ALLOSTERIC MODULATION OF A CANNABINOID G PROTEIN-COUPLED RECEPTOR: BINDING SITE ELUCIDATION AND RELATIONSHIP TO G PROTEIN SIGNALING*

Derek M. Shore1, Gemma L. Baillie2,3, Dow H. Hurst1, Frank Navas, III4, Herbert H. Seltzman4, Jahan P. Marcu5, Mary E. Abood5, Ruth A. Ross2,3, Patricia H. Reggio1

1Center for Drug Discovery, UNC Greensboro, Greensboro, NC 27402 USA
2Institute of Medical Sciences, University of Aberdeen, Aberdeen, AB25 2ZD Scotland, UK
3Department of Pharmacology & Toxicology, University of Toronto, Toronto, Ontario, Canada. M5S 1A8.
4Research Triangle Institute, Research Triangle Park, NC 27709 USA
5Department of Anatomy and Cell Biology and Center for Substance Abuse Research, Temple University, Philadelphia, PA 19140 USA

*Running title: The CB1 Receptor’s Allosteric Binding Site and Mechanism

To whom correspondence should be addressed: Patricia H. Reggio, Department of Chemistry and Biochemistry, Center for Drug Discovery, The University of North Carolina at Greensboro, Patricia A. Sullivan Science Building, PO Box 26170, Greensboro, NC 27402-6170, USA, Tel.: (336) 334-5333; Fax: (336) 334-5402; E-mail: phreggio@uncg.edu

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Background: Cannabinoid-1 (CB1) receptor allosteric modulator ORG27569 increases CP55,940 binding, yet antagonizes G protein signaling.

Results: ORG27569 binding, sterically blocks movement in CB1 extracellular loops and transmembrane helix 6 (TMH6).

Conclusion: ORG27569 increases CP55,940 binding by promoting an intermediate receptor conformation at which changes important for signaling are blocked.

Significance: This information may lead to new allosteric modulator rational design.

SUMMARY

The cannabinoid 1 (CB1) allosteric modulator, 5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)-ethyl]-amide (ORG27569), has the paradoxical effect of increasing the equilibrium binding of [3H](-)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxylpropyl] cyclohexan-1-ol (CP55,940, an orthosteric agonist), while at the same time decreasing its efficacy (in G protein-mediated signaling). ORG27569 also decreases basal signaling, acting as an inverse agonist for the G protein-mediated signaling pathway. In ligand displacement assays, ORG27569 can displace the CB1 antagonist/inverse agonist, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A).

The goal of this work was to identify ORG27569’s binding site at CB1. To this end, we used computational, synthesis, mutation, and functional studies to identify ORG27569’s binding site in the CB1 TMH3-6-7 region. This site is consistent with the results of K3.28192A, F3.36200A, W5.43279A, W6.48356A, and F3.25189A mutation studies, which revealed ORG27569’s binding site overlaps with our previously determined binding site of SR141716A, but extends extracellularly. Additionally, we identified a key electrostatic interaction between ORG27569’s piperidine ring nitrogen and K3.28192 that is important for ORG27569 to act as an inverse agonist. At this allosteric site, ORG27569 promotes an intermediate conformation of the CB1 receptor, explaining ORG27569’s ability to increase CP55,940’s equilibrium binding. This site also explains ORG27569’s ability to antagonize CP55,940’s efficacy in three complimentary ways: (1) ORG27569 sterically blocks movements of the
second extracellular loop that have been linked to receptor activation; (2) ORG27569 sterically blocks a key electrostatic interaction between the third extracellular loop residue K373 and D2.63; and (3) ORG27569 packs against TMH6, sterically hindering movements of this helix that have been shown to be important for receptor activation.

The G protein-coupled receptor (GPCR) superfamily of integral membrane proteins is composed of ~1,000 members (1) and comprises ~3% of the human genome (2). Considering their ubiquity and fundamental importance to cellular function, it is not surprising that ~60% of pharmaceuticals target GPCRs (3). Unfortunately, many of these drugs have numerous side effects, due to a lack of receptor subtype selectivity (4) and/or an interference with physiological signaling (5). This lack of receptor subtype selectivity is thought to be due (in part) to high sequence convergence at the orthosteric site— the binding site of most endogenous ligands and pharmaceutical drugs (6).

