

Allosteric Modulators of the CB₁ Cannabinoid Receptor: A Structural Update Review

Paula Morales, Pilar Goya, Nadine Jagerovic, and Laura Hernandez-Folgado*

Abstract

In 2005, the first evidence of an allosteric binding site at the CB₁R was provided by the identification of three indoles of the company Organon that were allosteric enhancers of agonist binding affinity and, functionally, allosteric inhibitors of agonist activity. Since then, structure–activity relationships of indoles as CB₁R modulators have been reported. Targeting the allosteric site on CB₁R, new families structurally based on urea and on 3-phenyltropane analogs of cocaine have been discovered as CB₁R-negative allosteric modulators (NAMs), respectively, by Prosidion and by the Research Triangle Park. Endogenous allosteric ligands of different nature have been identified more recently. Thus, the therapeutic neuroprotection application of lipoxin A4, an arachidonic acid derivative, as an allosteric enhancer of CB₁R activity has been confirmed *in vivo*. It was also the case of the steroid hormone, pregnenolone, whose negative allosteric effects on Δ^9 -tetrahydrocannabinol (Δ^9 -THC) were reproduced *in vivo* in a behavioral tetrad model and in food intake and memory impairment assays. Curiously, the peroxisome proliferator-activated receptor- γ agonist fenofibrate or polypeptides such as pepcan-12 have been shown to act on the endocannabinoid system through CB₁R allosteric modulation. The mechanistic bases of the effects of the phytocannabinoid cannabidiol (CBD) are still not fully explained. However, there is evidence that CBD behaves as an NAM of Δ^9 -THC- and 2-AG. Allosteric modulation at CB₁R offers new opportunities for therapeutic applications. Therefore, further understanding of the chemical features required for allosteric modulation as well as their orthosteric probe dependence may broaden novel approaches for fine-tuning the signaling pathways of the CB₁R.

Key words: CB₁R; synthetic cannabinoids

Introduction

One traditional way of designing new drugs is the so-called one target–one disease approach. Indeed, this is the case of many commercially available drugs, which interact with enzymes, receptors, or ionic channels among others. One of the most important targets are the G-protein-coupled receptors (GPCRs) for which agonists and antagonist/inverse agonists have been developed.

These traditional ligands bind to the same site as the endogenous ligand, the so-called orthosteric site. However, recently, research has shifted to compounds that

can interact with a different region of the receptor, termed the allosteric site, since this approach may result in pharmacological advantages such as higher specificity and thus reduced side effects. The best known mechanisms of action of these allosteric ligands are activation or inhibition of the receptor signaling, referred to as positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs). There are also neutral or silent ligands, silent allosteric modulators, which do bind to the allosteric site without affecting the response of the endogenous agonist. In addition, bitopic ligands that bind to both the orthosteric and

Instituto de Química Médica, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

*Address correspondence to: Dr. Laura Hernandez-Folgado, Instituto de Química Médica, Consejo Superior de Investigaciones Científicas, Calle Juan de la Cierva 3, Madrid E-28006, Spain. E-mail: lhernandez@iqm.csic.es

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allosteric sites have been described. In the last years, there has been considerable research in allosteric modulation of GPCRs both in academia and industry,^{1,2} and structural knowledge on how these interactions take place has advanced on the basis that some cocryystals of a ligand bound to an allosteric site have been obtained.³

In addition to two allosteric modulators marketed for HIV and hyperparathyroidism, recent potential therapeutic applications of allosteric modulators of GPCRs include central nervous system (CNS) disorders such as neurodegenerative diseases or schizophrenia⁴ and also pain for which PAMs of the μ -opioid receptor have been proposed.⁵

A particular kind of GPCRs are the cannabinoid CB₁ and CB₂ receptors (CB₁R and CB₂R), for which thousands of traditional ligands, belonging to many different chemical structures, have been synthesized and evaluated. However, this has had only limited success in bringing them to the clinic. In fact, only three natural derived medicines (Marinol™, Nabilone™, and Sativex™) are available largely due to their unwanted side effects.

