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Title:

Biased agonism and biased allosteric modulation at the CB_1 cannabinoid receptor

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Running title:

Biased signalling at CB_1 receptors

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List of nonstandard abbreviations:

CBR, cannabinoid receptor; GPCR, G protein-coupled receptor; Δ^9-THC, tetrahydrocannabinol; HU-210, 11-hydroxy-Δ^8-THC-dimethylheptyl; CP55940, 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol; WIN55,212-2, (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone; 2-AG, 2-arachidonoylglycerol; Anandamide, N-arachidonoylethanolamine; SR141716A, 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-

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yl)-1*H*-pyrazole-3-carboxamide; Org27569, 5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)ethyl]amide; LY320135, 4-[6-methoxy-2-(4-methoxyphenyl)1-benzofuran-3-carbonyl]benzonitrile; GTPγS, guanosine 5'-O-[gamma-thio]triphosphate; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; CHO, Chinese hamster ovary.

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Abstract

CB_1 cannabinoid receptors (CB_1Rs) are attractive therapeutic targets for numerous central nervous system disorders. However, clinical application of cannabinoid ligands has been hampered due to their adverse on-target effects. Ligand-biased signalling from, and allosteric modulation of, CB_1Rs offer pharmacological approaches that may enable the development of improved CB_1R drugs, through modulation of only therapeutically desirable CB_1R signalling pathways. There is growing evidence that CB_1Rs are subject to ligand-biased signalling and allosterism. Therefore, in the present study, we quantified ligand-biased signalling and allosteric modulation at CB_1Rs. Cannabinoid agonists displayed distinct biased signalling profiles at CB_1Rs. For instance, whereas 2-AG and WIN55,212-2 showed little preference for inhibition of cAMP and phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2), anandamide, methanandamide, CP55940 and HU-210 were biased towards cAMP inhibition. The small molecule allosteric modulator, Org27569, displayed biased allosteric effects by blocking cAMP inhibition mediated by all cannabinoid ligands tested, while having little or no effect on ERK1/2 phosphorylation mediated by a subset of these ligands. Org27569 also displayed negative binding cooperativity with [^{3}H]SR141716A, 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 CB_1Rs. Pregnenolone but not lipoxin A4 displaced [^{3}H]SR141716A, however, there was no functional interaction between either of these ligands and cannabinoid agonists. This study demonstrates an approach to validate and quantify ligand-biased signalling and allosteric modulation at CB_1Rs, revealing ligand-biased "fingerprints" that may ultimately allow the development of improved CB_1R-targeted therapies.

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Introduction

Cannabinoid receptors (CBRs) are members of the G protein-coupled (GPCR) superfamily and mediate the actions of a number of endogenous ligands (endocannabinoids). CB_1Rs are the most abundant GPCRs in the brain (Devane et al., 1988; Glass et al., 1997; Herkenham, 1991), although they are also found at lower levels in the testes, spleen and immune cells (Gerard et al., 1991; Kaminski et al., 1992). CB_2Rs are predominantly expressed in the periphery (Munro et al., 1993; Onaivi et al., 2006) but are also found in low levels in the brain (Atwood and Mackie, 2010; Onaivi et al., 2006).

CBRs have diverse roles, including inhibition of neurotransmitter release (Hashimotodani 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-arachidonylglycerol (2-AG). However, additional ligands have been suggested to act as endocannabinoids (Pertwee, 2005; Pertwee et al., 2010).

Despite the fact that CBRs bind multiple endogenous ligands, the functional significance of these interactions is not fully understood, although distinct physiological 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 CB_1R-mediated long term potentiation (LTP) and subsequent impairments in learning and memory (Basavarajappa et al., 2014). In contrast, elevated levels of 2-AG enhance CB_1R-mediated alteration of LTP, learning and memory (Pan et al., 2011).

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Besides the well-known Δ^9-tetrahydrocannabinol (Δ^9-THC; the main psychoactive component of marijuana), a number of exogenous cannabinoids (referred to herein as exocannabinoids) have been identified. These include the synthetic Δ^9-THC analogue, HU-210, the small molecule agonists, CP55940 and WIN55,212-2, the CB_1R-selective inverse agonist, SR141716A (rimonabant) (reviewed in Pertwee, 2005; Pertwee et al., 2010) and the allosteric modulators, Org27569 (Price et al., 2005) and PSNCBAM-1 (Horswill et al., 2007). These latter compounds are particularly intriguing because they potentiate agonist binding to the CB_1R, while inhibiting agonist activity in numerous functional assays (Horswill et al., 2007; Price et al., 2005). Other endogenous ligands, including pregnenolone, pepcans and lipoxin A4, have also recently been suggested to act allosterically at CB_1Rs (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 CB_1R ligands, adverse on-target effects have hampered their use in the clinic to date. Thus, selective activation of receptor signalling events that mediate desired effects at the expense of those that cause adverse effects is an essential goal for CB_1R-targeted therapeutics and may be achieved via the phenomenon of biased agonism. Biased agonism is driven through the propensity for different ligands to stabilise unique subsets of receptor states, with each state being able to couple to its own preferred intracellular signalling responses (Kenakin and Christopoulos, 2013). Both orthosteric and allosteric ligands have the propensity to impose biased signalling 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 (Kenakin and Christopoulos, 2013; Leach et al., 2015; Leach et al., 2007).

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Importantly, reversals in cannabinoid efficacy or potency, indicative of biased agonism, have been observed at CB_1Rs. For instance, whereas WIN55,212-2 activates all Gi subtypes (Gi1-3) via CB_1Rs, (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 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 is operative at CB_1Rs, no study has compared biased signalling 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 CB_1Rs, to ascertain potential biased cannabinoid "fingerprints" that may guide the development of more successful CB_1R-targeted therapies.

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Materials and Methods

Materials

(+)-WIN55,212-2, CP55940, HU-210, methanandamide, anandamide, LY320135 and JZL 184 were obtained from Tocris Bioscience (Bristol, UK), and Δ^9-THC from THC pharm (Frankfurt, Germany). 2-AG, SR141716A, Org27569, pregnenolone, forskolin and fatty acid free bovine serum albumin (BSA) were purchased from Sigma Aldrich (Australia). Hygromycin B was obtained from Roche (Mannheim, Germany). Lipofectamine 2000, foetal bovine serum (FBS) and cell culture media were all obtained from Invitrogen (Australia). The cAMP AlphaScreen® kit and [^{3}H]SR141716A (35-60 Ci/mmol) were obtained from Perkin Elmer (USA). *SureFire*® ERK1/2 phosphorylation kits were a kind gift from Dr Michael Crouch (TGR BioSciences, SA, Australia).