The discovery of ligands (i.e. allosteric modulators) that bind to an allosteric site has generated considerable interest. Allosteric sites are topographically distinct from the orthosteric site; classically, allosteric modulators are thought to influence the binding and/or efficacy of orthosteric ligands (7). Allosteric-based drugs could potentially have reduced side effects, due to the increased evolutionary divergence at allosteric sites (8). Additionally, allosteric modulators have been observed to direct signaling down specific second messenger pathways (i.e. biased agonism) (9). These results suggest that allosteric-based therapies have the potential for unprecedented receptor subtype selectivity and functional control.

The discovery of an allosteric site at the cannabinoid 1 (CB1) receptor has generated significant interest, due to the anticipated therapeutic potential of allosteric-based drugs. The CB1 receptor is a member of the Class A (rhodopsin-like) family of GPCRs; it is found primarily in the central nervous system (CNS) and is important in the regulation of neuronal activity. The CB1 receptor has been implicated in Alzheimer’s disease, cancer, obesity, and pain (10). Regrettably, drugs that target the CB1 receptor’s orthosteric site have failed due to unacceptable CNS-related side effects (11). CB1 allosteric modulators could potentially avoid these side effects, due to an anticipated increase in receptor subtype selectivity, as well as a predicted improvement in functional control (12). ORG27569 (see Figure 1), the prototypical CB1 allosteric modulator, has the paradoxical effect of increasing the equilibrium binding of CP55,940 (a CB1 agonist, see Figure 1), while concurrently antagonizing its G protein-mediated efficacy (13). Additionally, ORG27569 acts as an inverse agonist of G protein-mediated signaling (14).

In order to understand these seemingly contradictory effects, we have used computational methods together with mutagenesis, synthesis, and pharmacological studies to identify an allosteric binding site for ORG27569 at the CB1 receptor and to probe its relationship to G protein signaling effects. We show here that the ORG27569 binding site is located in the transmembrane helix (TMH) 3-6-7 region of the CB1 receptor, partially overlapping the SR141716A binding site, but extending extracellularly. We identify receptor residues that are crucial not only for ORG27569 binding in the presence of CP55,940, but also residues important to the inverse agonism that ORG27569 exhibits when applied alone. Finally, we relate the location of this binding site to the conformational changes associated with G protein activation on the extracellular side of CB1 that are blocked by ORG27569.

**EXPERIMENTAL PROCEDURES**

**Amino acid numbering**—The numbering scheme suggested by Ballesteros and Weinstein was employed here (15). In this system, the most highly conserved residue in each TMH is assigned a locant of 0.50. This number is preceded by the TMH number and followed by the absolute sequence number in superscript. All other residues in a TMH are numbered relative to this residue. Sequence numbers used are human CB1 sequence numbers (16).

**Chemistry**—Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Anhydrous solvents were obtained from Aldrich and used directly. All reactions involving air- or moisture-sensitive reagents were performed under a nitrogen atmosphere. Numbers in **bold** refer to compounds shown in Scheme 1.
Analytical thin-layer chromatography (TLC) was carried out on plates precoated with silica gel GHLF (250 µM thickness). TLC visualization was accomplished with a UV lamp. Silica gel chromatography was performed using RediSep prepacked silica gel cartridges. HPLC analyses were performed using a Waters Emperor chromatography system comprised of a 1525 Binary Pump, 2487 Dual 1 Absorbance Detector, and a 717 plus Autosampler using a Waters C-18 reverse phase XBridge column (5 µm; 4.6 x 100 mm; 254 nm; 1 mL/min).

4-Cyclohexylbenzaldehyde (2)—This compound was prepared according to the patent procedure described (17). To a dry 250-mL round bottom flask was added 1-bromo-4-cyclohexylbenzene (2.6 mL, 14.0 mmol) and anhydrous THF (16 mL) under nitrogen. The solution was cooled in a dry ice/acetone bath and n-butyllithium (1.6 M in hexane) (11 mL, 17.6 mmol, 1.26 eq) was added dropwise with stirring under nitrogen. The reaction mixture was stirred in the dry ice/acetone bath for 1 h. To the cold stirred reaction mixture was added dropwise anhydrous N,N-dimethylformamide (11 mL, 142 mmol, 10.1 eq). The reaction mixture was stirred in the dry ice/acetone bath for 1 h. The dry ice/acetone bath was replaced with an ice-water bath and the reaction mixture was allowed to warm to 0 °C. To the stirred cold reaction mixture was added saturated ammonium chloride. The quenched reaction mixture was transferred to a separatory funnel and extracted with EtOAc. The organic phase was separated, dried (MgSO₄), filtered, and the filtrate was concentrated to give the crude product as a yellow oil. ¹H NMR (300 MHz; CDCl₃): δ 9.97 (s, 1H), 7.81 (d, J = 8.1 Hz, 2H), 7.37 (d, J = 8.1 Hz, 2H), 2.59 (m, 1H), 1.69-1.98 (m, 5H), 1.16-1.54 (m, 5H).