One possibility to overcome the limitations of these traditional cannabinoids interacting with the orthosteric sites would be to use allosteric cannabinoid ligands. Despite the considerable research undertaken on traditional cannabinoid agonists and antagonists, there have been comparatively few reports on allosteric cannabinoid ligands. In this review, we will give an overview of the chemical structures that have been tested and proven to be allosteric cannabinoid ligands, but will not deeply deal with the pharmacological as-

pects of cannabinoid allosterism, which has been covered elsewhere.⁶⁻⁹ According to structural features, CB₁R allosteric modulators reported to date can be classified as follows: indole derivatives; urea derivatives; and structures acting as endogenous ligands and miscellaneous structures.

Indole Derivatives

The first evidence for an allosteric binding site on CB₁R was revealed in 2005 by pharmacological studies realized on three indoles from the company Organon Research, Org27569, Org29647, and Org27759 (Fig. 1).¹⁰ Among them, Org27569 has been more widely studied and is considered as a prototypical CB₁R allosteric modulator. Org27569 behaves as a positive allosteric enhancer of agonist binding, whereas in terms of functionality, Org27569 is considered a NAM. Lately, the effect of Org27569 as a NAM has been confirmed in a neuronal model of endocannabinoid synaptic transmission.¹¹ However, when tested *in vivo*, Org27569 lacked efficacy in modulating the action of orthosteric cannabinoids.¹² Thus, controversial pharmacological data led to a very recent study in which the allosteric effects of Org27569 at CB₁R have been revealed to be pathway specific and time dependent, suggesting a more complex process than initially proposed.¹³

The Org27569-binding site has been determined by Shore et al.¹⁴ using a combination of molecular modeling, mutation, and functional assays. This site was identified in the CB₁R transmembrane helix (TMH)3–6–7 region. Org27569 acts by modulating receptor activity through conformational changes in the receptor. The authors described how Org27569 sterically blocked

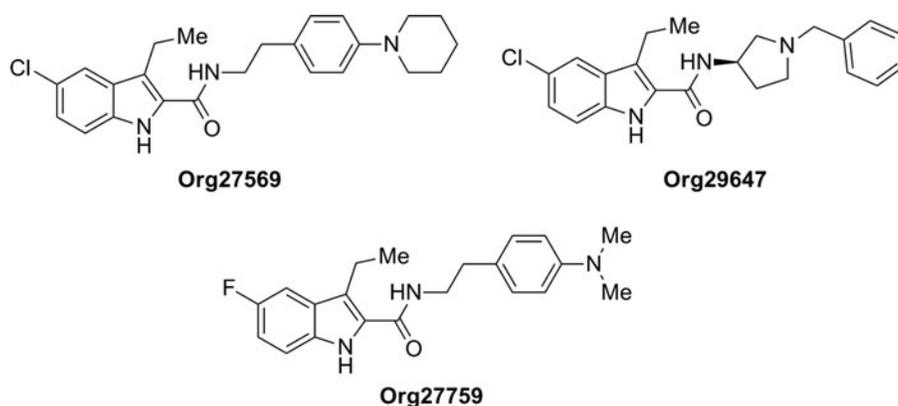


FIG. 1. Structure of indoles Org27569 (pharmacological profile in Table 1), Org29647, and Org27759.



movements of the extracellular loops and TMH6 that are involved in receptor activation. Recently, site-directed fluorescence labeling studies reported by Fay et al.¹⁵ showed that effectively TMH6 movements are associated with G-protein activation, whereas its attached helix eight could be involved in the binding of arrestin triggering the biased signaling pathway.

Following discovery of Org27569, structure–activity relationship (SAR) studies were reported by various research groups confirming that indole-2-carboxamide was a good scaffold for developing allosteric modulators for CB₁R. However, it was only in 2012 that Piscitelli et al.¹⁶ reported that the first indole-2-carboxamides structurally correlated with Org27569. The very close derivative of Org27759, the indole **1** (Fig. 2), was found to possess the highest positive stimulation of the series on the orthosteric agonist CP-55,940 binding at CB₁R.

A large number of Org27569 analogs have been identified in Kendall and Lu's laboratories.^{17–19} Among them, it is worthy to mention compound **2**, the 3-pentyl-1*H*-

indole-2-carboxamide derivative (Fig. 2), as an allosteric enhancer of [³H]-CP-55,940 binding with a markedly high binding cooperativity factor and potent antagonism of agonist-induced GTP γ S binding.¹⁸ Interestingly, the hexyl and the propyl derivatives of **2** induced β -arrestin 1-mediated pathway-biased signaling.¹⁹

Very recently, a novel series of 1*H*-indole 2-carboxamides has been reported by Nguyen et al.²⁰ The most potent compound **3** (Fig. 2) is reported as a NAM of CP-55,940 at CB₁R according to calcium mobilization assays.

