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Perspectives of Cannabinoid type 2 receptor (CB2R) ligands in neurodegenerative disorders: Structure Affinity Relationship (SAfiR) and Structure Activity Relationship (SAR) Studies

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Abstract

Up-regulation of CB2R on activated microglial cells, the first step in neurodegeneration, has been widely demonstrated, and this finding makes the receptor a promising target in the early diagnosis and treatment of several neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis (MS). The development of CB2R PET ligands could help demonstrate the neurodegenerative pathogenesis, thus providing useful tools for characterizing the role of neuroinflammation in the progression of these disorders. CB2R agonists and inverse agonists have emerged as neuroprotective agents and CB2R agonists have entered several clinical trials. CB2R ligands have therefore received great attention and different molecular scaffolds have been selected to target CB2R subtypes. This perspective is focused on structure-activity relationship (SAR) and structure-affinity relationship (SAfiR) studies performed on different scaffolds with the aim to identify the molecular features useful for the design of both therapeutic and diagnostic agents.

INTRODUCTION

The endogenous cannabinoid system (ECS) is a neuromodulatory system consisting of two G-protein coupled receptor subtypes (CB1R and CB2R), and endogenous ligands and enzymes responsible for their synthesis and degradation.[1] Cannabinoid type 1 receptor (CB1R) is the most abundant, and is mainly expressed in the central nervous system (CNS), in neurons and glial cells, where it modulates several functions such as memory and cognition, emotion and pain control.[2] In the peripheral tissue (lung, kidney, and liver) CB1R modulates metabolism and energy balance.[3] Cannabinoid type 2 receptor (CB2R) is mainly localized in the immune system (macrophages), and recent studies demonstrated its selective up-regulation in microglia in response to insults from neuroinflammation, as seen during neurodegeneration.[4]

Several pieces of evidence suggested the existence of two microglial activation states, M1 and M2, involved in the different stages of the neurodegenerative disorders. The M1 state is neurotoxic as it is characterized by the release of pro-inflammatory factors (IL-1β, IL-8, IL-6, TNF-α, and inducible NOS2), and contributes to secondary neuronal damage, cell death and demyelination, leading to neurodegeneration. The M2 state, defined "alternative activation", is neuroprotective as associated with the release of anti-inflammatory factors (IL-10, IL-4, and TGF-β).[5,6] The activation of CB2Rs on microglial cells has been shown to inhibit the release of pro-inflammatory cytokines and to induce the release of anti-inflammatory cytokines.[7] Therefore, the ECS might play a crucial role in microglial-derived neuroinflammation through the activation of CB2Rs, regulating distinct components of the brain's inflammatory response, including microglial cell proliferation, migration and differentiation into M1 or M2 phenotypes.

In view of all this, CB2R is emerging as a novel target for the treatment and early diagnosis of different neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis (MS).

In AD patients, CB2R is overexpressed in microglial cells surrounding beta-amyloid plaques[7]. *In vitro* and *in vivo* studies performed in different AD mouse models showed that CB2R agonists reduce

beta-amyloid levels and inhibit plaque deposition.[8-11] The up-regulation of CB2R has also been detected in microglial cells recruited to the nigrostriatal area in response to MPTP- or LPS-induced lesions in PD animal models[12,13] as well as in the *substantia nigra* of PD patients.[14,15] The involvement of CB2R in HD has been demonstrated by an *in vivo* study: *i*) CB2R gene deletion accelerated HD onset, exacerbating the pathology severity; *ii*) CB2R signaling in peripheral immune cells suppressed neurodegeneration and induced neuroprotection in HD mouse models; *iii*) CB2R activation modulated IL-6 levels that are responsible for the disease phenotypes in HD mice.[16] These findings support a direct link between CB2R signaling and the onset and severity of neurodegeneration in HD, providing a novel therapeutic strategy for this disease. Several studies also evaluated the analgesic efficacy of cannabinoids ligands in MS; recently, Fu et al. investigated the effect of the CB2R specific agonist 3-(1,1-dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol (JWH-133) in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS and observed its anti-hyperalgesic effects.[17] This was the first pre-clinical study suggesting CB2R as a novel target for the treatment of central pain in an animal model of multiple sclerosis.[17]

Therefore, CB2R ligands have received great attention, and different molecular scaffolds have been selected to target it. Structure Activity Relationship (SAR) and Structure Affinity Relationship (SAfiR) studies have been performed to identify the molecular features useful for the design of both therapeutic and diagnostic agents.

Recently, CB2R inverse agonists have emerged as new therapeutic strategy in neurodegenerative disorders, and new studies are ongoing to better understand their mechanism of action.[18]

Cannabinoid receptor ligands: the starting point

Δ^9-Tetrahydrocannabinol (Δ^9-THC, Fig. 1) is the principal psychoactive constituent of *Cannabis* sativa.[19] Δ^9-THC acts as a dual agonist for both cannabinoid receptors and thanks to this mechanism, it exhibits excellent analgesic effects.[20] Unfortunately, Δ^9-THC is associated with several psychotropic adverse effects, many of which are thought to be due to the activation of central CB1R.[21]

The occurrence of these adverse effects has limited the clinical application of all the cannabinoid dual agonists discovered in the last years, including compounds with structures that differ quite dramatically from Δ^9-THC. Starting from some well-known non-selective CBRs ligands, many efforts have been focused on developing CB2R selective derivatives, as they are devoid of CNS side effects[22,23] and play a pivotal role in analgesic and anti-inflammatory pathways. CB2R selective derivatives, which are the main subject of this Perspective, have been developed starting from certain important structural requirements shown by other classes of CBRs ligands (Fig. 1),[24-32] such as mono- or bicyclic core bearing heteroatoms (e.g. oxygen or nitrogen); bulky aliphatic or aromatic carboxamide groups; either alkyl or aryl or arylalkyl substituents.

Figure 1

1. SAfiR and SAR studies on different scaffolds

During the last three years, several studies have been focused on the research of the molecular determinants responsible for: *i*) the affinity towards CB2R; *ii*) the selectivity *versus* CB1R; *iii*) the CB2R activity profile (agonist, antagonist or inverse agonist). These results led to the design of novel CB2R ligands for the treatment and the early diagnosis of the neurodegenerative diseases.

Several scaffolds have been identified: oxoquinoline, naphthyridinone, quinolinedione, alkyloxycoumarin, indole, indazole, imidazopyridine, imidazopyrazine, benzimidazole, purine, thiophene, triazine, pyridinone, biphenyl, proline, and piperidine. All the reported small molecules targeting CB2R are characterized by the above mentioned scaffolds bound by different linkers (such as a carboxy, carboxamide, carboxylate or enamine function) to the adamantane tricycle or to its structurally simplified analogues. For each scaffold, the detailed SAR and SAfiR studies are reported in the following sections.

Oxoquinolines. In the last decade, Pasquini and co-workers developed a large series of quinolin-4(1*H*)-one-3-carboxamides with high affinity and selectivity towards CB2R.[33-38] As a result,

important information about SAR is available for this class. In general, the nature and the position of the substituent on the condensed benzene ring can influence the activity towards the CB2R subtype: an aliphatic group in the 6-position leads to compounds with agonist profile,[33,34] while an aryl or heteroaryl substituent in the same position leads to compounds with inverse agonist profile[34,35] (Fig. 2A). In a recent effort to improve the physicochemical properties (mainly the solubility in water) of quinolin-4(1*H*)-one-3-carboxamide derivatives, Mugnaini et al. converted the quinolin-4(1*H*)-one nucleus into a 4-hydroxy-quinolin-2(1*H*)-one. Such replacement led to derivatives with a slightly improved physicochemical profile, while a switch to inverse agonist activity was detected for alkyl-substituted 4-hydroxy-quinolin-2(1*H*)-ones. On the other hand, the aryl substituted ones were switched to CB2R agonists (Fig. 2B).[36,37] Many of the activities discussed here have been evaluated in a formalin test of acute peripheral and inflammatory pain in mice.

Figure 2

SAfiR studies on various quinolinone scaffolds are reported in Figure 3. Several substituents at the 4-oxoquinoline scaffold have been investigated, particularly at the N_1 atom (R_1), at the *N*-carboxammidic atom (R_2), and on the benzene ring (R_3).

- The substituent R_1 influences the binding capability to both CBRs: high CBRs affinities were mainly due to alkyl linear chains with the *n*-pentyl group leading to the best results.[37] However, also aryl and arylalkyl groups were tolerated, specially by CB2R.[38]
- 3-Carboxammide substituent (R_2) is involved both in the binding affinity and in the selectivity profile towards CBRs: a) bulky and lipophilic substituents allowed high CBRs affinities, with the highest CB2R affinity and selectivity associated to an adamantyl group; b) a fenchyl group resulted in high-affinity and non-selective derivatives; c) alkyls, phenylethyl, benzyl, and groups bearing heteroatoms led to low binding affinity.[33,34,38]

- Substituent R_3 on the condensed benzene ring has an important role in the affinity and selectivity profile. Compounds with affinity and selectivity for CB2R were obtained with a substituent in the 6-position (aryl, alkyl, alkenyl, and alkynyl) or in the 8-position (electron donating groups, EDG, such as $-CH_3$ or $-OCH_3$); by contrast, the lack of substituents or a substituent in the 7-position usually led to less selective derivatives.[37,34,37,38]

Figure 3

As mentioned before, further modifications have been performed in order to ameliorate the physicochemical properties and, in particular, aqueous solubility. In a first effort, attention was directed towards the 3-carboxamide functional group, and the bioisosteric heteroaryl 5-membered rings systems consisting of 1,2,4-, 1,3,4-oxadiazol, and 1,2,3-triazol were evaluated: 1,2,3-triazol resulted as the best bioisoster in terms of CB2R affinity, selectivity and physicochemical properties (K_i CB2 = 1.2 nM, $SI_{CB1/CB2}$ > 8620 for derivative bearing a N_1-pentyl and 6-(2-furyl) substituents).[39] In a second effort, the 4-oxoquinoline core was turned into the 4-hydroxy-2-oxoquinoline, showing both high CB2R affinity and selectivity and higher aqueous solubility.[36] Worth noting is that i) an aryl substituent in the 6-position was tolerated less than an alkyl substituent (e.g. derivative bearing a N_1-pentyl chain and either 2-furyl or *i*-propyl in the 6-position displayed K_i value for CB2R of 15 nM and 0.53 nM, respectively), and ii) the 2-oxoquinoline, in place of 4-oxoquinoline, displayed high CB2R affinity (in a subnanomolar range) but no selectivity,[36,40] whereas 4-hydroxy-2-oxoquinoline showed both high CB2R affinity and high selectivity *vs* CB1R.[36]

1,8-Naphthyridin-2(1*H*)-ones. Manera and colleagues developed a series of 1,8-naphthyridin-2(1*H*)-one-3-carboxamides.[40,41] Recently, starting from the nucleus reported in Figure 4 that bears a 3-(4-methylcyclohexyl)-carboxamide function and displays high CB2R affinities,,[40] some modifications have been performed to evaluate the effect of:[42,43]

1. different N_1-substituents (R_1), such as an aliphatic chain bearing an electronegative atom, a morpholinoethyl or *p*-fluorobenzyl groups;
2. 6-aryl and 6-bromo substituents (R_2);
3. hydroxy group in the 4-position.