1-Cyclohexyl-4-[(E)-2-nitroethenyl]benzene (3)—This compound was prepared according to a procedure (18) described for another substituted benzaldehyde. 4-Cyclohexylbenzaldehyde (2.4 g, 0.0127 mol), ammonium acetate (1.74 g, 0.0226 mol, 1.78 eq), and nitromethane (25 mL) were combined in a round bottom flask and the reaction mixture was heated at 100 °C with stirring under nitrogen for 2.5 h. The brown-orange reaction mixture was allowed to cool at room temperature and concentrated. The resulting oil was dissolved in EtOAc and the solution was washed with 1 N HCl followed by saturated NaHCO₃. The organic phase was separated, dried (MgSO₄) filtered, and the filtrate was concentrated to give an orange oil. The crude product was purified by flash chromatography over silica (80 g) with a hexane:EtOAc gradient (100:0 to 90:10) to give 1.27 g (43%) of the title compound as an orange oil. ¹H NMR (300 MHz; CDCl₃): δ 8.00 (d, J = 13.7 Hz, 1H), 7.57 (d, J = 13.7 Hz, 1H), 7.48 (d, J = 8.2 Hz, 2H), 7.29 (d, J = 8.2 Hz, 2H), 2.56 (m, 1H), 1.84 (m, 5H), 1.50-1.16 (m, 5H).

2-(4-Cyclohexylphenyl)ethan-1-amine (4)—This compound was prepared according to a procedure (19) described for the reduction of a nitrovinyl substituted indole. To a dry round bottom flask was added 1-cyclohexyl-4-[(E)-2-nitroethenyl]benzene (396 mg, 1.7 mmol) and anhydrous THF (24 mL). To the stirred solution was added lithium aluminum hydride (1 M in THF) (8 mL, 8 mmol, 4.7 eq) at room temperature under nitrogen. The reaction mixture was heated at reflux for 1 h. The reaction mixture was allowed to cool at room temperature and then cooled in an ice-water bath. To the stirred cold reaction mixture was slowly added water dropwise (0.3 mL) (H₂ was evolved) followed by 15% NaOH (0.3 mL) and water (0.9 mL). The aqueous mixture was filtered through a pad of celite and the pad was washed with EtOAc. The filtrate was partially concentrated in vacuo, washed with saturated NaHCO₃ followed by brine, dried (MgSO₄), filtered, and the filtrate was concentrated to give 0.314 g (90%) of the crude product as a gold-yellow oil. The crude product
was used directly without further purification. ES MS: 204 (M+H').

Ethyl 3-acetyl-5-chloro-1H-indole-2-carboxylate (6)—This compound was prepared according to a procedure (20) described for the corresponding 5-bromoindole analog. To a dry 500-mL round bottom flask was added ethyl-5-chloroindole-2-carboxylate (1.71 g, 0.0076 mol). The flask was capped with a rubber septum and purged with nitrogen. The indole ester was partially dissolved in anhydrous CH2Cl2 (40 mL). The stirred mixture was cooled in an ice-water bath and diethylaluminum chloride (1 M in hexane) (15 mL, 0.015 mol, 2.0 eq) was added slowly dropwise. The reaction mixture was stirred with cooling for 30 min. To the cold stirred reaction mixture was added dropwise a solution of acetyl chloride (1.1 mL, 0.0154 mol, 2.0 eq) in CH2Cl2 (40 mL). The reaction mixture was stirred under nitrogen in the ice-water bath for 6 h and then allowed to slowly warm to room temperature overnight. The reaction mixture was cooled in an ice-water bath and saturated NaHCO3 (100 mL) was slowly added. The quenched reaction mixture was extracted with CH2Cl2. The organic phase was separated, washed with brine, dried (MgSO4), filtered, and the filtrate was concentrated to give a gold-yellow solid. The crude product was purified by flash chromatography over silica (80 g) with dichloromethane to give 1.12 g (56%) of the desired product as a pale yellow solid. 1H NMR (300 MHz, CDCl3): δ 9.12 (br s, 1H), 8.12 (s, 1H), 7.34 (m, 2H), 4.48 (q, J = 7.1 Hz, 2H), 2.74 (s, 3H), 1.46 (t, J = 7.1 Hz, 3H). ES MS: 266 (M+H+), 288 (M+Na+), 264 (M-H+).

