± 2	>476
40	diallylamine	cyclohexyl	>10000	1352 ± 124	>7
41	diallylamine	adamantan-1-yl	>10000	158 ± 13	>63

[a]The data are expressed as the mean ± SEM of n = 4 independent experiments. The affinity values were calculated by using [^{3}H]CP-55,940 as radioligand on human CB_1CHO membranes[b], and human CB_2CHO membranes[c].

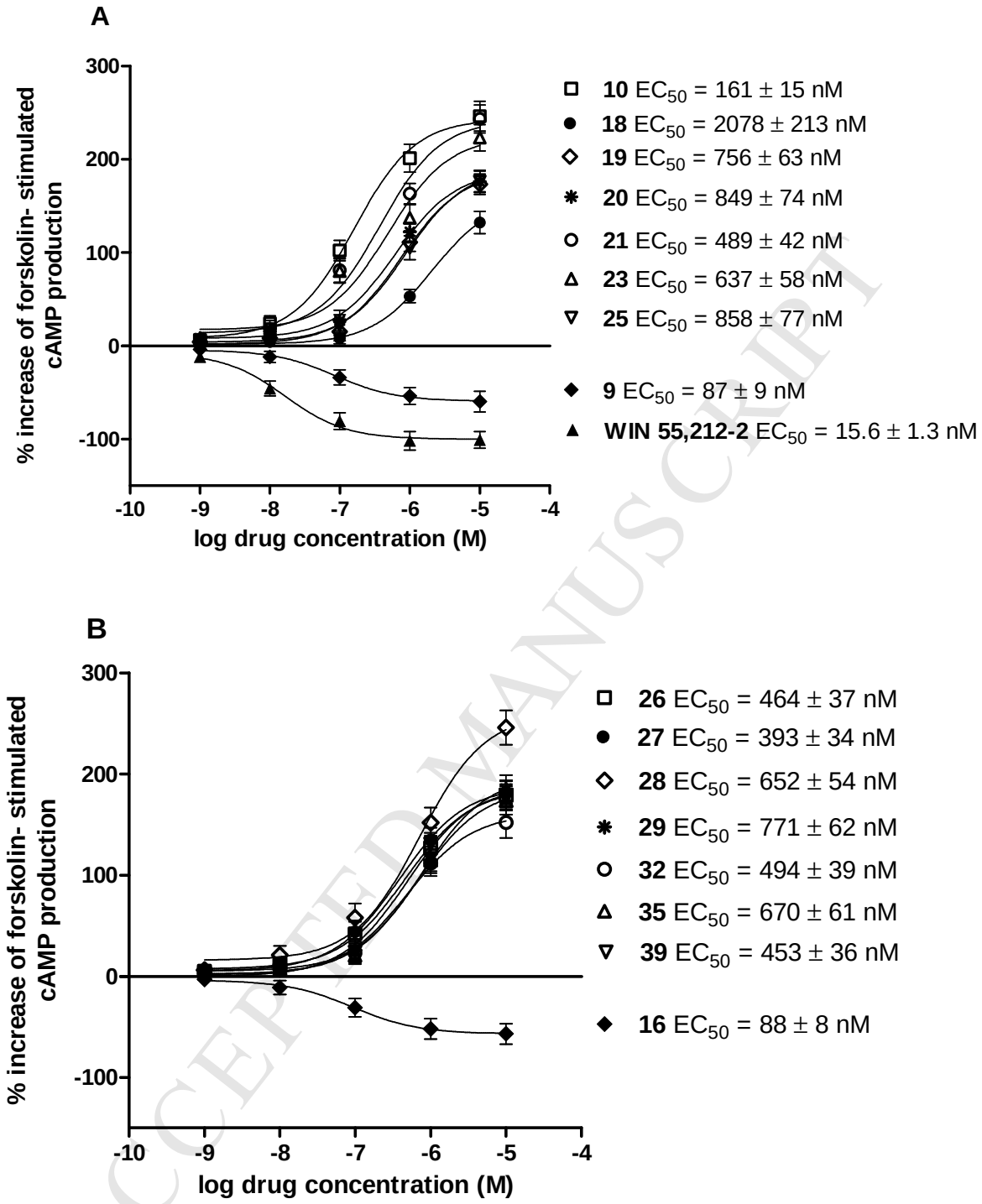


Figure 1. Effect of the new CB compounds on cAMP accumulation in comparison with the two previous derivatives 9 and 10, and the reference agonist WIN 55,212-2 in CB_2-transfected CHO cells. Values are reported as percentage of increase of forskolin-stimulated cAMP production and are expressed as mean ± SEM of three independent experiments performed in duplicate.

