Biased Agonism and Biased Allosteric Modulation at the CB₁ Cannabinoid Receptor

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Received March 24, 2015; accepted June 4, 2015

ABSTRACT

CB₁ cannabinoid receptors (CB₁Rs) are attractive therapeutic targets for numerous central nervous system disorders. However, clinical application of cannabinoid ligands has been hampered owing to their adverse on-target effects. Ligand-biased signaling from, and allosteric modulation of, CB₁Rs offer pharmacological approaches that may enable the development of improved CB₁R drugs, through modulation of only therapeutically desirable CB₁R signaling pathways. There is growing evidence that CB₁Rs are subject to ligand-biased signaling and allosterism. Therefore, in the present study, we quantified ligand-biased signaling and allosteric modulation at CB₁Rs. Cannabinoid agonists displayed distinct biased signaling profiles at CB₁Rs. For instance, whereas 2-arachidonylglycerol and WIN55,212-2 [(R⁻)⁻[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] showed little preference for inhibition of cAMP and phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2), [1H]SR141716A [5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide] exhibited minimal effects on binding of cannabinoid agonists. Furthermore, we highlight the need to validate the reported allosteric effects of the endogenous ligands lipoxin A₄ and pregnenolone at CB₁-Rs. Pregnenolone but not lipoxin A₄ displaced [1H]SR141716A, but there was no functional interaction between either of these ligands and cannabinoid agonists. This study demonstrates an approach to validating and quantifying ligand-biased signaling and allosteric modulation at CB₁Rs, revealing ligand-biased “fingerprints” that may ultimately allow the development of improved CB₁R-targeted therapies.

Introduction

Cannabinoid receptors (CBRs) are members of the G protein–coupled (GPCR) superfamily and mediate the actions of a number of endogenous ligands (endocannabinoids). CB₁Rs are the most abundant GPCRs in the brain (Devane et al., 1988; Herkenham, 1991; Glass et al., 1997), although they are also predominantly expressed in the periphery (Munro et al., 1993; Onaivi et al., 2006) but are also found in low levels in the brain (Onaivi et al., 2006; Atwood and Mackie, 2010). The principal endocannabinoids that facilitate these functions are the eicosanoids, N-arachidonylethanolamine (anandamide) and 2-arachidonylglycerol (2-AG). However, it has been hampered owing to their adverse on-target effects. Ligand-biased signaling from, and allosteric modulation of, CB₁Rs offer pharmacological approaches that may enable the development of improved CB₁R drugs, through modulation of only therapeutically desirable CB₁R signaling pathways. There is growing evidence that CB₁Rs are subject to ligand-biased signaling and allosterism. Therefore, in the present study, we quantified ligand-biased signaling and allosteric modulation at CB₁Rs. Cannabinoid agonists displayed distinct biased signaling profiles at CB₁Rs. For instance, whereas 2-arachidonylglycerol and WIN55,212-2 [(R⁻)⁻[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] showed little preference for inhibition of cAMP and phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2), [1H]SR141716A [5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide] exhibited minimal effects on binding of cannabinoid agonists. Furthermore, we highlight the need to validate the reported allosteric effects of the endogenous ligands lipoxin A₄ and pregnenolone at CB₁-Rs. Pregnenolone but not lipoxin A₄ displaced [1H]SR141716A, but there was no functional interaction between either of these ligands and cannabinoid agonists. This study demonstrates an approach to validating and quantifying ligand-biased signaling and allosteric modulation at CB₁Rs, revealing ligand-biased “fingerprints” that may ultimately allow the development of improved CB₁R-targeted therapies.

ABBREVIATIONS: 2-AG, 2-arachidonylglycerol; BSA, bovine serum albumin; CBR, cannabinoid receptor; CHO, Chinese hamster ovary; CP55940, 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GPCR, G protein–coupled receptor; HU-210, 11-hydroxy-Δ⁹-THC-dimethylheptyl; LY320135, 4-[6-[6-methoxy-2-(4-methoxyphenyl)-1-benzofuran-3-carbonyl]benzonitrile; Org27569, 5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-[4-piperidin-1-yl-phenyl]ethyl]amide; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PSNCBAM-1, N-[4-(chlorophenyl)-N'-[3-[6-[1-(pyrrolidin-1-yl)-2-pyridinyl]phenyl]urea]; SR141716A, 5-[(4-chlorophenyl)-1-([2,4-dichloro-phenyl]-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; WIN55,212-2, (R⁻)⁻[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; Δ⁹-THC, tetrahydrocannabinol.

This work was funded by a program grant of the National Health and Medical Research Council of Australia to P.M.S. and A.C. [ID 1055134]. A.C. and P.M.S. are Principal Research Fellows of the National Health and Medical Research Council of Australia [IDs 1041875 and 1050015, respectively]. dx.doi.org/10.1124/mol.115.099192. This article has supplemental material available at molpharm.aspetjournals.org.
been suggested that additional ligands act as endocannabinoids (Pertwee, 2005, 2010).

CBRs bind multiple endogenous ligands, but the functional significance of these interactions is not fully understood, although distinct physiologic roles for each endocannabinoid have been suggested. In fact, some endocannabinoids may mediate opposing effects, even when acting through the same receptor. For instance, elevated levels of 2-AG enhance CB1R-mediated alteration of long term potentiation, learning, and memory (Pan et al., 2011).

Besides the well known ∆9-tetrahydrocannabinol (∆9-THC, the main psychoactive component of marijuana), several exogenous cannabinoids (referred to herein as exocannabinoids) have been identified. These include the synthetic ∆9-THC analog HU-210 [11-hydroxy-∆9-THC-dimethylheptyl], the small-molecule agonist CP55940 [2-[(R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol] and WIN55,212-2 [(R)+-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-d:1,4-benzoxazin-6-yl]-1-naphthalenyl methanone], the CB1R-selective inverse agonist SR141716A [5-(4-chlorophenyl)-1,2,4-dichloro-phenyl]-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide] (rimonabant; reviewed in Pertwee, 2005, 2010), and the allosteric modulators Org27569 [5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl)-phenyl]amide] (Price et al., 2005) and PSCNBAM-1 [N(4-chlorophenyl)-N’-[3-(6-(1-pyridylidinyl)-2-pyridinyl)phenyl]urea] (Horswill et al., 2007). These latter compounds are particularly intriguing because they potentiate agonist binding to the CB1R, at the same time inhibiting agonist activity in numerous functional assays (Price et al., 2005; Horswill et al., 2007). Other endogenous ligands, including pregnenolone, pepcans, and lipoxin A4, may also act allosterically at CB1Rs, as was recently suggested (Bauer et al., 2012; Pamplona et al., 2012; Vallee et al., 2014). However, further studies are required to validate these putative allosteric effects.