Rationale for choice of signalling pathways and ligands

Like many downstream signalling pathways, CB_1R-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 CB_1R-mediated physiological roles. CB_1R modulation of cAMP signalling has been linked to neuronal remodelling, 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 (Berghuis et al., 2005; Rueda et al., 2002), and may play an important role in the development of tolerance and addiction to cannabinoids (Rubino et al., 2006). Furthermore, anandamide stimulation of ERK1/2 phosphorylation (pERK1/2)

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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 CB_1R ligands in cAMP inhibition and pERK1/2 assays, as they represent two important pathways that mediate CB_1R activity in the brain.

Cell lines

Flp-In Chinese Hamster Ovary (CHO) cells stably expressing human CB_1 cannabinoid receptors were generated according to the manufacturer's instruction (Invitrogen). Briefly, CB_1R in pEf5-frt-V5 was co-transfected with the pOG44 plasmid (encoding the Flp-In recombinase) at a ratio of 1:10 using lipofectamine 2000. The transfected cells were selected with 700 µg/ml of hygromycin B. Following selection, cells were maintained in DMEM containing 10% FBS, 16 mM HEPES and 700 µg/ml of hygromycin B.

Whole cell radioligand binding assays

CHO-hCB_1 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 ºC, 5% CO_2. The following day, media was removed and cells were washed twice with ice-cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4). 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 ºC. Non-specific binding was determined using 10 µM LY320135. Total binding was defined in the absence of unlabelled ligand.

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[^{3}H]SR141716A association kinetic assays were first performed to determine the time taken to reach [^{3}H]SR141716A equilibrium binding by incubating cells with an approximate K_d concentration (1 nM) of [^{3}H]SR141716A in the absence or presence of 10 µM LY320135 for different time intervals (0-360 min and overnight) at 4 ºC.

Homologous and heterologous competition experiments were next performed to determine the radioligand dissociation constant (K_d) and unlabelled competitor dissociation constants (K_i), respectively. Competition binding was performed by incubating cells with various concentrations of unlabelled cannabinoid ligands and 1 nM [^{3}H]SR141716A at 4 ºC for 6 h (based on the equilibrium time determined in association binding assays). To ensure that the cannabinoid agonists were not degraded by monoacylglycerol lipase (MAGL) in our Flp-In CHO-CB_1 cells, we first performed competition binding assays using 2-AG in the presence or absence of 100 nM JZL 184, a potent and selective MAGL inhibitor. A 30 min pre-incubation of cells with JZL 184 did not alter the pK_i value of 2-AG (Supplemental Fig. 1A and Supplemental Table 1), suggesting that the endocannabinoids were unlikely to be broken down in these assays, therefore subsequent assays were performed in the absence of JZL 184.

For interaction studies between the unlabelled competitor and allosteric modulators, all ligands were co-added and incubated together.

Assays were terminated by 2 rapid washes with ice cold 0.9% NaCl to remove unbound ligand. After the final wash, 100 µl of Optiphase supermix® scintillation fluid (Perkin Elmer, USA) was added to wells. Plates were shaken for 30 min and then radioactivity was measured for 1 min/well on a microbeta Counter (Perkin Elmer, USA).

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AlphaScreen® cAMP assays

CHO-hCB$_1$ cells were seeded at 50,000 cells per well into 96-well clear bottom culture plates, and incubated overnight at 37 ºC, 5% CO_2. The following day, cells were serum deprived for 1 h to minimize basal cAMP signalling, by replacing the growth media with serum-free DMEM/F12, containing 1mM IBMX and 0.5% w/v BSA. Cells were incubated with varying concentrations of cannabinoid compounds together with 1 µM forskolin for 30 min at 37 ºC 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 pre-incubated with varying concentrations of Org27569 for 10-15 min and with pregnenolone or lipoxin A4 for 10 min 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, 5mM HEPES, 0.3% Tween20 in milliQ water. Intracellular cAMP levels were determined using an AlphaScreen® cAMP kit (Perkin Elmer, USA). Plates were read on an Envision® plate reader (Perkin Elmer) after 2 h incubation in the dark at 37 °C using standard AlphaScreen® settings.

Extracellular signal regulated kinase 1/2 phosphorylation (pERK1/2) assays

CHO-hCB$_1$ 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

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were washed twice with 100 µl of PBS and incubated in serum-free DMEM, supplemented by 16 mM HEPES, at 37 °C for 5 h to minimise FBS-stimulated pERK1/2 levels. Cells were incubated with varying concentrations of cannabinoid compounds at 37 ºC 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, determined in time course assays (i.e. 2.5 min for anandamide and methanandamide and 5 min for all other cannabinoid ligands). For functional interaction studies with the allosteric modulators, cells were pre-incubated with varying concentrations of Org27569, pregnenolone or lipoxin A4 for 10-15 min before the addition of different concentrations of orthosteric ligands.

Phosphorylation of ERK1/2 at Thr202/Tyr204 was measured using AlphaScreen® SureFire® (Perkin Elmer, USA) as described previously (Nawaratne et al., 2008). Plates were read on an Envision plate reader (Perkin Elmer) after 1.5 h incubation at 37 °C in the dark using standard AlphaScreen® settings.

Data analysis

Nonlinear regression

Data were analysed 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 analyse specific binding of each ligand,

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

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

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inhibitor; and IC_{50} is the concentration of competitor that produces half the maximal response.

The equilibrium dissociation constant (K_i) of unlabelled 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,

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

Binding interaction experiments between [^{3}H]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 unlabelled ligand, respectively.

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

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

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

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agonist, CP55940, [B] represents the concentration of the antagonist, SR141716A, nH 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),

$$E = \frac{(E_{max} - Basal)}{1 + \left(\frac{\left(\frac{[A]}{10^{logK_A}} + 1 \right)}{\frac{10^{logR} \times [A]}{}} \right)} + Basal \qquad (5)$$

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, n is the unitless transducer slope and LogR 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),

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

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 Figures 1D and 1E, the mean ± 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, analysed 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, which denotes concentrations of agonist that are equipotent and equiactive in both assays.

Statistics

Values are expressed as means ± S.E.M. Mean values have been compared using one-way ANOVA with Bonferroni's multiple comparison test. A p value <0.05 was considered significant.