N_1-hydroxyalkyl derivatives showed higher CB2R affinity and higher selectivity toward CB1R than N_1-morpholinoethyl and N_1-*p*-fluorobenzyl derivatives. Moreover, elongation to five methylenes alkyl chain led to the best results (K_i CB2 = 3.6 nM, $SI_{CB1/CB2}$ > 2778). In the series of N_1-morpholinoethyl derivatives, a 6-aryl substituent increased CB1R affinity leading to less selective CB2R compounds, except for the 6-*p*-methoxyphenyl group, which displayed the highest selectivity index ($SI_{CB1/CB2}$ > 6902). In the 6-substituted N_1-*p*-fluorobenzyl derivatives, all modifications induced high affinity and high selectivity towards CB2R. The bromine atom in the 6-position caused slight changes in the CBRs affinities for both series, leading to a 500-fold higher selectivity for CB2R.[43] Functionalization in the 4-position of 1,8-naphthyridin-2(1*H*)-one with a hydroxy group gave different results depending on N_1-substitution: high CB1R and lower CB2R affinities were recorded by N_1-morpholinoethyl derivatives, whereas no change in the biological profile was detected in the corresponding N_1-*p*-fluorobenzyl derivatives.[42]

Figure 4

Importantly, according to SAR studies, substituents at the 6-position on the bicyclic core conferred an inverse agonist profile to CB2R ligands. On the other hand, compounds devoid of that substituent displayed an agonist profile.[43]

Quinoline-2,4(1*H*,3*H*)-diones. Han and co-workers developed a series of quinolone-2,4-diones characterized by the lack of the carboxamide function in the 3-position (substituted by an enamine function) and by the presence of two oxygen carbonyl groups in the 2- and 4-position of the quinoline

core (Fig. 5).[44] These compounds showed a quite clear SAR since a C5- or C8-substituent conferred CB2R agonist activity, whereas a C6- or C7-substituent conferred a CB2R antagonist/inverse agonist activity *in vitro*. As for the CB2R agonists (Fig. 5A), 8-substitution was better than 5-substitution both in terms of activity and selectivity. An 8-methyl group (EC_{50} CB2 = 0.0927 μM for derivative bearing *n*-butyl as R_1 cyclohexyl as R_2) led to better affinity and activity profiles than 8-ethyl, 8-methoxy, or 8-chloro substituents. Furthermore, in the 8-substitued series, the replacement of the N_1-butyl chain with either shorter or longer chain, cyclopropylmethyl, or an unsaturated chain led to a decrease both in the potency and in the selectivity for CB2R. The modification of the 3-enamine-cyclohexyl substituent (R_2) had different effects depending on the substituent: a) the bulky adamantly, cyclopropylmethyl, and cyclobutylmethyl groups caused a small decrease in the potency (EC_{50} CB2 = 0.127-0.317 μM); b) aryl or heteroaryl led to inactive compounds; c) four and five carbons chains and a cyclopentyl generated derivatives with high CB2R agonist potency and selectivity (EC_{50} CB2 = 0.0200-0.116 μM).[44]

As for the CB2R antagonists (Fig. 5B), compounds bearing a substituent in the 7-position displayed higher CB2R potency and selectivity than the 6-substituted ones; the 7-methyl derivative was found to be more active and selective than the 6-methyl, 7-methoxy, or 7-chloro derivatives (IC_{50} CB2 = 0.00903 μM for cyclohexylenamine derivative). Differently from the agonist derivatives, higher CB2R antagonist potency is associated to a N_1-pentyl chain and bulkier substituents on the enamine function.: the replacement of a cyclohexyl with an adamantyl led to a 20-fold increase of CB2R activity and selectivity (IC_{50} CB2 = 0.0817 μM).[44]

The agonist bearing R_1 as a butyl chain, R_2 as a cyclohexyl and R_3 as a methyl substituent (Fig 5A) was tested *in vivo* in EAE mouse model of multiple sclerosis; the results demonstrated a decrease in leukocyte infiltration in the spinal cord and a reduction of the extensive demyelination in white matter, indicating a potential therapeutic application of these quinoline-2,4(1*H*,3*H*)-dione derivatives in multiple sclerosis.[44]

Figure 5

8-Alkyloxycoumarin-3-carboxamides. A series of coumarin derivatives as novel selective ligands for CB2R has been recently developed by Han et al.[45] These compounds were designed on the basis of a CoMFA model built up on fifty-five known CB2R selective 2-pyridinone and 2-quinolinone derivatives. This study allowed for an understanding of the structural requirements responsible for the binding to the CB2R, clarifying that the NH group at the 1-position of both classes did not directly interact with the receptor. Therefore, the NH group was isosterically substituted with an oxygen atom leading to the conversion of the quinolinone scaffold to the coumarin one, while the carbonyl group on the 2-position was retained as a potential H-bond acceptor.[45]

SAR studies were carried out on this class of compounds. Firstly, as depicted in Figure 6, SAR investigations were focused on the R_1 substituent of the coumarin nucleus bearing as R_2 a cyclohexyl ring and no substituent in position 5, 6, or 7.[45] Different lengths of alkyl chains (from two to six atoms) and bulky groups (cyclopropylmethyl and benzyl) were evaluated and, among them, compounds with an 8-butyloxy group showed the best CB2R agonist activity (EC_{50} CB2 = 0.144 μM, $SI_{CB1/CB2}$ = 69.4). Thus an R_1 *n*-butyl chain was selected while the cyclohexyl ring on the amide function (R_2) was replaced by different aliphatic groups such as: *i*) smaller (cyclopropyl and cyclopropylmethyl) or bulkier (adamantyl) nuclei; *ii*) linear or branched alkyl chains; *iii*) polar substituents. These modifications on R_2 led to the following results:[45]

- cyclopropyl, cyclopropylmethyl or adamantyl insertion led to inactive compounds; a cyclopentyl group was associated with a 5.5-fold decrease in CB2R potency and selectivity;
- *n*-butyl or *tert*-butyl substituents were detrimental for CB2R activity and selectivity, whereas 3-carbon (*i*-propyl, EC_{50} CB2 = 0.103 μM, $SI_{CB1/CB2}$ = 97.1) and 5-carbon alkyl chains isomers (2-pentyl - EC_{50} CB2 = 0.321 μM, $SI_{CB1/CB2}$ = 31.2 - and 3-pentyl - EC_{50} CB2 = 0.130 μM, $SI_{CB1/CB2}$ = 76.9) led to compounds with high CB2R agonistic potency and selectivity;

- incorporation of the 3-carboxamide *N*-atom in a piperidine ring decreased agonist potency, indicating that the presence of a tertiary amide function leads to the loss of agonist activity.

In a second set of coumarin derivatives, modifications on the bicyclic core were performed by inserting 6-bromo or 7-methoxy groups as R_3. Interestingly and in accordance to the previously reported scaffolds, all these derivatives showed an antagonist profile. In the 6-bromo series, all the modifications on both 8-butyloxy and 3-carboxycyclohexylamide were detrimental for the antagonistic activity (IC_{50} CB2 = 0.090 μM, $SI_{CB1/CB2}$ = 111). The 7-methoxy derivatives better tolerated modifications on R_1 and R_2 without changes in the activity profile: the 8-pentyloxy substituent rather than 8-butyloxy caused only a 3-fold decrease in IC_{50}. As for the 3-carboxamide function, also in this second series of coumarin, 5-carbon alkyl chains were associated with high potencies, higher than the potency associated with the cyclohexyl group (IC_{50} CB2 = 0.019-0.022 μM, $SI_{CB1/CB2}$ > 454.5).[45]

Figure 6

Indoles. The indole scaffold is typical of several cannabinoid synthetic drugs. Recently different classes of 3-substituted *N*-alkylindole derivatives, such as *N*-alkyl-3-acylindoles, *N*-alkylindole-3-carboxylates, and *N*-alkylindole-3-carboxamides, were studied by Banister and colleagues.[46-48]

SAR studies are depicted in Figure 7 and they were focused on modifications at the 1- and 3- positions on the indole ring (R_1 and R_2).

A first study compared well known non-selective *N*-pentylindoles-3-substituted synthetic cannabinoids and their corresponding *N*-(5-fluoropentyl) analogues, suggesting that the terminal fluorination of R_1 generally enhanced the potency of CBRs activation keeping the non-selective profile.[46] Worth noting is the ability of a -OH group in the same position (5-position of *N*-pentyl chain) to increase CB1R EC_{50} of (1-pentyl-1*H*-indol-3-yl)(2,2,3,3-

tetramethylcyclopropyl)methanone (UR-144)[49] leading to 300-fold CB2R *vs* CB1R more selective derivative.[46]

Additional studies were performed and, in particular, a series of *N*-butyl-3-benzoylindoles and *N*-pentyl-3-benzoylindoles variously substituted on R_2-phenyl ring were screened towards CB1R and CB2R.[47] Interestingly, *N*-butyl substitution conferred functional selectivity towards CB2R ($SI_{CB1/CB2}$ = 31.2-42.2), while *N*-pentyl group increased CB1R potency and slightly decreased CB2R potency ($SI_{CB1/CB2}$ = 3.2-7.8). The presence and the position of a methoxy group on the benzoyl ring did not influence the activity but, in general, the substitution on the 2-position conferred the greatest potency at CB1R and CB2R for both *N*-butyl (EC_{50} CB1 = 178 nM, EC_{50} CB2 = 4.5 nM, $SI_{CB1/CB2}$ = 39.6) and *N*-pentyl (EC_{50} CB1 = 54 nM, EC_{50} CB2 = 6.9 nM, $SI_{CB1/CB2}$ = 7.8) series.[47]

Among the *N*-indole carboxamide drugs displaying cannabinoid mimetic effects, the valinate and *tert*-leucinate methyl esters were interesting because all these compounds acted as potent agonists at CB1R and CB2R. In the *tert*-leucinate functionalized compounds, the nature of *N*-alkyl substituent displayed a robust effect on CB1R potency (Fig. 7).[48]

Figure 7

Indazoles. An interesting class of cannabinoid compounds, inspired by recent Pfizer patents,[50,51] includes an indazole moiety substituted at the 1-position with various aliphatic, alicyclic, or aromatic groups (R_1), and at the 3-position with valine- or *tert*-leucine-derived carboxamides (R_2). In a recent study, Longworth et al. tested a series of 1-alkyl and 2-alkyl indazoles toward both cannabinoid receptors (Fig. 8). 1-Alkyl isomers showed high CB1R agonist activity displaying EC_{50} values in nanomolar range (2.1-7.8 nM). These indazoles derivatives also showed agonist activity in the nanomolar range towards CB2R, but their CB2R activity was 1.5-3.2-fold less potent than their CB1R activity. By contrast, 2-alkyl isomers displayed low potency toward both cannabinoid receptors.[52]

Figure 8

Imidazopyridines, imidazopyrazines and benzimidazoles. Starting from the indole derivative discovered at Merck Frosst, GW405833,[53] which displays CB2R nanomolar affinity and good selectivity *vs* CB1R (K_i CB2 = 10 nM, $SI_{CB1/CB2}$ = 200), Trotter and colleagues developed three series of new potential CB2R agonists differing for the substitution in the 1- and 3-position: Type I and Type II imidazopyridines and Type III imidazopyrazines (Fig. 9).[54]

Figure 9

These three series were tested for their activity towards both CBRs and for their binding to plasma proteins, because a higher plasma free fraction could be related with a higher ability to reach the CNS. Type I and Type II imidazopyridines generally displayed a CB2R agonist profile (IC_{50} CB2 = 0.3-44 nM) better than imidazopyrazine derivatives (IC_{50} CB2 = 36-139 nM), and the introduction of polar groups in R_2 increased plasma free fraction for all derivatives. As for the selectivity *vs* CB1R, all Type I derivatives showed moderate to high selectivity index ($SI_{CB1/CB2}$ > 200), retaining a small CB1R agonist activity (IC_{50} CB1 = 90-12700 nM); whereas, some Type II imidazopyridines and all imidazopyrazine derivatives showed a selectivity index in the same range, but with a complete loss of CB1R affinity (IC_{50} CB1 > 17000 nM). Therefore, the development of these three series suggests that CB2R agonist potency is modulated by modifications on the amide (R_1) and amino (R_2) functionalities, while the plasma protein binding ability is impacted by the presence of polar atoms.[54] Among these three series, compounds **1** and **2** depicted in Figure 10 showed well balanced activity, selectivity, and plasma protein binding and were selected for an *in vivo* study in a rat CFA (Complete Freund's adjuvant) hyperalgesia model. These two compounds exhibited different profiles in the rat CFA model and, although their concentrations in the plasma and in the CNS exceeded their IC_{50} values toward CB2R, only compound **1** showed a dose-dependent analgesic effect. To explain these

different *in vivo* behaviors, the analgesia seen for compound **1** was correlated to its residual capability to bind CB1R, which are more expressed in the CNS than CB2Rs.[54]