Despite the variety of CB1R ligands, adverse on-target effects have hampered their use in the clinic to date. Thus, selective activation of receptor signaling events that mediate desired effects at the expense of those that cause adverse effects is an essential goal for CB1R-targeted therapeutics and may be achieved via the phenomenon of biased agonism. Biased agonism is driven through the propensity for different ligands to stabilize unique subsets of receptor states, with important pathways that mediate CB1R activity in the brain. For instance, whereas WIN55,212-2 activates all Gi subtypes (Gi1–3) via CB1Rs, (R)-methanandamide acts as an agonist only at Gi3 and shows inverse agonism at Gi1 and Gi2 (Mukhopadhyay and Howlett, 2005), demonstrating a complete reversal in efficacy. In addition, Org27569 allosterically enhances CP55940-mediated phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2), whereas it inhibits JNK phosphorylation in hippocampal neurons (Ahn et al., 2012), demonstrating a reversal in the magnitude and direction of the allosteric effect. Significantly, exocannabinoid-mediated biased agonism may be operative in vivo. For instance, whereas WIN55,212-2 is 10 times more potent than ∆9-THC in producing hypoactivity in mice, ∆9-THC is approximately 10 times more potent than WIN55,212-2 in producing hypothermia (Abood and Martin, 1992).

Although biased agonism and allosterism are operative at CB1Rs, no study has compared biased signaling by multiple endocannabinoids in the same cellular background. Furthermore, the effect of an allosteric modulator on endocannabinoid-mediated bias has not been investigated in detail. Thus, in the current study, we used analytical methods to systematically evaluate biased agonism and allosterism at CB1Rs, to ascertain potential biased cannabinoid “fingerprints” that may guide development of more successful CB1R-targeted therapies.

Materials and Methods

(+)-WIN55,212-2, CP55940, HU-210, methanandamide, anandamide, L1320135 [4-[6-methoxy-2-(4-methoxyphenyl)benzofuran-3-carbonyl]benzotriazole], 5(8),6R-lipoxin A4, and JZL 184 [4-[bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester] were obtained from Tocris Bioscience (Bristol, UK), and ∆9-THC from THC Pharm GmbH (Frankfurt, Germany). 2-AG, SR141716A, Org27569, pregnenolone, forskolin, and fatty acid–free bovine serum albumin (BSA) were purchased from Sigma-Aldrich Australia (Castle Hill, NSW, Australia). Hygromycin B was obtained from Roche (Mannheim, Germany). Lipofectamine 2000, fetal bovine serum (FBS), and cell culture media were all obtained from Invitrogen/Life Technologies (Mulgrave, VIC, Australia). The cAMP AlphaScreen kit and [3H]SR141716A (35–60 Ci/nmol) were obtained from PerkinElmer (Waltham, MA). SureFire ERK1/2 phosphorylation kits were a kind gift from Dr. Michael Crouch (TGR BioSciences, Thebarton, SA, Australia).

Rationale for Choice of Signaling Pathways and Ligands

Like many downstream signaling pathways, CB1R-mediated modulation of cAMP and stimulation of pERK1/2 may arise from activation of multiple effectors, including distinct G proteins, accessory proteins, and even transactivation events. Nonetheless, both pathways serve important CB1R-mediated physiologic roles. CB1R modulation of cAMP signaling has been linked to neuronal remodeling, which may facilitate some of the psychomotor and neurotoxic effects of cannabinoids (Zhao and Song, 2001). The extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway is involved in regulation of cannabinoid-mediated neuronal migration and differentiation (Rueda et al., 2002; Berghuis et al., 2005) and may play an important role in the development of tolerance and addiction to cannabinoids (Rubino et al., 2006). Furthermore, anandamide stimulation of pERK1/2 and cAMP response element–binding protein (pCREB) are key regulators of synaptic plasticity, learning, and memory (Basavarajappa et al., 2014). Thus, in the present study, we chose to investigate the effects of CB1R ligands in cAMP inhibition and pERK1/2 assays, as they represent two important pathways that mediate CB1R activity in the brain.

Cell Lines

Flp-In Chinese hamster ovary (CHO) cells stably expressing human CB1 cannabinoid receptors were generated according to the manufacturer’s instruction (Invitrogen). Briefly, CB1R in pE5-FRT/V5 recombinase at a ratio of 1:10 using Lipofermantine 2000. The transfected cells were selected with 700 µg/ml of hygromycin B. Following selection, cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 16 mM HEPES, and 700 µg/ml of hygromycin B.
Whole Cell Radioligand Binding Assays

CHO-hCB1 cells were seeded at a density of 50,000 cells per well in 96-well tissue culture–treated isoplates (PerkinElmer), and incubated overnight at 37°C, 5% CO2. The following day, media was removed and cells were washed twice with ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.5 mM KH2PO4). Cells were then incubated with ligands in DMEM containing 5% w/v fatty acid–free BSA in a final volume of 200 μl at 4°C. Nonspecific binding was determined using 10 μM LY320135. Total binding was defined in the absence of unlabeled ligand.

[3H]SR141716A association kinetic assays were first performed to determine the time taken to reach [3H]SR141716A equilibrium binding by incubating cells with an approximate Kd concentration (1 nM) of [3H]SR141716A in the absence or presence of 10 μM LY320135 for different time intervals (0–90 minutes and overnight) at 4°C. Homologous and heterologous competition experiments were next performed to determine the radioligand dissociation constant (Kd) and unlabeled competitor dissociation constants (Ki), respectively. Competition binding was performed by incubating cells with various concentrations of unlabeled cannabinoid ligands and 1 nM [3H]SR141716A at 4°C for 6 hours (on the basis of the equilibrium time determined in association binding assays). To ensure that the cannabinoid agonists were not degraded by monoacylglycerol lipase in our Flp-In CHO-CB1 cells, we first performed competition binding assays using 2-AG in the presence or absence of 100 nM JZL 184, a potent and selective monoacylglycerol lipase inhibitor. A 30-minute preincubation of cells with JZL 184 did not alter the pEC50 value of 2-AG (Supplemental Fig. 1A and Supplemental Table 1), suggesting that the endocannabinoids probably would not to be broken down in these assays; therefore, subsequent assays were performed in the absence of JZL 184.

For interaction studies between the unlabeled competitor and allosteric modulators, all ligands were added and incubated together. Assays were terminated by two rapid washes with ice-cold 0.9% NaCl to remove unbound ligand. After the final wash, 100 μl of OptiPhase scintillation fluid (PerkinElmer) was added to wells. Plates were shaken for 30 minutes and then radioactivity was measured for 1 min/well on a MicroBeta counter (PerkinElmer).

AlphaScreen cAMP Assays

CHO-hCB1 cells were seeded at 50,000 cells per well into 96-well clear-bottom culture plates and incubated overnight at 37°C, 5% CO2. The following day, cells were serum deprived for 1 hour, to minimize basal cAMP signaling, by replacing the growth media with serum-free DMEM/F12, containing 1 mM 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione-1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione and 0.5% w/v BSA. Cells were incubated with varying concentrations of cannabinoid compounds together with 1 μM forskolin for 30 minutes at 37°C in a final volume of 100 μl. Control cells were treated with only forskolin or vehicle.