After a first generation of Org27569 derivatives, new series of 2-phenyl-1*H*-indoles have been identified as PAMs at CB₁R. Compound PAM1, also named as GAT211, displayed in Figure 2, exemplifies the series of 3-(2-nitro-1-arylethyl)-1*H*-indoles claimed by Thakur et al.²¹ as PAMs for CB₁R in cAMP functional assays. Due to the presence of a chiral center, GAT211 is a racemic mixture of two optical isomers (R and S) that were isolated using supercritical fluid chromatography.

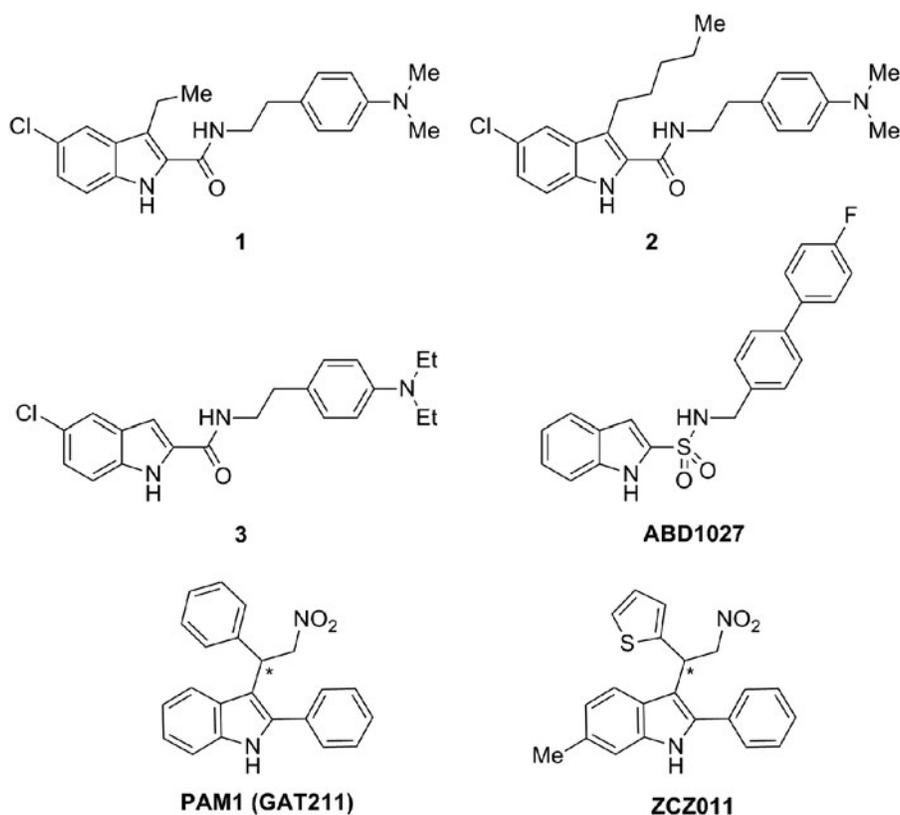


FIG. 2. Indole derivatives as CB₁R allosteric modulators: **1**, **2**, **3**, ABD1027, PAM1 (GAT211), and ZCZ011 (pharmacological profile in Table 1). *Race MIC mixture.



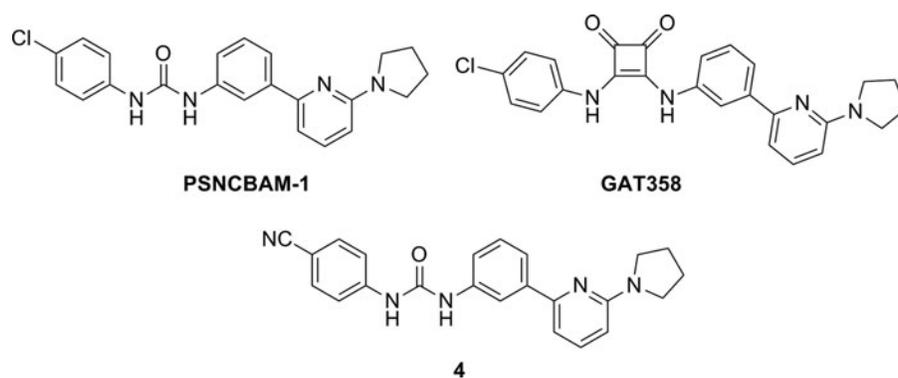


FIG. 3. PSNCBAM-1 and its analogs as CB₁R allosteric modulators: GAT358 and **4** (pharmacological profile in Table 1).

