

Biased Agonism and Biased Allosteric Modulation at the CB₁ Cannabinoid Receptor[§]

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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-arachidonoylglycerol 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), *N*-arachidonylethanolamine (anandamide), methanandamide, CP55940 [2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol], and HU-210

[11-hydroxy- Δ^8 -THC-dimethylheptyl] were biased toward cAMP inhibition. The small-molecule allosteric modulator Org27569 [5-chloro-3-ethyl-1*H*-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)ethyl]amide] displayed biased allosteric effects by blocking cAMP inhibition mediated by all cannabinoid ligands tested, at the same time having little or no effect on ERK1/2 phosphorylation mediated by a subset of these ligands. Org27569 also displayed negative binding cooperativity with [³H]SR141716A [5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide]; however, it had 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 CB₁Rs. Pregnenolone but not lipoxin A4 displaced [³H]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

found at lower levels in the testes, spleen, and immune cells (Gerard et al., 1991; Kaminski et al., 1992). CB₂Rs are 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).

CBRs have diverse roles, including inhibition of neurotransmitter release (Hashimoto et al., 2007); regulation of energy balance, metabolism, and cardiovascular function (Cota, 2007); bone formation (Bab and Zimmer, 2008); and immune cell responses (Massi et al., 2000; Sacerdote et al., 2000). The principal endocannabinoids that facilitate these functions are the eicosanoids, *N*-arachidonylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG). However, it has

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ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; BSA, bovine serum albumin; CBR, cannabinoid receptor; CHO, Chinese hamster ovary; CP55940, 2-[(1*R*,2*R*,5*R*)-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- Δ^8 -THC-dimethylheptyl; JZL 184, 4-[bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester; LY320135, 4-[6-methoxy-2-(4-methoxyphenyl)-1-benzofuran-3-carbonyl]benzotriazole; Org27569, 5-chloro-3-ethyl-1*H*-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-pyrrolidinyl)-2-pyridinyl]phenyl]urea; SR141716A, 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-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; Δ^9 -THC, tetrahydrocannabinol.

been suggested that additional ligands act as endocannabinoids (Pertwee, 2005, 2010).

CB₁R 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 anandamide levels result in diminished inhibition of CB₁R-mediated long term potentiation and subsequent impairments in learning and memory (Basavarajappa et al., 2014). In contrast, elevated levels of 2-AG enhance CB₁R-mediated alteration of long term potentiation, learning, and memory (Pan et al., 2011).

Besides the well known Δ^9 -tetrahydrocannabinol (Δ^9 -THC; the main psychoactive component of marijuana), several exogenous cannabinoids (referred to herein as exocannabinoids) have been identified. These include the synthetic Δ^9 -THC analog HU-210 [11-hydroxy- Δ^8 -THC-dimethylheptyl], the small-molecule agonists CP55940 [2-[(1*R*,2*R*,5*R*)-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-*de*]-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)-1*H*-pyrazole-3-carboxamide] (rimonabant; reviewed in Pertwee, 2005, 2010), and the allosteric modulators Org27569 [5-chloro-3-ethyl-1*H*-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)ethyl]amide] (Price et al., 2005) and PSNCBAM-1 [*N*-(4-chlorophenyl)-*N'*-[3-[6-(1-pyrrolidinyl)-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 each state being able to couple to its own preferred intracellular signaling responses (Kenakin and Christopoulos, 2013). Both orthosteric and allosteric ligands have the propensity to impose biased signaling at GPCRs. In the case of allosteric ligands, they may be biased allosteric agonists, or they may engender biased agonism on the orthosteric agonist(s) when both ligands occupy a GPCR simultaneously (Leach et al., 2007, 2015; Kenakin and Christopoulos, 2013).

Importantly, reversals in cannabinoid efficacy or potency, indicative of biased agonism, have been observed at CB₁Rs. For instance, whereas WIN55,212-2 activates all G_i subtypes (G_{i1-3}) via CB₁Rs, (*R*)-methanandamide acts as an agonist only at G_{i3} and shows inverse agonism at G_{i1} and G_{i2} (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 Δ^9 -THC in producing hypoactivity in mice, Δ^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, LY320135 [4-[6-methoxy-2-(4-methoxyphenyl)1-benzofuran-3-carbonyl]benzotrile], 5(*S*),6(*R*)-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 Δ^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/mmol) 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 psychoactive and neurotoxic effects of cannabinoids (Zhou 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

F1p-In Chinese hamster ovary (CHO) cells stably expressing human CB₁ cannabinoid receptors were generated according to the manufacturer's instruction (Invitrogen). Briefly, CB₁R in pE15-frt-V5 was cotransfected with the pOG44 plasmid (encoding the F1p-In recombinase) at a ratio of 1:10 using Lipofectamine 2000. The transfected cells were selected with 700 μ g/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 μ g/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 *K_d* concentration (1 nM) of [³H]SR141716A in the absence or presence of 10 μM LY320135 for different time intervals (0–360 minutes and overnight) at 4°C.