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Results

Endogenous and exogenous cannabinoids engender ligand-biased signalling at the CB_1R

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, cAMP and pERK1/2 assays in FlpIn CHO-CB_1R cells (Table 1). No functional response to cannabinoids was observed in untransfected FlpIn CHO cells, confirming the involvement of CB_1Rs in signalling mediated by cannabinoid ligands (data not shown).

The majority of radioligand binding studies at CB_1Rs reported in the literature have been performed in membrane preparations from recombinant CB_1R-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 signalling 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 internalised upon agonist binding.

[^{3}H]SR141716A association kinetic assays were first performed to determine the time at which radioligand equilibrium binding was reached, which was approximately 6 h after incubation of cells with 1 nM [^{3}H]SR141716A (Supplemental Fig. 2). Therefore, all subsequent binding experiments were terminated after 6 h incubation with ligands.

Homologous competition binding experiments were next performed to determine the affinity of [^{3}H]SR141716A and the number of [^{3}H]SR141716A binding sites present, which was estimated at 800,098 ± 1868 sites per cell. Unlabelled

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SR141716A did not completely displace [^{3}H]SR141716A binding in whole cells (unlabelled SR141716A displaced approximately 70% specific [^{3}H]SR141716A binding), which has previously been attributed to non-receptor radioligand binding events that occur even in the presence of an unlabelled competitive ligand (Wennerberg et al., 2010). Nonetheless, the pK_d calculated for [^{3}H]SR141716A in these assays (8.51 ± 0.35 Supplemental Fig. 3A) was in agreement with its reported pK_d value determined previously in both membrane preparations (Govaerts et al., 2004) and in whole HEK293 CB_1R 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 analysed using the Gaddum/Schild equation (equation 4), which estimated the SR141716A pA_2 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 (equation 2). Similar to homologous competition assays, cannabinoids did not fully displace [^{3}H]SR141716A binding (approximately 20% specific [^{3}H]SR141716A binding remained). Cannabinoid equilibrium dissociation constants can differ depending on whether antagonist or agonist radioligands are used (Govaerts et al., 2004; Thomas et al., 1998). Nonetheless, pK_i values determined in the current study for CP55940, HU-210, WIN55,212-2, Δ^9-THC, anandamide and methanandamide were in general agreement with those reported previously in membrane-based radioligand binding assays that employed [^{3}H]SR141716A (Bisogno et al., 2000; D'Antona et al., 2006; Muccioli et al., 2005; Thomas et al., 1998). There are no published pK_i values for 2-AG using [^{3}H]SR141716A. However, its estimated pK_i value in the current study is similar to a reported value using [^{3}H]CP55940

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(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 = Δ^9-THC > methanandamide = anandamide = 2-AG.

With the exception of WIN55,212-2, cannabinoid pEC_{50}s 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 = Δ^9-THC > methanandamide = anandamide = 2-AG. In cAMP assays (Fig. 1C, Table 1), HU-210, Δ^9-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 was 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 = Δ^9-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 signalling at CB_1Rs. Therefore, to better visualise the preference of each cannabinoid to activate CB_1R-mediated cAMP inhibition or pERK1/2, "bias plots" (Gregory et al., 2010; Kenakin et al., 2012) were constructed and are shown in Figure 1D 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

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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 visualisation purposes, they do not differentiate between true biased agonism and that imparted by "system bias" (e.g. the receptor coupling efficiency to different signalling proteins) or "observation bias" (e.g. assay conditions). Therefore, to quantify genuine biased agonism from the CB_1R, we fitted agonist concentration-response data to an operational model of agonism (Black and Leff, 1983) (equation 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 upon K_A values predetermined from radioligand binding assays (Rajagopal et al., 2011), whereas the second employs K_A estimates derived from the operational model of agonism (Kenakin et al., 2012). A comparison of analysing the current data using both approaches is shown in Table 2 and Table 3. To eliminate system or observation bias, we compared cannabinoid transduction ratios to that of 2-AG, in part because this endogenous CB_1R agonist exhibited little bias towards cAMP or pERK1/2. Therefore, the transduction ratio of each agonist was normalised to that determined for the reference agonist, 2-AG. The difference in the transduction ratios between 2-AG and each cannabinoid is shown in Table 2 and Table 3 (ΔLogR). The difference between the ΔLogR values for each pathway ($\Delta\Delta$LogR) was then quantified to determine the strength of cannabinoid-mediated receptor coupling towards each pathway (Kenakin et al., 2012). The inverse Log of $\Delta\Delta$LogR represents the bias factor of each agonist (Table 2 and Table 3). Bias factors in Table 2 and Table 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 towards cAMP inhibition over

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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 towards cAMP inhibition. CP55940, Δ^9-THC and anandamide also displayed a preference towards cAMP inhibition, although they did not reach statistical significance (Table 2 and Table 3).

Org276529 displays probe-dependent allosteric modulation at CB_1Rs

Previous studies have shown that Org27569 increases the binding of the CB_1R agonist, [^{3}H]CP55940, and displays negative binding cooperativity with the CB_1R inverse agonist [^{3}H]SR141716A (Baillie et al., 2013; Price et al., 2005). In binding assays, Org27569 had no effect on [^{3}H]WIN55,212-2 binding, although in functional assays there was evidence that it potentiated WIN55,212-2 binding (Baillie et al., 2013; Price et al., 2005). 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 Org27569 on [^{3}H]SR141716A, and then on displacement of [^{3}H]SR141716A by cannabinoids in FlpIn CHO-hCB_1 cells. Similar to 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 pK_i value of 5.8 ± 0.1 for Org27569 was determined by fitting the displacement data to a one-site inhibition mass action equation (equation 1).

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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 (equation 3) to determine the cooperativity between Org27569 and the cannabinoids (Table 4). Similar to 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 (Table 4, Fig. 2). This indicates neutral cooperativity between Org27569 and WIN55,212-2. However, a similar observation was also made for all other cannabinoids (Table 4, Fig. 2), 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 CB_1Rs, depending on the probe

Similar to binding studies, the allosteric activity of Org27569 on functional measures of CB_1R 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 CB_1Rs (Ahn et al., 2012; Baillie et al., 2013). Therefore, to evaluate the allosteric effects of Org27569 on different CB_1R-mediated signalling pathways, we extended previous studies by using endo- and exocannabinoid probes in functional interaction studies with Org27569.