Difference in pharmacology potencies of both enantiomers could be appreciated.

Ignatowska-Jankowska et al.²² reported the pharmacological properties of 6-methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole (ZCZ011; Fig. 2). ZCZ011 increased [³H]-CP-55,940 binding and potentiated anandamide (AEA)-stimulated signaling in [³⁵S]-GTP γ S binding, β -arrestin recruitment, and extracellular signal-regulated kinase (ERK) phosphorylation assays. A very interesting finding is that PAM effects of ZCZ011 could be confirmed by *in vivo* assays. The indole ZCZ011 exhibited antinociceptive effects in neuropathic and inflammatory pain models with no associated cannabimimetic effects.

Greig et al.²³ from the University of Aberdeen applied the bioisosteric replacement of carboxamide by sulfonamide leading to numerous *N*-arylalkyl-1*H*-indole-2-sulfonic amides claimed in a patent. Inventors reported that ABD1027 (Fig. 2) did not affect [³H]-CP-55,940 binding, although it inhibited agonist signaling in functional β -arrestin recruitment assays.

Urea Derivatives

In 2007, the company Prosidion identified a novel class of CB₁R allosteric compounds through high-throughput screening of a small library.²⁴ The lead optimized urea of this series, PSNCBAM-1 (Fig. 3), increased CB₁R agonist binding and behaved as an allosteric antagonist in [³⁵S]-GTP γ S binding and cAMP assays. In this study, acute food intake experiments provided the first *in vivo* data showing the efficacy of CB₁R allosteric antagonism similar to the antiobesity effects of the well-known CB₁R antagonist, SR141716.

In 2015, 8 years after the discovery of PSNCBAM-1, the first SAR studies on PSNCBAM-1 were published by German et al.²⁵ The resulting analogs showed similar pharmacological profiles to the parent NAM in binding and calcium mobilization assays. Structural modifications have focused on the pyridine and the 4-chlorophenyl groups. Substituted amine with small size alkyl chains showed to be preferred for pyridine substitution. Substitution in position 4 of the phenyl ring with an electrowithdrawing group was revealed to be important for activity (compound **4**; Fig. 3).

In the same year, Thakur et al.²⁶ claimed novel CB₁R allosteric modulators based on PSNCBAM-1 structure. SAR studies involving bioisosterism of urea were extensively examined with the synthesis and evaluation of carbamates, thioureas, 1,3,4-oxadiazol-2-amines, and 3,4-diaminocyclobut-3-ene-1,2-diones, leading to functionally selective NAMs. The 3,4-diaminocyclobut-3-ene-1,2-dione derivative, GAT358, was selected for *in vivo* behavioral tests that suggested minimal CB₁R inverse agonist-related side effects.

Endogenous CB₁R Allosteric Modulators

In addition to synthetic allosteric modulators categorized herein, endogenous molecules of diverse chemical nature have been identified as allosteric modulators of CB₁Rs.

One of these molecules is lipoxin A4 (Fig. 4), an oxygenated derivative of arachidonic acid involved in immune system regulation and known as a potent endogenous anti-inflammatory mediator. However, the specific effects of lipoxin A4 in the CNS were reported to be mediated by unknown mechanisms. In 2012, lipoxin A4 was proposed as an allosteric modulator of



