HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2018 September 15.

Published in final edited form as:

Neuropharmacology. 2017 September 15; 124: 3–12. doi:10.1016/j.neuropharm.2017.05.018.

Modulation of CB_1 Cannabinoid Receptor by Allosteric Ligands: Pharmacology and Therapeutic Opportunities

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Abstract

Cannabinoid pharmacology has been intensely studied because of cannabis' pervasive medicinal and non-medicinal uses as well as for the therapeutic potential of cannabinoid-based drugs for the treatment of pain, anxiety, substance abuse, obesity, cancer and neurodegenerative disorders. The identification of allosteric modulators of the cannabinoid receptor 1 (CB_1) has given a new direction to the development of cannabinoid-based therapeutics due to the many advantages offered by targeting allosteric site(s). Allosteric receptor modulators hold potential to develop subtype-specific and pathway-specific therapeutics. Here we briefly discuss the first-generation of allosteric modulators of CB_1 receptor, their structure-activity relationships, signaling pathways and the allosteric binding site(s) on the CB_1 receptor.

Keywords

CB_1; allosteric modulator; biased signaling; therapeutic potential

1. Introduction

Cannabinoid receptors are considered to be key regulators of nausea, obesity (Bellocchio et al., 2006; DiMarzo and Després, 2009), pain (Manzanares et al., 2006; Sagar et al., 2009), anxiety, depression, substance use disorders (Mackie et al., 2006) and neurodegenerative disorders such as Alzheimer's disease (Aso et al., 2014), and Parkinson's disease (Brotchie et al., 2003). Despite exhaustive research, few cannabis-based therapeutics have reached clinical use, although nabilone (Cesamet) (Frank et al., 2008), dronabinol (Marinol) (Pertwee et al., 2006) and a Δ^9-tetrahydrocannabinol (THC)/cannabidiol blend (Sativex) (Blake et al., 2006) are approved for the treatment of spasticity, nausea and pain in various countries. Conventional drug design has primarily targeted the orthosteric site of

cannabinoid receptors, creating ligands that compete with the endogenous cannabinoid, anandamide and 2-arachidonoyl-glycerol (2-AG), for binding to this site. However, psychoactive side effects are frequent and often preclude clinical usefulness for ligands that target these sites on the CB_1 cannabinoid receptor (CB_1) which is expressed in high numbers in many of the regions of the brain (Mackie et al., 2006).It is now well appreciated that allosteric sites exist on many G-protein coupled receptors (GPCRs), including CB_1 (Kenakin, 2012; Conn et al., 2014). Targeting these sites offers in principle several advantages such as greater subtype-selectivity (Conn et al., 2009), maintenance of spatial and temporal aspects of receptor activation and consequent attenuation of side effects (Conn et al., 2009; Burford et al., 2013). This manuscript focuses on the allosteric modulation of the CB_1 receptor and gives a structural and functional review of its known allosteric modulators and their outcome.

2. Allosteric modulators

Recent research in GPCRs has shifted focus to identifying ligands that bind to a site(s) topographically distinct from where the endogenous ligands bind (orthosteric site). These sites are called allosteric sites and the ligands that bind these sites to modulate receptor activity are called allosteric modulators.

2.1 Types of allosteric modulators

There are four types of allosteric modulators:

1. Potentiators or positive allosteric modulators (PAMs): ligands that increase receptor function. The positive modulation can be seen by an increase in agonist affinity or efficacy (Gentry et al., 2015). PAMs can also function by blocking desensitization of the receptor (Burford et al., 2013).
2. Allosteric antagonists or negative allosteric modulators (NAMs): ligands that decrease receptor function through a decrease in agonist affinity or efficacy (Gentry et al., 2015).
3. Allosteric agonists (ago-allosterics or ago-agonists): allosteric compounds that display positive modulation in the absence of the orthosteric ligand (Gentry et al., 2015).
4. Neutral allosteric ligands (NALs): ligands that bind at the allosteric site but do not modulate receptor function (Gentry et al., 2015).

2.2 Detection and quantification of allostery

Many different binding or functional assays can be used to detect and quantify allostery at GPCRs. One such assay is equilibrium binding which identifies two important parameters for allostery: K_B, the equilibrium dissociation constant which defines the affinity of an allosteric modulator for its receptor; and α, the cooperativity factor, which defines the magnitude and direction of impact the allosteric modulator and orthosteric ligand have on each other when both occupy the receptor. When $\alpha>1$, the ligand is a positive allosteric modulator whereas when $\alpha<1$, the ligand is a negative allosteric modulator. $\alpha=1$ denotes no

allosteric modulation. The two parameters are calculated based on an allosteric ternary complex model (Christopoulos et al., 2002; Price et al., 2005), according to the following equation:

$$Y=\frac{[A]}{[A]+K_A\frac{\left(1+\frac{[B]}{K_B}\right)}{\left(1+\frac{\alpha[B]}{K_B}\right)}}$$

where Y represents the fractional specific binding; K_A and K_B are the equilibrium dissociation constants for the orthosteric and allosteric ligands, respectively; [A] and [B] are the concentrations of the orthosteric and allosteric ligands, and α is the cooperativity factor. Another method to detect the above two parameters through binding assays is to perform kinetic assays to determine the impact of allosteric compounds on the association or dissociation of orthosteric ligands. PAMs increase the association kinetics or decrease the dissociation kinetics of orthosteric agonists. NAMs, on the other hand, increase the dissociation kinetics or decrease the association kinetics of orthosteric agonists (May et al., 2007).