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Org27569 completely abolished inhibition of cAMP formation stimulated by all tested cannabinoids (2-AG, anandamide, methanandamide, Δ^9-THC, WIN55,212-2, CP55940 and HU-210) (Fig. 3 and 4, panel A). Interestingly, however, in pERK1/2 assays, while 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 (Fig. 3 and 4, panel B). Furthermore, in contrast to previous studies (Ahn et al., 2012; Baillie et al., 2013), Org27569 on its own did not affect pERK1/2 signalling (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-signalling. 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 signalling.

Data were then fitted to an operational model of allosterism (equation 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 signalling efficacy between Org27569 and cannabinoid agonists, demonstrated by the large effect of Org27569 on the maximum signalling capacity (E_{max}) of the cannabinoids. The potency (pEC_{50}) of the cannabinoid agonists was unchanged in the absence and presence of Org27569.

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Pregnenolone, but not lipoxin A, displays weak activity at CB_1Rs

Pregnenolone was previously reported to act as an endogenous allosteric inhibitor at CB_1Rs because it reduced Δ^9-THC-induced activation of pERK1/2 in CHO-hCB_1 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 [^{3}H]CP55940 and [^{3}H]WIN55,212-2 (Vallee et al., 2014). Lipoxin A4 was also suggested as an endogenous allosteric potentiator at the CB_1R, where it enhanced anandamide, [^{3}H]CP55940 and [^{3}H]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_1Rs, we first investigated the effects of pregnenolone and lipoxin A4 on displacement of [^{3}H]SR141716A. Pregnenolone by itself caused a concentration-dependent decrease in [^{3}H]SR141716A binding. However, due to the incomplete displacement of [^{3}H]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 [^{3}H]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-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

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lack of modulation of CB_1R signalling by these compounds at concentrations up to 10 μM and 500 nM, respectively (Fig. 5). We also examined the actions of lipoxin A4 and pregnenolone on activation of CB_1R signalling 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.

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Discussion

This study quantified biased agonism, biased allosterism and probe-dependence by the small molecule, Org27569, at CB_1Rs, and highlights the need to validate the allosteric nature of two previously reported endogenous CB_1Rs ligands, pregnenolone and lipoxin A4.

Ligand-biased signalling has particular significance where multiple endogenous ligands bind to the same receptor. This is because each ligand has the potential to mediate unique physiological functions via stimulation of distinct intracellular signalling 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 CB_1Rs. Whereas 2-AG shows little preference for inhibition of cAMP formation and activation of pERK1/2, anandamide is approximately 7 times more biased towards cAMP inhibition. Methanandamide, the stable analogue of anandamide, showed a similar biased profile to that of anandamide, and was over 15 times more biased towards 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 due to their differential signalling at CB_1Rs. Furthermore, whereas cAMP inhibition has been linked to neurite remodelling (Zhou and Song, 2001), pERK1/2 signalling contributes to the regulation of neuronal migration and differentiation (Berghuis et al., 2005; Rubino et al., 2006; Rueda et al., 2002). Thus, CB_1R-mediated cAMP and

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pERK1/2 signalling may contribute to further distinct *in vivo* effects facilitated by cannabinoids.

We also showed that the exogenous cannabinoids displayed biased agonism at CB_1Rs. Similar to 2-AG, WIN55,212-2 displayed little bias towards cAMP inhibition or pERK1/2, whereas CP55940, Δ^9-THC and in particular HU-210, favoured 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 SAR between the small molecule cannabinoids and their biased profile. The findings of biased agonism at CB_1Rs supports the notion that CB1R therapeutics could selectively drive CB_1R signalling towards specific pathways, which may have important implications for the development of CB_1R-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 that employs K_A values predetermined in separate radioligand binding assays (Rajagopal et al., 2011), and another that 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.

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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 CB_1Rs 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 signalling properties of the orthosteric agonist (May et al., 2007). Further complexity may be added if the modulator exhibits probe- and/or pathway-dependent allosteric modulation. Indeed, Org27569 was previously shown to increase the binding of the CB_1R agonist, [^{3}H]CP55940, while having negative binding cooperativity with the inverse agonist, [^{3}H]SR141716A (Price et al., 2005). In contrast to the positive modulation of agonist binding, Org27569 inhibited agonist signalling in cAMP, [^{35}S]GTPγS 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 [^{3}H]SR141716A binding. However, it had little effect on [^{3}H]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 (Baillie et al., 2013; Price et al., 2005). 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 CP555940. In addition, the current study examined [^{3}H]SR141716A displacement by cannabinoid agonists in the

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presence of Org27569, whereas in previous studies the direct effect of Org27569 on binding of tritiated agonists ([^{3}H]CP55940 or [^{3}H]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, while 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, while only partially inhibiting 2-AG and WIN55,212-2 responses and having no significant effects on pERK1/2 activation by anandamide, methanandamide and Δ^9-THC. Thus, probe-dependence provides the opportunity to target selective signalling 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 signalling or has no effect is in contrast to these previous investigations. However, both Gi-dependent and independent mechanisms mediate CB_1R activation of pERK1/2 signalling (Ahn et al., 2012; Baillie et al., 2013; Bouaboula et al., 1995; Derkinderen et al., 2003; Sanchez et al.,

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1998; Sanchez et al., 2001) and the cell background and subsequent compliment of intracellular signalling proteins may therefore greatly influence the ability of Org27569 to modulate CB_1R signalling. Perceived biased agonism may also be dependent on receptor expression levels, with high expressing systems more likely 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 mins and subsequently returned back to baseline levels (Supplemental Figure 4). Although the peak pERK1/2 response was used to determine the signalling 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 signalling pathway studied, such that it displays pathway-specific, or biased, allosteric modulation at CB_1Rs. Whereas Org27569 had partial inhibitory or no effect on pERK1/2 signalling 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 CB_1R signalling, or biased allosterism. Previous studies reported biased allosteric effects of Org27569, as it antagonised inhibition of cAMP formation, stimulation of [^{35}S]GTPγS binding and JNK phosphorylation by CP55490, while 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 CB_1R upon immediate exposure, more prolonged contact with the receptor results in enhanced receptor desensitisation and a subsequent reduction in cell signalling events (Cawston

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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 CB_1Rs. 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 CB_1Rs. In contrast to previous findings (Vallee et al., 2014), we saw no inhibitory effect of pregnenolone on either Δ^9-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 CB_1R 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 compartmentalisation of receptor signalling 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 (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.

