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ALLOSTERIC MODULATORS TARGETING CANNABINOID CB1 AND CB2 RECEPTORS: IMPLICATIONS FOR DRUG DISCOVERY

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- **Abstract:** Allosteric modulators of cannabinoid receptors hold great therapeutic potential, as they do not possess intrinsic efficacy, but instead enhance or diminish the receptor's response of orthosteric ligands allowing for the tempering of cannabinoid receptor signaling without the desensitization, tolerance and dependence. Consequently, allosteric modulators of cannabinoid receptors have numerous advantages over the orthosteric ligands such as higher receptor type selectivity, probe dependence, biased signaling. In this way they provide more precise pharmacological modulation and then they have a great potential to separate the therapeutic benefits from side effects own of orthosteric ligands. In this review, we will cover recent progress for cannabinoid CB1 and CB2 receptor allosteric modulators and discuss the future promise of them.

- **Keywords:** allosteric modulator • endocannabinoid system • cannabinoid CB1 receptor • cannabinoid CB2 receptor • biased signaling • probe dependence • drug discovery

- **Main body of text:**

The endocannabinoid system (ECS) is a complex lipid signaling system widely distributed in the central and peripheral nervous system. It comprises at least two cannabinoid receptors (CBRs), (CB1Rs and CB2Rs), a class of lipids mediators called endocannabinoids (ECs), and their synthesizing and degrading enzymes, intracellular signaling pathways as well as transport systems [1,2]. Several other receptors such as GPR55 and GPR18 have been shown to recognize certain cannabinoids, but questions remain if they truly belong to the cannabinoid family [3]. A great interest for the development of drugs targeting CB1Rs and CB2Rs was engendered from the identification of ECS. Indeed, CBRs have huge potential as target for the treatment of a number of disorders [4-7]. For this reason, CB1R and CB2R modulators interacting with the CBR orthosteric sites have been developed and tested *in vitro* and *in vivo*. CB1Rs have a ubiquitous distribution, being found predominantly in the central nervous system (CNS) but also in peripheral nerve terminals and non-neuronal tissues [8-10]. CB1Rs are implicated in various disorders, such as obesity, mental illness, pain, multiple sclerosis and drug addiction. However, their direct modulation by agonists or antagonists has been associated with psychotropic effects (agonists) or depression, anxiety and suicidal ideation (antagonists), thus limiting the clinical development of such agents.

The expression of CB2Rs is limited to few areas in the CNS. They are predominantly found in immune cells where modulate cell migration and cytokine release. For these reasons CB2R agonists have been considered as promising drug candidates for the treatment of diseases with a neuroinflammatory component such as multiple sclerosis, amyotrophic lateral sclerosis, autoimmune diseases, osteoporosis, Huntington's disease, ischemic stroke, and diverse cancer types [11,12]. However, apart from a few synthetic CB2R agonists (from ClinicalTrials.gov: GW842166X, CP-55,940, S-777469 and JTE-907), none of the CB2R ligands developed have reached an advance stage of clinical trials as well [5,11]. Furthermore, immune dysfunction, due to the predominance of CB2Rs in immune cells, might cause immunosuppression.

One possibility to overcome the limitations of the traditional orthosteric CBR ligands would be the use of allosteric modulators that might constitute a promising new strategy to achieve potential therapeutic benefits avoiding inherent side effects of orthosteric ligands.

Allosteric ligands are capable to bind topographically distinct sites from the orthosteric binding sites, modifying the conformation of the receptor protein and modulating the affinity and/or efficacy of orthosteric ligands [13,14].

In the case of CB1Rs, an allosteric binding pocket has been identified and several computational studies have been reported. More specifically, Shore *et al.* in 2014 characterized the binding site of the allosteric modulator ORG27569 at the TMH3-6-7 region [15]. Regarding CB2Rs, a potential allosteric binding pocket adjacent to the orthosteric ligand-binding site has been postulated for the first time by Feng and co-workers in 2014 [16].

Concerning the mechanisms of action of the allosteric ligands, they can in general enhance or reduce the orthosteric ligand-driven signaling of CBRs. A positive allosteric modulator (PAM) enhances affinity, potency and/or efficacy of

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orthosteric ligands, while a negative allosteric modulator (NAM) produces an opposite effect (Figure 1) [13,17,18]. Moreover, allosteric modulator can act as neutral allosteric ligand (NAL) when it does not modulate orthosteric ligand activity, but competes with other PAM or NAM at the allosteric binding site (Figure 1) [18], or also may be a combination of PAM and NAM depending on the signaling output being measured [19]. Finally, allosteric ligands may possess an intrinsic agonist profile in the absence of an orthosteric ligand, in spite of binding to the allosteric site. In this case the allosteric ligand is termed ago-PAM.

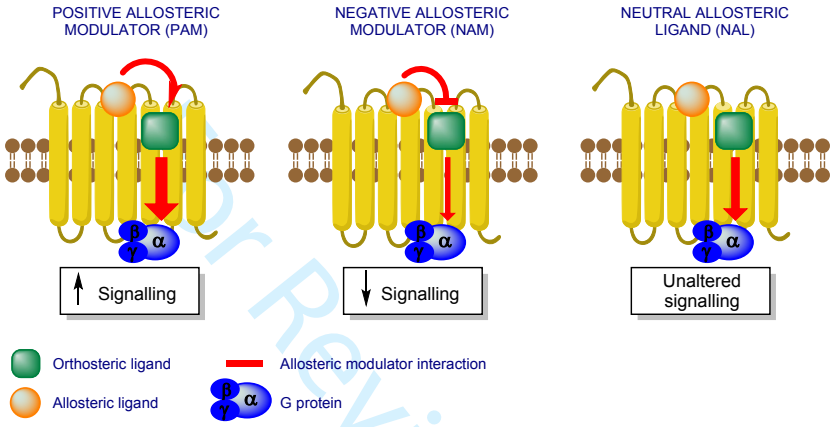

Figure 1. General classification of allosteric modulators.