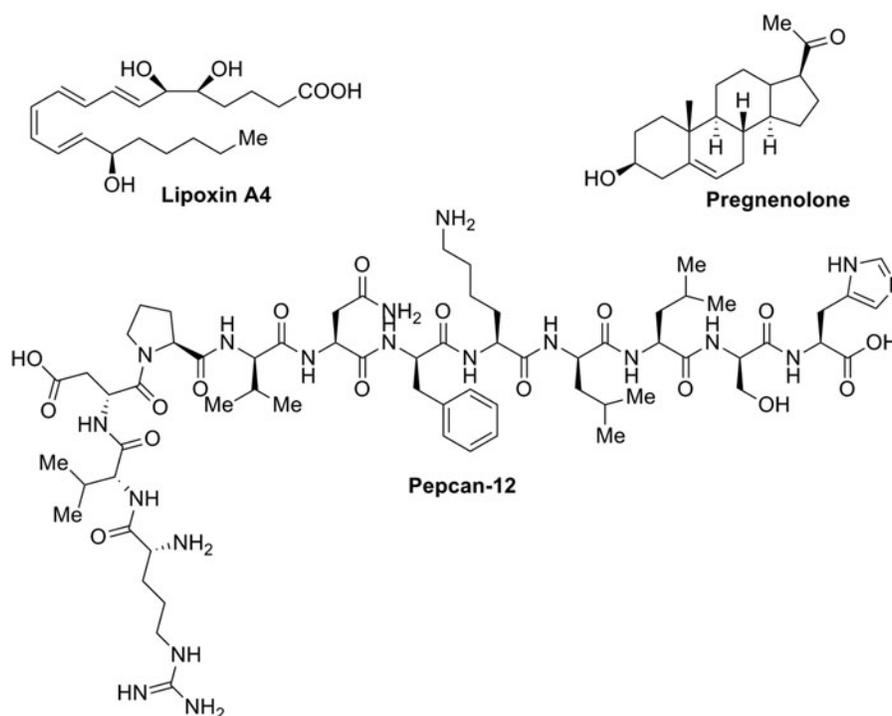


FIG. 4. Endogenous CB₁R allosteric modulators: lipoxin A4, pregnenolone, and pepcan-12 (pharmacological profile in Table 1).

CB₁R by Pamplona et al.²⁷ This lipid acted as a CB₁R PAM, enhancing receptor binding of AEA and [³H]-CP-55,940 and not only potentiating selectively AEA-versus 2-AG in HEK293-CB₁R cells but also in the behavioral tetrad tests. The authors confirmed the therapeutic application for neuroprotection of lipoxin A4 as an allosteric enhancer of CB₁R activity in an *in vivo* model of β -amyloid-induced spatial memory impairment. Recently, Staiker et al.¹¹ reported that lipoxin A4 surprisingly exhibited a CB₁R NAM profile, and no PAM as reported so far, in a neuronal model of 2-AG-mediated depolarization-induced suppression of excitation (DSE). Thus, this effect could be attributable to a potential probe dependence of lipoxin A4. Adding to the complexity, recent studies realized by Khajehali et al.¹³ on lipoxin A4 could not corroborate the PAM modulatory effects on either AEA- or CP-55,940-mediated cAMP inhibition in CHO-CB₁R cells.

Another putative endogenous allosteric modulator at the CB₁R is the steroid hormone pregnenolone (Fig. 4), a hydrophobic precursor for all C₁₈, C₁₉, and C₂₁ steroids directly synthesized from cholesterol. The therapeutic known targets for pregnenolone are

GABA and NMDA receptors.²⁸ However, in 2014, Vallée et al.²⁹ identified pregnenolone as an allosteric inhibitor at CB₁Rs decreasing Δ^9 -tetrahydrocannabinol (Δ^9 -THC)-induced ERK1/2 phosphorylation in CHO-CB₁R cells. Furthermore, these NAM effects on Δ^9 -THC were reproduced *in vivo* in a behavioral tetrad model, in food intake and memory impairment assays. In contrast, in the neuronal model reported by Staiker et al.,¹¹ pregnenolone failed to modulate 2-AG of synaptic transmission. Likewise, Khajehali et al.¹³ reported lack of modulatory effect of pregnenolone on either Δ^9 -THC- or WIN55,212-2-induced activation of ERK1/2 phosphorylation. These divergent data suggest probe- or pathway-dependent allosteric effects.

Until recently, synthetic or endogenous compounds acting on the endocannabinoid system (ECS) were of lipid nature. In 2012, Bauer et al.³⁰ described a 12-amino acid sequence, structurally corresponding to an N-terminal extended form of hemopressin, as a potent CB₁R NAM of orthosteric agonist-induced cAMP accumulation, [³⁵S]-GTP γ S binding, and receptor internalization. This polypeptide, named pepcan-12, has been recently localized in discrete cells of the