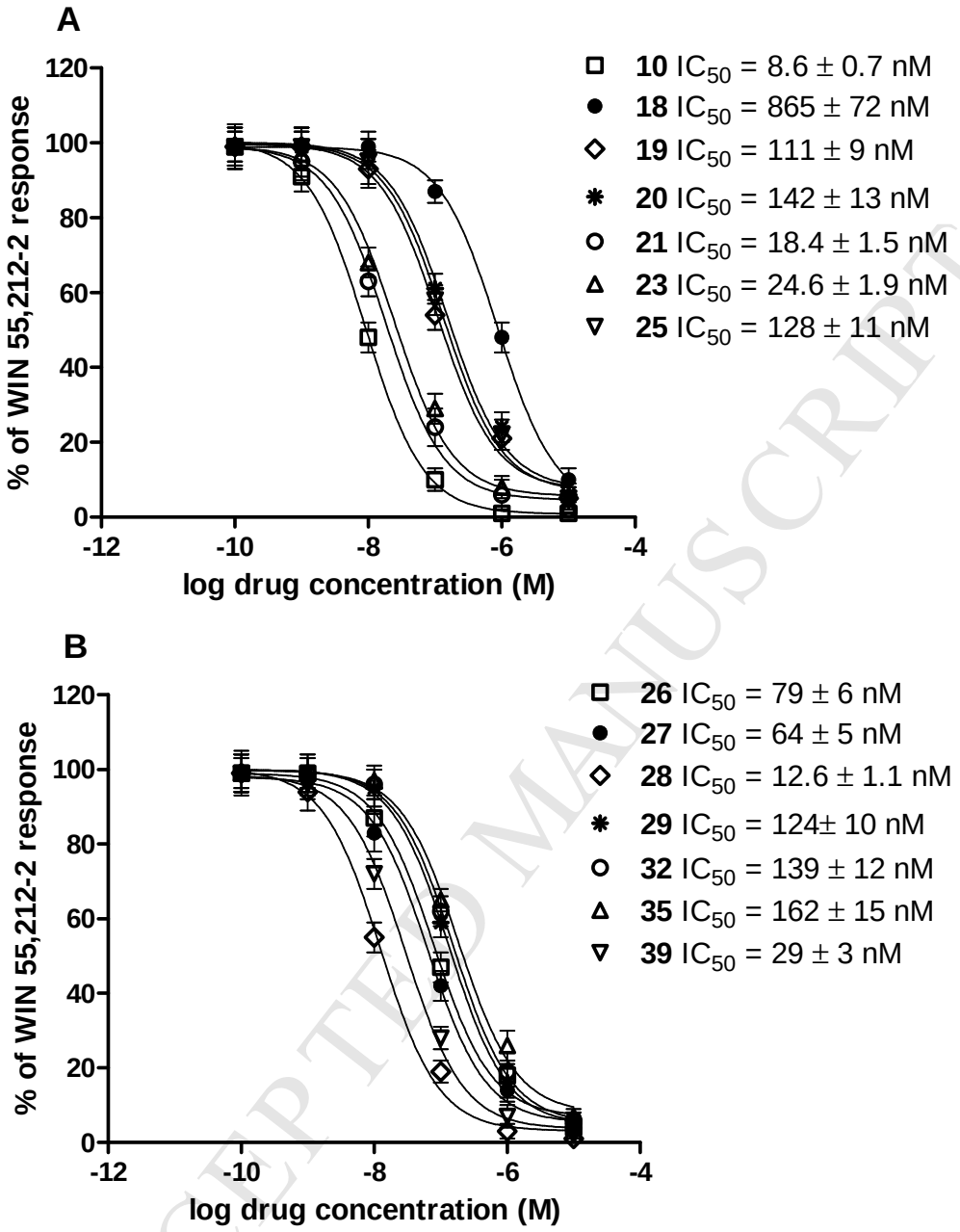


Figure 2. Antagonism of the new CB ligands evaluating their capability to counteract the inhibitory effect of WIN 55,212-2 (20 nM) on forskolin-stimulated cAMP production in CB_2-transfected CHO