Allosteric modulators, compared to orthosteric ligands, present several advantages: (1) temporal and spatial selective signaling. They exert their effects only in tissues where endogenous ligands are present and do not compete with endogenous ligands bound to orthosteric sites. Since the ECs are produced and released "on demand," allosteric modulators do not have long-lasting enhancing/blocking effects, allowing a further "fine-tuning" and decreasing the chances of side effects. (2) Receptor subtype selectivity; the sequence and structural diversity of allosteric sites compared to orthosteric ones allow for the development of selective allosteric GPCR modulators reducing off-target side effects. (3) Saturability; since they depend on endogenous ligands for signaling, an increasing concentration of allosteric modulators beyond saturation of the allosteric binding site does not increase the magnitude of the allosteric effect avoiding the risk of overdose ("ceiling effect") (4). Biased signaling/probe-dependence; allosteric modulators may also give probe-dependence and biased signaling, providing more precise pharmacological modulation (Figure 2). Indeed, the nature of the allosteric interaction is dependent on the orthosteric ligand (probe). Furthermore, allosteric ligands might induce and stabilize unique conformations of the orthosteric site inducing biased signalling by the orthosteric ligand that preferentially engages certain signaling interactions (e.g., G proteins) over other ones (e.g., β-arrestins) [20,21].

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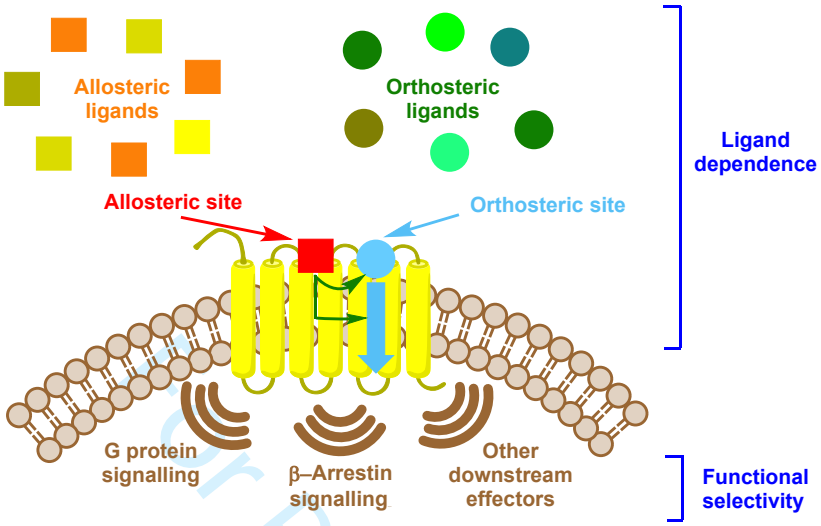

Figure 2. Allosteric modulation, probe dependence and biased signaling.

This review aims to provide a thorough overview of the recent medicinal chemistry efforts in the field of CBR allosteric modulation, focusing on the biased-signaling of the different modulators and on the future directions of the research.

CB1R ALLOSTERIC MODULATORS

EXOGENOUS ALLOSTERIC MODULATORS

Indole-2-carboxamides

The first class of CB1R allosteric modulators identified, consists of indole-2-carboxamide derivatives described in 2005 by Price *et al.* [22]. Org27569 (Figure 3), the most active and characterized compound of the series, became a prototypical compound for further studies. Allosteric modulators of this class are characterized by a divergent modulation at CB1Rs, behaving as PAM for binding affinity for orthosteric agonists and NAM for the cellular response [22]. The increase of the binding affinity for orthosteric agonists at CB1Rs induced by Org27569 has been demonstrated in different studies: firstly, Price *et al.* reported that Org27569 showed a positive binding cooperativity for the orthosteric ligand CP55,940 at CB1Rs in mouse brain membranes [22] and this result was also subsequently obtained in HEK293-hCB1 cells by Ahn *et al.* [23]. In a further study, Org27569 showed only small effect on WIN55,212-2 in mouse brain membranes, and in CHO-hCB1 cells, displaying the first hint of probe-dependence in the modulation of the binding affinity at CB1Rs [24]. This behaviour was supported by a subsequent study, where Org27569 slightly enhanced the binding of AEA and 2-AG in CHO-hCB1 cells [25]. Org27569 significantly, but not completely, proved to decrease the binding of CB1R antagonist/inverse agonist SR141716A in mouse brain membranes [22] and in HEK293-hCB1 cells, indicating a limited negative binding cooperativity [23]. Additionally, Org27569 provided a reduction of the dissociation rate constants of CP55,940, without influencing the rate of the different phases of dissociation, in mouse brain membranes, thus stabilizing the interaction between CB1Rs and the orthosteric agonist, confirming a PAM profile in binding assays [22].

Although Org27569 acts as PAM for binding, it reduces CP55,940-dependent G protein-coupling, producing an insurmountable antagonism of the receptor functionality, and inhibits CP55,940-dependent cAMP inhibition,

consistent with a behaviour as NAM [15,22,25,26]. In accordance to this behaviour, in [^{35}S]GTPγS functional assays, Org27569 resulted effective in removing CP55,940-induced inhibition of G protein coupling in mouse brain membranes and in HEK293-hCB1R cells [22,23]. Moreover, Org27569 suppressed the CB1R-depedent phosphorylation of JNK, while it promoted β-arrestin-1-mediated phosphorylation of ERK1/2 in HEK-hCB1 cells either alone or after co-treatment with CP55,940 [24,27]. Furthermore, Org27569 proved not to affect receptor internalization if administered alone in HEK 3HA-hCB1 cells [26]. On the other side, when Org27569 was tested using an inactive CB1R mutant (T210A), the internalization resulted promoted, and in co-administration with CP55,940, the process was even accelerated. This outcome was in agreement with the Org27569-stimulated promotion of an active state of the receptor, which is readily internalized, in spite of its inactivity in G protein-mediated signaling. This issue disclosed for the first time the possibility of Org27569 to promote biased signaling at CB1Rs [23,28,29]. Org27569 also demonstrated probe-dependence. In particular, it showed to be a more efficacious NAM for CP55,940 and AEA compared to the synthetic aminoalkylindole WIN55,212-2 in GTPγS, cAMP, and ERK1/2 assays [22,23,27]. Moreover, in cultured mouse autaptic hippocampal neurons, Org27569 behaved as NAM of 2-AG dependent inhibition of glutamate release [30].

In *in vivo assays*, Org27569 reduced food intake by both wildtype and CB1R knockout mice, showing that the anorectic effect most likely was not mediated by a CB1R-dependent mechanism [31]. Another study conducted in rats found that pre-treatment with Org27569 dose-dependently attenuated both cue- and drug-induced reinstatement of cocaine and methamphetamine-seeking behaviors [32]. However, the results obtained from *in vivo* and *in vitro* assays highlight the pharmacological complexity of Org27569 and, more in general, the difficulties associated with the pharmacological characterization of CB1R allosteric modulators.