Figure 10

In the effort to develop CB2R agonists and to better understand their involvement in CNS diseases, Nanda and colleagues converted the imidazopyridine scaffold mentioned above in a benzimidazole one.[55] As depicted in Figure 11, SAR studies on compounds bearing arylmethyl or aliphatic amides at the benzimidazole 2-position have been performed. Among arylmethyl amides, compounds bearing an ethyl as R_1 were found more potent and CB2R-selective than *N*-unsubstituted benzimidazoles. The most potent CB2R compound of this series was the derivative bearing a 2-chlorobenzyl substituent as R_2, showing low EC_{50} value (9.9 nM) and 92-fold selectivity over CB1R. All of the other performed substitutions on the aryl in R_2, including the introduction of heteroatoms, led to a general decrease of CB2R potency (EC_{50} = 40-1437 nM) and to the complete loss of CB1R potency, showing moderate to high selectivity *vs* CB1R subtype (as for the derivatives bearing a 2-fluorobenzyl, $SI_{CB1/CB2} > 377$ or a 3-chlorobenzyl, $SI_{CB1/CB2} > 425$). In the benzimidazoles bearing aliphatic amides, an ethyl group as R_1 led to less potent and selective derivatives. As for the R_2 substituent, a bulky and lipophilic adamantyl group led to the highest CB2R potency detected in the series (EC_{50} = 1.9 nM), but the selectivity over CB1R was only 65-fold. The replacement of the adamantyl nucleus with a *tert*-butyl methyl group slightly decreased CB2R agonist activity (EC_{50} = 14.7 nM), but led to a high selectivity over CB1R ($SI_{CB1/CB2} > 1156$). In order to decrease the lipophilicity of these ligands, aliphatic amides bearing hydroxy groups were also designed, and potent and selective CB2R agonists were developed (e.g. EC_{50} CB2 = 6.3 nM, $SI_{CB1/CB2} > 2698$ for derivative bearing a hydrogen as R_1 and a 2-(2,3-dihydroxy)-propyl as R_2). Nevertheless, none of these derivatives has been selected for *in vivo* studies and further optimization is needed to ameliorate the pharmacokinetic profile.[55]

Figure 11

Purines. Eli Lilly researchers screened several compounds on CB1R and CB2R identifying the thienopyrimidine derivative reported in Figure 12A as CB2R agonist displaying appreciable activity, but moderate selectivity toward the subtype 1. Several modifications have been performed on this scaffold to ameliorate the pharmacodynamic profile and to overcome the limits due to the poor *in vitro* metabolic stability and high *in vivo* clearance, leading to a new more polar purine scaffold (Fig. 12B).[56]

Figure 12

All these new ligands were potent CB2R agonists and displayed an excellent selectivity toward CB1R. Compound **3** reported in Figure 13 shows a good pharmacodynamic profile (appreciable activity and high CB2R selectivity), a good pharmacokinetic profile (brain penetrant since not a P-gp substrate), and good biopharmaceutical properties (high water-solubility).[56] With a chloro as R_3 substituent (Fig. 12B), SAR studies were carried out: the purine N_9- (R_1) and the piperazine $N_{4'}$- (R_2) atoms were functionalized in order to reduce GABA-gated chloride receptors[57] binding ability and ameliorate CYP metabolism. Selectivity profile, GABA-gated chloride receptors binding ability, and CYP metabolism susceptibility were influenced by the substituent in the 9-position of the purine core. Therefore, various hydroxyalkyl chains were selected because of the alternative metabolic routes other than CYP metabolism linked to -OH group. Compound **4** (Fig. 13) with a (*2R*)-3-hydroxy-2-propyl chain showed activity, selectivity, and pharmacokinetic profiles comparable with the lead compound **3** and no significant binding to GABA-gated chloride receptors. Moreover, compound **4** was found to be brain penetrant as it was not a P-gp substrate, and it showed a CB2R agonist profile in MIA (monoiodoacetic acid) induced joint pain assay.[56,58]

Figure 13

Thiophenes. In a CB2R focused screening approach, a thiophene nucleus was introduced as bioisoster of the CB2R-active indole scaffold. Benzo- and tetrahydrobenzo[b]thiophene derivatives (Fig. 14) have been developed and SAR studies have been performed on these two new scaffolds.[59,60] The results displayed that the presence of an amide function in the 2-position on the tetrahydrobenzo[b]thiophene nucleus is crucial for the binding to the CB2R; indeed, the corresponding amine and sulphonamide analogs do not show CB2R affinity. Compound with a 2-adamantylamide group shows the best affinity and selectivity toward CB2R and agonist profile (Fig. 14A).[59]

On the benzothiophene core, modifications in the 2- (R_1) and in the 3-position (R_2) have been introduced: alkyl chains or 4-methoxyphenyl as R_1, and an aromatic ketone as R_2 (Fig. 14B). On the basis of the substituent in the 3-position of benzothiophene (R_2), two different series were developed: the 1-naphthoyl-bearing and the 4-methoxybenzoyl-bearing series. The derivatives belonging to the latter series displayed high CB2R affinity and low CB1R affinity compared to the former series. These findings were corroborated by molecular docking studies: the 1-naphthoyl series lacks a π-cation interaction (between CB2R Lys 109 and the benzothiophene ring) that is characteristic of the 4-methoxybenzoyl series. Moreover, the naphthoyl moiety is able to interact with CB1R Phe 174 (5.43) through a strong T-shaped π-π interaction that is not found in docking poses of 4-methoxybenzoyl moiety. Compounds devoid of the alkyl chain showed different binding profiles explainable by different docking poses with CB2R: compound bearing a 1-naphthoyl moiety in the 2-position is able to interact with both Lys 109 and Thr 114 in CB2R, as for 3-(4-methoxybenzoyl) derivatives and showed good CB2R affinity and selectivity (K_i CB2 = 0.2 μM, $SI_{CB1/CB2} > 50$).[60]

Figure 14

1,3,5-Triazines. Yrjölä and co-workers developed a series of 1,3,5-Triazines as CB2R ligands, and SAR studies demonstrated that the presence of substituents in 2-, 4- and 6- positions exerted a specific role in the activity and selectivity profile towards CB2R (Fig. 15).[61,62] Indeed, the presence of an ethoxy substituent as R_1 and (4-methyl)-piperidine as R_3 led to potent CB2R compounds, and changes in these two positions usually induced a decrease in CB2R potency. By contrast, modifications in the 4-position (R_2) could be better tolerated, and bulky groups (e.g. adamantyl) generally caused an increase in the agonist potency.[61] Moreover, in order to obtain more water-soluble CB2R ligands, several modifications have been performed on the 1,3,5-triazine scaffold. While the ethoxy substituent was kept as R_1 and a cyclopentylamine or an adamantylamine group as R_2, more polar groups, such as morpholine, thiomorpholine, or substituted piperazines, were inserted in the 6-position as R_3. The best results were obtained with N_4-methyl or N_4-(2-fluoroethyl)-piperazine substituents that conferred potent and selective CB2R agonist profile compared to morpholine and thiomorpholine-1,1-dioxide.[62]

Figure 15

2-Pyridinone-3-carboxamides. This class of compounds was developed by Lucchesi et al. through a molecular simplification strategy starting from 1,8-naphthyridin-2(1*H*)-one-3-carboxamides.[40,63] SAR and SAfiR studies performed on this class of ligands are reported in Figure 16. Cyclohexyl, 4-methylcyclohexyl, or cycloheptyl rings were tested as R_1, and the best results in terms of CB2R affinity were found for derivatives bearing a cycloheptyl substituent. The presence of *m*- and *p*-iodobenzyl, morpholinoethyl,[38] butyric acid, butyric ester, alkyl, and hydroxyalkyl chains as R_2 were probed and all induced a reduction or a total loss of the affinity towards both CBR subtypes. The only exception was the *p*-fluorobenzyl derivative which displayed high CB2R affinity, but low CB2R selectivity (K_i CB2 = 7.8 nM, $SI_{CB1/CB2}$ = 5.5). Different modifications were also performed on the substituent in 5-position of 2-pyridinone scaffold (R_3), and both bromine and aryl groups led to an

increased affinity at both subtypes. The only exception was detected with the 5-*p*-methoxyphenyl substituent, which increased CB2R affinity whilst slightly decreasing CB1R affinity. Among all 2-pyridinone tested compounds, derivative with cycloheptyl as R_1, *p*-fluorobenzyl as R_2, and *p*-methoxyphenyl as R_3 showed the highest affinity and selectivity towards CB2R (K_i CB2 = 1.0 nM, $SI_{CB1/CB2}$ = 63).[63]

Substituent at the 5-position on 2-pyridinone scaffold (R_3) were also found pivotal for the activity profile, as it determined the switch from an agonist profile (absence of C5-substituent) to an antagonist/inverse agonist profile, indicating that bulky substituents in that position may play the same role as C6- and C7- substituent in the 1,8-naphthyridin-2(1*H*)-one-3-carboxamide scaffold (Fig. 16).[63]

Figure 16

Biphenylic carboxamides. During the last years, Bertini et al. developed a series of biphenylic carboxamides as bioisosteric modification of 2-pyridinones.[64,65] Starting from the scaffold depicted in Figure 17, three modifications have been performed in order to improve CB2R affinity and selectivity:

1. an aliphatic (methyl or *n*-butyl) or an aromatic (benzyl or *p*-fluorobenzyl) substituent as R_1;
2. cycloheptyl or 4-methylcyclohexyl as R_2;
3. hydrogen, fluorine, or methoxy groups as R_3.

The SAfiR studies demonstrated that:

1. the 5-*n*-butyl chain as R_1 substituent confers higher CB2R selectivity compared to the 5-methyl, benzyl, or *p*-fluorobenzyl counterparts;
2. the cycloheptylcarboxamide derivatives display higher CB2R affinity than 4-methylcyclohexylcarboxamide ligands;

3. hydrogen, fluorine, or methoxy groups as R_3 do not have a clear and unambiguous role in the binding ability.

As for the SAR, the substituents in 5- and 4'- positions (Figure 17) are the main responsible of the activity profile. Indeed, all compounds with a 4'-methoxy group (R_3) show a neutral competitive antagonist activity, while smaller 4'-substituents, such as hydrogen or fluorine, leads to full agonist or inverse agonist activity (depending on 5-substituent size R_1). A small methyl group in the 5-position is associated with a full agonist profile, while *n*-butyl, benzyl, and *p*-fluorobenzyl groups are associated with an inverse agonist profile.[64,65]

Figure 17

Prolines and piperidines. In an effort to establish new scaffolds useful to obtain new and selective CB2R ligands, Hickey et al. used a Computer Assisted Drug Design (CADD) approach based on two well-known series of CB2R ligands, diazepanes and β-sulfonylacetamides, characterized by the lack of the right balance between potency and drug-like properties (e.g. aqueous solubility and metabolic stability). Three new potential scaffolds were found: *N*-substituted-2-carboxamido-azetidine (**5**), -proline (**6**), and -piperidine (**7**) (Fig. 18).[66-68]

Figure 18

Figure 19 shows the SAR for the proline scaffold. An initial investigation allowed for the preferred configuration to be established for C_α as (*S*). Indeed, several (*S*)-isomers, with different R_1 and a 5-*tert*-butylisoxazol-3-yl as R_2, displayed full CB2R agonist activities with picomolar potencies and CB2R *vs* CB1R selectivities higher than 750-fold. By contrast, corresponding (*R*)-isomers displayed partial agonist profile with nanomolar potencies towards CB2R and less than 600-fold CB2R *vs* CB1R selectivities.[66] Moreover, the insertion of a methyl group on C_α of the proline ring did not

modify CB2R potency, but led to non-selective ligands. Several proline derivatives bearing different R_1 and R_2 substituents were also developed, and other modifications were performed to obtain derivatives with a good compromise between agonistic potency and drug-like properties (water-solubility and metabolic stability). The results of these further investigations are listed below:

- Phenyl and pyridine-2-yl bearing electron withdrawing groups (EWG, e.g. -Cl, -CF_3) as R_1 lead to comparable CB2R potencies in the picomolar range. Nevertheless, substituted-pyridine-2-yl groups cause an important increase in CB1R activity generating non-selective derivatives.[66] Cycloalkyl substituents at N_1 are also well tolerated with the cyclohexyl ring determining lower CB2R EC_{50} values than cyclopentyl and cyclobutyl rings. Moreover, tetrahydropyranyl substituent at N_1 displays 12-fold lower CB2R potencies compared to *N*-cyclohexyl ring, but a 9-fold increase in selectivity *vs* CB1R, suggesting a lower tolerance of CB1R towards polar groups in this position.[67]
- Among the 5-membered heteroaryl rings tested, 5-*tert*-butylisoxazol-3-yl and 3-*tert*-butylisoxazol-5-yl as R_2 appear as the most beneficial. Among bicyclic moieties, only 5-chloro-benzothiazol-2-yl fragment shows a CB2R activity in the low nanomolar range and a high selectivity *vs* CB1R.[67]
- Two main changes have been also performed on the proline core: the insertion of a hydroxy group on C_γ and the insertion of a carbonyl group on C_δ. *Trans*-γ-hydroxyproline derivatives show high CB2R potency and selectivity, and an improved metabolic stability in human liver microsomes than the not-hydroxylated derivatives. Also δ-oxoproline derivatives result as CB2R potent and selective agonists with improved drug-like properties, but cycloalkyls as R_1 lead to an unexpected loss of CB2R activity. All in all, because of the high metabolic stability and the high aqueous solubility, δ-oxoproline emerges as a new powerful scaffold for the development of new selective CB2R agonists.[67]