Complementing ligand binding assays, functional assays such cAMP assays and calcium assays can also be performed to identify allosteric interactions of ligands with the orthosteric ligand. Functional assays allow readouts that can easily be translated into therapeutic effects exerted by the allosteric modulator on the signaling pathway of interest (May et al., 2007). To accommodate the impact of allosteric modulators on the efficacy of orthosteric ligands in functional assays, an operational model of allosterism is used which allows quantification of allosteric effects on both affinity and efficacy (Price et al., 2005; May et al., 2007). The model quantifies impact of allostery on binding of orthosteric ligands by a cooperativity factor α and the allosteric impact on efficacy by parameter β. Two other parameters, τ_A and τ_B, relate the intrinsic efficacy of the orthosteric ligand and the allosteric ligand, respectively, independent of the other ligand. A composite cooperativity factor logαβ defines the combined modulation effects on affinity and efficacy of orthosteric ligands. The following equation describes the operational model of allosterism (Gregory et al., 2010):]

$$E=\frac{E_m(\tau_A[A](K_B+\alpha\beta[B])+\tau_B[B]K_A)^n}{([A]K_B+K_AK_B+K_A[B]+\alpha[A][B])^n+(\tau_A[A](K_B+\alpha\beta[B])+\tau_B[B]K_A)^n}$$

where E and E_m represents the effect and the maximum possible effect respectively and n represents the slope factor that governs the shape of the stimulus-response function. If the allosteric ligand has no intrinsic efficacy, the equation simplifies to the following (May et al., 2007; Gregory et al., 2010):

$$E=\frac{E_m\tau^n[A]^n\left(1+\frac{\alpha\beta[B]}{K_B}\right)^n}{\left[[A]\left(1+\frac{\alpha[B]}{K_B}\right)+K_A\left(1+\frac{[B]}{K_B}\right)\right]^n+\tau^n(A)^n\left(1+\frac{\alpha\beta[B]}{K_B}\right)^n}$$

2.3 Therapeutic potential of allosteric modulators

Allosteric ligands have a number of potential advantages over their orthosteric counterparts as therapeutic agents. First, allosteric modulators may show more subtype specificity than orthosteric ligands do. Since most allosteric sites are not constrained by high evolutionary pressure to maintain endogenous ligand binding, they may not have high sequence identity across species or between subtypes. Thus targeting these sites could help develop subtype-specific drugs (Conn et al., 2009), but also emphasizes the challenges of identifying and characterizing allosteric modulators across species. Subtype specificity of allosteric modulators has been documented for many GPCRs, including adenosine receptors (Goblyos et al., 2011) and metabotropic glutamate receptors (Lindsley et al., 2004). Second, allosteric sites offer an opportunity to develop therapeutics for receptors where targeting the orthosteric site has not yielded successful drugs. For example, for the GLP-1 receptor, no small molecule orthosteric agonists have been identified, yet allosteric agonists have been reported (Knudsen et al., 2007; Burford et al., 2011). Third, unlike orthosteric drugs, “pure” allosteric modulators (not ago-allosteric modulators) may have an effect only when the endogenous ligand is present and thus maintain the temporal and spatial characteristics of endogenous signaling. PAMs, for example, may amplify endogenous signaling without affecting its temporal regulation. This could be of great importance especially with neurotransmitters where signal timing is very important (Conn et al., 2009; Burford et al., 2011). Fourth, orthosteric ligands may cause significant desensitization/downregulation of receptors due to constant receptor activation. Since allosteric activity may not be constant, or may not activate cellular pathways leading to desensitization and down regulation, in some instances these allosteric modulators offer potential for lesser receptor desensitization and/or behavioral tolerance (May et al., 2007). Fifth, allosteric activity often has a ceiling effect, and thus can be used to develop ligands with better adverse effect profiles than efficacious orthosteric ligands, where increasing concentrations may lead to undesirable adverse events (Wild et al., 2013).

3. Allosteric modulators of the CB_1 receptor

Several allosteric modulators of CB_1 have been identified or proposed. This has opened possibilities to develop subtype-specific ligands since many of the orthosteric ligands for CB_1 alsobind CB_2 with significant affinities. Some of the known allosteric modulators for CB_1 include 5-chloro-3-ethyl-N-(4-(piperidin-1-yl)phenethyl)-1H-indole-2-carboxamide (ORG27569) (Price et al., 2005), 1-(4-chlorophenyl)-3-(3-(6-(pyrrolidin-1-yl)pyridin-2-yl)phenyl)urea (PSNCBAM-1) (Horswill et al., 2010), 3-(4-chlorophenyl)-5-(8-methyl-3-p-tolyl-8-azabicyclo[3.2.1]octan-2-yl)isoxazole (RTI-371) (Navarro et al., 2009), the endogenous ligand (5S,6R,9E,11Z,13E,15S)-5,6,15-trihydroxyicosa-9,11,13-trienoic acid (lipoxin A4) (Pamplona et al., 2012) and 3-(2-nitro-1-phenylethyl)-2-phenyl-1H-indole (GAT211) (as well as its (S-(-)) enantiomer, GAT229), all of which display positive allosteric modulation (PAMs) of CB_1 under some conditions. Ligands that display negative allosteric modulation (NAMs) under some conditions have also been reported, including Pepcans (Bauer et al., 2012), cannabidiol (Laprairie et al., 2015), pregnenolone (Vallee et al., 2014), and, likely, GW405833 (Dhopeshwarkar et al., 2017). The most frequently

encountered of these allosteric modulators are discussed below and some of the chemical structures are shown in Fig. 1.

3.1 ORG27569

The compound ORG27569, developed by Organon (now merged with Merck), was among the first CB_1 allosteric modulators to be identified and characterized. Since its discovery, the pharmacological profile of this molecule has been extensively studied. ORG27569 increases the binding of CP55,940, an orthosteric agonist of CB_1 and decreases the binding affinity of SR141716A (Rimonabant), an inverse agonist of CB_1, indicating that it acts as a PAM for the CB_1 receptor (Price et al., 2005; Ahn et al., 2012; Khajehali et al., 2015). Paradoxically, ORG27569 decreased G-protein coupling induced by CP55,940 in membranes expressing the CB_1 receptor (Price et al., 2005; Ross et al., 2007; Ahn et al. 2012; Khurana et al., 2014). To resolve this conundrum and address whether ORG27569 promotes an active state of the receptor or not, the Kendall group evaluated the impact of this compound on receptor internalization. Using the inactive mutant T210A CB_1 (D'Antona et al., 2006), which primarily expresses CB_1 at the cell surface, it was shown that co-treatment of ORG27569 with CP55,940 produced robust CB_1 internalization compared to treatment with CP55,940 alone. Furthermore, ORG27569 induced receptor internalization even in the absence of CP55,940. This indicated that although ORG27569 inhibits G-protein coupling, it rapidly promotes a receptor conformation that increases its internalization (Ahn et al. 2012; Ahn et al. 2013).This is a challenging issue to examine since the wild-type CB_1 is largely localized on intracellular vesicles in the absence of agonist. It is also consistent with constitutive activities (Leterrier et al., 2004) or in the presence of endogenous agonist (Howlett, A. et al., 2011), all of which may contribute to some of the seemingly confounding results (Cawston et al., 2013; Gamage et al., 2016) including recycling of internalized receptors. For example, Cawston et al. (2013) indicate that treatment with allosteric modulators ORG27569 and PSNCBAM-1 antagonized CP55,940 induced inhibition of cAMP levels and suggest that this allosteric modulator may cause the receptor to favor inactive conformations and traffic accordingly. These results further emphasize the complexity of GPCRs, their allosteric modulation, and the challenge of interpreting assays that may only provide one aspect of signaling rather than more global interconnecting phenomena.