Since the discovery of Org27569, several structure–activity-relationship (SAR) studies have been performed on the indole-2-carboxamide scaffold. These studies highlighted the key structural requirements of indole-2-carboxamides for allosteric modulation of CB1Rs. The carboxamide function is crucial for the activity, since its replacement with an ester group resulted in a loss of the allosteric modulation of agonist binding [33]. The spacer between the carboxamide function and the phenyl ring cannot be shortened [34] or elongated [35] and cannot be replaced by different linkers (such as phenyl or piperidinyl) to maintain allosteric activity [36].

The substituent at the 4 position on the phenyl ring should be a basic nitrogen, with the best results achieved with -$N(Me)_2$ and -$N(Et)_2$ groups. A further elongation of the N-alkyl chains leads to a reduction of the activity, indicating a limited spatial tolerance at this position. On the other side -NO_2, -Cl, -OMe, -H, -*t*Bu, -morpholinyl or acyl groups provide a loss of the activity in binding assays, demonstrating the importance of electronic effects [34]. Regarding the position of the substituent, when the -$N(Me)_2$ group was moved at the 3 position of the phenyl ring a decrease of the activity was observed. At this position, smaller groups (e.g. -Cl) are preferred, due to a more limited spatial tolerance. Moreover, the di-substitution at positions 3 and 4 does not lead to an improvement of the activity and in some cases the compounds resulted inactive [36].

The indole ring is critical, indeed, when it is replaced by other heteroaromatic rings, such as benzofuran or benzimidazole, the allosteric activity was abolished or reduced [34,37]. Furthermore, the NH of indole ring must be unsubstituted [27].

At the position 5 of the indole ring an electron-withdrawing group, with a preference for a halogen or an isothiocyanate, is required for allosteric activity [33,38]. In particular, the isothiocyanate group provided good activity and resulted in a more potent compound than Org27569 in β-arrestin 1 recruitment and cAMP accumulation assays, with functional selectivity towards β-arrestin 1 recruitment [35]. Finally, at the position 3 of the indole ring a lower linear alkyl chain is preferred, whereas its substitution with aryl or aliphatic cycles reduces the allosteric activity [34,38].

The indole 2-carboxamide class represented by the parental compound Org27569, provides a scaffold endowed with a PAM nature for the binding of orthosteric agonists and a mixed ago-NAM profile for CB1Rs functional activity, with the possibility to achieve inverse agonist activity. These compounds are characterized by ligand-dependence, showing different amplitude with different orthosteric probes and by a functional selectivity, proving to inhibit the G protein-mediated signaling cascade and to stimulate ERK1/2 phosphorylation through a G-protein independent

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pathway. This feature could be successfully exploited to reach high degrees of selectivity in activating the desired signaling cascade and to provide a "fine tuning" of CB1R functionality.

Diaryl ureas

The second class of CB1R allosteric modulators comprises compound PSNCBAM-1 (Figure 3), which was discovered in 2007 through a high-throughput screening [39]. PSNCBAM-1 is diaryl urea derivative, structurally unrelated to Org27569. Similar to Org27569, PSNCBAM-1 proved to enhance the binding affinity of CB1R agonist [^{3}H]CP55,940 in a dose-dependent manner and significantly, but incompletely, decreased the binding of the CB1R inverse agonist [^{3}H]SR141716A, showing a PAM profile in binding assays [24,39]. Like Org27569, PSCBAM-1 displayed probe-dependence, enhancing the binding affinity of different orthosteric agonists with the same trend but a different amplitude of the effect. In fact, the effect was of little entity for WIN55,212-2 [24], whereas it was much higher for CP55,940 [39].

Like Org27569, PSCBAM-1 behaves as a non-competitive functional antagonist, decreasing the cellular response induced by orthosteric agonists but, on the other hand, is not endowed with constitutional activity and so it can be defined as a pure allosteric modulator of CB1Rs [39]. PSNCBAM-1 revealed to decrease in a dose-dependent manner CB1R signaling induced by CP55,940 and, with different ranges, by WIN55,212-2, AEA and 2-AG, showing probe-dependence [24]. Furthermore, PSNCBAM-1 exhibited no intrinsic negative regulation of the constitutive activity of CB1Rs and did not behave as an inverse agonist [39]. Similar to Org27569, PSNCBAM-1 dose-dependently reduced [^{35}S]GTPγS binding induced by the [^{3}H]CP55,940 [24], and it also inhibited Δ^9-THC-induced [^{35}S]-GTPγS binding [40]. PSNCBAM-1 was found to display functional selectivity *via* the β-arrestin-1 mediated pathway, as evidenced in the ERK1/2 phosphorylation assay [24]. In cAMP accumulation assays, in HEK293-hCB1R cells, PSNCBAM-1 inverted the inhibition induced by CP55,940 and AEA, thus proving to efficiently antagonize signaling transduction both at G protein and at second messengers level [39]. In rat cerebellum, PSNCBAM-1 did not have any effect on the reduction of release of presynaptic GABA, induced by WIN55,212-2, whereas it efficiently removed the inhibitory effect provoked by CP55,940, and prevented the antagonist AM251 to go beyond the basal levels [41].

PSNCBAM-1 was also administered *in vivo* to rats, proving to decrease the food intake and the gain of body weight (without any visible effects as far as toxicity and behaviour disturbs) [39]) and also to reduce the reinstatement of cocaine and methamphetamine seeking [42].

Several structural modifications of the diarylurea scaffold were performed, in order to extend the structure-activity relationships studies (Figure 3). The results indicated that the pyrrolidine moiety is not fundamental for the activity. It can be replaced with a N,N-dimethylamino function, showing good activity in calcium mobilization assays [43] or totally removed, without changing the type/level of activity. In particular, the lack of the pyrrolidine moiety gives more metabolically stable compounds, thus providing higher concentrations in the CNS [42]. The replacement of the pyrrolidine group with different heterocyclic rings is tolerated, anyway, electro-donating groups are preferred at this position [43].

The chlorine atom at position 4 of the phenyl ring can be efficiently substituted with other electron-withdrawing groups (such as -CN or -F), whereas the activity in binding assays is highly decreased in the presence of electro-donating groups [43]. Electro-withdrawing groups at positions 3 and/or 2 produce a decrease of the activity and a 3,4-disubstituted derivative is generally more active than a 3,5- and a 2,6-disubstituted one, but still it is worse than the corresponding mono-substituted analogue at the position 4; trisubstituted analogues are, instead, inactive [43,44]. Bulky substituents, such as a phenyl or a benzophenone groups, proved to dampen the activity, confirming a limited spatial tolerance at this position [42]. Rigid and hindered systems in replacement of this phenyl ring, such as naphtyl groups and tricyclic systems lead to a reduction of the activity in calcium mobilization assays [43].