Figure 19

Similar modifications have been performed on piperidine scaffold: Figure 20 shows SAR studies for piperidine derivatives and (*S*)-enantiomers show the best activity profile, as for the proline derivatives.[68]

- Regarding the substituent in position N_1 (R_1), phenyl and benzyl bearing EWG lead to high CB2R agonist activity and selectivity, and similar results are obtained with cycloalkyls and heterocycloalkyls linked by a methylene to the central core which is depicted in Figure 20. In order to ameliorate the physicochemical properties, amide derivatives involving N_1 have been developed, and the best results in terms of both pharmacodynamic and physicochemical profiles are found for tetrahydropyran-4-yl-methylcarboxamide and thiomorpholinedioxydecarboxamide as R_1.[68]
- Carboxamide substituent in the 2-position of the piperidine ring (R_2) shows the same behavior as in the proline scaffold: *tert*-butylisoxazol and chlorobenzothiazol isomers are well tolerated. The modifications of the *tert*-butyl substituent on the isoxazolyl ring generally lead to a decrease in CB2R potency. Unexpectedly, 3-phenyl-1,2,4-thiadiazol-5-yl as R_2 shows a good balance between CB2R activity/selectivity and physicochemical properties.[68]
- In contrast to δ-oxoproline, a carbonyl group within the piperidine core is not tolerated.[68]

Figure 20

Among these two series, the proline-based derivative (**8**) and the piperidine-based derivative (**9**) depicted in Figure 21 have been selected for *in vivo* evaluation in a streptozotocin-induced diabetic neuropathy rat model. Compared to vehicle treated animals, both compounds demonstrated a dose-responsive reversal of mechanical hyperalgesia and a full reversal was observed respectively at 30 mg/kg of compound **8** and at 60 mg/kg of compound **9**.[67,68]

Figure 21

Docking studies

Small structural changes are reported as being responsible for the switch from agonist to antagonist/inverse agonist of derivatives sharing the same scaffold. In the 1,8-naphthyridin-2(1*H*)-one, quinoline-2,4(1*H*,3*H*)-dione and 8-alkyloxycoumarin-3-carboxamide derivatives (Fig. 4-6), C6- or C7-substitution led to an antagonist/inverse agonist profile, whereas either a substituent in the 5- or 8-position or the complete absence of substituents on the condensed benzene ring led to an agonist profile.[43-45] To better understand the reasons for these different biological results, molecular docking studies were carried out on each scaffold and similar results were obtained with all of them confirming previous studies on other GPCRs, including CB1R[69] and CB2R.[70] As depicted in the exemplificative Figure 22 for quinolindione derivatives,[44] the main difference between docking simulated structural models of CB2R-agonist and CB2R-antagonist is the relative position of highly conserved residues F3.36 (117) and W6.48 (258): in the CB2R-agonist complex, the side chains of these amino acids may form a parallel π-π interaction (Fig. 22A-B); whereas, in the CB2R-antagonist complex, W6.48's indole ring is rotated, having a T-shaped π-π interaction (Fig. 22C-D). Hence, a substituent in the 6- or 7-position may switch the activity from agonism to antagonism due to the ability to deeply extend in the binding pocket and to block the switch toggle change of W6.48 (258) that is necessary for the CB2R transition from the inactive (F3.36-W6.48 T-shaped π-π interaction) to the active conformation (F3.36-W6.48 parallel π-π interaction).[43-45]

Figure 22

CB2R ligands as diagnostic tools: PET studies

Several studies demonstrated an up-regulation of CB2R on activated microglial cells rendering the receptor a promising target to monitor neuroinflammatory changes involved in neurodegenerative

disorders.[71] The development of CB2R PET ligands could help in improving the understanding about neurodegenerative pathogenesis and could provide useful tools for characterizing the role of neuroinflammation in the progression of these disorders and for the monitoring of therapy. Several CB2R radioligands have been published (Fig. 23) but to date none of them has been employed in clinical practice because of limitations such as a) low selectivity, b) high lipophilicity, c) low brain uptake as P-gp substrates, d) brain penetrant radiometabolites, and e) absence of specific animals or human models.

Figure 23

We previously described a series of 2,4,6-trisubstitued-1,3,5-triazine as CB2R agonists with different polarity[61,62] and, among them, 4-(2-fluoroethyl)-piperazin-1-yl derivative (**10**) was [^{18}F]-radiolabeled because of its promising *in vitro* profile.[62] This compound showed a good metabolic stability in rat liver homogenate but the *in vivo* results were not encouraging: after intravenous injection, the tracer showed a rapid elimination in urine (<15 min), and HPLC analyses revealed that the sample consisted of hydrophilic radioactive metabolites and a low amount (<6%) of non-metabolized compound. The detected radioactivity levels in the brain reflected the low plasma levels of the tracer. Nevertheless, it is not possible to conclude that the biodistribution of this 1,3,5-triazine derivative could limit its pharmacological applications, as other studies are needed to evaluate the biodistribution in pathological conditions associated to CB2R overexpression.[62] A different [^{18}F]-1,3,5-triazine derivative (**11**) was developed by Hortala et al. and displayed good brain uptake which increased after the administration of LPS.[72] Based on these findings, the 1,3,5-triazine scaffold was confirmed to be a suitable template in the design of highly potent CB2R agonists with reasonable water solubility for promising PET tracers.

Slavik et al. reported the ^{11}C-labeled 4-oxo-quinoline derivative **12** ([^{11}C]RS-016), that displayed high CB2R binding affinity and no affinity toward CB1R (K_i > 10000 nM).[73] In addition, it displayed:

i) low lipophilicity; *ii*) good radiochemical properties (high specific binding to CB2R in *in vitro* autoradiography on rodent spleen tissue); *iii*) good *in vitro* and *in vivo* stability; iv) high and specific brain uptake in *in vivo* PET imaging using a LPS-induced neuroinflammation mouse model with high expression level of CB2R.[73] RS-016 was confirmed to be a promising PET tracer for imaging CB2R in human, but further works are currently ongoing to evaluate the usefulness of this radiotracer in other neuroinflammation animal models. Starting from derivative **12**, the corresponding fluoroethyl derivative **13** ([^{18}F]RS-126) was developed and pharmacologically evaluated toward CB2R as [^{18}F]-radiolabeled *in vitro* and *in vivo*.[74] In autoradiography experiments, **13** showed high specific binding toward CB2R in rat spleen tissue and no degradation product was identified. *In vivo*, rat spleen samples revealed the 81% of intact compound at 15 min post injection, and displacement studies confirmed selective binding and 79% specificity to spleen tissue. However, CB2R expression in the brain of LPS-treated mice could not be detected. This could be attributed to the relative low specific activity of **13** and to a moderately enhanced CB2R expression in LPS-treated mice. However, it was not proved that the radioligand crossed the blood brain barrier (BBB).[74]

As reported before, a new class of thiophene amide derivatives emerged as potent and selective CB2R agonists,[59] and two thiophene based radioligands, **14** ([^{11}C]AAT-015) and **15** ([^{11}C]AAT-778), have been described by Haider et al. for CB2R imaging.[75] Both derivatives were designed with an ester function which allowed the generation of the precursor for ^{11}C labeling. Binding affinity studies revealed K_i values in the nanomolar range for CB2R and in the micromolar range for CB1R. The introduction of the hydrophilic OH in the adamantyl moiety and the introduction of a greater rigidity by ring closure did not affect the CB2R affinity, and the lipophilicity in AAT-015 was significantly reduced. Despite the reduction of lipophilicity, the *in vitro* autoradiography experiment showed high non-specific binding in mouse and rat spleen tissues. PET studies confirmed the lack of specificity toward CB2R physiologically expressed in the spleen, and therefore this could be explained by high plasma protein binding and rapid metabolism. Thus, both thiophene ligands were not useful for *in*

vivo imaging of CB2R but may be an interesting starting point in the development of CB2R PET radioligands.[76]

Conclusions

Herein we reported a summary of all the best CB2R ligand structures bearing a great variety of scaffolds. Oxoquinoline (**a**), 1,8-naphthyridin-2(1H)-one (**b**,**c**), 2-pyridinone (**d**), and biphenyl (**e**) scaffolds appeared to be responsible of the highest affinity towards CB2R subtype (Figure 24).

Figure 24

All of them bear a carboxamide function linked to a hindered cycloalkyl ring. From these structures, it appears that:

- the presence of a bicycle ring in the ***core*** of the molecule is not pivotal for CB2R affinity. Indeed, the 2-pyridinone scaffold (Fig. 24d) displays an affinity comparable to that of the other reported scaffolds (Fig. 24a-c);
- the presence of *N* atom in the ***core*** is not needed as from the biphenyl compounds (Fig. 24e), although ring bearing the *N* atom are endowed with higher affinity (> 10-fold);
- the presence of a carbonyl group in the ***core,*** mainly in the 2- or 4-position, improves CB2R affinity;
- functionalization of the 1-position with linear alkyl chains, heterocycloalkyls, or arylalkyl rings leads to high affinity CB2R interactions.

As for the activity profile, little modifications can deeply influence the activity of the compounds switching the profile from agonist to inverse agonist or antagonist. As an example, in the 4-oxo-quinoline series, the presence of an alkyl or aryl substituents in the 6-position can differently modulate the activity from agonist (alkyl) to inverse agonist (aryl); in the 2-oxo-quinoline scaffold the presence of a hydroxyl group in the 4-position completely reverses the activity profile with respect to the 4-

oxo-quinoline series (6-aryl derivatives as agonists, 6-alkyl derivatives as inverse agonists). Molecular docking studies have helped to explain the shift from agonism to antagonism due to these small structural changes.

Activity profiles of all the scaffolds mentioned in this perspective are listed in Table 1, and the best agonist profile has been observed for proline derivatives that emerged as a new powerful pharmacophore for the development of new selective CB2R agonists.

Overall, this work has summarized the SAfiR and SAR studies conducted on a number of different classes of CB2R ligands that have been developed so far with the aim of shedding light on the most promising scaffolds for the production of powerful and selective CB2R ligands to be exploited in neurodegenerative diseases either for therapeutic or diagnostic purposes.

Table 1

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Elena Capparelli is a researcher of the Spinoff Biofordrug srl at the Department of Pharmacy and she collaborates as a researcher/lecturer with the Catholic University “Our Lady of Good Counsel” (AL). Elena obtained her PhD in Pharmaceutical Sciences from the University of Bari (Italy) in March 2012 under the supervision of Prof. Nicola Antonio Colabufo. Her PhD project focused on the design, synthesis and biological evaluation of new P-glycoprotein ligands as useful tools in PET imaging for oncology and early diagnosis of Alzheimer’s disease.

Carmen Abate is an Assistant Professor at the University of Bari from 2009 in the Department of Pharmacy. She graduated in 1998 in Medicinal Chemistry dealing with the synthesis of sigma receptors ligands as part of her doctorate. From 2002 to 2003 she worked as Associate Researcher to

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ABBREVIATIONS USED

AD, Alzheimer's disease; BBB, blood-brain barrier; CADD, computer-assisted drug design; CB1R (CB1), cannabinoid type 1 receptor; CB2R (CB2), cannabinoid type 2 receptor; CBRs, cannabinoid

receptors; CFA, complete Freund's adjuvant; CNS, central nervous system; CoMFA, comparative molecular field analysis; CYP, cytochrome P; EAE, experimental autoimmune encephalomyelitis; ECS, endogenous cannabinoid system; EDG, electron donating group; EWG, electron withdrawing group; GPCR, G-protein coupled receptor; HD, Huntington's disease; IL, interleukin; LPS, lipopolysaccharide; MIA, monoiodoacetic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; NOS2, nitric oxide synthase 2; PD, Parkinson's disease; PET, positron emission tomography; SAfiR, structure affinity relationship; SAR, structure activity relationship; SI = Selectivity index, calculated as $K_{i\ CB1}/Ki_{\ CB2}$ or $EC_{50\ CB1}/EC_{50\ CB2}$; TGFα/β, tumor growth factor α/β; Δ^9-THC, Δ^9-Tetrahydrocannabinol.

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Legends of Figures

Figure 1. CBRs ligands.