Similar to findings in radioligand binding assays, there was no significant difference between the potency or efficacy of 2-AG in the presence or absence of JZL 184 in assays measuring inhibition of cAMP formation (Supplemental Fig. 1B).

For functional interaction studies with the allosteric modulators, cells were preincubated with varying concentrations of Org27569 for 10–15 minutes and with pregnenolone or lipoxin A4 for 10 minutes before the addition of different concentrations of orthosteric ligands. The assays were terminated by adding 50 μl of 100% ethanol and the cells were lysed using 0.1% BSA, 5 mM HEPES, and 0.3% Tween 20 in Milli-Q water. Intracellular cAMP levels were determined using an AlphaScreen cAMP kit (PerkinElmer). Plates were read on an EnVision plate reader (PerkinElmer) after a 2-hour incubation in the dark at 37°C using standard AlphaScreen settings.

Extracellular Signal-Regulated Kinase 1/2 Phosphorylation Assays

CHO-hCB1 cells were seeded at 50,000 cells/well into 96-well clear-bottom culture plates and grown overnight in complete medium. The following day, cells were washed twice with 100 μl of phosphate buffered saline and incubated in serum-free DMEM, supplemented by 16 mM HEPES, at 37°C for 5 hours to minimize FBS-stimulated pERK1/2 levels. Cells were incubated with varying concentrations of cannabinoid compounds at 37°C in a final volume of 200 μl. In all experiments, 10% FBS was used as a positive control, and vehicle controls were also included. The assays were terminated by addition of 100 μl SureFire lysis buffer at the time at which maximum pERK1/2 was stimulated, as determined in time-course assays (i.e., 2.5 minutes for anandamide and methanandamide and 5 minutes for all other cannabinoid ligands). For functional interaction studies with the allosteric modulators, cells were preincubated with varying concentrations of Org27569, pregnenolone, or lipoxin A4 for 10–15 minutes before the addition of different concentrations of orthosteric ligands.

Phosphorylation of ERK1/2 at Thr202/Tyr204 was determined using standard AlphaScreen settings.

Data Analysis

Nonlinear Regression. Data were analyzed using Prism 6 (GraphPad, San Diego, CA). For competition binding experiments with orthosteric ligands, a one-site binding equation (Motulsky and Christopoulos, 2004) was used to analyze specific binding of each ligand,

\[
Y = \frac{(Top - Bottom)}{1 + 10^{\frac{logEC50 - logIC50}{Top - Bottom}}} + Bottom
\]

where Y represents the percentage of specific binding; Top and Bottom denote the maximal and minimal asymptotes of the curve, respectively; \([I]\) is the concentration of inhibitor; and IC50 is the concentration of competitor that produces half the maximal response.

The equilibrium dissociation constant (Kd) of unlabeled ligand was calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973), where the radioligand Kd was constrained to that derived from homologous competition binding assays,

\[
K_d = \frac{IC50}{1 + \frac{IC50}{Kd}}
\]

Binding interaction experiments between [3H]SR141716A, cannabinoic ligands, and Org27569 were fitted to the following allosteric ternary complex model (Ehlert, 1988; Leach et al., 2010),

\[
Y = \frac{B_{max}[A]}{[A] + \frac{K_d}{\alpha[B] + K_d} + \frac{K_a}{\beta[B] + K_a} + \frac{K_{la}}{K_{la}[B]}}
\]

where Kd, Ka, and Kla denote the equilibrium dissociation constants of the radioligand, the orthosteric ligand, and the allosteric ligand, respectively; \([A], [B], \text{ and } [I]\) denote their respective concentrations; Bmax is the total number of receptors; and \(\alpha\) and \(\beta\) are the cooperativity factors between the allosteric ligand and radioligand or unlabeled ligand, respectively.

Studies of functional interaction between SR141716A and CP55940 in cAMP assays were fitted to the following equation (Motulsky and Christopoulos, 2004),

\[
Y = Bottom + \frac{(Top - Bottom)}{\left(1 + \frac{K^H}{\left[\frac{[B]}{10^{-pA2}}\right]^s}\right)^{nH}}
\]

where Top represents the maximal asymptote of the curves; Bottom represents the lowest asymptote (basal response) of the curves; logEC50 represents the logarithm of the agonist EC50 in the absence of antagonist,
The majority of radioligand binding studies at CB1Rs reported in the literature have been performed in membrane preparations from recombinant CB1R-expressing cells or murine brain extracts. Thus, to determine the equilibrium dissociation constant of cannabinoids under conditions that more closely resembled those used for functional signaling assays, we performed whole cell radioligand binding assays. However, in contrast to functional assays that were performed at 37°C, it was necessary to perform radioligand binding assays at 3°C.
at 4°C to ensure that receptors were not internalized upon agonist binding.

[0] association kinetic assays were first performed to determine the time at which radioligand equilibrium binding was reached, which was approximately 6 hours after incubation of cells with 1 nM [0]SR141716A (Supplemental Fig. 2). Therefore, all subsequent binding experiments were terminated after a 6-hour incubation with ligands.

Homologous competition binding experiments were next performed to determine the affinity of [0]SR141716A and the number of [0]SR141716A binding sites present, which was estimated at 800,098 ± 1868 sites per cell. Unlabeled SR141716A did not completely displace [0]SR141716A binding in whole cells (unlabeled SR141716A displaced approximately 70% of specific [0]SR141716A binding), which had previously been attributed to nonreceptor radioligand binding events that occur even in the presence of an unlabeled competitive ligand (Wennerberg et al., 2010). Nonetheless, the pKd calculated for [0]SR141716A in these assays (8.51 ± 0.35; Supplemental Fig. 3A) was in agreement with its reported pKd value determined previously both in membrane preparations (Govaerts et al., 2004) and in whole HEK293 CB1R cells (Wennerberg et al., 2011). To confirm this pKd, functional interaction studies between SR141716A and cannabinoid agonists in cAMP versus pERK1/2 assays may be indicative of ligand-biased signaling at CB1Rs. Therefore, to better visualize the preference of each cannabinoid to activate CB1R-mediated cAMP inhibition or pERK1/2, we constructed “bias plots” (Gregory et al., 2010; Kenakin et al., 2012), shown in Fig. 1, D and E. Bias plots represent the response of the receptor to equimolar concentrations of agonist in pERK1/2 (x-axis) versus inhibition of cAMP formation (y-axis). Curves that lie either side of the line of identity (shown by the dotted line and denoting equipotent and equiactive agonist concentrations in both assays) highlight preferential coupling to one pathway over the other. It is again apparent from these plots that 2-AG and WIN55,212-2 show little preference for coupling to either pathway, whereas methanandamide and HU-210, in particular, preferentially mediate inhibition of cAMP.