Ethyl 5-chloro-3-ethyl-1H-indole-2-carboxylate (7)—This compound was prepared according to a procedure (20) described for the corresponding 5-bromoindole analog. To a stirred solution of ethyl 3-acetyl-5-chloro-1H-indole-2-carboxylate (1.1 g, 0.0041 mol) in CH2Cl2 (40 mL) was added triethylsilane (2.6 mL, 0.0163 mol, 4 eq) at room temperature under nitrogen. After 4 h, the reaction mixture was poured into water and the aqueous mixture was extracted with EtOAc. The organic extract was washed with water followed by brine, dried (MgSO4), filtered, and the filtrate was partially concentrated to give a suspension. To the suspension was added EtOAc. Fumes were released upon addition of EtOAc, which suggested that trifluoroacetic acid remained. The organic solution was washed with saturated NaHCO3 followed by brine. The organic phase was separated, dried (MgSO4), filtered, and the filtrate was concentrated to give a wet yellow solid. The solid was washed with hexane to give 0.893 g (87%) of the desired product as a pale yellow solid. 1H NMR (300 MHz, CDCl3): δ 8.71 (br s, 1H), 7.66 (m, 1H), 7.30 (d, J = 7.1 Hz, 1H), 7.26 (dd, J = 1.9 Hz, 8.8 Hz, 1H), 4.43 (q, J = 7.1 Hz, 2H), 3.07 (q, J = 7.5 Hz, 2H), 1.43 (t, J = 7.1 Hz, 3H), 1.26 (t, J = 7.5 Hz, 3H). ES MS: 250 (M-H').

5-Chloro-3-ethyl-1H-indole-2-carboxylic acid (8)—To a stirred solution of ethyl 5-chloro-3-ethyl-1H-indole-2-carboxylate (405 mg, 1.61 mmol) in 1,4-dioxane (12 mL) was added 1N NaOH (8 mL, 8 mmol, 5 eq). The reaction mixture was heated under nitrogen at 120 °C for 30 min. The reaction mixture was allowed to cool at room temperature. To the stirred reaction mixture was added 1 N HCl to pH ~3 (indicator strip). The acidic mixture was transferred to a separatory funnel and extracted with EtOAc. The organic phase was separated, washed with brine, dried (MgSO4), filtered, and the filtrate was concentrated to give 0.39 g of a white solid. The solid was dried at 80 °C under vacuum to give 0.376 g. 1H NMR indicated the sample contains 1,4-dioxane (14% by wt.). Final yield of the desired product (less 1,4-dioxane) was 0.323 g (90%). 1H NMR (300 MHz, CDCl3): δ 8.73 (br s, 1H), 7.69 (m, 1H), 7.30 (m, 2H), 3.13 (q, J = 7.5 Hz, 2H), 1.30 (t, J = 7.5 Hz, 3H). ES MS: 222 (M-H').

5-Chloro-N-[2-(4-cyclohexylphenyl)ethyl]-3-ethyl-1H-indole-2-carboxamide (9)—To a stirred mixture of 5-chloro-3-ethyl-1H-indole-2-carboxylic acid (77 mg, 0.34 mmol), 1,3-dicyclohexylcarbodiimide (81 mg, 0.39 mmol, 1.15 eq), and 1-hydroxybenzotriazole hydrate (52 mg, 0.38 mmol, 1.13 eq) in anhydrous dichloromethane (12 mL) was added dropwise a solution of crude 2-(4-cyclohexylphenyl)ethan-1-amine (147 mg) in CH2Cl2 (4 mL) at room temperature under nitrogen. After 22 h, the reaction mixture was filtered through a pad of celite and the pad was washed with dichloromethane. The filtrate was concentrated to give a cloudy yellow oil. To the oil was added dichloromethane and the turbid mixture was filtered. The filtrate was applied to a
silica column (24 g) and purified by flash chromatography with a hexane:EtOAc gradient (100:0 to 50:50) to give a white solid which was dried under vacuum at 70 °C.

**HPLC Conditions**—Waters XBridge C-18 reverse phase column; 5 μm; 4.6 x 100 mm; 1 mL/min; CH3CN:Water (90:10) indicated the compound was 94% pure. The solid was triturated at room temperature with EtOAc followed by MeOH (2X), and hexane (2X), and then dried under vacuum at 70 °C to give 23.2 mg (17%) of the title compound as a white solid. HPLC analysis indicated the compound was 98.4% pure.