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Selectively targeting specific CB_1R-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 CB_1Rs, its *in vitro* effects do not necessarily translate into *in vivo* effects. While in mice Org27569 reduced food intake, it did so independently of CB_1Rs (Gamage et al., 2014). Furthermore, it did not modulate anandamide, CP55940 or Δ^9-THC-induced analgesia, catalepsy, or hypothermia. In rats, however, Org27569 inhibited hypothermia produced by CP55940 and anandamide, while having no effect on CP55940-induced catalepsy and anti-nociception. Org27569 also decreased food intake in rats. However, the involvement of CB_1Rs in these effects is unclear (Ding et al., 2014). This suggests that *in vitro* drug activity at CB_1Rs 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 signalling bias and allosteric modulation at CB_1Rs. 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* CB_1R pharmacology.

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Authorship contributions

Participated in research design: Khajehali, Christopoulos and Leach.

Conducted experiments: Khajehali

Performed data analysis: Khajehali and Leach

Wrote or contributed to the writing of the manuscript: Khajehali, Malone, Glass, Sexton, Christopoulos and Leach

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Figure Legends

Figure 1. Cannabinoids are biased agonists at CB_1Rs. (A) Endogenous and exogenous cannabinoids inhibit the binding of the radiolabelled inverse agonist, [^{3}H]SR141716, in a concentration-dependent manner. Data points represent the mean + S.E.M. from at least 4 independent experiments performed in triplicate. Competition binding curves are the best of a one-site binding equation (equation 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 signalling via inhibition of cAMP formation or pERK1/2 (see methods section for construction of bias plots). Data points represent the mean + S.E.M. response to equimolar cannabinoid concentrations in cAMP (y axis) versus pERK1/2 (x axis) assays, from at least three experiments performed in triplicate. Curves through the points are the agonist concentration-response curves analysed using a three parameter Hill equation. Data points and curves that lie either side of the line of identity (dotted line) reflect signalling preference towards one pathway over the other. Figures A-E represent the grouped data from at least three experiments performed in triplicate.

Figure 2. Org27569 displays probe dependence at CB_1Rs. Binding interaction studies between Org27569 and (A) endogenous-like cannabinoids, and (B) exogenous cannabinoids in CHO-hCB_1 cells indicate that whereas Org27569 inhibits the binding of the radiolabelled inverse agonist, [^{3}H]SR141716, it has little effect on the ability of unlabelled cannabinoids to inhibit [^{3}H]SR141716 binding. Data points represent mean values + S.E.M. from at least three experiments carried out in triplicate. Curves

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through the data points are the best fit of the data to an allosteric ternary complex model (equation 3).

Figure 3. Org27569 exhibits pathway- and probe-dependent allosteric effects on endocannabinoid-mediated signalling. Effects of Org27569 on CB_1R-mediated inhibition of cAMP formation (A) and activation of pERK1/2 (B) in CHO-hCB_1 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 (equation 6) to the data.

Figure 4. Org27569 exhibits pathway- and probe-dependent allosteric effects on exocannabinoid-mediated signalling. Effects of Org27569 on CB_1R-mediated inhibition of cAMP formation (A) and activation of pERK1/2 (B) in CHO-hCB_1 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 (equation 6) to the data.

Figure 5. Pregnenolone, but not lipoxin A, alters [^{3}H]SR141716A binding. Effect of pregnenolone and lipoxinA4 on [^{3}H]SR141716A binding (A) and the interaction between pregnenolone and Δ^9-THC in pERK1/2 assays (B), and between lipoxin A4 and anandamide in cAMP assays (C). All experiments were conducted in CHO-hCB_1 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.

Tables

Table 1. Binding affinity (pK_i), potency (pEC_{50}) and relative efficacy (E_{max}) of cannabinoids in cAMP and pERK1/2 assays in CHO-hCB_1R cells. Values represent the mean ± S.E.M. from at least four experiments performed in triplicate.

	Radioligand binding	cAMP		pERK1/2	
Ligand	**pK_i**	**pEC_{50} (EC_{50} nM)**	**E_{max}[a]**	**pEC_{50} (EC_{50} nM)**	**E_{max}[b]**
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)

[a] % maximum inhibition of forskolin response

[b] % maximum FBS response

*p < 0.05, two-way ANOVA with Bonferroni's multiple comparison test between changes in pEC50 values for cannabinoid ligands in cAMP versus pERK1/2 assays

Table 2. Biased agonism by cannabinoids. LogR (τ/K_A), ΔLogR ($\Delta\tau/K_A$), ΔΔLogR ($\Delta\Delta\tau/K_A$) ratios and bias factors (BF) for cannabinoid ligands, relative to 2-AG, at the CB_1R were obtained by fitting the data to an operational model of agonism (equation 5). Values represent the mean ± S.E.M. from at least four independent experiments carried out in triplicate.

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

*$p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison test between ΔΔLogR for 2-AG compared to other cannabinoids.

Table 3. Biased agonism by cannabinoids. LogR (τ/K_A), ΔLogR ($\Delta\tau/K_A$), ΔΔLogR ($\Delta\Delta\tau/K_A$) ratios and bias factors (BF) for cannabinoid ligands, relative to 2-AG, at the CB_1R were obtained by fitting the data to an operational model of agonism (equation 5), where the K_A was fixed to that predetermined in radioligand binding assays. Values represent the mean ± S.E.M. from at least four independent experiments carried out in triplicate.

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

*$p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison test between ΔΔLogR for 2-AG compared to other cannabinoids.

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 (equation 3) to determine the cooperativity between Org27569 and cannabinoids agonists (Logα'). The binding cooperativity between [^{3}H]SR141716A and Org27569 (Logα) was highly negative (>-10) and could not be defined due to complete inhibition of [^{3}H]SR141716A binding by Org27569. The negative logarithm of the radioligand dissociation constant was fixed to that determined from homologous competition binding experiments (pK_a 8.5). Values represent the mean ± S.E.M. from at least three experiments performed in triplicate.