Although bias plots are useful for visualization purposes, they do not differentiate between true biased agonism and that imparted by “system bias” (e.g., the receptor coupling efficiency to different signaling proteins) or “observation bias” (e.g., assay conditions). Therefore, to quantify genuine biased agonism from the CB1R, we fitted agonist concentration-response data

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Radioligand Binding PKd</th>
<th>cAMP pEC50</th>
<th>E_max</th>
<th>pERK1/2 pEC50</th>
<th>E_max</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP55940</td>
<td>7.4 ± 0.2</td>
<td>8.1 ± 0.2</td>
<td>2.79</td>
<td>7.5 ± 0.1</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>HU-210</td>
<td>7.7 ± 0.2</td>
<td>9.0 ± 0.2</td>
<td>1.01</td>
<td>7.5 ± 0.1*</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>6.3 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>3.93</td>
<td>7.0 ± 0.2</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>2-AG</td>
<td>5.8 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>2.79</td>
<td>5.5 ± 0.2</td>
<td>41 ± 5</td>
</tr>
</tbody>
</table>

n = 4–6 (pKd); n = 6–8 (cAMP); n = 4–8 (pERK1/2).

*P < 0.05, two-way analysis of variance with Bonferroni’s multiple comparison test between changes in pEC50 values for cannabinoid ligands in cAMP versus pERK1/2 assays.

Percentage of maximum inhibition of forskolin response.

Percentage maximum FBS response.
to an operational model of agonism (Black and Leff, 1983) (eq. 5) to determine the transduction ratio, \( R = \frac{\tau}{K_A} \) (Kenakin et al., 2012), of each cannabinoid. Two approaches have been described to calculate the transduction ratio. The first relies on \( K_A \) values predetermined from radioligand binding assays (Rajagopal et al., 2011), and the second employs \( K_A \) estimates derived from the operational model of agonism (Kenakin et al., 2012). For a comparison of the two approaches, Tables 2 and 3 show analyses of the current data. To eliminate system or observation bias, we compared cannabinoid transduction ratios to that of 2-AG, in part because this endogenous CB1R agonist exhibited little bias toward cAMP or pERK1/2. Therefore, the transduction ratio of each agonist was normalized to that determined for the reference agonist 2-AG. The difference in the transduction ratios between 2-AG and each cannabinoid is shown in Tables 2 and 3 (\( \Delta \log R \)). The difference between the \( \Delta \log R \) values for each pathway (\( \Delta \Delta \log R \)) was then quantified to determine the strength of cannabinoid-mediated receptor coupling toward each pathway (Kenakin et al., 2012). The inverse Log of \( \Delta \Delta \log R \) represents the bias factor of each agonist (Tables 2 and 3). Bias factors in Tables 2 and 3 equal to 1 demonstrate that the cannabinoid promotes the same coupling preference as 2-AG. Bias factors greater than 1 indicate that the cannabinoid preferentially promotes receptor coupling toward cAMP inhibition over pERK1/2 relative to 2-AG. The bias factors again highlight that whereas WIN55,212-2 demonstrates a similar profile to 2-AG with a bias factor not dissimilar from 1, HU-210 and methanandamide exhibit strong bias toward cAMP inhibition. CP55940, \( \Delta^2 \)-THC, and anandamide also displayed a preference toward cAMP inhibition, although it did not reach statistical significance (Tables 2 and 3). 

**Org276529 Displays Probe-Dependent Allosteric Modulation at CB1Rs.** Previous studies have shown that Org27569 increases the binding of the CB1R agonist, \(^{[3]}H\)CP55940, and displays negative binding cooperativity with the CB1R inverse agonist \(^{[3]}H\)SR141716A (Price et al., 2005; Baillie et al., 2013). In binding assays, Org27569 had little effect on the CB1R were obtained by fitting the data to an operational model of agonism (eq. 5). Values represent the mean \( \pm \) S.E.M. from at least four independent experiments carried out in triplicate.

**TABLE 2**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( cAMP )</th>
<th>( pERK1/2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \log R )</td>
<td>( \Delta \log R )</td>
</tr>
<tr>
<td>CP55940</td>
<td>8.3 ( \pm ) 0.2</td>
<td>2.6 ( \pm ) 0.2</td>
</tr>
<tr>
<td>HU-210</td>
<td>8.8 ( \pm ) 0.2</td>
<td>3.1 ( \pm ) 0.2</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>6.9 ( \pm ) 0.2</td>
<td>1.2 ( \pm ) 0.2</td>
</tr>
<tr>
<td>( \Delta^2 )-THC</td>
<td>7.1 ( \pm ) 0.3</td>
<td>1.4 ( \pm ) 0.3</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>6.9 ( \pm ) 0.2</td>
<td>1.2 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Anandamide</td>
<td>6.3 ( \pm ) 0.2</td>
<td>0.6 ( \pm ) 0.2</td>
</tr>
<tr>
<td>2-AG</td>
<td>5.7 ( \pm ) 0.2</td>
<td>0.0 ( \pm ) 0.2</td>
</tr>
</tbody>
</table>

*\( ^* \) \( P < 0.05 \), one-way analysis of variance with Bonferroni’s multiple comparison test between \( \Delta \Delta \log R \) for 2-AG compared with other cannabinoids.

We first validated the effects of Org27569 on \(^{[3]}H\)SR141716A, and then on displacement of \(^{[3]}H\)SR141716A by cannabinoids in FlpIn CHO-hCB1 cells. As in previous findings (Price et al., 2005), Org27569 fully inhibited \(^{[3]}H\)SR141716A binding, indicating high negative allosteric cooperativity between these two ligands (Supplemental Fig. 3C). The displacement of \(^{[3]}H\)SR141716A binding was indistinguishable from a competitive interaction with the receptor, suggesting very high negative cooperativity between Org27569 and \(^{[3]}H\)SR141716A. Therefore, the \( P \) value of 5.8 \( \pm \) 0.1 for Org27569 was determined by fitting the displacement data to a one-site inhibition mass action equation (eq. 1).

To determine the effect of Org27569 on other cannabinoids, binding interaction studies were performed by measuring cannabinoid displacement of \(^{[3]}H\)SR141716A in the absence and presence of various Org27569 concentrations. Data from these experiments were fitted to an allosteric ternary complex model (eq. 3) to determine the cooperativity between Org27569 and the cannabinoids (Table 4). As in previous findings (Baillie et al., 2013), Org27569 had little effect on the binding of WIN55,212-2, indicated by little effect on WIN55,212-2–mediated displacement of \(^{[3]}H\)SR141716A (Fig. 2; Table 4). This indicates neutral cooperativity between Org27569 and WIN55,212-2. However, a similar observation was also made for all other cannabinoids (Fig. 2; Table 4), including CP55940. Therefore, in contrast to its strong negative cooperativity with \(^{[3]}H\)SR141716A, Org27569 displayed close to neutral cooperativity with all other cannabinoids, demonstrating probe dependence.