Homologous and heterologous competition experiments were next performed to determine the radioligand dissociation constant (*K_d*) and unlabeled competitor dissociation constants (*K_i*), 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 p*K_i* value of 2-AG (Supplemental Fig. 1A and Supplemental Table 1), suggesting that the endocannabinoids probably would not 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 Supermix 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 measured using AlphaScreen SureFire (PerkinElmer) as described previously (Nawaratne et al., 2008). Plates were read on an EnVision plate reader (PerkinElmer) after a 1.5-hour incubation at 37°C in the dark 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,

$$Y = \frac{(Top - Bottom)}{1 + 10^{(\log[I] - \log IC_{50})}} + Bottom \quad (1)$$

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 *IC*₅₀ is the concentration of competitor that produces half the maximal response.

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

$$K_i = \frac{IC_{50}}{1 + \frac{[Radioligand]}{K_d}} \quad (2)$$

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

$$Y = \frac{B_{max}[A]}{[A] + \left[\frac{K_A K_B}{\alpha [B] + K_B} \right] \left[1 + \frac{[I]}{K_I} + \frac{[B]}{K_B} + \frac{\alpha [I][B]}{K_I K_B} \right]} \quad (3)$$

where *K_A*, *K_B*, and *K_I* denote the equilibrium dissociation constants of the radioligand, the orthosteric ligand, and the allosteric ligand, respectively; [*A*], [*B*], and [*I*] denote their respective concentrations; *B*_{max} is the total number of receptors; and α' and α 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),

$$Y = Bottom + \frac{(Top - Bottom)}{1 + \left(\frac{10^{\log EC_{50}} \left[1 + \left(\frac{[B]}{10^{-pA_2}} \right)^{\alpha} \right]^{\frac{1}{n_H}}}{[A]} \right)} \quad (4)$$

where *Top* represents the maximal asymptote of the curves; *Bottom* represents the lowest asymptote (basal response) of the curves; log*EC*₅₀ represents the logarithm of the agonist *EC*₅₀ in the absence of antagonist,

[A] represents the concentration of the agonist, CP55940; [B] represents the concentration of the antagonist, SR141716A; n_H represents the Hill slope of the agonist curve; S represents the Schild slope for the antagonist; and pA_2 represents the negative logarithm of the concentration of antagonist that shifts the agonist EC_{50} by a factor of 2. When fitting data to this equation, the estimated Schild slope was not significantly different from unity, therefore it was constrained as such and the estimate of pA_2 represents the antagonist equilibrium dissociation constant.

Concentration-response data generated from cAMP and pERK1/2 assays were fitted to a three-parameter concentration-response equation or to the following operational model of agonism (Black and Leff, 1983; van der Westhuizen et al., 2014),

$$E = \frac{(E_{\max} - Basal)}{1 + \left(\frac{\left(\frac{[A]}{10^{\log K_A}} + 1 \right)}{10^{\log R} \times [A]} \right)} + Basal \quad (5)$$

where E_{\max} is the maximal possible system response (the top plateau of the dose-response curve obtained for the full agonist, CP55940), $Basal$ is the response in the absence of agonist, K_A denotes the equilibrium dissociation constant of the agonist (A), which was fixed to that determined in radioligand binding assays, [A] is the concentration of agonist, and $\log R$ is the logarithm of the transduction ratio, which is an index of the coupling efficacy of the agonist and is defined by τ/K_A (Kenakin et al., 2012). τ incorporates the intrinsic efficacy of the ligand, the total density of receptors, and the efficiency of stimulus-response coupling.

To define allosteric effects on intrinsic efficacy of orthosteric ligands (β), data from functional interaction experiments were fitted to the following operational model of allosterism (Leach et al., 2007),

$$Effect = \frac{E_{\max}(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{(K_B[A] + K_A K_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \quad (6)$$

where, τ_A and τ_B denote the efficacy of orthosteric and allosteric ligands, respectively; α and β denote allosteric effects on orthosteric ligand binding affinity and efficacy, respectively. K_A and K_B are the equilibrium dissociation constant of orthosteric and allosteric ligands, respectively; and [A] and [B] denote their respective concentrations. E_{\max} is the maximal possible system response, and n is the slope factor of the transducer function.

Bias Plots. For the generation of “bias plots” shown in Fig. 1, D and E, the mean \pm S.E.M. response of the receptor to equimolar concentrations of agonist in pERK1/2 (x -axis) versus inhibition of cAMP formation (y -axis) were plotted against one another. Nonlinear regression curves that defined the cannabinoid agonist concentration-response in pERK1/2 and cAMP assays, analyzed using a three-parameter Hill equation, were plotted against one another, with pERK1/2 on the x -axis and cAMP on the y -axis. A line of identity was generated that denotes concentrations of agonist that are equipotent and equiactive in both assays.

Statistics

Values are expressed as means \pm S.E.M. Mean values have been compared using one-way analysis of variance with Bonferroni's multiple comparison test. A P value < 0.05 was considered significant.