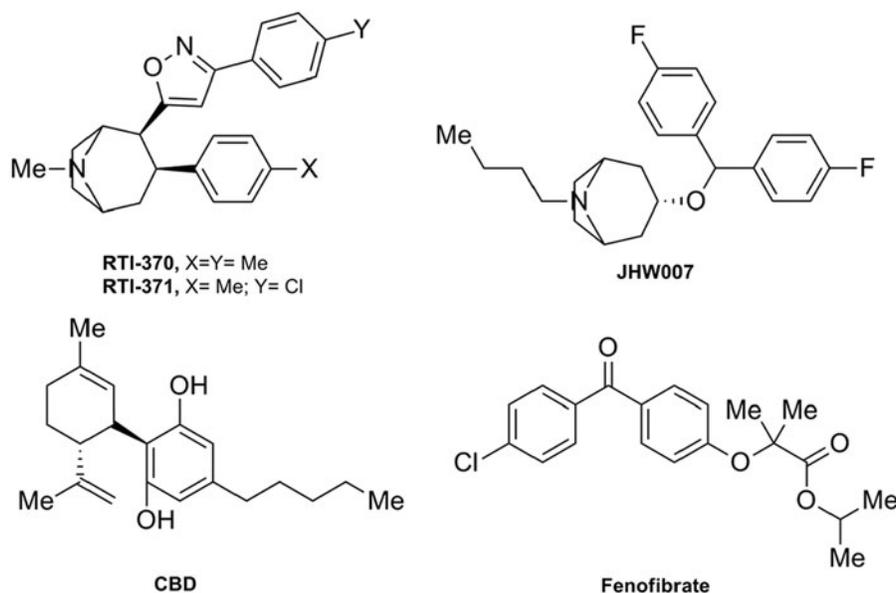


FIG. 5. Miscellaneous CB₁R allosteric modulators: RTI370, RTI371, JHW007, cannabidiol (CBD), and fenofibrate (pharmacological profile in Table 1).

CNS and adrenal gland.³¹ It is worth mentioning that this endocannabinoid peptide was previously reported to interact with CB₁R as an agonist.³² Furthermore, the NAM profile of pepcan-12 has been confirmed in the DSE study of 2-AG synaptic transmission performed by Staiker et al.¹¹

Miscellaneous CB₁R Allosteric Modulators

The interest gained in this field led to the identification of an assorted range of other small molecules that also display CB₁R allosteric modulation.

In 2009, researchers from the Research Triangle Park screened several 3-phenyltropane analogs of cocaine for their activity at the CB₁R.³³ These molecules had been previously characterized as dopamine transporter (DAT) inhibitors.³⁴ Among this series, the tropanes, RTI-371 and RTI-370, and the benzotropine JHW007 (Fig. 5) stand out as PAMs of CP-55,940-induced CB₁R activity in a cell-based calcium mobilization assay. RTI-371 was found to be of special interest because it is a potent and selective DAT inhibitor that lacks cocaine-like behavioral effects and abuse liability. According to the authors, these atypical cocaine-related effects could be due to positive allosteric modulation of the CB₁R.³³ This study highlights the potential of the 3-phenyltropane scaffold for the development of novel CB₁R PAMs.

The nonpsychoactive phytocannabinoid cannabidiol (CBD; Fig. 5) has shown diverse promising therapeutic applications through multiple biological targets. CBD is known to interact with many nonendocannabinoid signaling systems such as the opioid receptors, the transient channel receptor, transient receptor potential channel 1, or the nuclear receptor, peroxisome proliferator-activated receptor (PPAR)- γ , among others. CBD does not bind to the orthosteric CB₁R binding site; however, it has been shown to display antagonism of CB₁R agonists *in vitro*.^{35–37} More recently, Laprairie et al.³⁸ provided evidence that CBD behaved as an NAM of Δ^9 -THC- and 2-AG-dependent CB₁R internalization, β -arrestin recruitment, and phospholipase C β 3- and ERK1/2-phosphorylation. These results may explain some of the *in vivo* effects of this promising nonpsychoactive compound providing novel insights in the intriguing pharmacology of CBD.

The close relationship between the ECS and the PPARs led to explore the activity of the PPAR- α agonist, fenofibrate³⁹ (Fig. 5), in the ECS. This fibrate is an amphipathic molecule that acts as a prodrug formed by fenofibric acid linked to an isopropyl ester. Priestley et al.⁴⁰ reported the pharmacological profile of fenofibrate at CBRs. [³⁵S]-GTP γ S binding experiments revealed partial agonism of fenofibrate at CB₁R and full agonism at CB₂R. Moreover, at higher concentrations, this PPAR- α ligand was



also able to significantly decrease [³⁵S]-GTP γ S binding of CP-55,940 acting as an NAM of CB₁R. Thus, the authors suggested two possible interpretations. Fenofibrate could be considered a bitopic ligand at CB₁R since it binds both orthosteric and allosteric sites or fenofibrate could be an allosteric CB₁R modulator that could be able to produce by itself a functional response by interacting with a non-orthosteric site at CB₁R.