**Org276529 Displays Pathway-Dependent Allosteric Modulation at CB1Rs, Depending on the Probe.** Similar to binding studies, the allosteric activity of Org27569 on functional measures of CB1R activity has been shown previously to depend on the orthosteric probe used, such that it increases CP55940-induced pERK1/2, without affecting the WIN55,212-2–mediated response (Baillie et al., 2013). Org27569 has also previously been shown to display pathway-specific, or biased, allosteric modulation at CB1Rs (Ahn et al., 2012; Baillie et al., 2013). Therefore, to evaluate the allosteric effects of Org27569 on different CB1R-mediated signaling pathways, we extended previous studies by using endo- and exocannabinoid probes in functional interaction studies with Org27569.

**Org27569 completely abolished inhibition of cAMP formation stimulated by all tested cannabinoids (2-AG, anandamide,**

**Biased Signaling at CB1 Receptors 373**
mephamanhdamide, $\Delta^0$-THC, WIN55,212-2, CP55940, and HU-210; Figs. 3A and 4A). Interestingly, however, in pERK1/2 assays, whereas Org27569 abolished the response to HU-210 and CP55940, it had no significant effect on activation of pERK1/2 by anandamide, methanandamide, and $\Delta^9$-THC, and only partially inhibited 2-AG– and WIN55,212-2–induced pERK1/2 activation (Figs. 3B and 4B). Furthermore, in contrast to previous studies (Ahn et al., 2012; Baillie et al., 2013), Org27569 on its own did not affect pERK1/2 signaling (Supplemental Fig. 4). It is interesting to note that WIN55,212-2 and 2-AG displayed a similar pattern of modulation by Org27569, considering that they also showed a similar pattern of ligand biased-signaling. Our results demonstrate probe-dependent and biased allosteric effects of Org27569, whereby it negatively modulates cAMP inhibition by all cannabinoids, and some, but not all, cannabinoid-mediated pERK1/2 signaling.

Data were then fitted to an operational model of allostery (eq. 6) to determine the functional cooperativity between Org27569 and the cannabinoids (Table 5). The binding affinities of the orthosteric ligand and the modulator were fixed to the values determined in radioligand binding assays, and $\alpha$ was fixed to 1 to reflect the neutral binding cooperativity. This enabled determination of the functional cooperativity ($\beta$) between Org27569 and the cannabinoids (Table 5). The $\beta$ values close to 0 indicate very strong negative modulation of signaling efficacy between Org27569 and cannabinoid agonists, demonstrated by the large effect of Org27569 on the maximum signaling capacity ($E_{\text{max}}$) of the cannabinoids. The potency ($pEC_{50}$) of the cannabinoid agonists was unchanged in the absence and presence of Org27569.

**Pregnenolone, but Not Lipoxin A, Displays Weak Activity at CB$_2$Rs.** Pregnenolone was previously reported to act as an endogenous allosteric inhibitor at CB$_2$Rs because it reduced $\Delta^9$-THC–induced activation of pERK1/2 in CHO-hCB$_1$ cells (Vallee et al., 2014). Pregnenolone also inhibited hypovactivity, antinociception, hypothemia, catalepsy, food intake and memory impairment produced by $\Delta^9$-THC in mice, and blocked the effects of $\Delta^9$-THC on release of glutamate and dopamine. However, it had no effect on equilibrium binding of $[^3H]$CP55940 and $[^3H]$WIN55,212-2 (Vallee et al., 2014). Lipoxin A4 was also suggested as an endogenous allosteric potentiator at the CB$_2$R, where it enhanced anandamide, $[^3H]$CP55940 and $[^3H]$WIN55,212-2 binding, and potentiated anandamide-mediated cAMP inhibition (Pamplona et al., 2012).

Thus, to examine the potential allosteric activity of these ligands at CB$_2$Rs, we first investigated the effects of pregnenolone and lipoxin A4 on displacement of $[^3H]$SR141716A. Pregnenolone by itself caused a concentration-dependent decrease in $[^3H]$SR141716A binding. However, owing to the incomplete displacement of $[^3H]$SR141716A by pregnenolone at the maximum concentration that could be used in the assay, it was not determined whether pregnenolone acted in a competitive or allosteric manner. At the maximum concentration possible in the assay, lipoxin A4 did not alter the binding of $[^3H]$SR141716A (Fig. 5).

We next performed functional interaction studies between each putative modulator and the cannabinoids previously shown to be modulated by either pregnenolone or lipoxin A4. In contrast to studies reporting attenuation of $\Delta^9$-THC–induced activation of pERK1/2 by pregnenolone in CHO-hCB$_1$ cells (Vallee et al., 2014), and enhancement of anandamide-mediated inhibition of cAMP formation by lipoxin A4 in HEK-CB$_1$ cells (Pamplona et al., 2012), our results revealed a complete lack of modulation of CB$_2$R signaling by these compounds at concentrations up to 10 $\mu$M and 500 nM, respectively (Fig. 5). We also examined the actions of lipoxin

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**TABLE 3**
Biased agonism by cannabinoids ($K_i$ predetermined from radioligand binding assays)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>cAMP LogR</th>
<th>$\Delta$LogR</th>
<th>pERK1/2 LogR</th>
<th>$\Delta$LogR</th>
<th>cAMP/pERK1/2 BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP55940</td>
<td>8.3 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>7.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>0.6 ± 0.2 3.8</td>
</tr>
<tr>
<td>HU-210</td>
<td>8.8 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>7.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.3 ± 0.2 20.7</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>6.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>6.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.2 ± 0.2 1.6</td>
</tr>
<tr>
<td>$\Delta^9$THC</td>
<td>7.1 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.2 6.0</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>6.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>5.8 ± 0.1</td>
<td>-0.1 ± 0.1</td>
<td>1.3 ± 0.2 18.2</td>
</tr>
<tr>
<td>Anandamide</td>
<td>6.2 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>5.8 ± 0.1</td>
<td>-0.1 ± 0.1</td>
<td>0.8 ± 0.2 4.5</td>
</tr>
<tr>
<td>2-AG</td>
<td>5.7 ± 0.2</td>
<td>0.0 ± 0.2</td>
<td>5.9 ± 0.1</td>
<td>0.0 ± 0.1</td>
<td>0.0 ± 0.2 1.0</td>
</tr>
</tbody>
</table>

*S* < 0.05, one-way analysis of variance with Bonferroni’s multiple comparison test between $\Delta$LogR for 2-AG compared with other cannabinoids.