Another important characteristic exhibited by allosteric modulators is ligand (or probe) dependence. The impact of ORG27569 on G-protein coupling activity with three classes of orthosteric agonists i.e. CP55,940 (classical cannabinoid), WIN55,212-2 (aminoalkylindole) and anandamide (endocannabinoid) was examined (Baillie et al., 2013). In GTPγS assays in mouse brain membranes, ORG27569 displayed a concentration-dependent decrease in E_{max} of G-protein coupling induced by CP55,940 (Price et al.,2005: Ahn et al., 2012; Baillie et al., 2013; Khurana et al., 2014), WIN55,212-2 and anandamide (Baillie et al., 2013). However, a lesser reduction in GTPγS levels was seen with WIN55,212-2-induced G-protein coupling levels than those with CP55,940 and anandamide (Baillie et al., 2013). This issue has practical implications, because while it is often easier to screen potential allosteric modulators by evaluating their impact on binding and signaling of potent synthetic cannabinoids, what matters most for their development as therapeutic agents is how they affect signaling of endogenous cannabinoids.

The effect of ORG27569 on CB_1 agonist-mediated inhibition of forskolin-stimulated cAMP production in cell membranes expressing CB_1 has also been reported (Baillie et al., 2013). ORG27569 completely abolished the inhibition of CP55,940-mediated inhibition of forskolin-stimulated cAMP production at 10 nM ORG27569 (Baillie et al., 2013; Khajehali et al., 2015). However, it was less effective in reducing WIN55,212-2-mediated inhibition of forskolin-stimulated cAMP production (Baillie et al., 2013).

3.1.1 Impact on signaling pathways by ORG27569—Kendall and colleagues evaluated the impact of ORG27569 on signaling including via phosphorylation of ERK1/2 and JNK1/2, which are members of the MAPK family (Ahn et al., 2012). Typically, activation of the CB_1 receptor has been known to cause a G-protein mediated phosphorylation of JNK1/2 (Howlett 2005; Turu & Hunyady 2010). Treatment of CB_1-expressing cells with CP55,940 resulted in phosphorylation of JNK1/2, which was abrogated by co-treatment with ORG27569. This is consistent with the G-protein coupling inhibition demonstrated by ORG27569 (Price et al.,2005: Ahn et al., 2012; Baillie et al., 2013; Khurana et al., 2014). Also, ERK1/2 can be phosphorylated by CB_1 via G-protein dependent and independent pathways. Treatment of HEK293 cells expressing the CB_1 receptor with ORG27569 showed an increase in phosphorylation of ERK1/2. This increase in ERK1/2 phosphorylation was not abrogated by treatment of cells with pertussis-toxin (PTX) indicating that ERK1/2 phosphorylation by ORG27569 is not G-protein mediated. The role of other signaling proteins such as β-arrestins in the G-protein-independent ERK1/2 phosphorylation was evaluated via siRNA co-transfection (Ahn et al., 2013). It was demonstrated that β-arrestin 1 mediates ERK1/2 phosphorylation by ORG27569 but other results have also been reported (Gamadge et al., 2016). This was the first indication that allosteric modulators such as ORG27569 hold promise to develop subtype- and pathway-selective CB_1 therapeutics. ORG27569 demonstrates probe dependence in its impact on ERK1/2 phosphorylation since no significant effect was seen on WIN55,212-2 mediated ERK1/2 phosphorylation levels (Baillie et al., 2013). Using the mouse autaptic hippocampal culture model, Straiker and colleagues found that ORG27569 was also a negative allosteric modulator of 2-AG-mediated inhibition of glutamatergic neurotransmission (Straiker et al., 2015). Since ORG27569 may be a biased allosteric modulator (Ahn et al., 2013; Baillie et al., 2013; Khajehali et al., 2015), its analysis is especially challenging; competition due to affinity differences by a G-protein mediated orthosteric agonist and an arrestin–mediated allosteric modulator must be considered. Furthermore, binding of an allosteric modulator to a topological site that precludes G-protein coupling is also possible (Gentry et al., 2015; Stornaiuolo et al., 2015).

3.1.2 Structure-activity relationships of ORG27569—Several groups have investigated the key structural requirements of indole-2-carboxamides for allosteric modulation of CB_1 receptors. Critical structural factors include the following:

1. C3 alkyl chain length is critical. An n-propyl chain substitution enhances the allosteric modulation of orthosteric ligand binding, whereas an n-hexyl chain enhances the affinity of the allosteric modulator for CB_1 (Khurana et al., 2014). Substitution with short chain alkyl groups such as methyl improves allostery at CB_1 in calcium mobilization assays (Nguyen et al., 2015).

2. Length of the linker between the amide bond and the phenyl ring is critical. Only an ethylene substitution is tolerated, and any increase or decrease in linker length results in complete loss of allosteric activity (Mahmoud et al., 2013; Khurana et al., 2014).

3. Electron-withdrawing substituent at the C5 position of the indole ring is important. A fluoro substitution at the C5 position demonstrates improved allostery over a chloro substitution (Nguyen et al., 2015).

4. Changes in substituents on the phenyl ring influences both the affinity and efficacy of allosteric modulation with a *N,N*-dimethyl amino group demonstrating improved allostery relative to piperidinyl moiety (Mahmoud et al., 2013; Khurana et al., 2014).