The pyridine ring can be replaced by pyrimidine ring [44], phenyl ring [45] or other functionalized heteroaryl systems [42]. The urea group is required for the activity, in fact its replacement with an amide function provides a reduction of the activity both in binding and in functional assays [45]. Finally, the diaryl system can be efficiently modified with the insertion of an amine spatial linker; this derivative reported by Bertini *et al.*, showed good activity both in binding

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assays and in SRE functional assays (where it showed a higher potency than PSNCBAM-1) possibly creating a new point of interaction with the receptor [45].
To summarize, the allosteric modulation of the CB1Rs by PSNCBAM-1 and its derivatives is similar to that of Org27569, showing a dependence on the orthosteric ligands, receptor sources, cell types, pathways and time frame over which the signaling responses occurred [42].

2-Phenylindoles

ZCZ011 (Figure 3) is a representative compound of the 2-phenylindole class. It was identified immediately after the discovery of Org27569 and it is characterized by a complex pharmacological profile. ZCZ011 increases the binding affinity for CP55,940 and WIN55,212-2 at CB1Rs, decreasing the binding affinity of the inverse agonist SR141716A in mouse brain membranes and then behaving as a PAM in binding assays.
Its PAM activity was also showed in functional assays, indeed ZCZ011 in [^{35}S]GTPγS functional assays proved to increase the efficacy of AEA and of CP55,940 in mouse brain membranes without exerting any effect if administered alone. In cAMP accumulation assays, ZCZ011 slightly enhanced AEA-, but not CP55,940-induced inhibition of cAMP production, however it behaved as an agonist if administered in the absence of orthosteric agonists in CHO-hCB1R cells. Compound ZCZ011 increased AEA-induced phosphorylation of ERK 1/2, and enhanced the potency of CP55,940, being inactive if administered alone in CHO-hCB1R cells [46]. In β-arrestin recruitment assays, ZCZ011 potentiated the action of AEA in mouse brain membranes, maintaining the activity also if administered alone. In another study reported by Mitjavila *et al.* [47], ZCZ011 confirmed its mixed agonist and PAM properties, differently inhibiting the neurotransmission in diverse neurons. In some cases, it behaved as PAM, increasing the action of 2-AG, but in other cases ZCZ011 proved to behave as a direct agonist. This behaviour could be due to its racemic nature, since distinct activities could be attributed to the single enantiomers [47]. Moreover, in a recently published study, ZCZ011 proved to behave as a PAM in binding assays only at low concentrations (lower than 100 nM), and to behave as an agonist at higher doses, displacing the orthosteric agonist, confirming its mixed activity [48]. Finally, in well-established murine models of neuropathic and inflammatory pain, ZCZ011 did not produce psychoactive effect but reverses mechanical and cold allodynia for a fairly long period with CB1R mediate mechanism [46].
To summarize, compound ZCZ011 displays a PAM profile, which comprises both its influence on binding and on functionality of CB1R, differently from the well-known allosteric modulators Org27569 and PSNCBAM-1, which possess divergent activities. Moreover, in some cases, it shows direct agonist activity, demonstrating to possess a mixed profile, which could may be ascribed to its racemic structure. Indeed, the enantiomers have yet to be individually tested and no evidence of functional selectivity has been provided to date.
GAT211 (Figure 3) is a close analogue of ZCZ011 firstly reported by Lapraire *et al.* in 2017 [49]. Since its racemic composition, its enantiopure derivatives GAT228 (*R*) and GAT229 (*S*) have been isolated and furtherly characterized (Figure 3). The racemic mixture GAT211 can be defined as ago-allosteric modulator since it possesses a complex and mixed behaviour. It was reported to increase binding affinity for CP55,940 at CB1R in CHO-hCB1R membranes and in mouse brain membranes, elongating its dissociation times. Moreover, in the presence of GAT211, the binding affinity of SR141716A in CHO-hCB1R membranes resulted decreased, demonstrating a PAM profile [49]. In mouse vas deferens, GAT211 (1 μM) was found to increase the AEA-induced inhibition of contractions, exerting no effect in the absence of orthosteric agonists. The same results were obtained in cAMP accumulation assays in HEK293A-hCB1R cells, where GAT211 displayed again a PAM activity (potentiating the inhibition induced by CP55,940) without exerting any effect if administered alone [49]. On the other hand, GAT211 proved to increase β-arrestin 2 recruitment mediated by AEA, 2-AG and CP55,940 both in HEK293A-hCB1R and in Neuro2a cells, and to maintain this function also in the absence of any orthosteric agonist, in a concentration-dependent manner, thus proving to possess an ago-PAM mixed activity [49]. This profile was confirmed also in PLCβ3 and ERK phosphorylation functional assays, where GAT211 enhanced the activity of CP55,940, 2-AG and AEA, maintaining this effect also in their absence. GAT211 showed also a probe-dependent activity, being more potent in increasing 2-AG and AEA functionality over CP55,940 [49]. Finally, GAT211 proved to be effective against chemotherapy-induced pain in mice [50].

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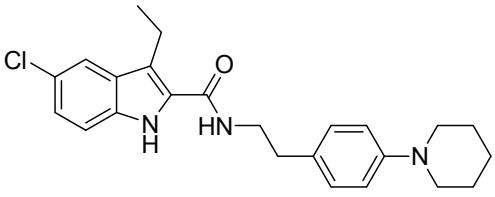
GAT228, which is the *R* enantiopure form of GAT211, does not influence the binding affinity for CP55,940 at CB1Rs but showed a small enhancement of binding at a supra-pharmacological concentration. As far as the functional assays, GAT228 did not influence the inhibition produced by orthosteric agonists in cAMP accumulation assays indicating to be inactive as a PAM, but it had a slight effect in increasing β-arrestin 2 recruitment induced by CP55,940 in HEK293A-hCB1R cells. Moreover, it decreased cAMP accumulation but increased β-arrestin 2 recruitment over the basal levels, without competing for the orthosteric site. Additionally, it increased the phosphorylation of ERK and PLCβ3 in HEK293A-hCB1R cells. Like GAT211, its *R* enantiomer possesses a probe-dependence for endocannabinoids over synthetic orthosteric agonists [49].