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**TABLE 4**
Binding parameters for the allosteric interaction between Org27569 and cannabinoid agonists determined in binding interaction experiments

Data were fitted to an allosteric ternary complex model (eq. 3) to determine the cooperativity between Org27569 and cannabinoid agonists (Log$\alpha$). The binding cooperativity between $[^3H]$SR141716A and Org27569 (Log$\beta$) was highly negative (−10) and could not be defined owing to complete inhibition of $[^3H]$SR141716A binding by Org27569. The negative logarithm of the radioligand dissociation constant was fixed to that determined from homologous competition binding experiments (p$K_i$, 8.5). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

| Ligand         | Log$\alpha$ | pK$\alpha$ | pK$\beta$ | Bmax |$
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>CP55940</td>
<td>0.5 ± 0.1</td>
<td>3.2</td>
<td>5.4 ± 0.2</td>
<td>391 ± 11.1</td>
</tr>
<tr>
<td>HU-210</td>
<td>0.0 ± 0.1</td>
<td>1.6</td>
<td>5.6 ± 0.1</td>
<td>393 ± 15.3</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>0.6 ± 0.1</td>
<td>5.4</td>
<td>5.2 ± 0.2</td>
<td>427 ± 16.8</td>
</tr>
<tr>
<td>$\Delta^9$THC</td>
<td>0.3 ± 0.2</td>
<td>2.0</td>
<td>6.7 ± 0.1</td>
<td>393 ± 16.2</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>0.3 ± 0.2</td>
<td>2.0</td>
<td>5.5 ± 0.2</td>
<td>405 ± 12.4</td>
</tr>
<tr>
<td>Anandamide</td>
<td>0.3 ± 0.2</td>
<td>2.0</td>
<td>5.0 ± 0.0</td>
<td>411 ± 8.02</td>
</tr>
<tr>
<td>2-AG</td>
<td>0.5 ± 0.2</td>
<td>2.2</td>
<td>5.0 ± 0.6</td>
<td>399 ± 8.88</td>
</tr>
</tbody>
</table>

$\alpha$ Logarithm of the binding cooperativity factor between Org27569 and cannabinoid agonists.

$\beta$ Negative logarithm of the allosteric modulator dissociation constant.

$B_{\text{max}}$ The maximum number of $[^3H]$SR141716A binding sites expressed as percentage of specific binding.
A4 and pregnenolone on activation of CB₁R signaling by other cannabinoid ligands. However, pregnenolone also did not inhibit pERK1/2 activation by WIN55,212-2, and lipoxin A4 did not alter cAMP inhibition by CP55940 (Supplemental Fig. 5). Therefore, the reported allosteric effects of pregnenolone and lipoxin A4 were not verified in the current study.

**Discussion**

This study quantified biased agonism, biased allosterism and probe-dependence by the small molecule Org27569 at CB₁Rs, and highlights the need to validate the allosteric nature of two previously reported endogenous CB₁R ligands, pregnenolone and lipoxin A4.

Ligand-biased signaling has particular significance where multiple endogenous ligands bind to the same receptor. This is because each ligand has the potential to mediate unique physiologic functions via stimulation of distinct intracellular signaling pathways. Accordingly, the first important finding of this study is the demonstration that the endocannabinoids, 2-AG and anandamide, display distinct biased agonism profiles at CB₁Rs. Whereas 2-AG shows little preference for inhibition of cAMP formation and activation of pERK1/2, anandamide is approximately seven times more biased toward cAMP inhibition. Methanandamide, the stable analog of anandamide, showed a similar biased profile to that of anandamide, and was over 15 times more biased toward inhibition of cAMP. 2-AG and anandamide are structurally related, indicating that subtle differences in endocannabinoid structure may influence biased agonism. Both CAMP and ERK pathways are the key regulators of synaptic plasticity, learning, and memory (Basavarajappa et al., 2014). Thus, the opposing effects of 2-AG and anandamide on learning and memory (Basavarajappa et al., 2014; Pan et al., 2011) may in part be attributable to their differential signaling at CB₁Rs. Furthermore, whereas CAMP inhibition has been linked to neurite remodeling (Zhou and Song, 2001), pERK1/2 signaling contributes to the regulation of neuronal migration and differentiation (Rueda et al., 2002; Berghuis et al., 2005;...
facilitated by cannabinoids. Signaling may contribute to further distinct in vivo effects on exocannabinoid-mediated signaling. Effects of Org27569 on CB1R-mediated cAMP and pERK1/2 (B) in CHO-hCB1 cells. Data points represent mean values ± S.E.M. from at least three experiments carried out in triplicate. Curves are the best fit of an operational model of allosterism (eq. 6) to the data.

Rubino et al., 2006). Thus, CB1R-mediated cAMP and pERK1/2 signaling may contribute to further distinct in vivo effects facilitated by cannabinoids.

We also showed that the exogenous cannabinoids displayed biased agonism at CB1Rs. As with 2-AG, WIN55,212-2 displayed little bias toward cAMP inhibition or pERK1/2, whereas CP55940, Δ9-THC, and in particular HU-210 favored inhibition of cAMP over activation of pERK1/2. It is interesting to note that CP55940 and HU-210 are both based on the structure of Δ9-THC, whereas WIN55,212-2 is structurally distinct, which hints at some degree of structure-activity relationship between the small-molecule cannabinoids and their biased profile. The findings of biased agonism at CB1Rs supports the notion that CB1R therapeutics could selectively drive CB1R signaling toward specific pathways, which may have important implications for the development of CB1R-targeted treatments for numerous disorders, including pain (Iversen and Chapman, 2002), multiple sclerosis (Pertwee, 2002), obesity (Horvath, 2003), nicotine addiction (Le Foll and Goldberg, 2005), and Parkinson's disease (Segovia et al., 2003).

Of note, we compared two related methods to calculate the biased agonism described above; one employs $K_A$ values predetermined in separate radioligand binding assays (Rajagopal et al., 2011), and another calculates $\tau$ and $K_A$ values from the same functional data set (Kenakin et al., 2012). Because it is assumed that biased agonism is "characterized by different affinities and/or different intrinsic efficacies for different receptor active states" (Kenakin et al., 2012), the latter method is advantageous (and in fact essential) if $K_A$ differs between pathways. However, this approach can only be used if the maximal system response can be defined, which is usually achieved in the presence of a full agonist. If only partial agonists are available, the only option is to fix the $K_A$ to that predetermined in radioligand binding assays. Our analysis shows that the bias factors calculated herein are almost identical when using either method. Therefore, in this instance, bias appears to arise from differences in cannabinoid efficacy, and not affinity, between pathways.

An alternative approach to gaining selectivity in the actions of therapies that target CB1Rs is through the use of allosteric modulators. Rather than directly mimicking or blocking the actions of endogenous agonists that bind to the orthosteric site, allosteric modulators can fine-tune pharmacological agonist responses by altering the binding and/or signaling properties of the orthosteric agonist (May et al., 2007). Further complexity may be added if the modulator exhibits allosteric properties of the orthosteric agonist (May et al., 2007).