Ligand	**Logα'[a] [α']**	**pK_b [b]**	**pK_i [c]**	**Bmax[d]**
CP55940	0.5 ± 1.0 [3.2]	5.4 ± 0.1	6.8 ± 0.6	391 ± 11.1
HU-210	0.0 ± 0.7 [1.0]	5.6 ± 0.1	7.0 ± 0.3	393 ± 15.3
WIN55,212-2	0.6 ± 0.5 [4.0]	5.2 ± 0.1	5.8 ± 0.2	427 ± 16.8
Δ^9-THC	0.3 ±0.2 [2.0]	5.6 ± 0.1	6.7 ± 0.1	393 ± 16.2
Methanandamide	0.3 ± 0.6 [2.0]	5.4 ± 0.1	5.5 ± 0.2	405 ± 12.4
Anandamide	0.3 ± 0.4 [2.0]	5.4 ± 0.0	5.2 ± 0.1	411 ± 8.02
2-AG	0.5 ± 0.2 [3.2]	5.4 ± 0.1	5.9 ± 0.6	399 ± 9.88

[a] Logarithm of the binding cooperativity factor between Org27569 and cannabinoid agonists

[b] The negative logarithm of the allosteric modulator dissociation constant

[c] The negative logarithm of the competing orthosteric ligand dissociation constant

[d] The maximum number of [^{3}H]SR141716A binding sites expressed as % specific binding

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. Logα (binding cooperativity) was fixed to 0 to reflect the near neutral cooperativity between Org27569 and cannabinoid agonists. Log τ_B 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.

	Logβ^a [β]		**Log$\tau_A{}^b$[τ_A]**	
Ligand	**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]

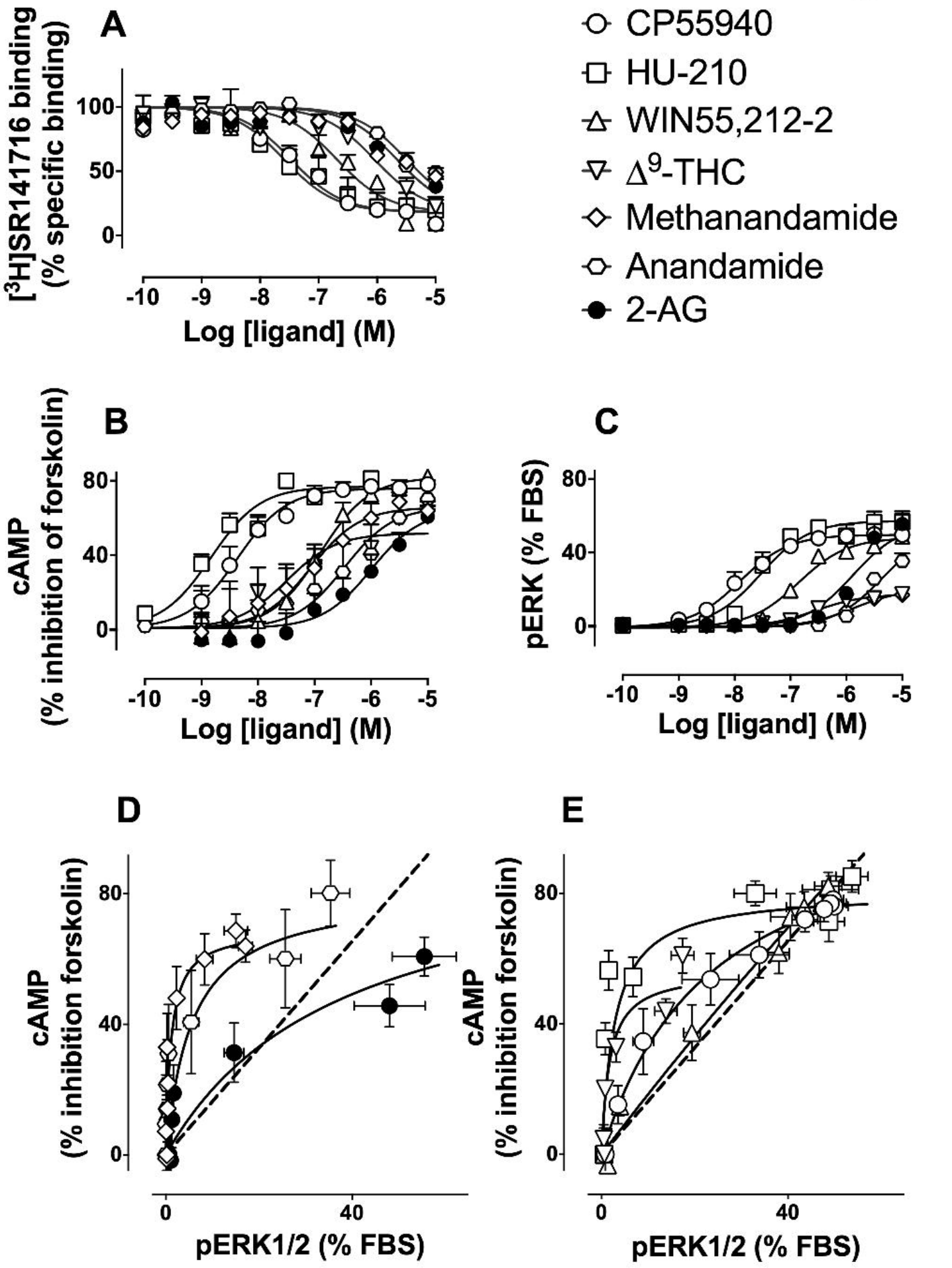
Anandamide	-0.1 ± 0.1 [0.8]	>-10 [~ 0][c]	0.8 ± 0.1 [6.3]	0.9 ± 0.3 [7.9]
2-AG	-0.4 ± 0.1 [0.4]	>-10 [~ 0][c]	0.0 ± 0.2 [1.0]	0.2 ± 0.2 [1.6]