3.2 PSNCBAM-1

PSNCBAM-1 is an allosteric modulator that is structurally distinct from ORG27569. The two compounds show similar profiles for their impact on CP55,940 binding and its G-protein coupling, i.e. increased binding of CP55,940, together with antagonism of CP55,940 induced G-protein coupling (Horswill et al, 2010; Khurana et al., 2017). Similar to ORG27569, PSNCBAM-1 demonstrates biased signaling via β-arrestin 1 as seen in ERK1/2 phosphorylation studies (Khurana et al., 2017). Like ORG25769, PSNCBAM-1 behaves as an inhibitor of 2-AG-mediated inhibition of synaptic transmission (Straiker et al., 2015). In a rat model, PSNCBAM-1 decreased both food intake and body weight gain (Horswill et al., 2010).

3.2.1 Structure-activity relationships of PSNCBAM-1—Structure-activity relationships (SAR) have been established for PSNCBAM-1, which may be modified to improve allostery. German and colleagues (German et al., 2014) utilized calcium mobilization assays whereas Kendall and group utilized radioligand binding assays to generate a SAR for PSNCBAM-1 (Khurana et al., 2017). Key findings of the SAR include (1) Non-cyclic substitutions such as a dimethylamino group at the 2-pyrrolidinylpyridine position of PSNCBAM-1 are more favored at this position (German et al., 2014), and (2) there needs to be an electron-withdrawing substituent at the 4-chorophenyl position with a cyano group being more potent than a chloro group (German et al., 2014; Khurana et al., 2017). However, other electron-withdrawing groups such as COOH, CF_3, acetyl or ethoxyacyl groups do not demonstrate high affinity or cooperativity in radioligand binding assays compared to the cyano group (Khurana et al., 2017). (3) The position of the electron-withdrawing group is critical. Change in the cyano group from *para* to *meta* position drastically reduced affinity and efficacy of the allosteric modulator (Khurana et al., 2017).

3.3 Pepcans

Pepcans are largely produced and released in the CNS by noradrenergic neurons (Hofer et al. 2015). Bauer and colleagues (Bauer et al., 2012), in their efforts to isolate CB_1 binding peptides from mouse brain and mouse and human plasma samples, identified pepcans that demonstrated negative allosteric modulation of the CB_1 receptor. A peptide Hpα (α-hemoglobin-derived peptide hemopressin) with the amino acid sequence PVNFKLSH was

previously isolated from rat brain membranes and found to exhibit inverse agonist effects at the CB_1 receptor (Heimann et al. 2007). Other peptides that were later identified were RVD-Hpα and VD-Hpα (Gomez et al., 2009). Bauer and colleagues generated monoclonal antibodies that identified the C-terminal regions of RVD-Hpα to isolate peptides that included N-terminally extended forms of RVD-Hpα that may bind the CB_1 receptor and designated them as Pepcans-12 to 23 depending on the peptide length. In radioligand displacement assays and dissociation kinetic assays, Pepcan-12 demonstrated negative allosteric modulation of CP55,940 and WIN55,212-2 as shown by partial displacement of the orthosteric ligands and an increase in the dissociation rate constants.

3.3.1 Impact on signaling pathways by Pepcan-12—The impact of Pepcan-12 on G-protein-mediated pathways was investigated by GTPγS assays and cAMP assays. Pepcan-12, by itself, did not impact the basal G-protein coupling efficacy in mouse brain membrane preparations. However, the peptide decreases HU-210-induced G-protein coupling efficacy (E_{max}) by 20% (Bauer et al., 2012). To investigate the impact of Pepcan-12 on endocannabinoid-induced G-protein coupling, the modulation of 2-AG mediated G-protein coupling by Pepcans was investigated. Pepcan-12 completely abrogated the G-protein coupling efficacy of 80 nM 2-AG (Bauer et al., 2012) and substantially attenuated 2-AG mediated inhibition of glutamate release from hippocampal neurons (Straiker et al., 2015), thus demonstrating the negative allosterism of endocannabinoid signaling exhibited by these naturally occurring peptides.

In assays examining G_s-mediated cAMP *accumulation* by CB_1 orthosteric ligands in a recombinant cell system, Pepcan-12 demonstrated concentration-dependent inhibition of cAMP accumulation efficacy by the synthetic agonist WIN55,212-2 and endocannabinoid 2-AG, thus further supporting that Pepcan-12 is a NAM of the CB_1 receptor for this signaling pathway as well (Bauer et al., 2012).

3.4 Lipoxin A4

Lipoxin A4 (LXA4) is an endogenously produced CB_1 receptor allosteric modulator. LXA4 demonstrated positive allosteric modulation of the CB_1 receptor both in vitro and in vivo (Pamplona et al., 2012). It has been previously shown that in mouse models, intracerebroventricular injections of LXA4 elicits cannabimimetic catalepsy, which is blocked by treatment with the CB_1 inverse agonist SR141716A (Pamplona at al., 2010). Displacement binding studies with SR141716A and cAMP accumulation assays were performed to evaluate direct binding of LXA4 to the CB_1 receptor, which demonstrated that the effects of LXA4 were not consistent with orthosteric agonist activation of CB_1 (Pamplona et al., 2012). To evaluate whether allosteric CB_1 activation might explain the cannabimimetic effects of LXA4, this compound and subeffective doses of two endocannabinoids, anandamide or 2-AG, were injected in mice. The results showed that anandamide-induced catalepsy was potentiated by LXA4. Decreasing the in vivo synthesis of LXA4 by injecting 5-lipoxygenase (LOX) inhibitors resulted in loss of anandamide activity in vivo. In binding assays, LXA4 potentiated the binding of CP55,940 and WIN55212-2 with a higher modulation of CP55,940 binding than WIN55212-2, thus demonstrating probe dependence (Pamplona et al., 2012).