Conclusions and Perspectives

The psychoactive side effects generated by activation of CB₁R in the brain have limited the use of orthosteric

CB₁R ligands as drugs. However, this receptor plays an important role in diverse processes such as pain, cognition, and metabolism. Alternative targets within the ECS have been proposed, including CB₂R, the fatty acid amide hydrolase, or the monoacylglycerol lipase. Allosteric modulation at CB₁R offers new opportunities for therapeutic applications. Even though the first CB₁R allosteric ligand, Org27569, was identified in 2005, significant interest by the research community only started few years ago. Then, there have been rapidly increasing research efforts directed into CB₁R allosterism during these last 3 years. This review reflects the

Table 1. Summary of CB₁R Allosteric Modulators and Their Cannabinoid Pharmacological Profile

Compound	CB ₁ R/CB ₂ R orthosteric modulation	CB ₁ R allosteric modulation	CB ₁ R allosterism	
			Functional outcome (orthosteric ligand)	References
Org27569	CB ₁ R inverse agonist ⁶	NAM	[³⁵ S]-GTP γ S binding assay ^a (CP-55,940; AEA), cAMP assay, β -arrestin recruitment ^a (CP-55,940; WIN55,212, AEA)	Price et al. 2005 ¹⁰
		PAM	ERK1/2 phosphorylation ^a (CP-55,940)	Baillie et al. 2013 ⁶
		NAM	ERK1/2 phosphorylation ^a (2-AG)	Khajehali et al. 2015 ¹³
		NAM	DSE ^c (2-AG)	Straiker et al. 2015 ¹¹
2	—	NAM	[³⁵ S]-GTP γ S binding assay ^b (CP-55,940)	Mahmoud et al. 2013 ¹⁸
3	—	NAM	Ca ²⁺ mobilization assay ^a (CP-55,940)	Nguyen et al. 2015 ²⁰
PAM1 (GAT211)	—	PAM	cAMP assay (CP-55,940)	Thakur et al. 2013 ²¹
ZCZ011	CB ₁ R agonist ²²	PAM	[³⁵ S]-GTP γ S binding assay ^d (CP-55,940; AEA)	Ignatowska-Jankowska et al. 2015 ²²
		NE	cAMP assay ^a (CP-55,940; AEA)	
		PAM	ERK1/2 phosphorylation ^a (CP-55,940; AEA)	
		PAM	β -arrestin recruitment ^b (AEA)	
ABD1027	—	NAM	β -arrestin recruitment ^b (CP-55,940)	Greig et al. 2012 ²³
PSNCBAM-1	CB ₁ R partial inverse agonist ²⁴	NAM	[³⁵ S]-GTP γ S binding assay ^b (CP-55,940; AEA), cAMP assay ^b (CP-55,940; AEA)	Horswill et al. 2007 ²⁴
		NAM	[³⁵ S]-GTP γ S binding assay ^e (CP-55,940; WIN55,212)	Wang et al. 2011 ⁴¹
		NAM	β -arrestin recruitment ^a (CP-55,940; WIN55,212)	Baillie et al. 2013 ⁶
		NAM	DSE ^c (2-AG)	Straiker et al. 2015 ¹¹
4	—	NAM	Ca ²⁺ mobilization assay ^f (CP-55,940)	German et al. 2014 ²⁵
GAT358	—	NAM	cAMP assay (CP-55,940), β -arrestin recruitment ^b (CP-55,940)	Thakur et al. 2015 ²⁶
Lipoxin A4	NE ²⁷	PAM	cAMP assay ^b (AEA)	Pamplona et al. 2012 ²⁷
		NAM	DSE ^c (2-AG)	Straiker et al. 2015 ¹¹
		NE	cAMP assay ^a (AEA; CP-55,940)	Khajehali et al. 2015 ¹³
Pregnenolone	—	NAM	ERK1/2 phosphorylation ^a (Δ^9 -THC)	Vallée et al. 2014 ²⁹
		NE	DSE ^c (2-AG)	Straiker et al. 2015 ¹¹
		NE	ERK1/2 phosphorylation ^a (Δ^9 -THC, WIN55,212)	Khajehali et al. 2015 ¹³
Pepcan 12	CB ₁ R agonist ³²	NAM	cAMP assay, [³⁵ S]-GTP γ S binding, CB ₁ R internalization ^a (2-AG, WIN55,212, HU210)	Bauer et al. 2012 ³⁰
		NAM	DSE ^c (2-AG)	Straiker et al. 2015 ¹¹
RTI-371	NE ³³	PAM	Ca ²⁺ mobilization assay ^a (CP-55,940)	Navarro et al. 2009 ³³
CBD	Weak CB ₁ R/CB ₂ R antagonist ^{36,37}	NAM	PLC β 3- and ERK1/2 phosphorylation, β -arrestin recruitment ^{b,9} (Δ^9 -THC, 2-AG)	Laprairie et al. 2015 ³⁸
		NAM	[³⁵ S]-GTP γ S binding ^a (CP-55,940)	Priestley et al. 2015 ⁴⁰