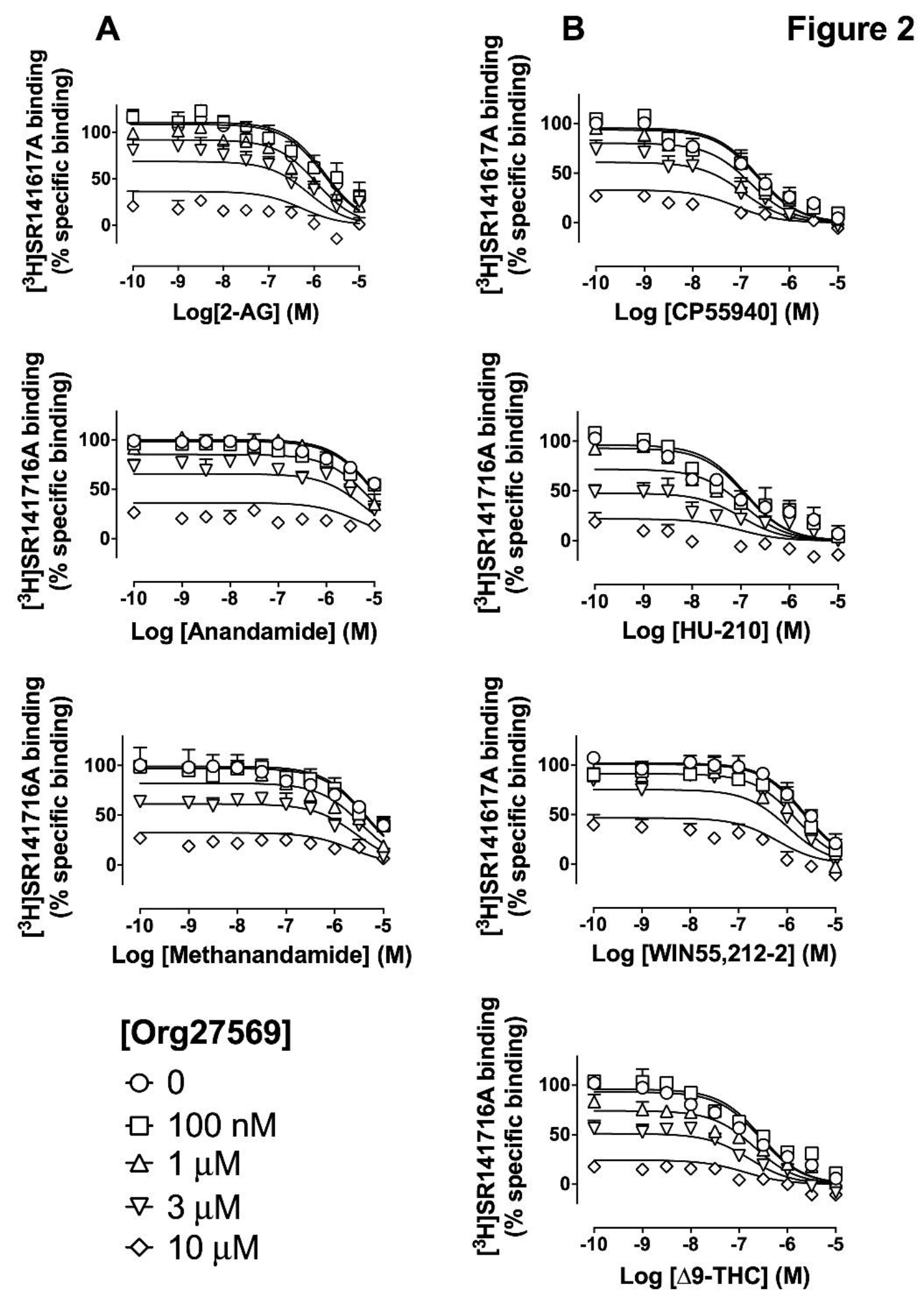
[a] Logarithm of the activation cooperativity factor between Org27569 and cannabinoid agonists

[b] Logarithm of the functional efficacy

[c] Logβ was allowed to float in the analysis, but could not be determined due to the very high negative cooperativity

Figure 1


A
[3H]SR141716 binding (% specific binding)
100
50
0
-10 -9 -8 -7 -6 -5
Log [ligand] (M)
CP55940
HU-210
WIN55,212-2
Δ9-THC
Methanandamide
Anandamide
2-AG
B
cAMP (% inhibition of forskolin)
80
40
0
-10 -9 -8 -7 -6 -5
Log [ligand] (M)
C
pERK (% FBS)
80
40
0
-10 -9 -8 -7 -6 -5
Log [ligand] (M)
D
cAMP (% inhibition forskolin)
80
40
0
0
40
pERK1/2 (% FBS)
E
cAMP (% inhibition forskolin)
80
40
0
0
40
pERK1/2 (% FBS)


A
B
Figure 2
[3H]SR141617A binding (% specific binding)
100
50
0
-10 -9 -8 -7 -6 -5
Log[2-AG] (M)
Log [CP55940] (M)
[3H]SR141716A binding (% specific binding)
Log [Anandamide] (M)
Log [HU-210] (M)
Log [Methanandamide] (M)
Log [WIN55,212-2] (M)
Log [Δ9-THC] (M)
[Org27569]
0
100 nM
1 μM
3 μM
10 μM

A
B
Figure 3
cAMP (% forskolin)
150
100
50
0
-10 -9 -8 -7 -6 -5
Log [2-AG] (M)
pERK1/2 (% FBS)
80
40
0
Log [2-AG] (M)
Log [Anandamide] (M)
Log [Methanandamide] (M)
[Org27569]
0
100 nM
300 nM
1 μM
3 μM
10 μM