3.4.1 Impact on signaling pathways by Lipoxin A4—Not only does LXA4 impact orthosteric ligand binding but it also positively modulates $G_{i/o}$ mediated inhibition of cAMP levels (Pamplona et al., 2012).LXA4 alone did not impact cAMP levels in concentrations between 0.1 nM and 1 μM. However, LXA4 positively modulates the potency of anandamide in decreasing the cAMP levels induced by forskolin. However, in another study, LXA4 failed to augment anandamide-induced suppression of cAMP levels (Khajeheli et al. 2015). 2-AG mediated inhibition of forskolin-induced cAMP was modestly attenuated by LXA4 (Pamplona et al., 2012), while LXA4 did not alter 2-AG mediated inhibition of glutamatergic synaptic transmission in cultured mouse hippocampal neurons (Straiker et al., 2015).

3.5 GAT211 and its enantiomers

GAT211 is an interesting racemic compound that has both direct agonist and positive allosteric properties. Examination of the two enantiomers of GAT211, GAT228 (R) and GAT229 (S), was able to assign allosteric agonism activity to GAT228 and PAM activity to GAT229 (Laprairie et al., 2017). GAT229 appears to be a PAM for a wide range of CB1 agonists, including anandamide, 2-AG, and CP55,940 (Laprairie et al., 2017).

3.6 Pregnenolone

Pregnenolone is best known as an intermediate in sterol synthesis (Miller & Auchus, 2011). However, a very interesting study found that THC increased pregnenolone levels, and that pregnenolone attenuated many of the actions of THC mediated by CB_1 cannabinoid receptors. Conversely, preventing pregnenolone synthesis increased the potency and/or efficacy of THC in a range of behavioral tests (Vallee et al., 2014).Pharmacological investigations were consistent with allosteric modulation (i.e., no effect on equilibrium binding of WIN55,212-2 or CP55,940) in a biased fashion (i.e., suppressed THC inhibition of mitochondrial respiration and activation of ERK1/2 phosphorylation, but did not affect THC-mediated inhibition of cAMP accumulation). A subsequent study (Khajehali et al., 2015), also using hCB_1-expressing CHO cells found that high concentrations of pregnenolone decreased equilibrium binding of SR141716A, but did not inhibit THC activation of ERK1/2 phosphorylation. Further investigations are needed to resolve this discrepancy.

3.7 Cannabidiol

Cannabidiol (CBD) is a cannabinoid compound present in varying quantities in cannabis preparations (ElSohly et al., 2016) and has attracted substantial recent interest as a possible therapeutic in some forms of pediatric epilepsy (Leo et al., 2016; Reddy 2017) as well as in schizophrenia (Leweke et al., 2012).In addition to its possible therapeutic use in pediatric epilepsy, CBD has long been studied as a modifier of THC actions (Niesink et al., 2013).The combination of CBD with THC was thoroughly explored during the development of nabiximols (Sativex), with the ~1:1 combination of CBD and THC found to be generally better tolerated and more efficacious than equivalent doses of THC alone (Wade et al., 2003; Russo et al., 2008; Johnson et al., 2010). Multiple mechanisms of action of CBD have been reported (see review: Campos et al., 2016), however recent studies suggest that CBD can act

as a negative allosteric modulator of CB_1 signaling (Laprairie et al., 2015).Negative allosterism of CB_1 was observed in multiple systems and ligands at CBD concentrations between 500 nM and 1000 nM. Of note, in pediatric epilepsy patients receiving 20 mg/kg of CBD daily, plasma concentrations reach ~1000 nM (Wong et al., 2016).

4. Biased signaling by CB_1 allosteric modulators

It is well appreciated that GPCR signaling is pluridimensional in nature (Fig. 2) and different ligands can evoke different receptor conformations that generate varied pharmacological responses (Luttrell et al., 2014). Some allosteric modulators such as ORG27569 and PSNCBAM-1 increase orthosteric agonist binding, yet decrease G-protein signaling by agonists. For example, ORG27569 is biased for β-arrestin-1 mediated pathways (Ahn et al., 2013). With the recent publication of crystal structures for inverse agonist-bound CB_1 (Hua et al., 2016; Shao et al., 2016), it will be intriguing to elucidate the differences in conformation exhibited by CB_1, when bound to ORG27569 and how this elicits biased signaling by this allosteric modulator.

As one way to address this question, Farrens and colleagues utilized site directed fluorescence labeling techniques to investigate changes in transmembrane helix (TM) movements upon binding of ORG27569 (Fay et al., 2015). They discovered that upon binding to the CB_1 receptor, the outward TM6 movements that are necessary to activate the receptor by exposing the binding site crevice for G-protein are blocked by ORG27569, thus explaining why ORG27569 inhibits G-protein coupling (Fay et al., 2012). Further, the group investigated movements in helix 8 (H8) upon ORG27569 binding. Biased signaling by β-arrestins has been proposed previously to involve movements in H8 to allow arrestin binding. Changes in fluorescence upon ORG27569 binding demonstrated increased H8 and TM7 movements, which are not observed with the orthosteric inverse agonist SR141716A. The authors, thus concluded that ORG27569 induces a conformation different from an inactive state conformation with blocked TM6 movements that explain the inhibition of G-protein coupling and high H8 and TM7 movements that may explain the biased β-arrestin signaling by ORG27569 (Fay et al., 2015). It will be interesting to determine if other β-arrestin-biased conformations including those induced by orthosteric ligands (Franks et al., 2014; Laprairie et al., 2016), result in a similar conformation.

5. Allosteric binding site(s) on the CB_1 receptor?

With the many allosteric modulators identified for the CB_1 receptor so far, one of the most important developments was the identification of the site where some of these allosteric modulators may bind. Identification of the allosteric binding site(s) allows an understanding of the mechanisms by which allosteric modulators such as ORG27569 display a contradictory profile i.e it increases CP55,940 binding while decreasing G-protein coupling associated with CP55,940 (Price et al., 2005; Ahn et al., 2012). Identifying the allosteric binding pockets will also allow structure-based drug design for modulators with improved allostery at the CB_1 receptor.