cells. Values are expressed as mean ± SEM of three independent experiments performed in duplicate.

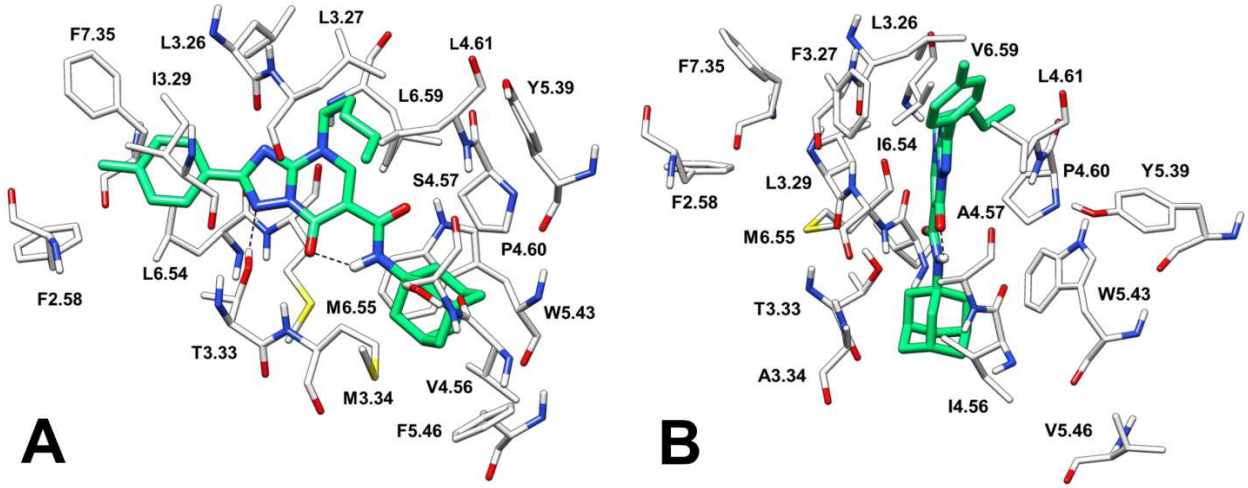


Figure 3. Energy minimized complex of compound **28** docked into CB_2 (A) and CB_1 (B) receptors