![Image](https://example.com/image.png)

**Fig. 4.** Org27569 exhibits pathway- and probe-dependent allosteric effects on exocannabinoid-mediated signaling. Effects of Org27569 on CB1R-mediated inhibition of cAMP formation (A) and activation of pERK1/2 (B) in CHO-hCB1 cells. Data points represent mean values ± S.E.M. from at least three experiments carried out in triplicate. Curves are the best fit of an operational model of allosterism (eq. 6) to the data.

### TABLE 5

Operational model parameters for the functional interaction between Org27569 and cannabinoid agonists

$p_{C^A}$ (cannabinoid equilibrium dissociation constant) and $p_{K^A}$ (Org27569 equilibrium dissociation constant) were fixed to values determined in binding assays. Log $t$ (binding cooperativity) was fixed to 0 to reflect the near neutral cooperativity assumed that biased agonism is "characterized by different affinities and/or different intrinsic efficacies for different receptor active states" (Kenakin et al., 2012), the latter method is advantageous (and in fact essential) if $K_A$ differs between pathways. However, this approach can only be used if the maximal system response can be defined, which is usually achieved in the presence of a full agonist. If only partial agonists are available, the only option is to fix the $K_A$ to that predetermined in radioligand binding assays. Our analysis shows that the bias factors calculated herein are almost identical when using either method. Therefore, in this instance, bias appears to arise from differences in cannabinoid efficacy, and not affinity, between pathways.

An alternative approach to gaining selectivity in the actions of therapies that target CB1Rs is through the use of allosteric modulators. Rather than directly mimicking or blocking the actions of endogenous agonists that bind to the orthosteric site, allosteric modulators can fine-tune pharmacological agonist responses by altering the binding and/or signaling properties of the orthosteric agonist (May et al., 2007). Further complexity may be added if the modulator exhibits allosteric properties of the orthosteric agonist (May et al., 2007).

![Image](https://example.com/image.png)

**Fig. 4.** Org27569 exhibits pathway- and probe-dependent allosteric effects on exocannabinoid-mediated signaling. Effects of Org27569 on CB1R-mediated inhibition of cAMP formation (A) and activation of pERK1/2 (B) in CHO-hCB1 cells. Data points represent mean values ± S.E.M. from at least three experiments carried out in triplicate. Curves are the best fit of an operational model of allosterism (eq. 6) to the data.

#### TABLE 5

Operational model parameters for the functional interaction between Org27569 and cannabinoid agonists

- $p_{C^A}$ (cannabinoid equilibrium dissociation constant) and $p_{K^A}$ (Org27569 equilibrium dissociation constant) were fixed to values determined in binding assays. Log $t$ (binding cooperativity) was fixed to 0 to reflect the near neutral cooperativity between Org27569 and cannabinoid agonists. Log $\tau$ was fixed to 1000 to reflect the lack of Org27569 efficacy in these assays. Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$p_{ERK1/2}$ - $\log t$</th>
<th>$cAMP$ - $\log t$</th>
<th>$p_{ERK1/2}$ - $\log {\tau}_t$</th>
<th>$cAMP$ - $\log {\tau}_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP55940</td>
<td>$&gt;-10$ [10]</td>
<td>$-10$ ± 0.5 [0.1]</td>
<td>$0.3$ ± 0.1 [2.0]</td>
<td>$0.3$ ± 0.4 [2.0]</td>
</tr>
<tr>
<td>HU-210</td>
<td>$0.6$ ± 0.2 [0.3]</td>
<td>$-10$ [10]</td>
<td>$0.3$ ± 0.6 [2.0]</td>
<td>$0.7$ ± 0.8 [5.0]</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>$0.1$ ± 0.0 [0.8]</td>
<td>$-10$ [10]</td>
<td>$0.1$ ± 0.1 [1.3]</td>
<td>$0.1$ ± 0.8 [1.3]</td>
</tr>
<tr>
<td>Δ9-THC</td>
<td>$0.1$ ± 0.1 [0.8]</td>
<td>$-10$ [10]</td>
<td>$0.8$ ± 0.0 [6.3]</td>
<td>$0.4$ ± 0.2 [2.5]</td>
</tr>
<tr>
<td>Methanandamide</td>
<td>$0.1$ ± 0.1 [0.8]</td>
<td>$-10$ [10]</td>
<td>$0.8$ ± 0.1 [6.3]</td>
<td>$0.9$ ± 0.3 [7.9]</td>
</tr>
<tr>
<td>Anandamide</td>
<td>$0.1$ ± 0.1 [0.8]</td>
<td>$-10$ [10]</td>
<td>$0.8$ ± 0.1 [6.3]</td>
<td>$0.9$ ± 0.3 [7.9]</td>
</tr>
</tbody>
</table>

Logarithm of the activation cooperativity factor between Org27569 and cannabinoid agonists.

Logarithm of the functional efficacy.

Log was allowed to float in the analysis, but could not be determined owing to the very high negative cooperativity.
probe- and/or pathway-dependent allosteric modulation. Indeed, Org27569 was previously shown to increase the binding of the CB₁R agonist [³H]CP55940 at the same time having negative binding cooperativity with the inverse agonist [³H]SR141716A (Price et al., 2005). In contrast to the positive modulation of agonist binding, Org27569 inhibited agonist signaling in cAMP, guanosine 5'-O-[³-thio]triphosphate, and reporter gene assays (Price et al., 2005). However, it demonstrated agonist activity in pERK1/2 assays (Ahn et al., 2012; Baillie et al., 2013) and exhibited weak potentiation of cannabinoid-mediated pERK1/2 (Baillie et al., 2013). In line with previous findings, the current study demonstrated that Org27569 reduced [³H]SR141716A binding. However, it had little effect on [³H]SR141716A displacement by CP55940 or other cannabinoid agonists. This may be explained by the use of whole cells in the present study versus mouse brain membrane preparations used in previous studies (Price et al., 2005; Baillie et al., 2013). Different receptor populations may be present in whole cells versus membranes (e.g., different proportions of receptor–G protein complexes), reducing the positive cooperativity between Org27569 and CP55940. In addition, the current study examined [³H]SR141716A displacement by cannabinoid agonists in the presence of Org27569, whereas in previous studies the direct effect of Org27569 on binding of tritiated agonists ([³H]CP55940 or [³H]WIN55,212-2) was investigated. Therefore, the presence of the inverse agonist in our study may change the equilibrium of high and low affinity binding sites, influencing the activity of Org27569. Overall, our results suggest that the modulatory effect of Org27569 may in part be dependent on the population of receptor conformations present.

The probe-dependent effects of Org27569 extend to functional assays, demonstrated previously by its ability to modulate CP55940-induced activation of pERK1/2 and inhibition of cAMP formation, at the same time having weak or no effect on responses to WIN55,212-2 (Baillie et al., 2013). We further investigated this by screening a wider range of cannabinoid ligands. Our results clearly show that Org27569 displays probe-dependence by fully inhibiting pERK1/2 activation by HU-210 and CP55940, although only partially inhibiting 2-AG and WIN55,212-2 responses and having no significant effects on pERK1/2 activation by anandamide, methanandamide, and Δ⁶-THC. Thus, probe-dependence provides the opportunity to target selective signaling pathways using distinct combinations of allosteric and orthosteric ligands.