Different research groups have attempted to identify the putative allosteric binding sites of the CB_1 receptor. Such sites, however, do not appear to overlap. Fay and colleagues (Fay et al., 2012) investigated how different conformations of the receptor are induced by ORG27569, which could explain the molecular pharmacology of this agent, including receptor activation, enhancement of CP55,940 binding, together with inhibition of G-protein coupling. Site-specific fluorescent labeling of the receptor was done to identify conformational changes in CB_1 upon binding of ORG27569. Further investigations by this group identified a disulfide bond in the N-terminus of the receptor that plays an important role in the allosteric effects seen with ORG27569 and PSNCBAM-1. The disulfide bond was formed between Cys98 and Cys107. Interestingly, these authors found that reduction of this amino-terminal disulfide bond enhanced the cooperativity effects seen with ORG27569 and PSNCBAM-1. The authors suggested that allosteric modulators may bind to this region and alter the dissociation of the orthosteric ligand CP55,940, thus enhancing CP55,940 binding (Fay et al., 2013). However, the studies lacked direct evidence to show the impact of the disulfide bridge on binding affinities of the allosteric ligands, suggesting that the disulfide bond may play an indirect effect in the allostery at CB_1 receptor.

Shore and colleagues utilized molecular modeling, mutagenesis and G-protein coupling assays to investigate the allosteric binding pocket for ORG27569 (Shore et al., 2013). The approach for the identification of the allosteric site was based on the observation that ORG27569, in ligand displacement assays, decreases the equilibrium binding of SR141716A, an inverse agonist of CB_1 receptor. This may be because the receptor active state is enhanced and/or because ORG27569 may have an overlapping binding site with SR141716A. Computational modeling of ORG27569 on the CB_1 receptor was done with docking the ligand manually in the TMH3-6-7 region. Modeling data suggested that the binding site for ORG27569 overlaps with the binding of SR141716A. Residues $K192^{3.28}$, $F200^{3.36}$, $W279^{5.43}$ and $W356^{6.48}$, which have previously been reported to be important for SR141716A binding were mutated to alanine and tested for their impact on G-protein coupling efficacy of CP55,940 with ORG27569. Only the K192A mutant showed an increase in G-protein coupling in presence of the ORG27569 compared to CB_1 wild-type, suggesting this amino acid plays an important role in the binding of ORG27569. The computational modeling suggested that the piperidine nitrogen of ORG27569 is involved in forming hydrogen bonding with K192. This was the first attempt at identification of the allosteric site for the CB_1 receptor. However, since this residue is conserved between CB_1 and CB_2, this mutation does not describe why ORG27569 binds to CB_1 and not CB_2. The computational modeling also predicts F268 from the EC2 loop to form aromatic stacking interactions with the indole-ring of ORG27569. No mutagenesis studies were reported for this residue. Interestingly, other class A GPCRs have also demonstrated the role of extracellular loops in the binding of allosteric modulators. C_7/3-phth, for example, a negative allosteric modulator of the orthosteric antagonist *N*-methylscopolamine (NMS), for M2 muscarinic receptors exhibited cationic-Π interactions between the ammonium groups on the ligand and the aromatic residues in the extracellular ligand-binding vestibule, in molecular dynamic simulations. These simulations suggested two mechanisms for cooperativity between the orthosteric and allosteric ligands. First, electrostatic repulsions between the cationic orthosteric ligand NMS and C_7/3-phth decrease the binding affinity of

one in the presence of other. Second, the presence of one ligand affects the shape of the binding site of the other, thereby controlling the cooperativity effects. For example, the wide and bulky PAM alcuronium demonstrated that when bound to the receptor, it exhibits a conformation with wide open orthosteric and allosteric binding sites. However, the NAM C_7/3-phth demonstrates extensive binding contacts in a closed conformation, causing a closed conformation of the orthosteric site (Dror et al., 2013; Langmead et al., 2014).

Stornaiuolo and colleagues identified an entirely different site of binding for ORG27569 (Stornaiuolo et al., 2015). The group utilized computational modeling techniques using the sphingosine 1-phosphate receptor to build a homology model of the CB_1 receptor and to identify five different possible allosteric binding sites. Out of all the sites evaluated, the few residues that were different in CB_1 and CB_2 were mutated in CB_1 to the corresponding residues in CB_2 receptor. Fluorescence binding assays were done using a fluorescent tetra-methyl-rhodamine (TAMRA) labeled form of the CB_1 inverse agonist, AM251, namely T1117. It has been previously demonstrated by the same group (Bruno et al., 2014) that binding of T1117 to CB_1 wild-type is negatively impacted by ORG27569. Loss of T1117 binding would suggest that the mutations are not important for ORG27569 binding. If however, no change in T1117 binding is seen in the presence of ORG27569, the mutation is considered important for ORG27569 binding. Photoactivatable analogs of ORG27569 were also utilized to crosslink with CB_1 and residues important for binding were then evaluated based on mass spectrometry. The authors identified an intracellular binding site for ORG27569 (Stornaiuolo et al., 2015). This was in contrast to the previously identified more extracellular site(s) identified. Mutational analysis identified three mutations $C^{1.55}Y$, $H^{2.41}L$ and $F^{4.46}L$ (P2 in the intracellular regions of TM1, 2 and 4), that demonstrated no loss of T1117 binding upon incubation with ORG27569, suggesting these residues to be important for binding of ORG27569. The mass spectrometry data identified two serines, $S^{2.45}$ and $S^{3.42}$, to be important for the binding of ORG27569.

Thus, overall, different researchers have identified different sites of binding of ORG27569 and none agree with each other (Fay et al., 2012; Shore et al., 2013; Stornaiuolo et al., 2015) or perhaps there is more than one site. Also, no investigations into the binding site for PSNCBAM-1 have been reported.

Nonetheless, these studies lay the foundation for the identification of the putative allosteric binding site(s) on the CB_1 receptor. With the recently published crystal structures of the CB_1 receptor bound to inverse agonists (Hua et al., 2016; Shao et al., 2016), the community awaits for crystal structures of CB_1 bound to allosteric modulators and for advanced computational studies (e.g. molecular dynamics simulations) integrating crystal structure information with site-directed mutagenesis which may shed new light on the allosteric binding sites on the CB_1 receptor. Alternatively, photoactivatable analogues of indole-2-carboxamides such as ORG27569 with functionalities such as benzophenone, aliphatic or phenyl azide and phenyltrifluoromethyldiazrine have also been reported which may provide novel tools for mapping the allosteric binding site(s) on the CB_1 receptor (Qiao et al., 2016).