Results

Endogenous and Exogenous Cannabinoids Engender Ligand-Biased Signaling at the CB₁R. First, we determined the binding affinity (pK_i), functional potency (pEC_{50}), and maximal agonist effect (E_{\max}) of endogenous and exogenous cannabinoids using whole cell radioligand binding,

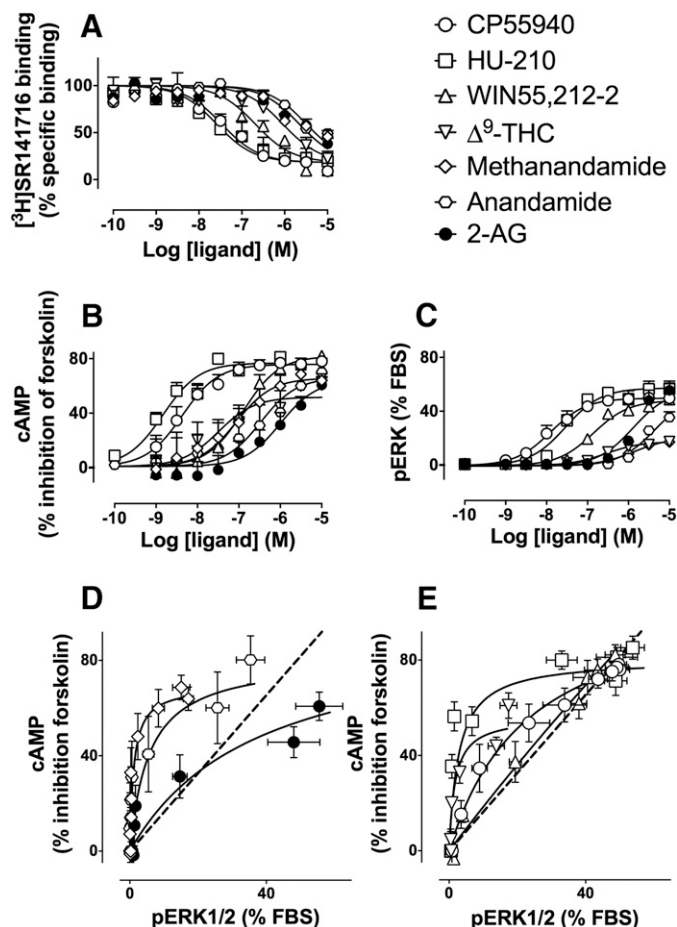


Fig. 1. Cannabinoids are biased agonists at CB₁R. (A) Endogenous and exogenous cannabinoids inhibit the binding of the radiolabeled inverse agonist [³H]SR141716 in a concentration-dependent manner. Data points represent the mean \pm S.E.M. from at least four independent experiments performed in triplicate. Competition-binding curves are the best of a one-site binding equation (eq. 1) to the data. (B) Endogenous and exogenous cannabinoids mediate inhibition of cAMP synthesis and (C) pERK1/2. Curves are the best fit to the standard three-parameter Hill equation. (D) and (E) demonstrate “bias plots” that denote the preference of endogenous and exogenous cannabinoids for signaling via inhibition of cAMP formation or pERK1/2 (see *Materials and Methods* for construction of bias plots). Data points represent the mean \pm S.E.M. response to equimolar cannabinoid concentrations in cAMP (y -axis) versus pERK1/2 (y -axis) assays, from at least three experiments performed in triplicate. Curves through the points are the agonist concentration-response curves analyzed using a three-parameter Hill equation. Data points and curves that lie on either side of the line of identity (dotted line) reflect signaling preference toward one pathway over the other. (A–E) represent the grouped data from at least three experiments performed in triplicate.

cAMP, and pERK1/2 assays in FlpIn CHO-CB₁R cells (Table 1). No functional response to cannabinoids was observed in untransfected FlpIn CHO cells, confirming the involvement of CB₁R in signaling mediated by cannabinoid ligands (unpublished data).

The majority of radioligand binding studies at CB₁R 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 4°C to ensure that receptors were not internalized upon agonist binding.

[³H]SR141716A 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 [³H]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 [³H]SR141716A and the number of [³H]SR141716A binding sites present, which was estimated at 800,098 ± 1868 sites per cell. Unlabeled SR141716A did not completely displace [³H]SR141716A binding in whole cells (unlabeled SR141716A displaced approximately 70% of specific [³H]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 pK_d calculated for [³H]SR141716A in these assays (8.51 ± 0.35; Supplemental Fig. 3A) was in agreement with its reported pK_d value determined previously both in membrane preparations (Govaerts et al., 2004) and in whole HEK293 CB₁R cells (Wennerberg et al., 2011). To confirm this pK_d, functional interaction studies between SR141716A and CP55940 in cAMP assays were performed (Supplemental Fig. 3B). Data were analyzed using the Gaddum/Schild equation (eq. 4), which estimated the SR141716A pA₂ to be 8.89 ± 0.37, *n* = 3, with a Schild slope not significantly different to 1.