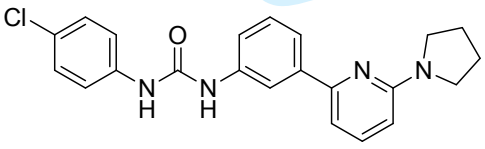
In binding assays, GAT229 (the *S* enantiopure form of GAT211) proved to increase the binding affinity at CB1Rs for CP55,940, in CHO-hCB1R membranes, with a greater effect if compared to GAT211. In cAMP and β-arrestin 2 recruitment functional assays, GAT229 behaved as a PAM possessing a functional selectivity towards cAMP production over β-arrestin recruitment. Moreover, GAT229 at 1 μM was found to improve ERK and PLCβ3 phosphorylation induced by 2-AG, AEA and CP55,940 in HEK293A-hCB1R and Neuro2a cells. In the absence of orthosteric agonists, it showed no activity, confirming its pure PAM nature, and no probe-selectivity was observed [49]. This behaviour was also confirmed by an *in vivo* study, where GAT229 was co-administered with WIN55,212-2 in eye at 0.2%, proving to increase its ability to reduce intraocular pressure in mice [51]. These data seem to ascribe the ago-allosteric properties of GAT211 to GAT228 and the pure PAM behaviour to GAT229, showing that CB1R is sensitive towards different enantiomers [49].

The GAT family is endowed with biased signaling towards cAMP production over β-arrestin recruitment but, given the mixed properties of GAT211 and GAT228, the pharmacological profile of GAT229 which behaves as a pure PAM allosteric modulator at CB1Rs, could be simpler and more easily modulated.

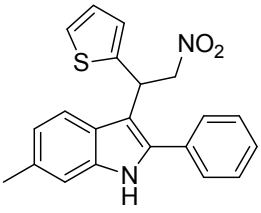
Currently, a limited SAR of the 2-phenyl indole class is reported [52,53]. These studies showed that the 2-aryl indole nucleus is essential for allosteric modulation effects, and the 2-phenyl moiety can be substituted at the *para* position with halogens. The di-substituted ethyl group at the 3-position of the indole ring is important for allosteric activity. The thiophene group can be replaced by a pyridine ring, while its replacement with a phenyl ring reduces the activity. The 2-nitro group can be replaced only by cyano group or trifluoromethyl group. On the contrary, when it was substituted by a carboxylic acid, an ester, an amide or an amino group, the activity was abolished. Finally, the replacement of the 6-methyl group with a chlorine or a hydrogen is tolerated.



ORG27569



PSNCBAM-1



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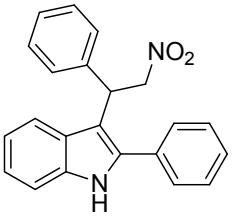

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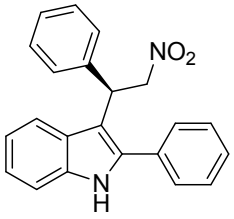

GAT228

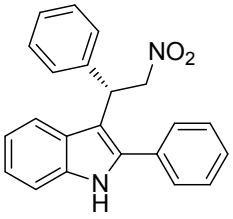

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Figure 3. Chemical structure of exogenous CB1R allosteric modulators.

ENDOGENOUS ALLOSTERIC MODULATORS

Pregnenolone

The neurosteroid pregnenolone (Figure 4), biosynthetic precursor for steroid hormones, emerged as a possible allosteric modulator of CB1Rs. THC and WIN,55-212-2 proved to enhance pregnenolone levels, activating an autocrine/paracrine loop regulating GPCRs [54].

In hCB1 cells, pregnenolone does not influence the binding affinity at CB1R for CP55,940 and WIN55,212-2 [54], indicating a lack of binding cooperativity with orthosteric agonists, and can partially decrease the binding affinity for SR141716A in a dose-dependent manner [25].

On the other hand, pregnenolone inhibits the THC-induced ERK1/2 phosphorylation in CHO-hCB1 cells, but it is ineffective in the accumulation of cAMP and in [^{35}S]GTPγS functional assays [40], giving evidence of a possible functional selectivity [54]. Conversely, pregnenolone does not inhibit WIN55,212-2-induced phosphorylation of ERK1/2 in CHO-hCB1 cells, thus displaying also a probe-dependence [25]. This neurosteroid is ineffective in modulating synaptic signaling of 2-AG in autaptic neurons [30], but showed activity in potentiating GABAergic and glutamatergic transmission inhibited by THC in rat nucleus accumbens [55], and in counteracting WIN55,212-2-induced inhibition of synaptic transmission in rat hippocampus [56].

Also in *in vivo* tests, pregnenolone gave good results. It blocks the increase of food-intake and memory impairments induced by THC in rats and mice, without showing any effect if administered alone [54]. Further studies proved that pregnenolone blocks the THC-induced impairment of social interactions, hyperlocomotion effects and psychotic-like state in mice [57].

Given the results reported to date, pregnenolone behaves as NAM for the CB1Rs functionality, displaying probe-dependence and functional selectivity towards ERK1/2 phosphorylation over G protein-mediated signaling pathways.

Fenofibrate

It has already been reported that many cannabinoids may present a pleiotropic pharmacology interacting not only with CBRs but also with other different receptors, such as GPR55, the transient receptor potential cation channel subfamily V member 1 (TRPV1) and peroxisome proliferator-activated receptors (PPARs) [58].

Among PPARs ligands, one class consists in fibrate compounds in which fenofibrate (Figure 4) is included. Prestley *et al.* [59] demonstrated partial agonism of fenofibrate at CB1Rs and full agonism at CB2Rs. In addition, it was also demonstrated that fenofibrate behaves as CB1R NAM at high concentrations. More specifically, fenofibrate provoked a reversal in CB1R GTPγS binding, leading to a ball-shape concentration curve. The same biphasic curves were also present in ERK1/2 phosphorylation and β-arrestin recrutiment responses.

These results suggest that fenofibrate might act at both orthosteric and allosteric sites of the CB1Rs but at different concentrations and potencies. It is worth mentioning that some clinical effects of fenofibrate may be due to its interaction with CBRs rather than PPARs. No biased signaling activity has been reported for its allosteric activity.