Previous studies indicated that whereas Org27569 inhibited CP55490-induced inhibition of cAMP production, it weakly potentiated CP55940-stimulated pERK1/2 (Baillie et al., 2013). Org27569 was also an allosteric agonist in pERK1/2 assays (Ahn et al., 2012; Baillie et al., 2013). Our finding that Org27569 negatively modulates cannabinoid-induced pERK1/2 signaling or has no effect is in contrast to previous investigations. However, both G₁-dependent and independent mechanisms mediate CB₁R activation of pERK1/2 signaling (Bouaboula et al., 1995; Sanchez et al., 1998, 2001; Derkinderen et al., 2003; Ahn et al., 2012; Baillie et al., 2013) and the cell background and subsequent complement of intracellular signaling proteins may therefore greatly influence the ability of Org27569 to modulate CB₁R signaling. Perceived biased agonism may also be dependent on receptor expression levels, with high expressing systems having a greater tendency to couple to pathways that are undetectable in lower expressing systems. Alternatively, these differences may reflect distinct pERK1/2 pools (e.g., nuclear versus cytosolic) captured in the different studies, which may be differentially modulated by Org27569. Furthermore, cannabinoid-mediated pERK1/2 is transient in nature, which in the present study peaked at 2.5–5 minutes and subsequently returned back to baseline levels (Supplemental Fig. 4). Although the peak pERK1/2 response was used to determine the signaling of cannabinoids in concentration-response studies, the time at which the response is measured may influence perceived bias.

The allosteric activity of Org27569 is also dependent on the signaling pathway studied, such that it displays pathway-specific, or biased, allosteric modulation at CB₁Rs. Whereas Org27569 had partial inhibitory or no effect on pERK1/2 signaling by some of the cannabinoids tested, it completely abolished inhibition of cAMP formation by every agonist. This may in part highlight pathway-specific modulation of CB₁R signaling, or biased allosterism. Previous studies reported biased allosteric effects of Org27569, as it antagonized inhibition of cAMP formation, stimulation of guanosine 5'-O-[³-thio]triphosphate formation, and activation by HU-210 and CP55940, although only partially inhibiting 2-AG and WIN55,212-2 responses and having no significant effects on pERK1/2 activation by anandamide, methanandamide, and Δ⁶-THC. Thus, probe-dependence provides the opportunity to target selective signaling pathways using distinct combinations of allosteric and orthosteric ligands.
binding, and JNK phosphorylation by CP55490, at the same time potentiating cannabinoid-induced activation of pERK1/2 (Ahn et al., 2012; Bailie et al., 2013). However, the allosteric effects of Org27569 are somewhat time-dependent. Thus, whereas Org27569 does not appear to modulate the CB1R upon immediate exposure, more prolonged contact with the receptor results in enhanced receptor desensitization and a subsequent reduction in cell signaling events (Cawston et al., 2013). Thus, differences in time points used to study the activity of Org27569 may influence the extent of the observed Org27579 allosteric effects.

In addition to the small-molecule allosteric modulators such as Org27579, lipoxin A4 and pregnenolone have more recently been proposed to act as endogenous allosteric ligands at CB1Rs. Thus, we evaluated their allosteric nature. Previous binding interaction studies demonstrated no effect of pregnenolone on equilibrium binding of \(^{3}H\)CP55940 and \(^{3}H\)WIN55,212-2 (Vallee et al., 2014). However, our results demonstrate displacement of \(^{3}H\)SR141716A by pregnenolone, suggesting an interaction between these two ligands at CB1Rs. In contrast to previous findings (Vallee et al., 2014), we saw no inhibitory effect of pregnenolone on either \(\Delta^{2}\)-THC– or WIN55,212-2–induced activation of pERK1/2. This suggests that either pregnenolone has probe- or pathway-dependent allosteric effects or that it displaces \(^{3}H\)SR141716A in a competitive or even in a CB1R-independent manner, such as disruption of the cell membrane. Indeed, lipids are now emerging as important putative allosteric modulators of GPCRs (van der Westhuizen et al., 2015). Although lipids can directly interact with GPCRs, they may also alter GPCR activity by influencing the physical properties of the membrane in which they sit, or by promoting compartmentalization of receptor signaling by contributing to cellular components such as caveolae and lipid rafts (van der Westhuizen et al., 2015).

Also, in contrast to previous studies, where lipoxin A4 partially inhibited \(^{3}H\)SR141716A binding and enhanced \(^{3}H\)SR141716A displacement by anandamide (Pampolina et al., 2012), it had no effect in our binding studies. Furthermore, our results demonstrated no enhancing effects of lipoxin A4 on anandamide- or CP55940-mediated inhibition of cAMP. Therefore, the potential allosteric effects of pregnenolone and lipoxin A4 warrant further investigation.

Selectively targeting specific CB1R-coupled pathways to the exclusion of others with biased agonists or allosteric modulators is a potential means to gain therapeutic advantages for the treatment of a number of conditions. Although Org27569 is a biased allosteric modulator at CB1Rs, its in vitro effects do not necessarily translate into in vivo effects. Whereas in mice Org27569 reduced food intake, it did so independently of CB1Rs (Gamage et al., 2014). Furthermore, it did not modulate anandamide, CP55940, or \(\Delta^{2}\)-THC–induced analgesia, catalepsy, or hypothermia. In rats, however, Org27569 inhibited hypothermia produced by CP55940 and anandamide, at the same time having no effect on CP55940-induced catalepsy and antinociception. Org27569 also decreased food intake in rats. However, the involvement of CB1Rs in these effects is unclear (Ding et al., 2014). This suggests that in vitro drug activity at CB1Rs must be further rigorously validated in relevant cell lines, tissues, and whole animals to assess the correlation between observed cell-based pharmacology and subsequent in vivo effects and to determine desirable pharmacological profiles of allosteric/orthosteric combinations. To this end, the present study demonstrates an approach that can quantitatively evaluate signaling bias and allosteric modulation at CB1Rs. This serves as an initial step in determining ligand-biased “fingerprints” that may be useful when assessing the correlation between in vitro and in vivo CB1R pharmacology.

Acknowledgments

The authors thank Dr. Michael Crouch for donating the SureFire pERK1/2 assay kits used in this study.

Authorship Contributions

Participated in research design: Khajehali, Christopoulos, Leach.
Conducted experiments: Khajehali.
Performed data analysis: Khajehali, Leach.
Wrote or contributed to the writing of the manuscript: Khajehali, Malone, Glass, Sexton, Christopoulos, Leach.

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Biased Signaling at CB1 Receptors


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