Figure 2. Effect of R substituent on 4-quinolin-4(1*H*)-one (**A**) and on 4-hydroxy-quinolin-2(1*H*)-ones (**B**).

Figure 3. SAfiR studies on oxo-quinoline scaffolds.

Figure 4. SAfiR studies on 1,8-naphthyridin-2-one scaffold.

Figure 5. SAR studies on quinoline-2,4-dione; **A**: CB2R agonists; **B**: CB2R antagonists.

Figure 6. SAR studies on coumarine-3-carboxamides.

Figure 7. SAR studies on indoles scaffold.

Figure 8. SAR studies on indazoles scaffold.

Figure 9. Imidazopyridine and imidazopyrazine scaffolds.

Figure 10. Imidazopyridines **1** and **2** evaluated in an *in vivo* rat CFA hyperalgesia model.

Figure 11. SAR studies on benzimidazole scaffold.

Figure 12. Thienopyrimidine scaffold (A) and new purine scaffold (B).

Figure 13. Purine derivatives.

Figure 14. Tetrahydrobenzothiophene compound with high CB2R affinity and selectivity (**A**); SAfiR studies on benzothiophene scaffold (**B**).

Figure 15. SAR studies on 1,3,5-triazine scaffold.

Figure 16. SAR and SAfiR studies on 2-oxo-piridine scaffold.

Figure 17. SAR and SAfiR studies on biphenylic carboxamides.

Figure 18. *N*-substituted-2-carboxamido-azetidine (**3**), *N*-substituted-2-carboxamido-proline (**4**), and *N*-substituted-3-carboxamido-piperidine (**5**).

Figure 19. SAR studies on proline derivatives.

Figure 20. SAR studies on piperidine derivatives.

Figure 21. Proline (**8**) and piperidine (**9**) derivatives evaluated in a rat neuropathy model.

Figure 22. Docking results of CB2R-agonists (**A**-**B**) and CB2R-antagonist (**C**-**D**) complexes. The F117/W258 carbon atoms are colored light green. Picture from reference 44.

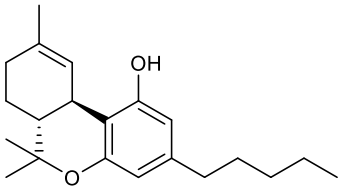
Figure 23. CB2R PET radiotracers.

Figure 24. Derivatives bearing different scaffolds leading to the best CB2R affinity results.

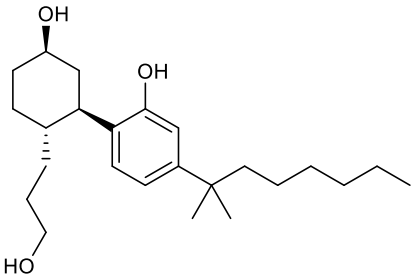
Table 1. Summary of the activity profiles.

TOC. Summary of all the modifications aimed to improve CB2R affinity.

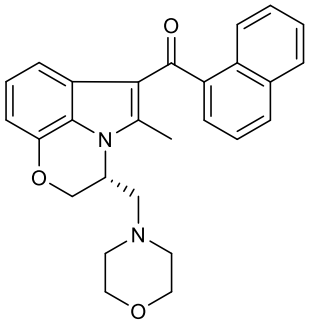
Figure 1.



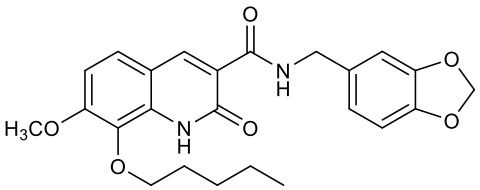
Δ^9-THC
Dual agonist[19,20]



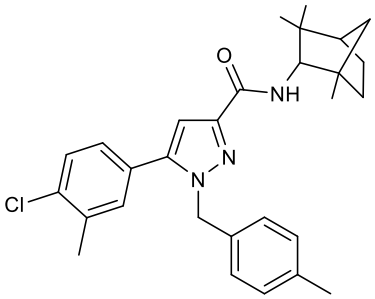
CP55940
Dual agonist[24]



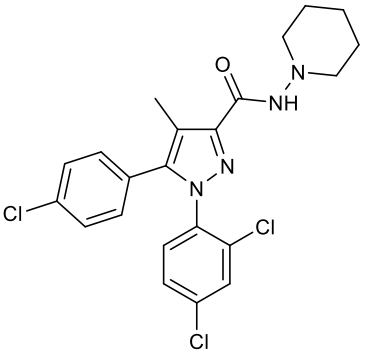
WIN55212-2
Dual agonist[25]



JTE-907
CB2R inverse agonist[26]



SR144528
CB2R inverse agonist[27,28]



SR141716A
CB1R antagonist[29]

Figure 2.

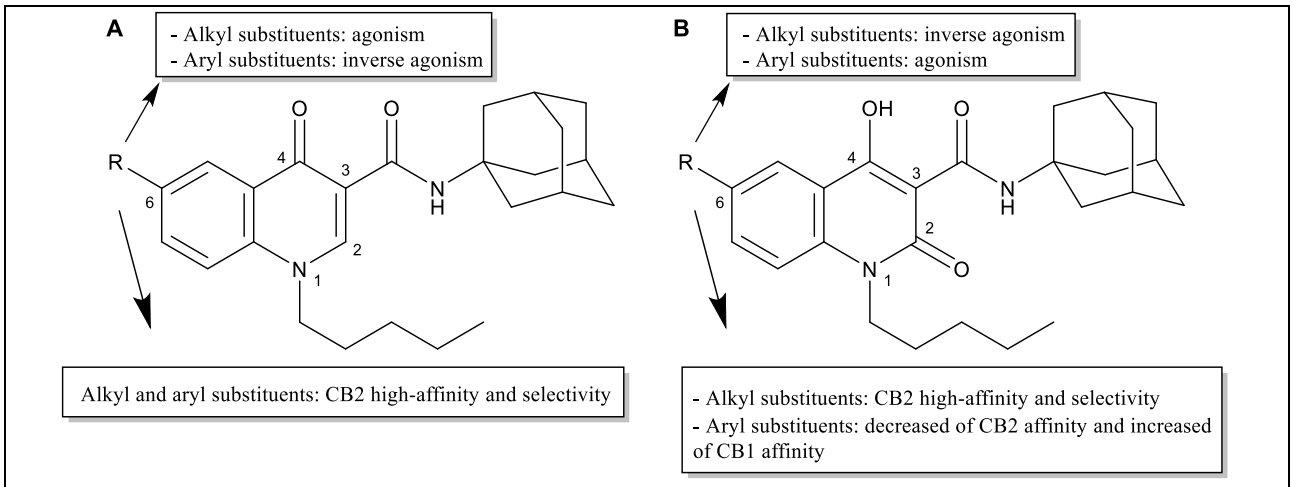


Figure 3.

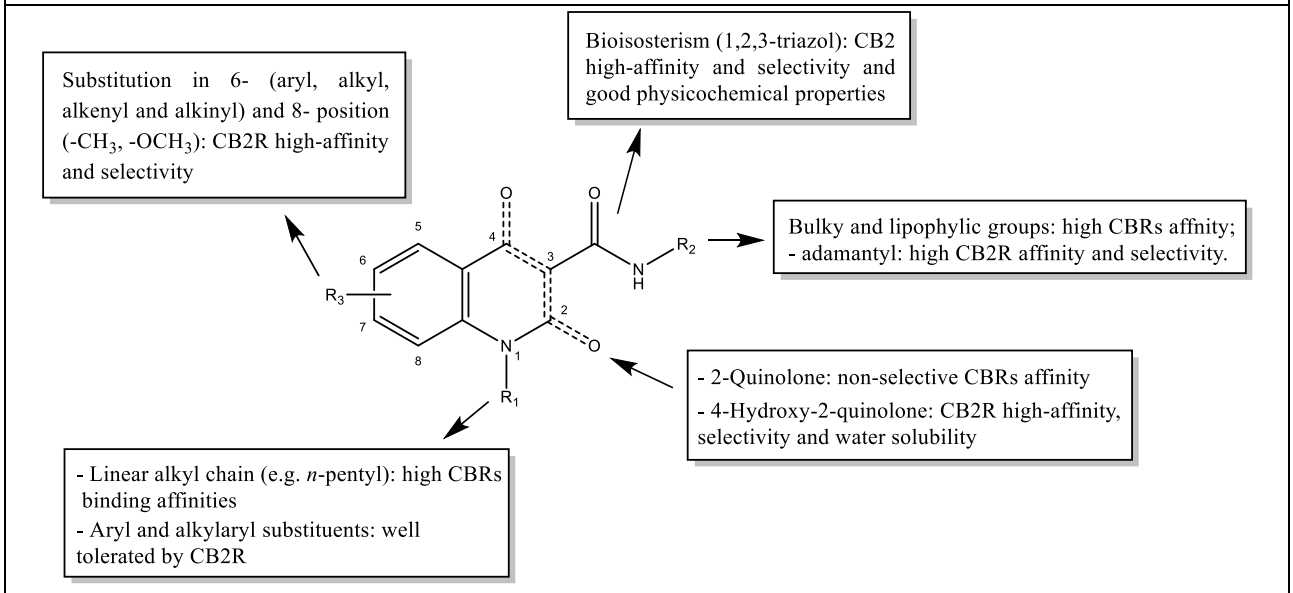


Figure 4.

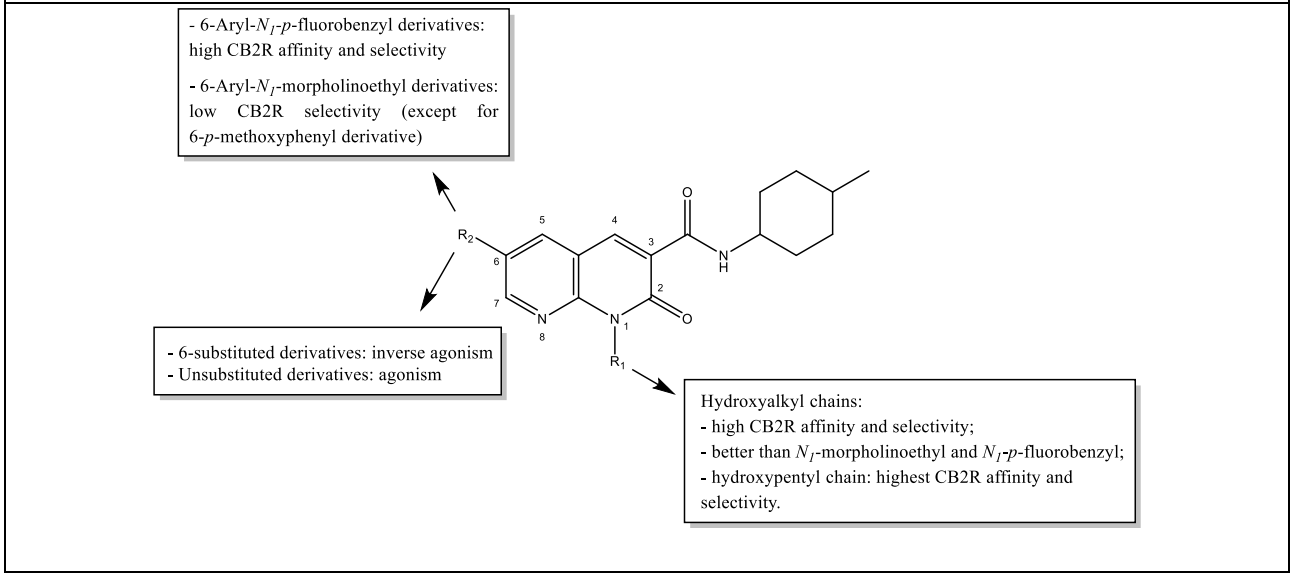


Figure 5.