Lipoxin A4

Among all the endogenous lipoxins, lipoxin A4 (LXA4) (Figure 4) is certainly the most studied because of its involvement in immune system regulation and its implications in the resolution of inflammation [60,61]. Pamplona *et al.* in 2012 studied the interaction between LXA4 and CB1R [62]. In binding assays, LXA4 enhanced the affinity of AEA, CP55,940 and WIN55,212-2 to CB1Rs. Regarding functional assays, LXA4 enhanced the potency of AEA and 2-

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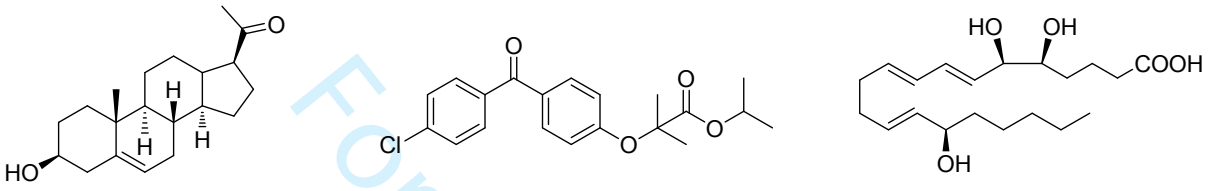
AG in decreasing cAMP levels in HEK cells transfected with mouse CB1Rs.
These results confirmed LXA4 as PAM at CB1Rs. The same authors reported also the therapeutic application for neuroprotection of lipoxin A4 as CB1R PAM in an *in vivo* model of β-amyloid-induced spatial memory impairment [62].
Furthermore, it was reported that LXA4 synthesis is increased following inflammatory stimuli and it might act as a homeostatic check to inflammation [30,62]. Thus LXA4 can be considered as endogenous CB1R PAM providing evidence for the physiological relevance of cannabinoid allosteric modulation as a potential therapeutic target.



Pregnenolone **Fenofibrate** **Lipoxin A4**

Figure 4. Chemical structure of endogenous CB1R allosteric modulators.

CB2R ALLOSTERIC MODULATORS

Pepcan-12

Pepcan-12 (RVD-hemopressin) (Figure 5) is an endogenous dodecapeptide derived from α-hemoglobin, which was firstly isolated from mouse brain extracts [63,64]. In the periphery, pepcan-12 is present in human plasma, kidney, liver and adrenal glands [64,65]. Furthermore, it was reported that Pepcan-12 acts as NAM at CB1Rs. Indeed, in a concentration-dependent binding assay, pepcan-12 was able to partially displace both [^{3}H]CP55,940 and [^{3}H]WIN55,212-2, indicating an allosteric interaction. In order to define its allosteric behavior, Pepcan-12 cooperativity factor (α) was calculated and resulted less than 1 as expected for an allosteric compound [66]. Regarding functional assays, pepcan-12 response was firstly analyzed in G protein-dependent signaling pathways. As all the allosteric modulators, it was ineffective by itself in changing basal [^{35}S]GTPγS binding at CB1Rs, which instead resulted reduced in the presence of the orthosteric agonist 2-AG at three different concentration. Pepcan-12 was also tested in a cAMP assay where it reduced the accumulation induced by the orthosteric ligands WIN55,212-2 and 2-AG and, also in this case, it was totally ineffective if tested alone. Regarding G-protein independent pathways, Pepcan-12 reduced the CB1R internalization induced by WIN55,212-2 and 2-AG. To make sure it did not affect CB1R response through the modulation of the levels of AEA and 2-AG, Pepcan-12 was tested for its ability to induce the hydrolysis of 2-AG and AEA, but it was found not to affect ECs degradation [66].
More recently, following the discovery of the relatively high concentration of pepcan-12 in peripheral organs, Petrucci *et al.* in 2017 assessed its action also on the mostly peripheral CB2Rs, demonstrating, unexpectedly, its positive allosteric modulation activity on these receptors [67]. Indeed, it increased significantly the receptor binding of the two orthosteric agonists [^{3}H]CP55,940 and [^{3}H]WIN55,212-2. This positive allosteric effect was quantified by the corresponding binding cooperativity factor (α) which was higher than 1, confirming the positive cooperation between pepcan-12 and both orthosteric ligands. In order to establish its functional properties, pepcan 12 was studied for its effects on key signaling events prompted by the CB2R agonists CP55,940 and 2-AG, measuring [^{35}S]GTPγS receptor binding, cAMP production, β-arrestin recruitment and receptor internalization. Regarding the G

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protein signaling, pepcan-12 was found to be able to potentiate [^{35}S]GTPγS binding and the signaling through cAMP of both CP55,940 and 2-AG. The same assays were performed in the absence of a CB2R agonist and pepcan-12 did not affect CB2R signaling as expected by an allosteric ligand. Interestingly, unlike the previous signaling pathways, CP-55,940 responses induced by β-arrestin recruitment were not altered by pepcan-12 as well as receptor internalization [67].

Considering all these results, since pepcan-12 is a CB1R NAM but also a CB2R PAM, it might be able to shift the CB1R/CB2R activation ratio towards CB2R, leading eventually to positive beneficial effects on liver and kidney and hopefully other organs. To date, there are no proves of biased signaling properties of pepcan-12 at CB1Rs; on the other, it has been clearly demonstrated that its PAM activity at CB2Rs is specifically mediated through $G_{\alpha i}$ proteins [67].

Cannabidiol

Cannabidiol (CBD) (Figure 5) is a non-psychoactive constituent of the cannabis plant with low affinity for both CBRs. In 2007 was reported the first evidence of its activity as antagonist of the CB2Rs, provided through [^{35}S]GTPγS binding assays [68]. Few years later, CBD was found to be a NAM at CB1Rs [69]. Indeed, in functional assays, CBD was able to reduce both G protein-dependent signaling pathways and the recruitment of β-arrestin 2 by the two agonists 2-AG and Δ^9-THC. Importantly, no biased activity was detected using HEK cells as CBD effects did not differ in the three pathways. Interestingly, using STHdh (medium spiny projection neurons expressing wild-type CB1Rs) cells, CBD showed biased signaling for PLC β rather than β-arrestin and ERK1/2. Moreover, in these cells CBD resulted to be a more potent NAM for β-arrestin recruitment when THC was used as orthosteric probe rather than 2-AG. Conversely, no orthosteric probe-dependence was detected in the case of the other pathways. CBD NAM activity might explain its action as antidepressant, antipsychotic and antiepileptic providing new insights into its medicinal value [69]. In 2017, Martinez-Panilla *et al*. demonstrated through a HTRF-mediated binding assay and traditional binding assay using WIN55,212-2 as orthosteric probe, that CBD is a CB2R allosteric modulator [70]. More specifically, CBD is a NAM since it blocks the effect of the orthosteric ligand JWH-133. Indeed, regarding functional assays, low concentrations of CBD can decrease the response of JWH-133 in cAMP, pERK1/2 signaling and in the activation of MAP kinase pathway. No biased signaling evidence was reported in the study. Moreover, another indication of its allosteric behavior consists in the lack of any effects when tested alone [70].