Heterologous competition assays were subsequently performed to determine the equilibrium dissociation constant of cannabinoid agonists in whole cell radioligand binding assays (eq. 2). As with homologous competition assays, cannabinoids did not fully displace [³H]SR141716A binding (approximately 20% specific [³H]SR141716A binding remained). Cannabinoid equilibrium dissociation constants can differ depending on whether antagonist or agonist radioligands are used (Thomas et al., 1998; Govaerts et al., 2004). Nonetheless, pK_i values determined in the current study for CP55940, HU-210, WIN55,212-2, Δ⁹-THC, anandamide, and methanandamide were in general agreement with those reported previously in membrane-based radioligand binding assays that employed [³H]SR141716A (Thomas et al., 1998; Bisogno et al., 2000; Muccioli et al., 2005; D'Antona et al., 2006). There are no

published pK_i values for 2-AG using [³H]SR141716A. However, its estimated pK_i value in the current study is similar to a reported value using [³H]CP55940 (Sugiura et al., 1995). The rank order of cannabinoid agonist affinity determined in heterologous competition assays (Fig. 1A; Table 1) from highest to lowest was HU-210 = CP55940 > WIN55,212-2 = Δ⁹-THC > methanandamide = anandamide = 2-AG.

With the exception of WIN55,212-2, cannabinoid pEC₅₀s generally matched their estimated pK_i values in pERK1/2 assays. Thus, the rank order of cannabinoid potency in pERK1/2 assays (Fig. 1B; Table 1) was HU-210 = CP55940 > WIN55,212-2 = Δ⁹-THC > methanandamide = anandamide = 2-AG. In cAMP assays (Fig. 1C; Table 1), HU-210, Δ⁹-THC, methanandamide, and anandamide demonstrated a greater potency and/or E_{max} than in pERK1/2 assays (although anandamide did not reach statistical significance). In contrast, the potency and efficacy of CP55940, WIN55,212-2, and 2-AG were comparable in both cAMP and pERK1/2 assays (Table 1). Thus, the rank order of agonist potency in cAMP assays was HU-210 > CP55940 > WIN55,212-2 = Δ⁹-THC > methanandamide = anandamide = 2-AG.

The change in potency and/or efficacy of some but not all 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 1

Binding affinity (pK_i), potency (pEC₅₀), and relative efficacy (E_{max}) of cannabinoids in cAMP and pERK1/2 assays in CHO-hCB₁R cells

Values represent the mean ± S.E.M. from at least four experiments performed in triplicate.

Ligand	Radioligand Binding pK _i	cAMP		pERK1/2	
		pEC ₅₀	E _{max} ^a	pEC ₅₀	E _{max} ^b
		EC ₅₀ nM	%	EC ₅₀ nM	%
CP55940	7.4 ± 0.2	8.1 ± 0.2 (7.9)	82 ± 6	7.7 ± 0.2 (19)	51 ± 2
HU-210	7.7 ± 0.2	9.0 ± 0.2 (1.0)	81 ± 5	7.5 ± 0.1* (31)	57 ± 4
WIN55,212-2	6.3 ± 0.1	7.1 ± 0.1 (79)	86 ± 4	7.0 ± 0.2 (107)	52 ± 5
Δ ⁹ -THC	6.6 ± 0.2	7.6 ± 0.1 (25)	52 ± 5	6.4 ± 0.1* (371)	19 ± 3
Methanandamide	5.9 ± 0.1	6.8 ± 0.4 (158)	71 ± 4	5.9 ± 0.1* (1230)	26 ± 6
Anandamide	5.5 ± 0.2	6.1 ± 0.2 (794)	74 ± 9	5.5 ± 0.2 (3162)	~40
2-AG	5.6 ± 0.1	5.9 ± 0.2 (1259)	64 ± 11	5.8 ± 0.1 (1585)	~60

n = 4–6 (pK_i); *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

^aPercentage of maximum inhibition of forskolin response.

^bPercentage maximum FBS response.

to an operational model of agonism (Black and Leff, 1983) (eq. 5) to determine the transduction ratio, $R = \tau/K_A$ (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 ($\Delta\text{Log}R$). The difference between the $\Delta\text{Log}R$ values for each pathway ($\Delta\Delta\text{Log}R$) was then quantified to determine the strength of cannabinoid-mediated receptor coupling toward each pathway (Kenakin et al., 2012). The inverse Log of $\Delta\Delta\text{Log}R$ 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, Δ^9 -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 Org276569 increases the binding of the CB₁R agonist, [³H]CP55940, and displays negative binding cooperativity with the CB₁R inverse agonist [³H]SR141716A (Price et al., 2005; Baillie et al., 2013). In binding assays, Org276569 had no effect on [³H]WIN55,212-2 binding, although in functional assays there was evidence that it potentiated WIN55,212-2 binding (Price et al., 2005; Baillie et al., 2013). Therefore, we further sought to evaluate the potential for Org276529 to display probe dependence with both endogenous and exogenous cannabinoids.

We first validated the effects of Org276569 on [³H]SR141716A, and then on displacement of [³H]SR141716A by cannabinoids in FlpIn CHO-hCB₁ cells. As in previous findings (Price et al., 2005), Org276569 fully inhibited [³H]SR141716A binding, indicating high negative allosteric cooperativity between these two ligands (Supplemental Fig. 3C). The displacement of [³H]SR141716A binding was indistinguishable from a competitive interaction with the receptor, suggesting very high negative cooperativity between Org276569 and [³H]SR141716A. Therefore, the pK_i value of 5.8 ± 0.1 for Org276569 was determined by fitting the displacement data to a one-site inhibition mass action equation (eq. 1).