6. Therapeutic potential of allosteric modulators of the CB_1 receptor

It is becoming evident that CB_1 allosteric modulators may demonstrate therapeutic potential. PSNCBAM-1, for example, elicits acute hypophagic effects (Horswill et al., 2007) and antagonizes neuronal excitability (Wang et al., 2010), properties which may lead to treatments of obesity and some central nervous system (CNS) disorders. Pregnenolone has demonstrated promise in blocking the psychotic-like symptoms such as THC-impaired cognitive function (Busquets-Garcia et al., 2017).Lipoxin A4 has been shown to protect neuronal cells from β-amyloid-induced neurotoxicity (Pamplona et al., 2012), thus demonstrating possible therapeutic potential for the treatment of Alzheimer's disease. Moreover, the PAM ZCZ011, enhances agonist binding and attenuates neuropathic pain in a mouse model without cannabimimetic side effects (Ignatowska-Jankowska et al., 2015). Similarly, in a chemotherapy-induced painful neuropathy model, the PAM GAT211 produced CB_1-mediated analgesia, without tolerance or CB_1-mediated dependence, even with repeated dosing (Slivicki et al., 2016). The potential therapeutic impact of ORG27569 remains unsettled. ORG27569 reduced food consumption in both CB_1 (−/−) and CB_1 (+/+) mice, questioning the role of CB1 receptors in the hypophagic effects seen with ORG27569. Similarly, the cataleptic, antinociceptive and hypothermic effects associated with THC or CP55,940 were not altered by administration of ORG27569 which raises issues of translation of pharmacologic effects seen at a molecular level to whole animals (Gamage et al., 2014; Ding et al., 2014). On the other hand, ORG27569 resulted in dose-dependent decreases in both cue- and drug-induced reinstatement of cocaine- and methamphetamine-seeking behavior in rat models, thus demonstrating that functional antagonism of CB_1 receptors by ORG27569 may hold promise for developing effective therapeutics for drug addiction (Jing et al., 2014).

With increasing evidence of some allosteric modulators of CB_1 such as indole-2-carboxamides demonstrating biased signaling, manipulation of selective signaling pathways in a spatially and temporally restricted fashion can be achieved and this may be therapeutically beneficial. An example of this potential is shown by the angiotensin II receptor. This receptor may exhibit G-protein dependent or β-arrestin-dependent signaling. Biased agonism of this receptor to promote β-arrestin mediated effects may provide a beneficial cytoprotective response and obviate a deleterious increase in blood pressure that is observed when this receptor signals in a G-protein manner (Kenakin et al., 2010). This offers tremendous opportunities for developing precision drugs for many disorders that involve CB_1 receptors.

7. Future considerations

The identification of allosteric modulators of the CB_1 receptor (Fig. 3) has provided new opportunities to develop therapeutics that are subtype-specific and in some cases, pathway-specific. This allows an appreciation of the pluridimensional nature of GPCR signaling and how different ligands can induce different conformations and this differential signaling can be utilized for the treatment of human diseases. Further identification of the allosteric binding site(s) can be pursued as it will further help in the strategic design of small

molecules for lead identification and optimization, which is currently hindered by the paucity of reliable models of the CB_1 receptor.

Acknowledgments

This work was supported in part by NIH Grant DA039942 (to D.A.K.), DA021696 (to K.M.), DA012413 and DP1DA031387 (to D.P.).

Abbreviations

CB_1	cannabinoid receptor one
GPCR	G-protein coupled receptors
PAM	positive allosteric modulators
NAM	negative allosteric modulators
SAM	silent allosteric modulators
PTX	pertussis-toxin
2-AG	2-arachidonylglycerol
LXA4	lipoxin A4
LOX	5-lipoxygenase
TM	transmembrane helices
CNS	central nervous system
THC	Δ^9-tetrahydrocannabinol

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HIGHLIGHTS

- Modulation of the CB_1 receptor by allosteric modulators is an area of intense research.
- Receptor subtype and pathway specificity can be achieved by allosteric modulators of the CB_1 receptor.
- Different allosteric modulators and their impact on signaling pathways is revealed though complexities are apparent.
- Putative allosteric binding site(s) on the CB_1 receptor are examined.