Since the different activities of CBD, synthetic CBD derivatives were synthesized in order to elucidate the structure–activity relationship of CBD at the CBRs [71]. Several modifications on different pharmacophoric positions of CBD scaffold have been accomplished, one of those was the substitution of the alkyl chain with a 1,1'-dimethyl epthyl (DMH) lipophilic chain [72]. CBD-DMH (Figure 5) was tested for both binding and functional activity on CBRs. Initially, CBD-DMH was observed to increase the efficacy, the potency and the binding affinity of CP55,940 at the CB1Rs, but to reduce the potency of Org27569. Moreover, CBD-DMH was tested in binding assays where it enhanced [^{3}H]-CP55,940 binding to CB1R at low concentrations, but it reduced ligand binding at higher concentration. In functional assays, CBD-DMH displayed agonist activity in cAMP assays while, regarding β-arrestin, it displayed an activity comparable to an agonist and a PAM (agoPAM). Regarding the CB2R, CBD-DMH decreased the binding of CP-55,940. Its allosteric activity was confirmed through functional assays that also highlighted that the allosteric activity of CBD-DMH at CB2R was pathway-specific. Indeed, it resulted a PAM in cAMP signaling but a NAM in β-arrestin recruitment. Although the mechanism is not clear, CBD-DMH was thought to promote a conformational change in CB2R which favors G-protein-dependent signaling rather than β-arrestin-dependent signaling [71].

Interestingly, in the same study, cAMP luciferase reporter assay and a bioluminescence resonance energy transfer (BRET) assay for measuring β-arrestin 1 recruitment, were used for determining the functional activity of CBD-DMH and CBD. Conversely to the study reported by Martinez-Panilla *et al.* in 2017 [70] (who demonstrated its NAM activity at the CB2Rs), in these conditions CBD resulted a partial CB2R agonist [71].

In the light of all these results, more information about the activity of CBD and CBD-DMH are required. Their ago-allosteric activity is very challenging and the NAM/PAM nature of CBD-DMH may result in a switch in the response of G-protein signaling or β-arrestin pathway.

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2-oxo-pyridin-3-cycloheptanecarboxamide derivative C2

N-[5-Bromo-1,2-dihydro-1-(4'-fluorobenzyl)-4-methyl-2-oxo-pyridin-3-yl]cycloheptanecarboxamide (C2, Figure 5) is the first synthetic allosteric modulator of the CB2Rs reported in literature [73]. It belongs to a new class of 2-oxopyridine-3-carboxamide derivatives obtained reversing the amide moiety of a previous series of compounds presenting an orthosteric activity on CBRs [74]. C2 allosteric behavior has been demonstrated through binding and functional assays. More specifically, it was reported to enhance the binding of [^{3}H] CP55,940 on both CBRs suggesting that C2 may be a CB1R and CB2R PAM. However, on CB1Rs the CP55,940-induced stimulation of [^{35}S]GTPγS binding was not significantly altered by C2, suggesting that although compound C2 increases the binding of the orthosteric ligand CP55,940 to CB1Rs, this enhancement does not affect the ability of CP55,940 to activate CB1Rs. However, the data reported are not sufficient to indicate C2 as NALs for CB1Rs. Conversely, the ability of CP55,940 to stimulate [^{35}S]GTPγS binding to CB2R was significantly enhanced by C2, whereas it did not produce any effect by itself. Interestingly, C2 significantly improved the ability of 2-AG, but not of AEA, to stimulate [^{35}S]GTPγS binding to CB2R, also showing a probe-dependent activity. Then, derivative C2 would be able to activate CB2Rs by low concentrations of endogenously released 2-AG, and it could also potentiate the pharmacological activity of MAGL inhibitors. Interestingly, oral administration of C2 was efficient in cold allodynia assays, and this effect was demonstrated to be predominantly mediated by the CB2Rs. Finally, C2 potentiated the antiallodynic effect of a MAGL inhibitor which previously showed to be efficacious in the same model of neuropathic pain [71].

All these data strongly demonstrate that C2 behaves as a CB2R PAM both *in vivo* and *in vitro*. However, further studies should be carried out with the aim to highlight different behaviors at other CB1R- and CB2R-mediated signaling pathways and then to study the possibility of C2 to promote biased signaling.

Potential CB2R allosteric ligands

At the 9th Conference on Cannabinoids in Medicine in 2017 at Cologne, the pyrazole carboxamide IQM311 was presented as a new potential CB2R allosteric modulator by Jagerovic and co-workers [75]. Its allosteric activity was investigated through [^{3}H]CP55,940 and [^{35}S]GTPγS binding assays in CHO cells stably transfected with hCB2R and hCB1R. It resulted to be a CB2R PAM at 1 μM without affecting CP55,940 dissociation rate, but a weak CB2R inverse agonist at higher concentrations (10 μM). However, its action is not selective towards CB2R since at higher concentrations (10 μM) it can also target CB1Rs, behaving as an agonist or inverse agonist. Moreover, Rajasekaran in his PhD thesis in 2011 [76] reported Dihydrogambogic acid (DHGA, Figure 5) and trans-β-caryophyllene (TBC, Figure 5) as potential CB2R NAM at micromolar concentration since they altered the dissociation kinetics of [^{3}H]CP55,940 and [^{3}H]WIN55,212-2 in a probe-dependent way. However, the activity of these compounds cannot be described in details since both these research have not been published yet.