To determine the effect of Org276569 on other cannabinoids, binding interaction studies were performed by measuring cannabinoid displacement of [³H]SR141716A in the absence and presence of various Org276569 concentrations. Data from these experiments were fitted to an allosteric ternary complex model (eq. 3) to determine the cooperativity between Org276569 and the cannabinoids (Table 4). As in previous findings (Baillie et al., 2013), Org276569 had little effect on the binding of WIN55,212-2, indicated by little effect on WIN55,212-2-mediated displacement of [³H]SR141716A (Fig. 2; Table 4). This indicates neutral cooperativity between Org276569 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 [³H]SR141716A, Org276569 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 Org276569 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). Org276569 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 Org276569 on different CB₁R-mediated signaling pathways, we extended previous studies by using endo- and exocannabinoid probes in functional interaction studies with Org276569.

Org276569 completely abolished inhibition of cAMP formation stimulated by all tested cannabinoids (2-AG, anandamide,

TABLE 2

Biased agonism by cannabinoids (K_A estimated from the operational model of agonism)

$\text{Log}R$ (τ/K_A), $\Delta\text{Log}R$ ($\Delta\tau/K_A$), $\Delta\Delta\text{Log}R$ ($\Delta\Delta\tau/K_A$) ratios and bias factors (BF) for cannabinoid ligands, relative to 2-AG, at 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.

Ligand	cAMP		pERK1/2		cAMP/pERK1/2	
	LogR	$\Delta\text{Log}R$	LogR	$\Delta\text{Log}R$	$\Delta\Delta\text{Log}R$	BF
CP55940	8.3 \pm 0.2	2.6 \pm 0.2	7.9 \pm 0.1	2.0 \pm 0.1	0.6 \pm 0.2	3.9
HU-210	8.8 \pm 0.2	3.1 \pm 0.2	7.6 \pm 0.1	1.8 \pm 0.1	1.3 \pm 0.2*	20.8
WIN55,212-2	6.9 \pm 0.2	1.2 \pm 0.2	6.8 \pm 0.1	1.0 \pm 0.1	0.2 \pm 0.2	1.7
Δ^9 -THC	7.1 \pm 0.3	1.4 \pm 0.3	6.5 \pm 0.2	0.6 \pm 0.2	0.8 \pm 0.4	5.6
Methanandamide	6.9 \pm 0.2	1.2 \pm 0.2	5.9 \pm 0.2	0.0 \pm 0.2	1.2 \pm 0.3*	15.1
Anandamide	6.3 \pm 0.2	0.6 \pm 0.2	5.6 \pm 0.1	-0.3 \pm 0.1	0.8 \pm 0.2	6.8
2-AG	5.7 \pm 0.2	0.0 \pm 0.2	5.9 \pm 0.1	0.0 \pm 0.1	0.0 \pm 0.2	1.0

* $P < 0.05$, one-way analysis of variance with Bonferroni's multiple comparison test between $\Delta\Delta\text{Log}R$ for 2-AG compared with other cannabinoids.

TABLE 3

Biased agonism by cannabinoids (K_A predetermined from radioligand binding assays)
 LogR (τ/K_A), ΔLogR ($\Delta\tau/K_A$), $\Delta\Delta\text{LogR}$ ($\Delta\Delta\tau/K_A$) ratios and bias factors for cannabinoid ligands, relative to 2-AG, at the CB₁R were obtained by fitting the data to an operational model of agonism (eq. 5), where K_A was fixed to that predetermined in radioligand binding assays. Values represent the mean \pm S.E.M. from at least four independent experiments carried out in triplicate.

Ligand	cAMP		pERK1/2		cAMP/pERK1/2	
	LogR	ΔLogR	LogR	ΔLogR	$\Delta\Delta\text{LogR}$	BF
CP55940	8.3 \pm 0.2	2.6 \pm 0.2	7.9 \pm 0.1	2.0 \pm 0.1	0.6 \pm 0.2	3.8
HU-210	8.8 \pm 0.2	3.1 \pm 0.2	7.6 \pm 0.1	1.8 \pm 0.1	1.3 \pm 0.2*	20.7
WIN55,212-2	6.9 \pm 0.2	1.2 \pm 0.2	6.8 \pm 0.1	1.0 \pm 0.1	0.2 \pm 0.2	1.6
Δ^9 -THC	7.1 \pm 0.2	1.4 \pm 0.2	6.4 \pm 0.1	0.6 \pm 0.1	0.8 \pm 0.2	6.0
Methanandamide	6.9 \pm 0.2	1.2 \pm 0.2	5.8 \pm 0.1	-0.1 \pm 0.1	1.3 \pm 0.2*	18.2
Anandamide	6.2 \pm 0.2	0.6 \pm 0.2	5.8 \pm 0.1	-0.1 \pm 0.1	0.8 \pm 0.2	4.5
2-AG	5.7 \pm 0.2	0.0 \pm 0.2	5.9 \pm 0.1	0.0 \pm 0.1	0.0 \pm 0.2	1.0