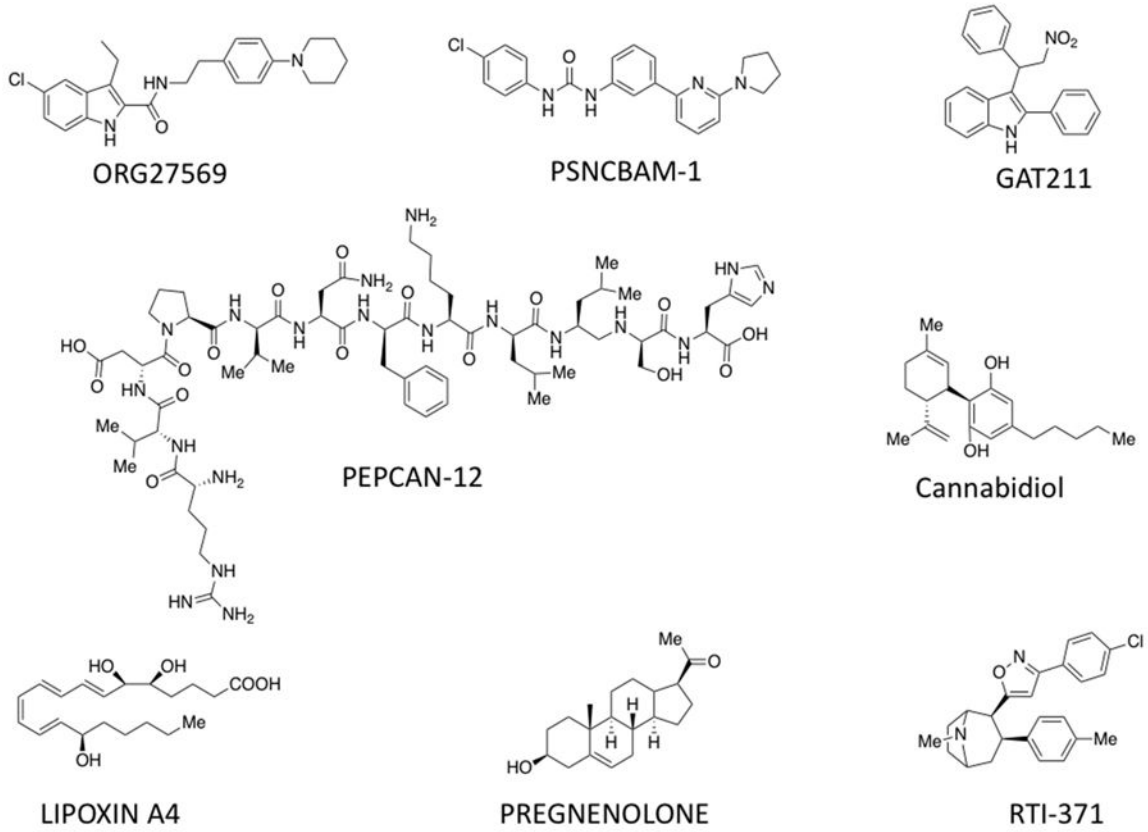

Fig. 1. Some well-studied allosteric modulators of the CB_1 receptor

The figure shows the chemical structure of ORG27569, PSNCBAM-1, GAT211, Pepcan-12, Cannabidiol, Lipoxin A4, Pregnenolone and RTI-371. These compounds have been reported to have allosteric modulation of the CB_1 receptor for different signaling pathways. The compounds pregnenolone, lipoxin A4 and pepcan-12 represent endogenous compounds; cannabidiol is naturally occurring and ORG27569, PSNCBAM-1, GAT211 and RTI-371 are synthesized compounds.

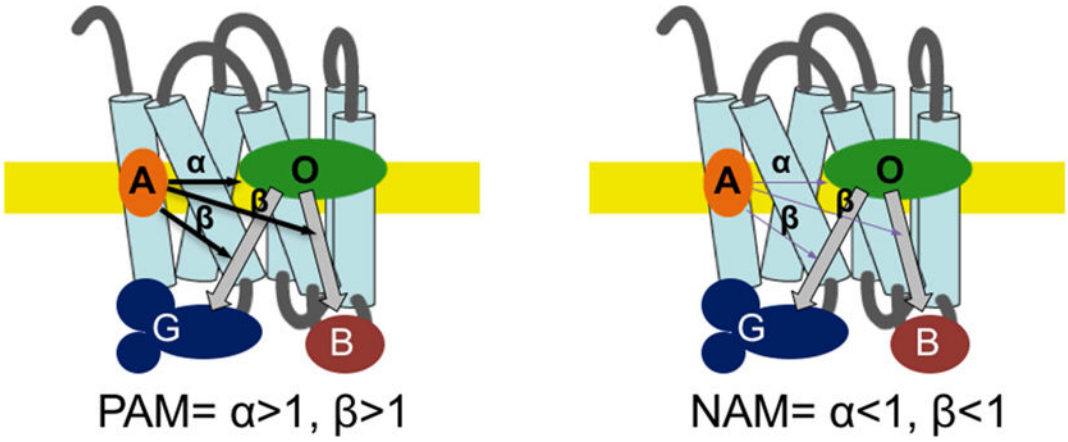

Fig. 2. Schematic depiction of allosteric modulation of a GPCR

Classically, an orthosteric agonist (O) binds to the GPCR and facilitates signaling through several G protein (only one shown for clarity) and non-G protein pathways (G and B, respectively). The relative strength of signaling often varies among signaling pathways (functional selectivity).An allosteric modulator (A) will bind to the receptor at a site independent from the orthosteric site. The allosteric modulator may positively (PAM) or negatively (NAM) influence orthosteric ligand binding (α) and/or signaling pathways (β), as depicted by thick and thin arrows and discussed in the text.

Compound	Agonist ?	PAM?	NAM?	In vivo	Comments
ORG27569	No	Yes	?	No	First characterized allosteric modulator
GAT211	Yes	Yes	No	Yes	Racemic mixture of GAT228 and GAT229
GAT228	Yes	No	No	?	R enantiomer of GAT211, positive allosteric modulator
GAT229	No	Yes	No	?	S enantiomer of GAT211, allosteric agonist
ZCZ011	Yes	Yes	No	Yes	Racemic mix
PSNCBAM-1	No	Yes	No	Yes	Potent
Lipoxin A4	No	Yes	No	Yes ($-CB_1$)	Low efficacy, generalizable?
Pepcan-12	No	No	Yes	?	Peptide, derived from α_1 subunit of haemoglobin family
Pregnenolone	No	No	Yes	Yes	Possibly specific for THC, generalizable?
Cannabidiol	No	No	Yes	Likely	CBD likely engages multiple targets

Fig. 3. Allosteric compounds for the CB_1 receptor and their classification as PAMs or NAMs or agonists based on activity

Since a compound can show positive allosterism for one pathway and negative or no allosteric activity for others, defining the status of a compound as a PAM or NAM or agonist is challenging. For example, ORG27569 demonstrated positive allosteric modulation for agonist CP55,940 binding and was internalized as expected for an activated receptor (Price et al., 2005; Ahn et al., 2012) and thus may be classified as a PAM. However, this is an inhibitor of G-protein coupling (Price et al., 2005; Mahmoud et al., 2013) and demonstrates negative modulation of CP55,940 induced calcium mobilization (Nguyen et al., 2015) and thus is also reported as a NAM. Similarly, PSNCBAM-1 demonstrated PAM activity for CP55,940 binding (Horswill et al., 2007) and CP55,940 induced ERK1/2 phosphorylation (Khurana et al., 2017). However, it demonstrates negative modulation of G-protein coupling (Horswill et al., 2007) and CP55,940 induced calcium mobilization (German et al., 2014) and thus acts as inhibitor for these signaling pathways, emphasizing that different signaling pathways can be modulated in opposite directions by an allosteric modulator. Thus, when speaking of an allosteric modulator it is helpful to specify the pathway(s) being discussed. The *In Vivo* column identifies if the allosteric modulator has in vivo activity consistent with allosteric modulation. The *Comments* column highlights particularly notable aspects of the allosteric modulator. See text for details. Abbreviations: AM, allosteric modulator.