^aCHO-CB₁R cells.

^bHEK293-CB₁R.

^cHippocampal neurons.

^dMouse brain membranes.

^eCerebellar membranes.

^fRD-HGA16-CB₁R cells.

⁹STHdh^{Q7/Q7}-CB₁R.

Δ^9 -THC, Δ^9 -tetrahydrocannabinol; AEA, anandamide; CBD, cannabidiol; DSE, depolarization-induced suppression of excitation; ERK, extracellular signal-regulated kinase; NAM, negative allosteric modulator; NE, no effect; PAM, positive allosteric modulator; PLC, phospholipase C.



structural diversity and nature of the CB₁R allosteric modulators identified so far. In particular, there has been an increasing identification of endogenous allosteric modulators that offer new insights in the intriguing pharmacology of CB₁R. In Table 1, a summary of the pharmacological profile of diverse CB₁R allosteric modulators is displayed. It is worth mentioning that in several of the studies discussed above, there is no correlation between the different cell-based pharmacological data. Moreover, the *in vitro* data do not always translate to *in vivo* effects. These divergent functional data may account for signaling-specific allosteric modulation as well as orthosteric probe dependence. Subsequently, there is a clear need for developing more allosteric pharmacological tools for understanding this complex pharmacology.

The characterization of small molecules as allosteric modulators of CB₁Rs provides the basis for future design and synthesis of optimized allosteric modulators. Efforts need to be made to expand these scaffolds and obtain structural information about the ligand–allosteric site interactions. Further understanding of the chemical features required for allosteric modulation as well as their orthosteric probe dependence may broaden novel approaches for fine-tuning the signaling pathways of the CB₁R.

Acknowledgments

The authors gratefully acknowledge research support from Spanish Grants SAF2012-400075-C02-02 of the Spanish Ministry and S2010/BMD-2308 of CAM. P.M. is recipient of a fellowship JAE-Pre-2010-01119 from “Junta para la Ampliación de Estudios” cofinanced by FSE.

Author Disclosure Statement

No competing financial interests exist.

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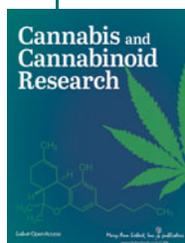
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Cite this article as: Morales P, Goya P, Jagerovic N, Hernandez-Folgado L (2016) Allosteric modulators of the CB₁ cannabinoid receptor: a structural update review, *Cannabis and Cannabinoid Research* 1.1, 22–30, DOI: 10.1089/can.2015.0005.

Abbreviations Used

2-AG = 2-arachidonoylglycerol
AEA = anandamide
CB₁R = cannabinoid receptor 1
CB₂R = cannabinoid receptor 2
CBD = cannabidiol
CNS = central nervous system
DAT = dopamine transporter
DSE = depolarization-induced suppression of excitation
ECS = endocannabinoid system
ERK = extracellular signal-regulated kinase
GPCR = G-protein-coupled receptor
NAM = negative allosteric modulator
PAM = positive allosteric modulator
PLC = phospholipase C
PPAR = peroxisome proliferator-activated receptor
SAR = structure–activity relationship
TMH = transmembrane helix
 Δ^9 -THC = Δ^9 -tetrahydrocannabinol

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