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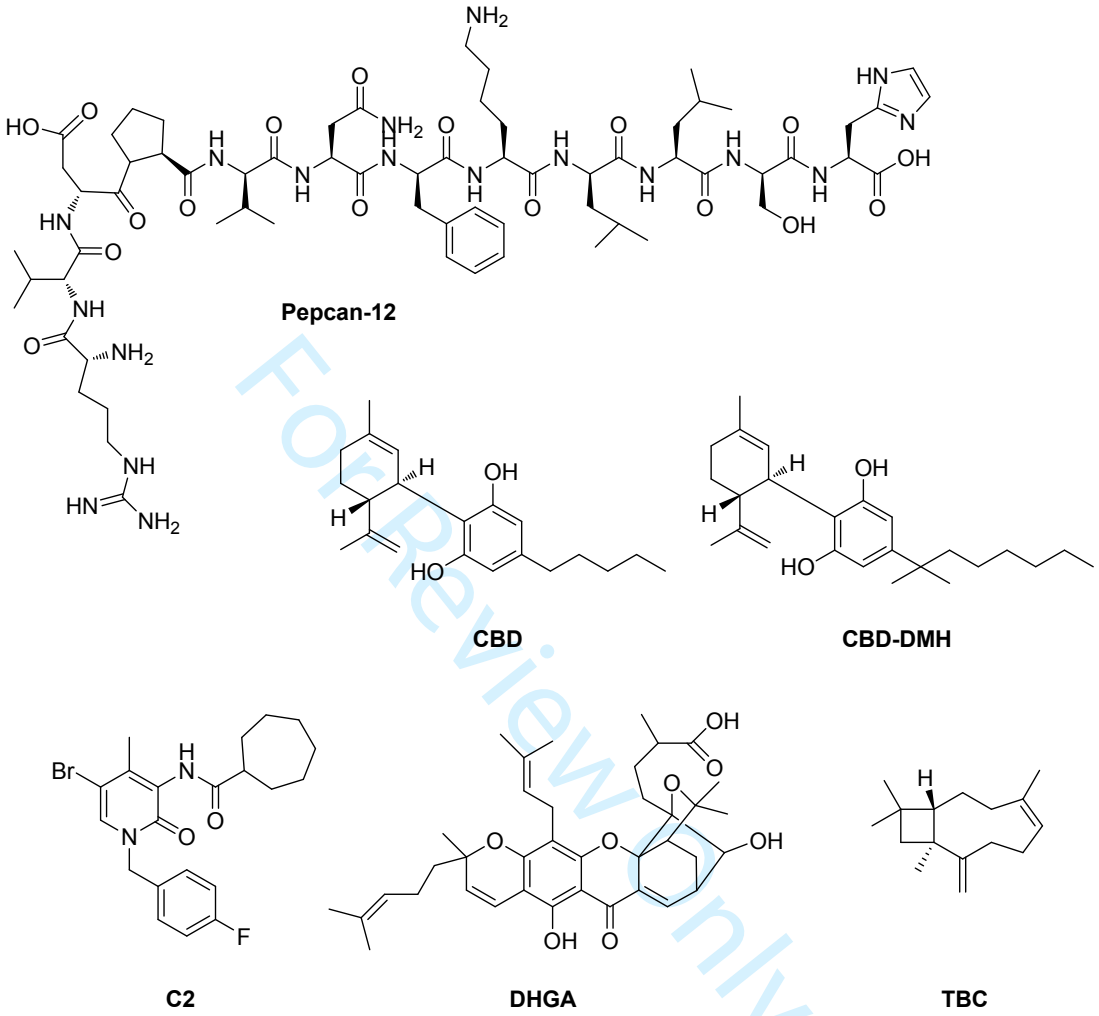

Figure 5. Chemical structure of CB2R allosteric modulators.

- **Future Perspective:**

The CBRs are attractive targets for drug discovery due to their involvement in many physiological and pathological processes. Historically, cannabis-based drug discovery has focused on the development of orthosteric ligands that interact with the active site to which endogenous cannabinoids bind. However, the research fulfilled over the last decades, has shown significant difficulties in translating CBR orthosteric ligands into druggable candidates. This difficulty is mainly due to the adverse effects associated with CBR orthosteric ligands. Currently, there are several evidences that CBRs can be modulated through allosteric mechanisms. Different scaffolds have been identified as CB1R allosteric modulators, while very few compounds have been reported as CB2R allosteric modulators. CBR allosteric modulators may be very promising therapeutic agents characterized by numerous advantages over the

orthosteric ligands, such as higher receptor type selectivity, probe dependence, and no modification of the activity of ECs. Furthermore, CBR allosteric modulators can selectively handle the signaling pathways through biased signaling. These features suggest that CBR allosteric modulators have a great potential to separate therapeutic benefits from side effects own of orthosteric ligands and might to be determinant to identify the signaling factors that cause the side effects mediated by activation or downregulation of CBRs, in particular of CB1Rs. However, some considerations must be made: 1) the CBRs allosteric mechanisms is very complex, therefore, the type of modulation, the orthosteric probe, the pathway, and the model system should be stated as clearly as possible for each allosteric modulator. Moreover, receptor specificity should be well determined because receptor subtype overlap exists even at the allosteric sites [70,77]. Thus more efforts should be focused on the bioassays used for allosteric evaluation considering these difficulties; 2) the *in vivo* efficacy should be defined in acute and chronic disease models to evidence the behavior as CBR allosteric modulators; 3) efforts should be made to define the binding site/s of allosteric modulators through mutagenesis and modeling studies. These findings might facilitate determining structural requirements for allosteric binding, as well as G-protein activation, and β–arrestin interaction; 4) Finally, allosteric ligands could have a lower affinity than ligands targeted to the orthosteric binding site. A solution to the latter problem could be the development of bitopic ligands bearing the pharmacophore of both orthosteric and allosteric ligands connected by a linker. Because of their dual pharmacophore nature, bitopic ligands can provide both greater selectivity through interaction with an allosteric site and higher affinity through concomitant engagement of the orthosteric site.

- **Executive Summary:**
- Allosteric modulators represent a new strategy to achieve potential therapeutic benefits avoiding inherent side effects of orthosteric ligands.
- Biased signaling is one of the principal advantages of allosteric ligands. It consists in the ability of allosteric modulators to stabilize determined conformations of CBRs induced by orthosteric ligands which, in this way, engage certain signaling pathways rather than others.
- There are several classes of CB1R allosteric modulators, some of them deeply studied for years. They mostly have been characterized through *in vitro* and *in vivo* assays useful to eventually highlight their biased properties. Conversely, little is known about CB2R allosteric modulations. Indeed, there are few modulators reported in the literature and, in general, they need more tests in order to clarify their biased signaling properties. There is a huge need of CB2R allosteric modulators which might be useful also for computational studies regarding the pharmacology and crystal structure of the CB2R's allosteric site.
- Bitopic ligands may constitute a future promising new strategy. Indeed, they are characterized by a dual pharmacophore nature which means that they present both the orthosteric and allosteric pharmacophore group connected by a linker. This feature might lead to a greater selectivity and higher affinity because of their concomitant interaction with both the orthosteric and the allosteric site.

- **Figure legends**

  Figure 1. General classification of allosteric modulators.

  Figure 2. Allosteric modulation, probe dependence and biased signaling.

  Figure 3. Chemical structure of exogenous CB1R allosteric modulators.

  Figure 4. Chemical structure of endogenous CB1R allosteric modulators.

  Figure 5. Chemical structure of CB2R allosteric modulators.

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**** This paper presents in silico docking study of the crystal structure of CB1 receptor on endogenous and natural hydrophobic ligands that act as positive allosteric and negative allosteric modulators**.