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

methanandamide, Δ^9 -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 Δ^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 allosterism (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 α was fixed to 1 to reflect the neutral binding cooperativity. This enabled determination of the functional cooperativity (β) between Org27569 and the cannabinoids (Table 5). The β 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_{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 CB₁Rs. Pregnenolone was previously reported to act as an endogenous allosteric inhibitor at CB₁Rs because it reduced Δ^9 -THC-induced activation of pERK1/2 in CHO-hCB₁ cells (Vallee et al., 2014). Pregnenolone also inhibited hypoactivity, antinociception, hypothermia, catalepsy, food intake and memory impairment produced by Δ^9 -THC in mice, and blocked the effects of Δ^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 CB₁R, 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 CB₁Rs, 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 Δ^9 -THC-induced activation of pERK1/2 by pregnenolone in CHO-hCB₁ cells (Vallee et al., 2014), and enhancement of anandamide-mediated inhibition of cAMP formation by lipoxin A4 in HEK-CB₁ cells (Pamplona et al., 2012), our results revealed a complete lack of modulation of CB₁R signaling by these compounds at concentrations up to 10 μM and 500 nM, respectively (Fig. 5). We also examined the actions of lipoxin

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 ($\text{Log}\alpha'$). The binding cooperativity between [³H]SR141716A and Org27569 ($\text{Log}\alpha$) 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 ($\text{p}K_A$ 8.5). Values represent the mean \pm S.E.M. from at least three experiments performed in triplicate.

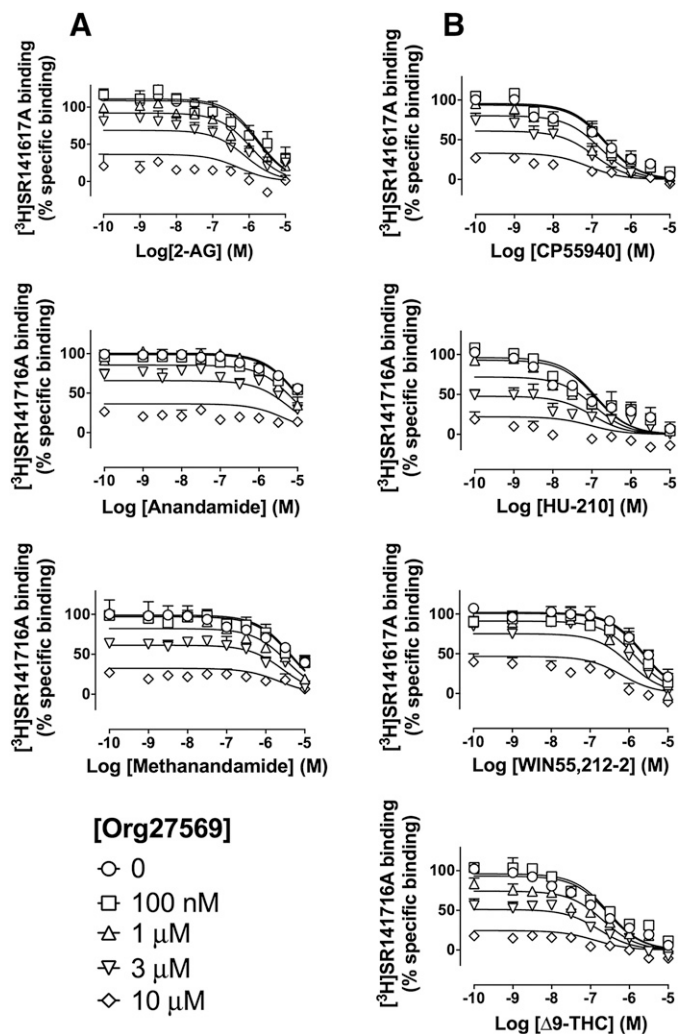
Ligand	$\text{Log}\alpha'^a$ [α']	$\text{p}K_B^b$	$\text{p}K_i^c$	B_{max}^d
CP55940	0.5 \pm 1.0 [3.2]	5.4 \pm 0.1	6.8 \pm 0.6	391 \pm 11.1
HU-210	0.0 \pm 0.7 [1.0]	5.6 \pm 0.1	7.0 \pm 0.3	393 \pm 15.3
WIN55,212-2	0.6 \pm 0.5 [4.0]	5.2 \pm 0.1	5.8 \pm 0.2	427 \pm 16.8
Δ^9 -THC	0.3 \pm 0.2 [2.0]	5.6 \pm 0.1	6.7 \pm 0.1	393 \pm 16.2
Methanandamide	0.3 \pm 0.6 [2.0]	5.4 \pm 0.1	5.5 \pm 0.2	405 \pm 12.4
Anandamide	0.3 \pm 0.4 [2.0]	5.4 \pm 0.0	5.2 \pm 0.1	411 \pm 8.02
2-AG	0.5 \pm 0.2 [3.2]	5.4 \pm 0.1	5.9 \pm 0.6	399 \pm 9.88

^aLogarithm of the binding cooperativity factor between Org27569 and cannabinoid agonists.