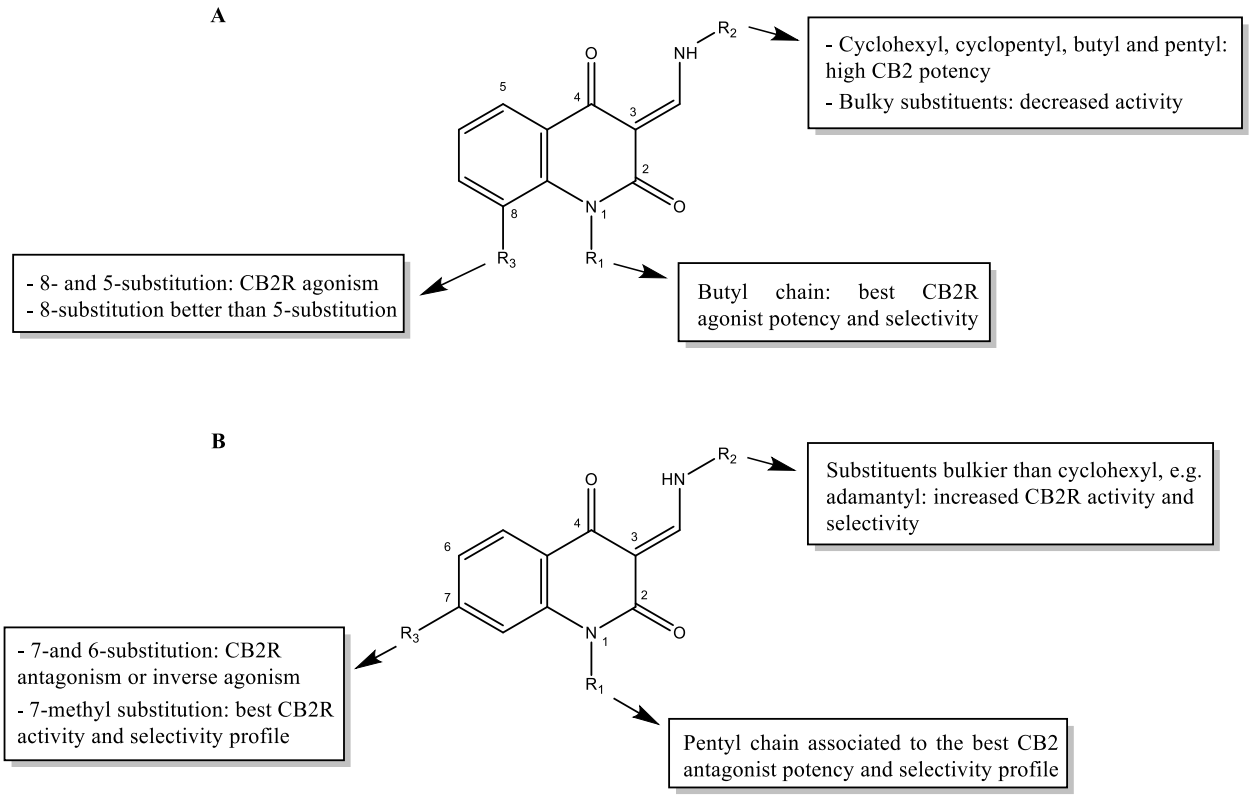


Figure 6.

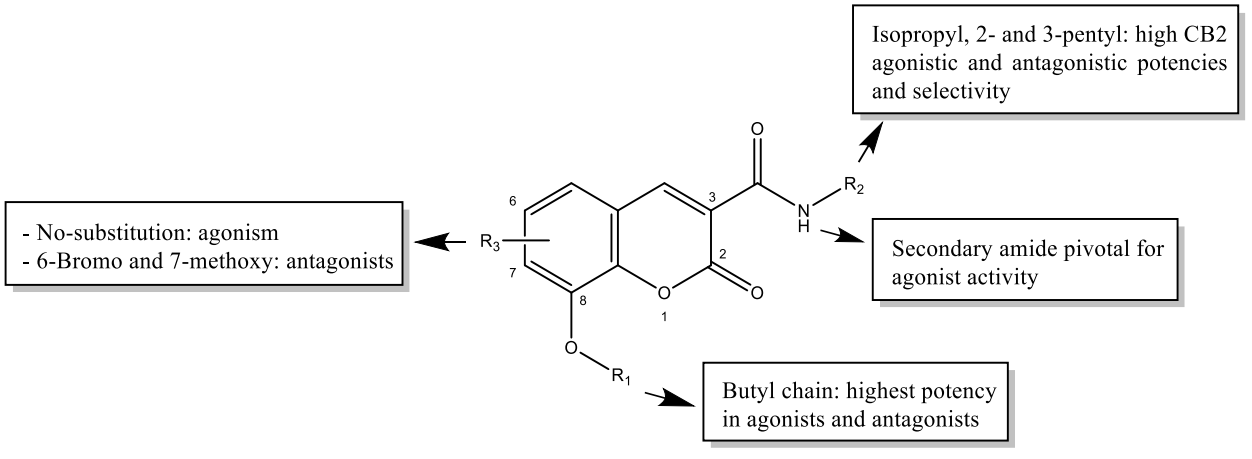


Figure 7.

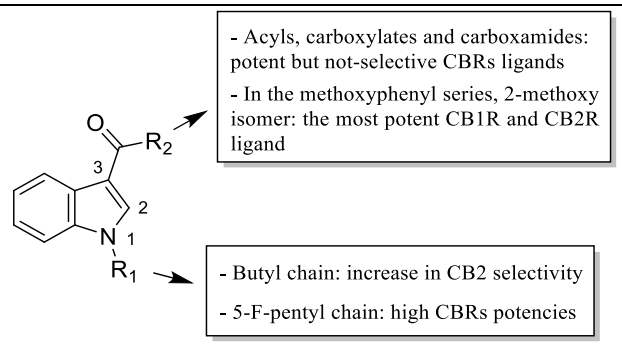


Figure 8.

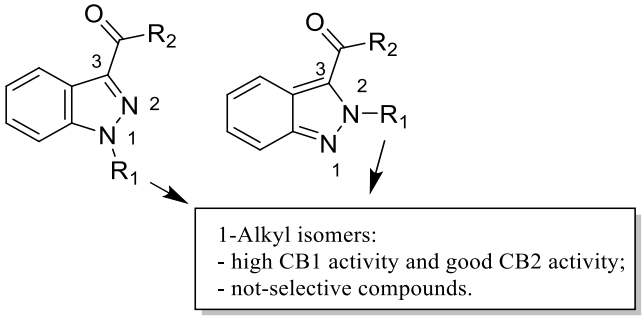


Figure 9.

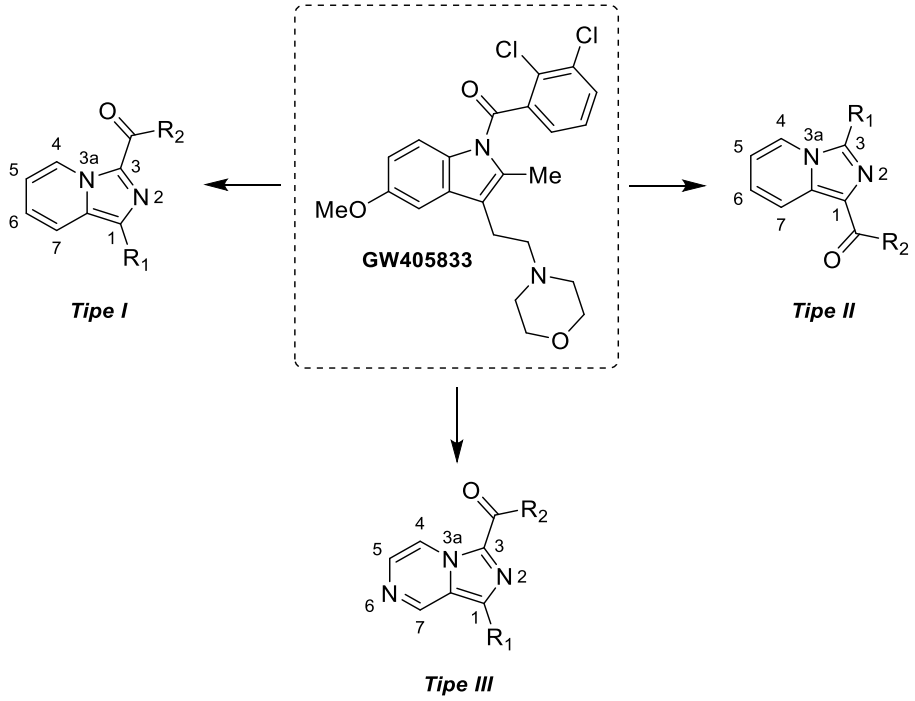
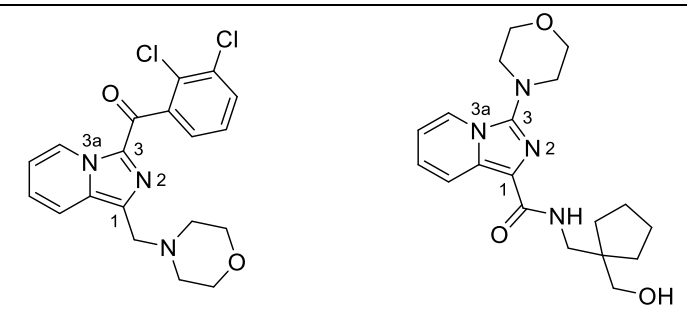


Figure 10.



1
IC_{50} CB1 = 5 nM
IC_{50} CB2 = 2565 nM
SI (CB1/CB2) = 513
Plasma free fraction = 24%

2
IC_{50} CB1 = 33 nM
IC_{50} CB2 > 17000 nM
SI (CB1/CB2) > 515
Plasma free fraction = 16%

Figure 11.

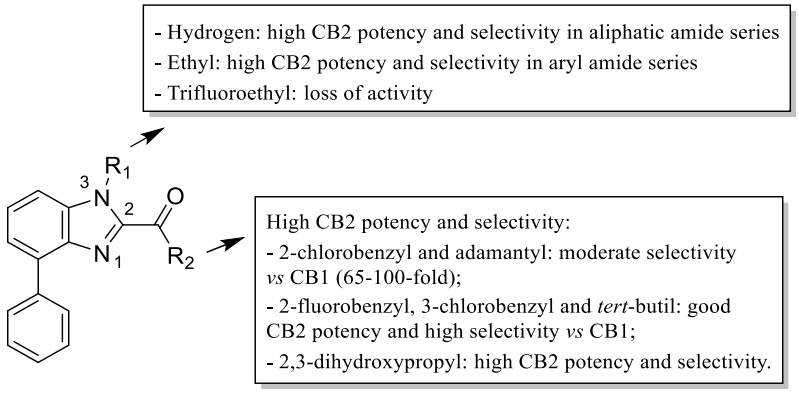


Figure 12.

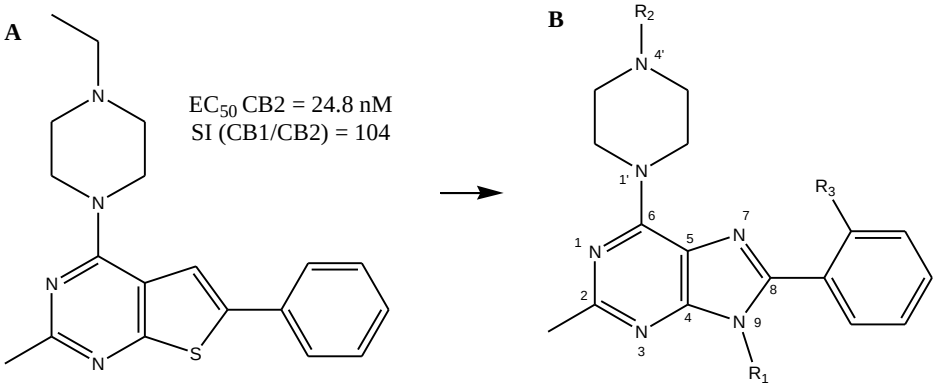
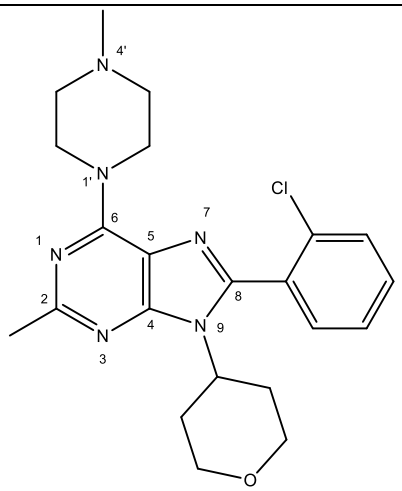
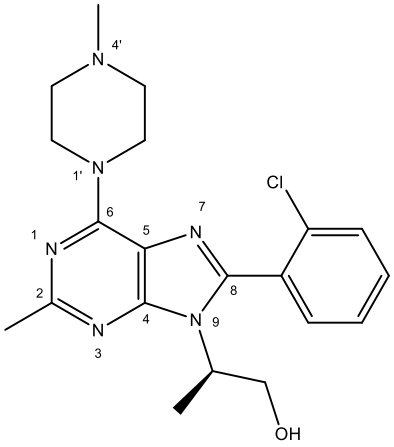


Figure 13.