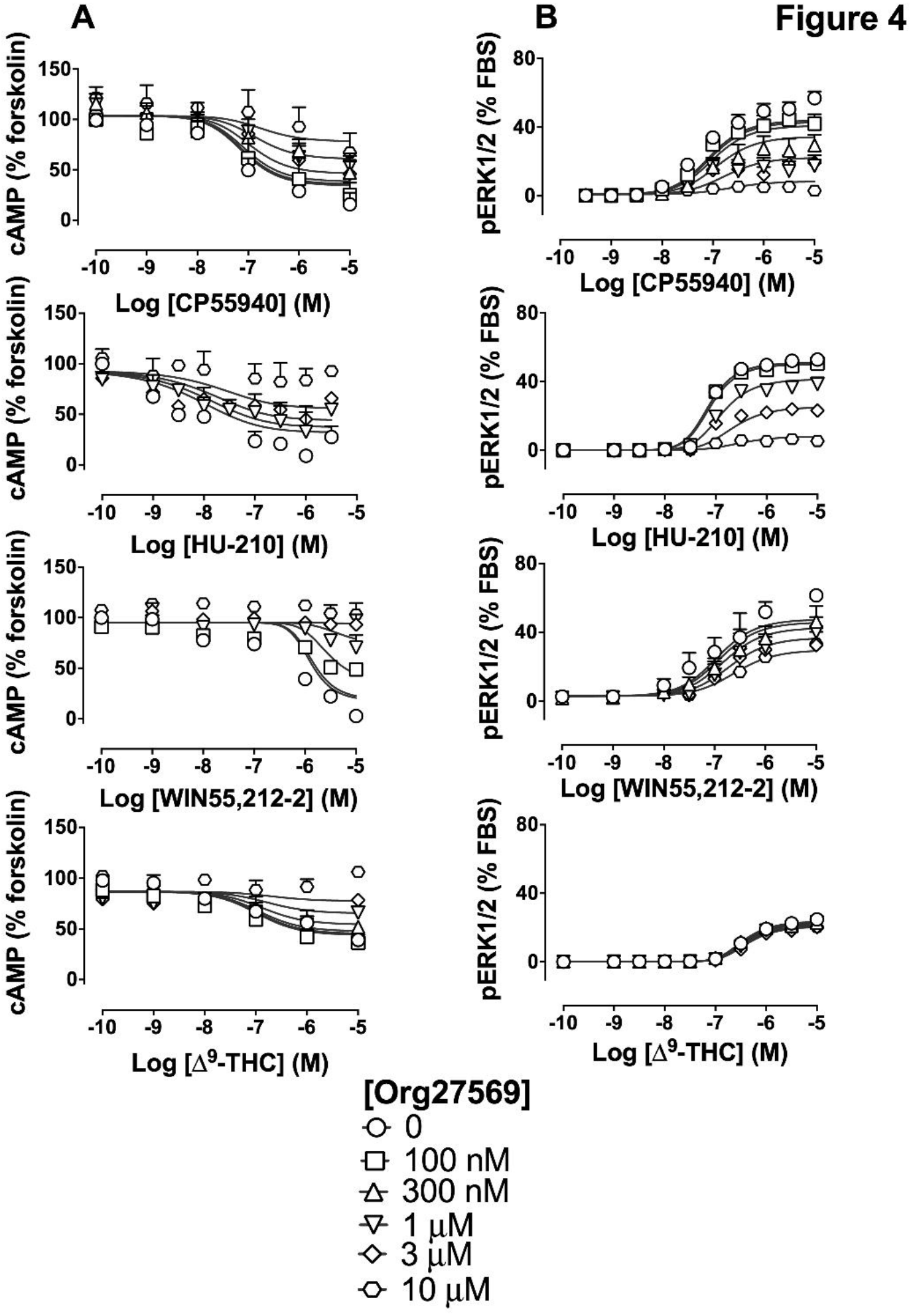
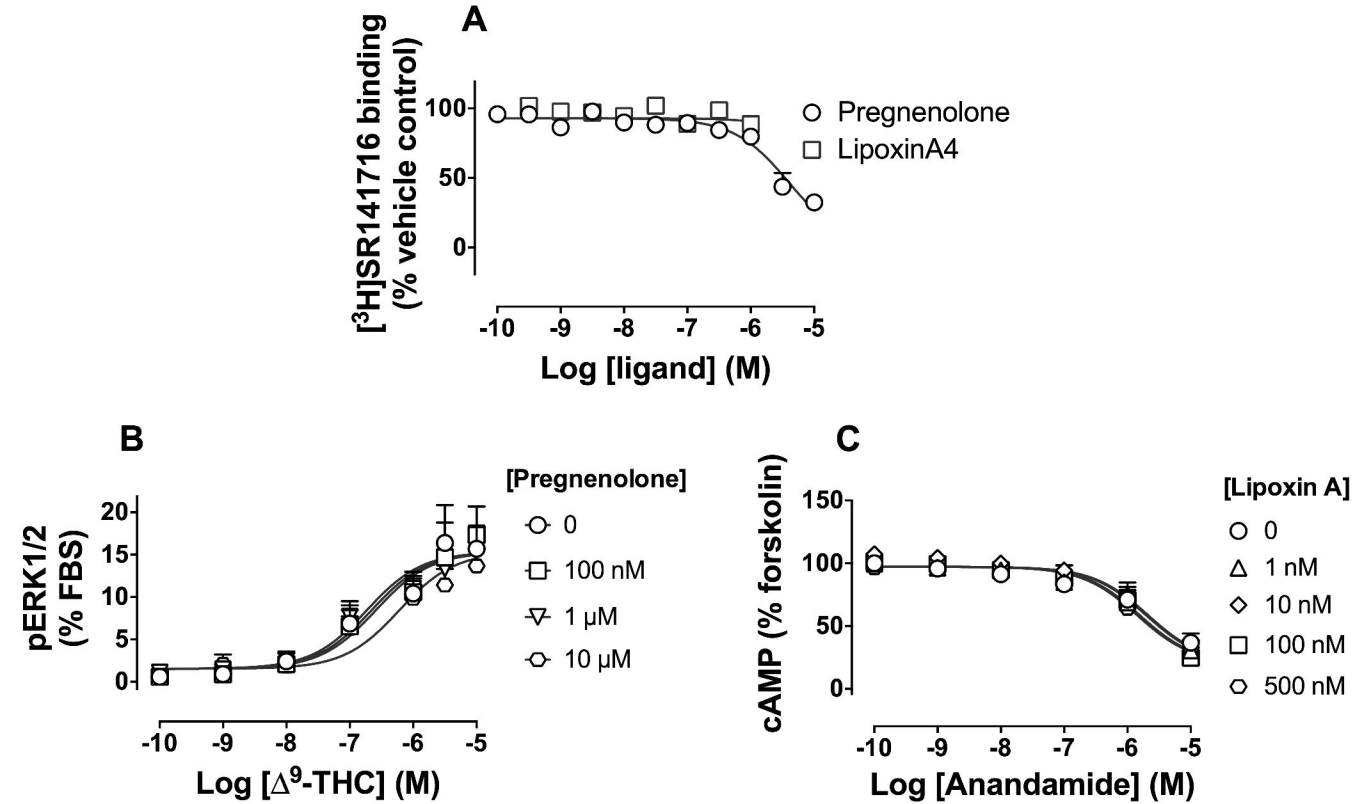

A
B
Figure 4
cAMP (% forskolin)
pERK1/2 (% FBS)
Log [CP55940] (M)
Log [HU-210] (M)
Log [WIN55,212-2] (M)
Log [Δ9-THC] (M)
[Org27569]
0
100 nM
300 nM
1 μM
3 μM
10 μM

Figure 5


A
[3H]SR141716 binding (% vehicle control)
100
50
0
-10
-9
-8
-7
-6
-5
Log [ligand] (M)
Pregnenolone
LipoxinA4
B
pERK1/2 (% FBS)
20
15
10
5
0
Log [Δ9-THC] (M)
[Pregnenolone]
0
100 nM
1 μM
10 μM
C
cAMP (% forskolin)
150
100
50
0
Log [Anandamide] (M)
[Lipoxin A]
0
1 nM
10 nM
100 nM
500 nM