^bThe negative logarithm of the allosteric modulator dissociation constant.

^cThe negative logarithm of the competing orthosteric ligand dissociation constant.

^dThe maximum number of [³H]SR141716A binding sites expressed as percentage of specific binding.

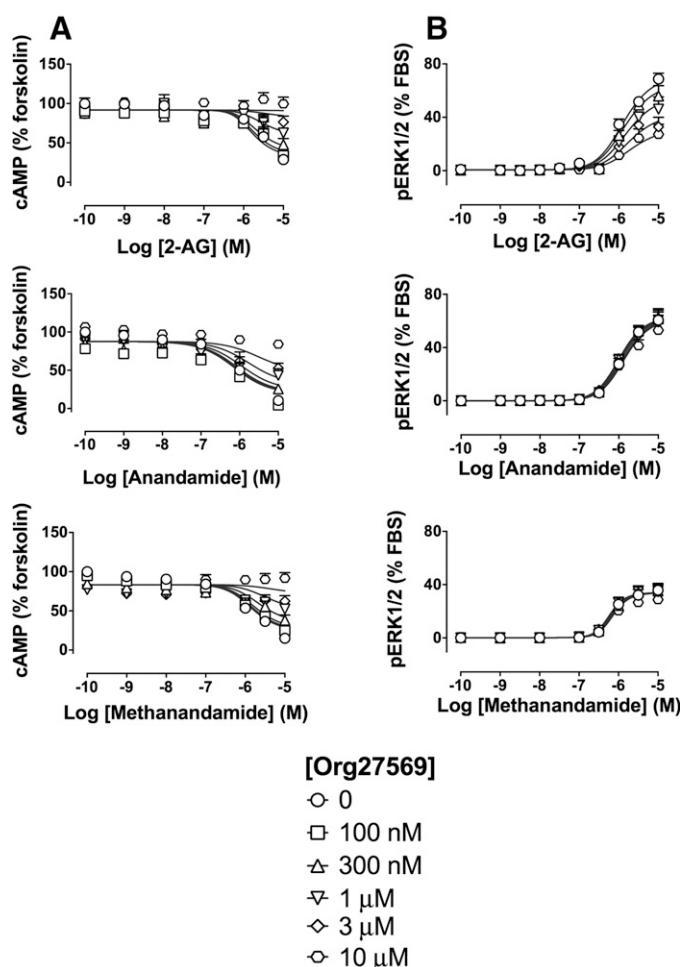


A4 and pregnenolone on activation of CB₁R signaling by other cannabinoid ligands. However, pregnenolone also did not inhibit pERK1/2 activation by WIN55,212-2, and lipoxin A4 did not alter cAMP inhibition by CP55940 (Supplemental Fig. 5). Therefore, the reported allosteric effects of pregnenolone and 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 CB₁R_s, 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 CB₁R_s. 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 CB₁R_s. 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;

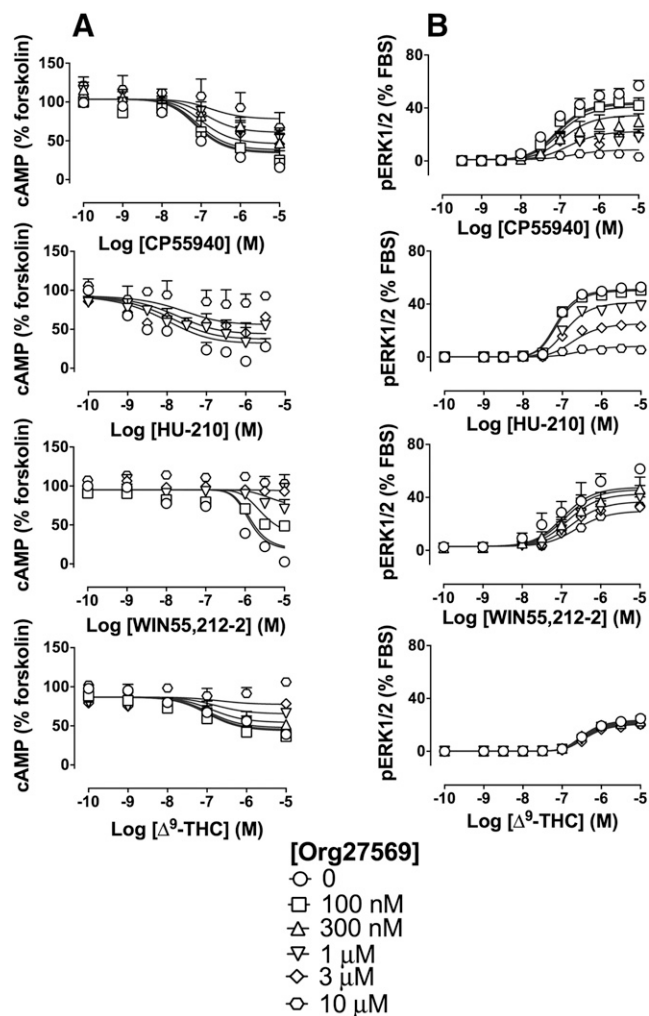


Fig. 4. Org27569 exhibits pathway- and probe-dependent allosteric effects on exocannabinoid-mediated signaling. Effects of Org27569 on CB₁R-mediated inhibition of cAMP formation (A) and activation of pERK1/2 (B) in CHO-hCB₁ cells. Data points represent mean values + S.E.M. from at least three experiments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