3

EC_{50} CB1 > 10^5 nM
EC_{50} CB2 = 20.1 nM
SI (CB1/CB2) > 5000

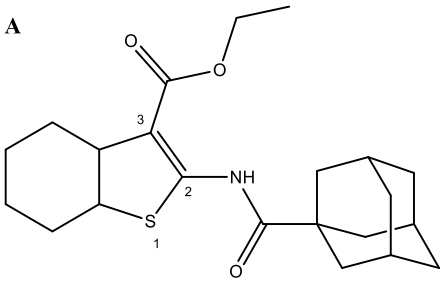


4

EC_{50} CB1 > 10^5 nM
EC_{50} CB2 = 22.4 nM
SI (CB1/CB2) > 4000

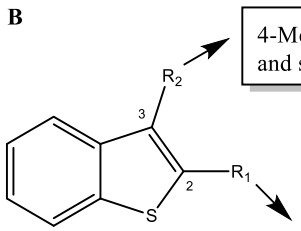
Figure 14.

A



K_i CB1 = 1008 nM
K_i CB2 = 2.15 nM
SI (CB1/CB2) = 469

B



4-Methoxybenzoyl: high CB2R affinity and selectivity

- Alkyl groups: high CB2 affinity
- 1-Naftoyl group: high CB2R affinity and selectivity

Figure 15.

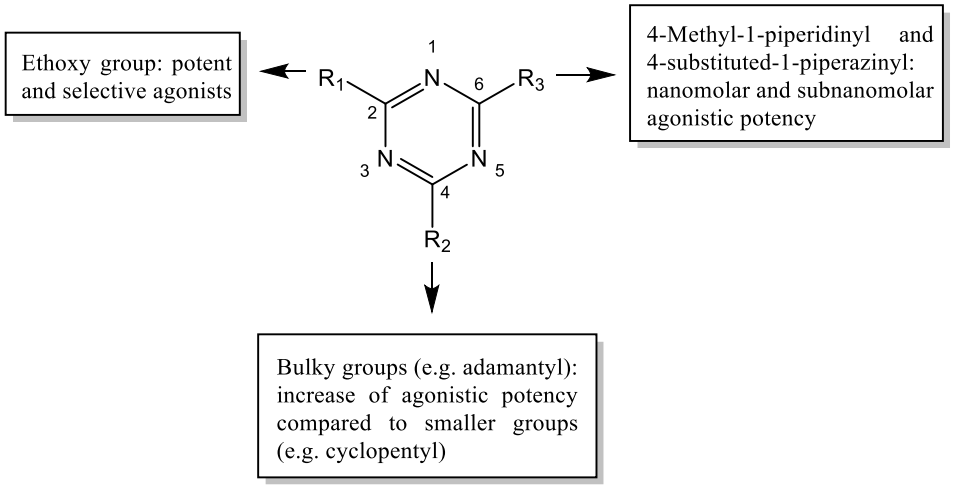


Figure 16.

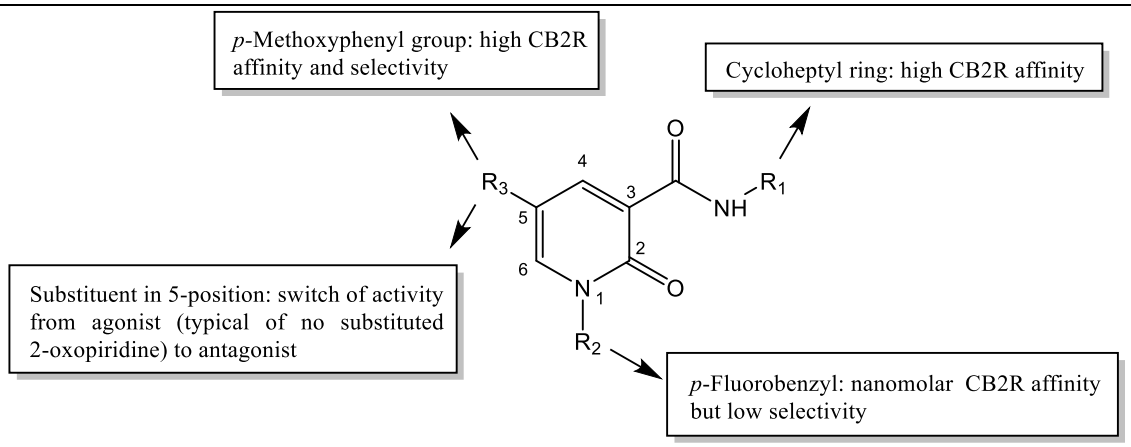


Figure 17.

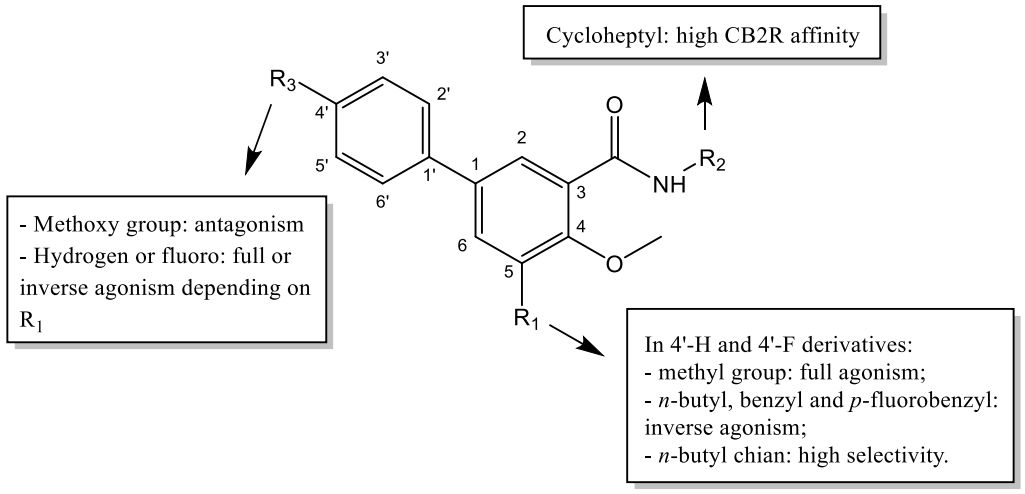
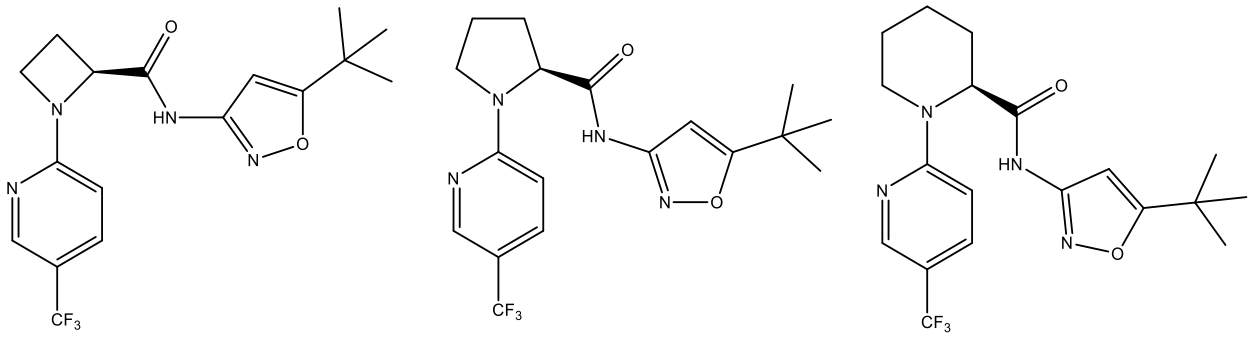


Figure 18.



5
EC_{50} CB1 = 1400 nM
EC_{50} CB2 = 1.15 nM
SI (CB1/CB2) = 1217

6
EC_{50} CB1 = 53 nM
EC_{50} CB2 = 0.07 nM
SI (CB1/CB2) = 757

7
EC_{50} CB1 = 250 nM
EC_{50} CB2 = 0.09 nM
SI (CB1/CB2) = 2778

Figure 19.

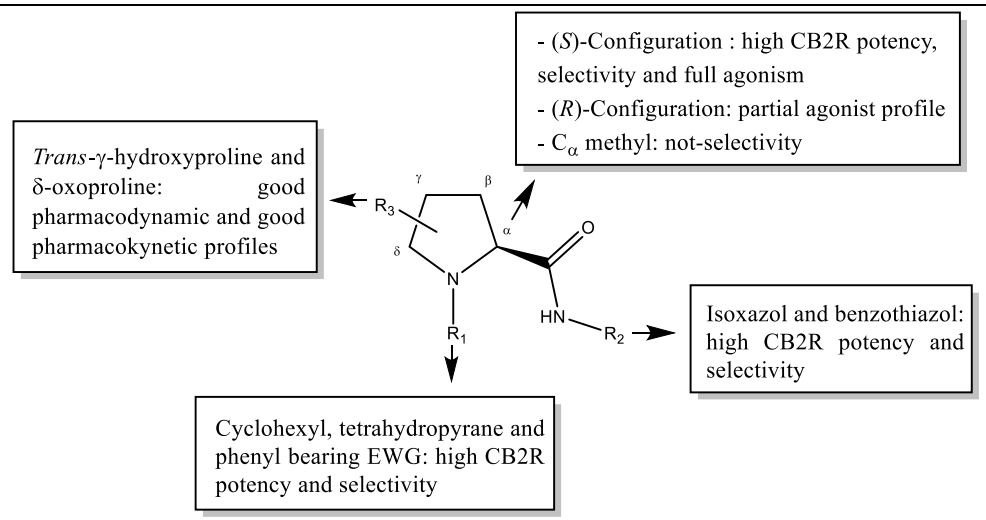


Figure 20.

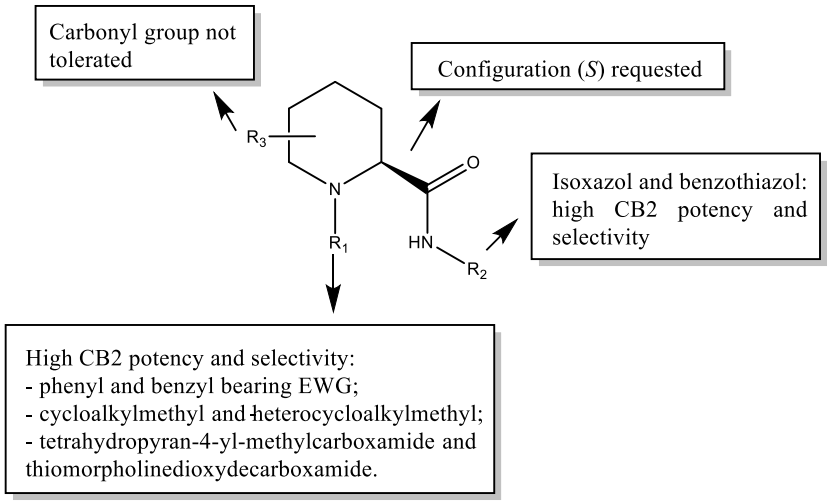
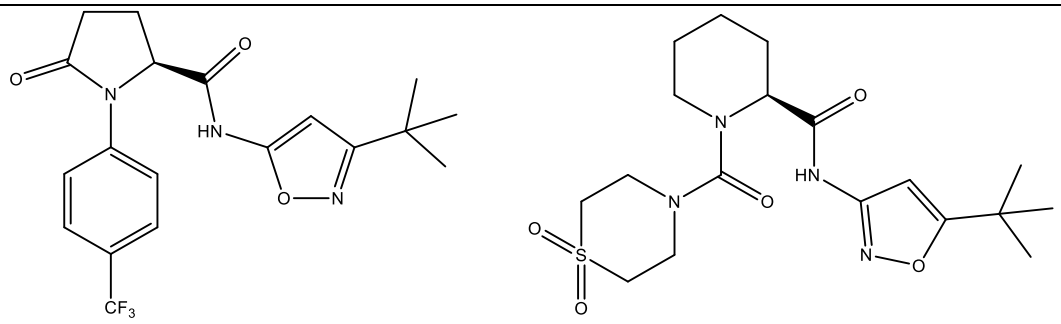


Figure 21.



8

EC_{50} CB1 (nM) > 50000 nM
EC_{50} CB2 (nM) = 1.4 nM
SI (CB1/CB2) > 35700

9

EC_{50} CB1 (nM) > 20000 nM
EC_{50} CB2 (nM) = 11 nM
SI (CB1/CB2) > 1800

Figure 22.