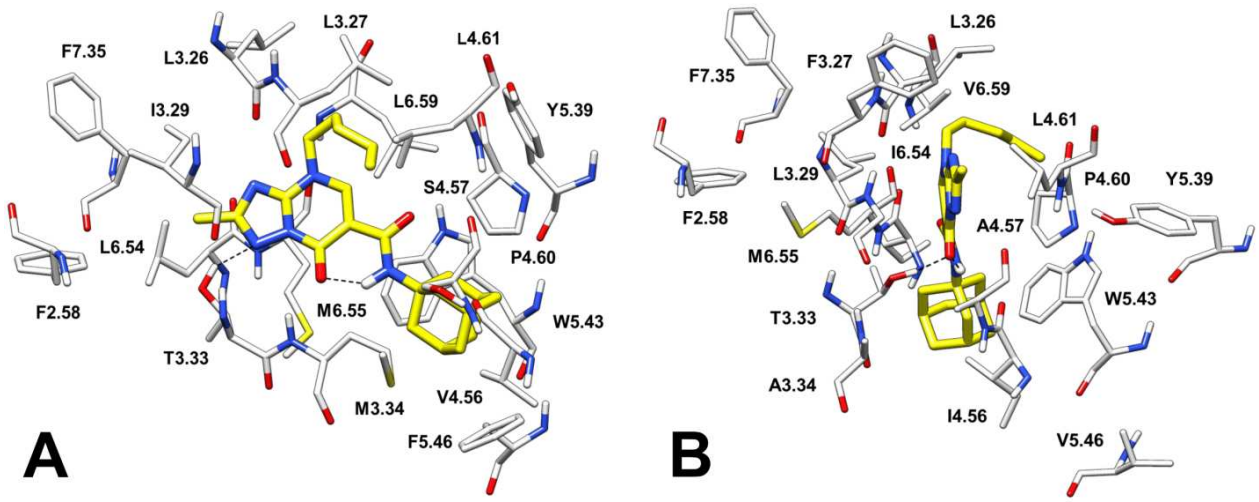


Figure 4. Energy minimized complex of compound **18** docked into CB_2 (A) and CB_1 (B) receptors

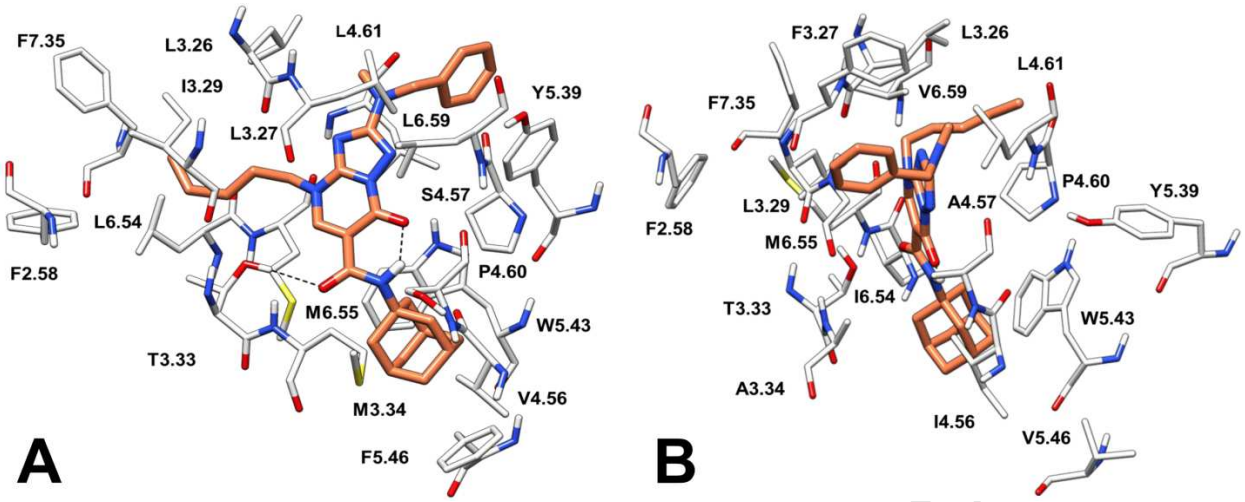


Figure 5. Energy minimized complex of compound **39** docked into CB_2 (A) and CB_1 (B) receptors

Highlights

- Novel triazolo[1,5-*a*]pyrimidine-6-carboxamide derivatives were synthesized.
- Affinity (K_i, nM) on human CB_1 and CB_2 receptors of the novel derivatives is reported.
- The most active tiazolopyrimidines exhibited greater selectivity than reference compound.
- Introduction of 2-substitutents at triazolopyrimidine core changes the functional activity.
- The new ligands were able to significantly increase cAMP production acting as inverse agonists.