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

TABLE 5

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

pK_A (cannabinoid equilibrium dissociation constant) and pK_B (Org27569 equilibrium dissociation constant) were fixed to values determined in binding assays. $\text{Log} \alpha$ (binding cooperativity) was fixed to 0 to reflect the near neutral cooperativity between Org27569 and cannabinoid agonists. $\text{Log} \tau_B$ was fixed to -1000 to reflect the lack of Org27569 efficacy in these assays. Values represent the mean \pm S.E.M. from at least three experiments performed in triplicate.

Ligand	$\text{Log} \beta^a$ [β]		$\text{Log} \tau_A^b$ [τ_A]	
	pERK1/2	cAMP	pERK1/2	cAMP
CP55940	> -10 [~ 0]	-1.0 ± 0.5 [0.1]	0.3 ± 0.1 [2.0]	0.3 ± 0.4 [2.0]
HU-210	-1.0 ± 0.1 [0.1]	> -10 [~ 0] ^c	0.3 ± 0.0 [2.0]	0.7 ± 0.8 [5.0]
WIN55,212-2	-0.6 ± 0.2 [0.3]	> -10 [~ 0] ^c	0.4 ± 0.3 [2.5]	0.3 ± 0.1 [2.0]
Δ^9 -THC	-0.1 ± 0.0 [0.8]	> -10 [~ 0] ^c	0.1 ± 0.1 [1.3]	0.1 ± 0.8 [1.3]
Methanandamide	-0.1 ± 0.1 [0.8]	> -10 [~ 0] ^c	0.8 ± 0.0 [6.3]	0.4 ± 0.2 [2.5]
Anandamide	-0.1 ± 0.1 [0.8]	> -10 [~ 0] ^c	0.8 ± 0.1 [6.3]	0.9 ± 0.3 [7.9]
2-AG	-0.4 ± 0.1 [0.4]	> -10 [~ 0] ^c	0.0 ± 0.2 [1.0]	0.2 ± 0.2 [1.6]

^aLogarithm of the activation cooperativity factor between Org27569 and cannabinoid agonists.

^bLogarithm of the functional efficacy.

^c $\text{Log} \beta$ was allowed to float in the analysis, but could not be determined owing to the very high negative cooperativity.

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, Δ^9 -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 Δ^9 -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 τ 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

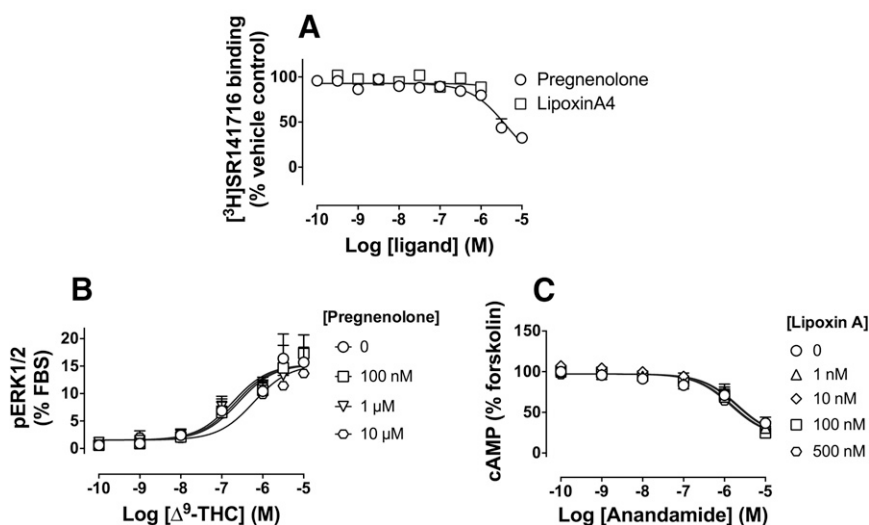


Fig. 5. Pregnenolone, but not lipoxin A, alters [³H]SR141716A binding. Effect of pregnenolone and lipoxin A4 on [³H]SR141716A binding (A) and the interaction between pregnenolone and Δ⁹-THC in pERK1/2 assays (B), and between lipoxin A4 and anandamide in cAMP assays (C). All experiments were conducted 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 the standard three-parameter Hill equation to the data.

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-[γ-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 Δ⁹-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 CP55940-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 G_i-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-[γ-thio]triphosphate

binding, and JNK phosphorylation by CP55490, at the same time potentiating cannabinoid-induced activation of pERK1/2 (Ahn et al., 2012; Baillie 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 Org27569 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 [³H]CP55940 and [³H]WIN55,212-2 (Vallee et al., 2014). However, our results demonstrate displacement of [³H]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 Δ⁹-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 [³H]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 [³H]SR141716A binding and enhanced [³H]SR141716A displacement by anandamide (Pamplona 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 Δ⁹-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.

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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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