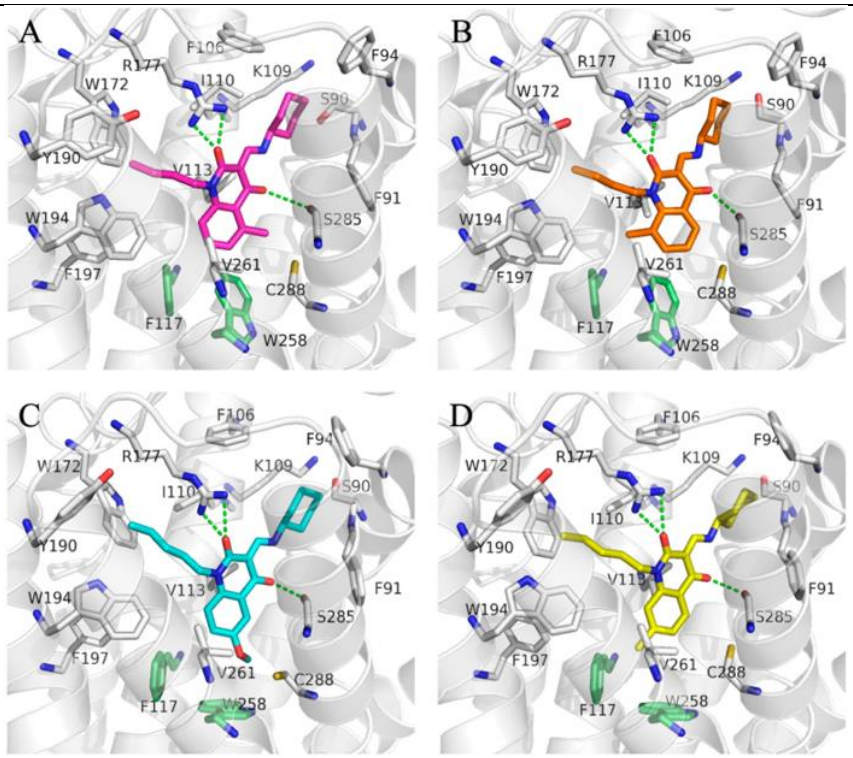
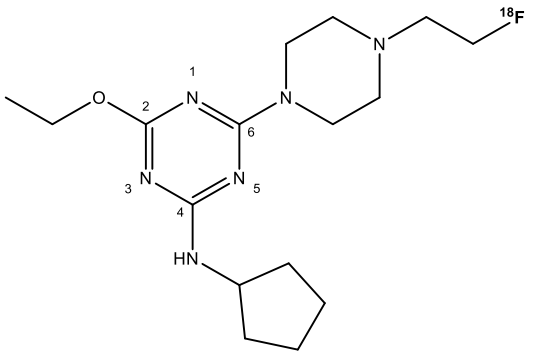
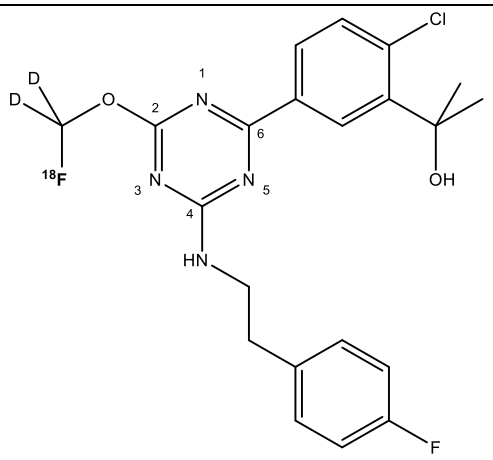

A
F106
R177
K109
F94
W172
I110
S90
Y190
V113
F91
W194
S285
F197
V261
C288
F117
W258
B
F106
R177
F94
W172
I110
K109
S90
Y190
V113
F91
W194
S285
F197
V261
C288
F117
W258
C
F106
F94
W172
R177
K109
I110
S90
Y190
V113
F91
W194
S285
F197
V261
C288
F117
W258
D
F106
F94
W172
R177
K109
S90
I110
Y190
V113
F91
W194
S285
F197
V261
C288
F117
W258

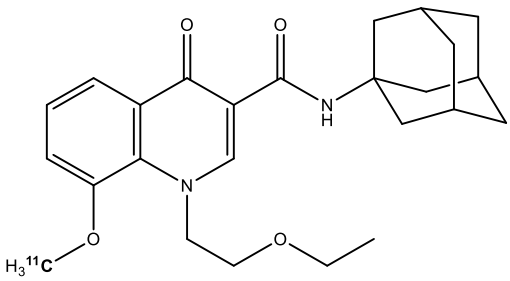
Figure 23.



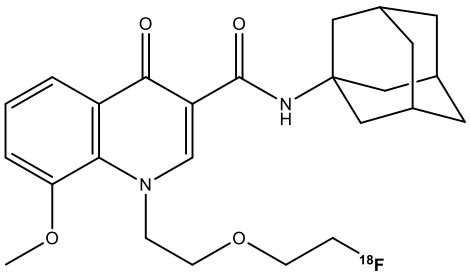
10
EC_{50} CB1 = - nM
EC_{50} CB2 = 40 nM
SI (CB1/CB2) = -



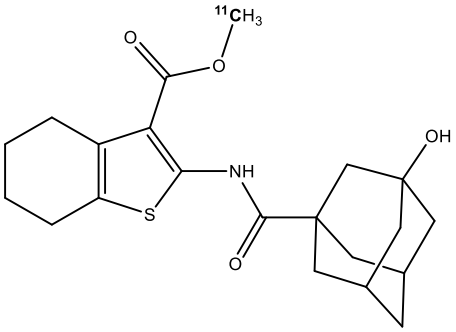
11
IC_{50} CB1 = 180 nM
IC_{50} CB2 = 1.4 nM
SI (CB1/CB2) = 128.6



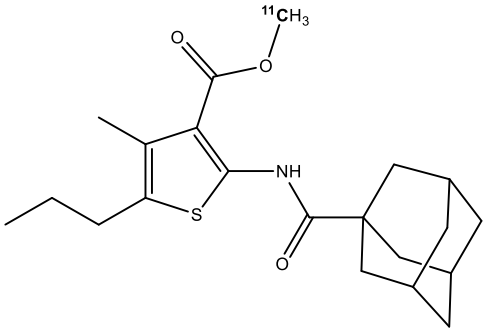
12
K_i CB1 > 10000 nM
K_i CB2 = 0.7 nM
SI (CB1/CB2) > 14285



13
K_i CB1 > 10000 nM
K_i CB2 = 1.2 nM
SI (CB1/CB2) > 8333



14
K_i CB1 = 1000 nM
K_i CB2 = 3.3 nM
SI (CB1/CB2) = 303



15
K_i CB1 = 1100 nM
K_i CB2 = 4.3 nM
SI (CB1/CB2) = 255.8

Figure 24.

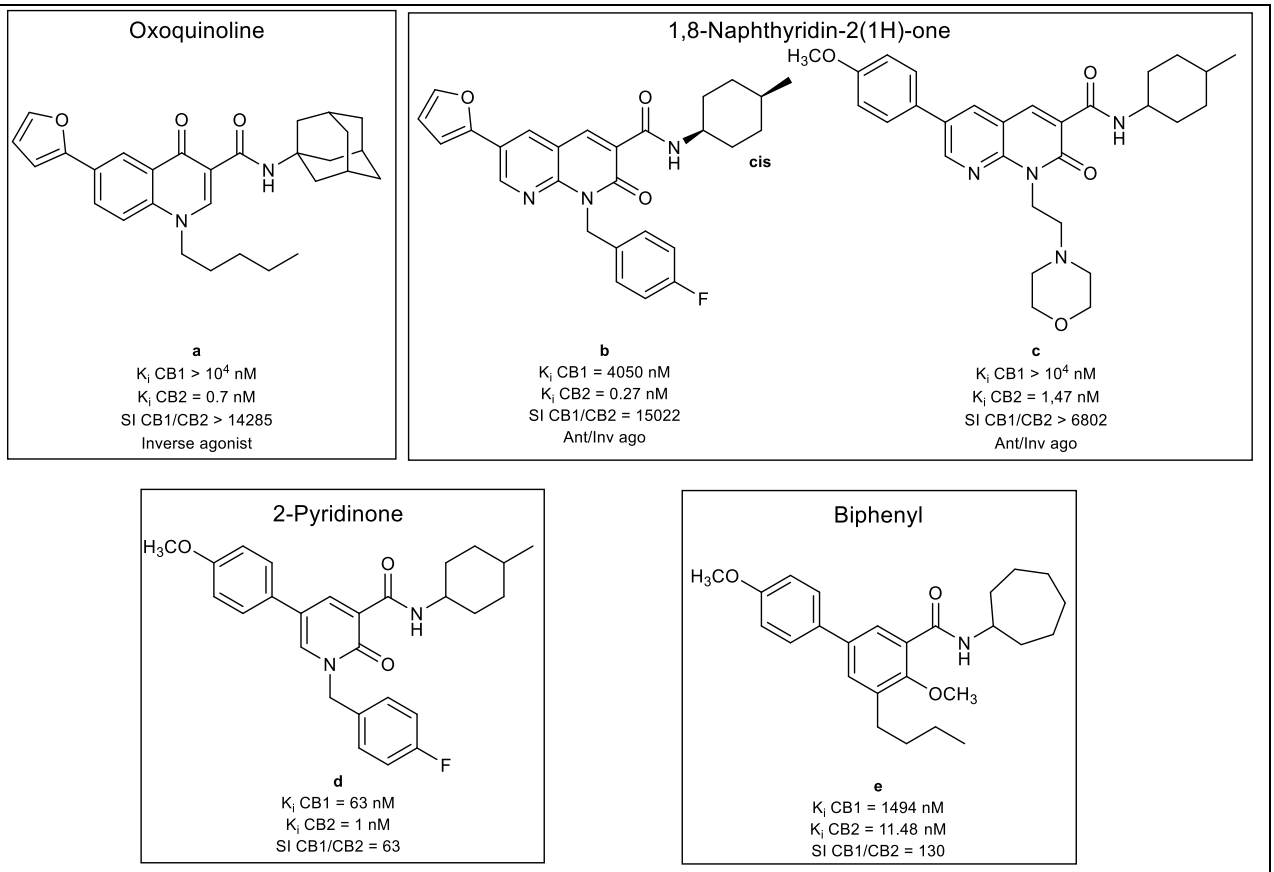

Oxoquinoline
a
K_i CB1 > 10^4 nM
K_i CB2 = 0.7 nM
SI CB1/CB2 > 14285
Inverse agonist
1,8-Naphthyridin-2(1H)-one
cis
b
K_i CB1 = 4050 nM
K_i CB2 = 0.27 nM
SI CB1/CB2 = 15022
Ant/Inv ago
c
K_i CB1 > 10^4 nM
K_i CB2 = 1,47 nM
SI CB1/CB2 > 6802
Ant/Inv ago
2-Pyridinone
d
K_i CB1 = 63 nM
K_i CB2 = 1 nM
SI CB1/CB2 = 63
Biphenyl
e
K_i CB1 = 1494 nM
K_i CB2 = 11.48 nM
SI CB1/CB2 = 130

Table 1

Scaffold	Substitution	Activity
4-Oxoquinoline	6-Alkyl	Agonist
	6-Aryl	Inverse agonist
4-Hydroxy-2-oxoquinoline	6-Alkyl	Inverse agonist
	6-Aryl	Agonist
1,8-Naphtyridin-2-one	5,6,7-unsubstituted	Agonist
	6-substituted	Inverse agonist
Quinolin-2,4-dione	5- or 8-substituted	Agonist
	6- or 7-substituted	Antagonist
Coumarin	5,6,7-unsubstituted	Agonist
	6- or 7-substituted	Antagonist
Indole		Agonist
Indazole		Agonist
Imidazopyridine		Agonist
Imidazopyrazine		Agonist
Benzimidazole		Agonist
Purine		Agonist
1,3,5-Triazine		Agonist
2-Pyridone	5-unsubstituted	Agonist
	5-substituted	Antagonist/Inverse agonist
Biphenyl	4’-methoxy	Antagonist
	4’-H or 4’-F-5-methyl	Agonist
	4’-H or 4’-F-5-butyl/benzyl-*p*-fluorobenzyl	Inverse agonist
Proline		Agonist
Piperidine		Agonist

Table of Contents graphic