1H NMR (300 MHz; CD3OD): δ 7.57 (d, J = 1.9 Hz, 1H), 7.33 (d, J = 8.7 Hz, 1H), 7.17 (m, 5H), 3.63 (t, J = 7.3 Hz, 2H), 2.93 (m, 4H), 2.47 (m, 1H), 1.79 (m, 5H), 1.53-1.22 (m, 5H), 1.16 (t, J = 7.5 Hz, 3H). HRMS analysis calc. for C25H28ClN2O: 407.1890 (M-H+) Observed: 407.1904.

**HEK293 cells**—HEK293 cells were stably transfected with varying CB1 receptor mutations: K3.28192A, F3.36200A, W5.43797A, W6.48356A, F3.25189A, and wild-type. These transfected cell lines express the receptor at approximately 1 pmol/mg protein as previously described (21,22). Cells were maintained at 37°C and 5% CO2 in DMEM + 4.5g/L Glucose supplemented with 10% Foetal bovine serum, 0.7 mg/ml G418 and 0.6% penicillin-streptomycin. These cells were passed approximately twice a week using non-enzymatic cell dissociation solution. When using these cells in the [35S]GTPyS binding assay, cells were starved of foetal bovine serum for 24 hours before being scraped and frozen in a pellet at -20°C.

**[35S]GTPyS binding assay**—0.5mg/ml cell membranes were incubated with the agonist with vehicle or modulator for 60 minutes at 30 °C in assay buffer (50 mM Tris-HCl; 50 mM Tris-Base; 5 mM MgCl2; 1 mM EDTA; 100 mM NaCl; 1 mM DTT; 0.1% BSA) for at least 24 hours. Each reaction tube was washed five times with a 1.2 ml aliquot of ice-cold wash buffer. The filters were oven-dried for at least 60 minutes and then placed in 4 ml of scintillation fluid (Ultima Gold XR, Packard). Radioactivity was quantified by liquid scintillation spectrometry.

**Molecular modeling: conformational analysis of allosteric modulators**—A complete conformational analysis of ORG27569 and PHR015 was performed using ab initio Hartree-Fock calculations at the 6-31G* level as encoded in the Spartan molecular modeling program (Wavefunction, Inc., Irvine, CA), as previously described (23). Specifically, HF 6-31G* 6-fold conformer searches were performed for all rotatable bonds. In each conformer search, local energy minima were identified by rotation of a subject torsion angle through 360° in 60° increments (6-fold search), followed by HF 6-31G* energy minimization of each rotamer generated. To calculate the difference in energy between the global minimum energy conformer of each compound and its final docked conformation, rotatable bonds in the global minimum energy conformer were driven to their corresponding value in the final docked conformation and the single-point energy of the resultant structure was calculated at the HF 6-31G* level. The global minimum energy conformers of ORG27569 and PHR015 were compared by superimposing the two structures on all heavy atoms (see Figure 6C).

**Docking of ORG27569 in the presence of CP55,940**—The results of equilibrium binding assays from the Ross, Kendall, and Ferrans groups (9,13,24), as well as recent structural studies also from the Ferrans group (24), strongly suggest that ORG27569 is inducing an R* conformation of the CB1 receptor (i.e. an intermediate receptor conformation that can bind agonists, yet does not signal in G protein-mediated pathways). However, before docking the allosteric modulators in our previously published model of the CB1R* (active state) receptor (with CP55,940 docked in its global minimum energy conformation, with respect to its ring systems) (25), the extracellular (EC) loops were temporarily removed from the model; this was done to allow a better exploration of potential allosteric binding sites. In addition, the N (residues 1-111) and C (residues 414-472) termini were truncated in our model, as mutation results from...
the Farrens group have shown that ORG27569 is able to antagonize the efficacy of CP55,940 at wild-type levels (in G protein-mediated pathways) at mutant CB₁ receptors in which both the N and C termini have been truncated (24).

ORG27569 (in its global minimum energy conformation) was manually docked in the TMH3-6-7 region of the receptor; this is consistent with the results of equilibrium binding assays that suggest that ORG27569 displaces SR141716A (but not other orthosteric ligands) (13). The automatic docking program, Glide (version 5.7, Schrödinger, LLC, New York, NY, 2011), was then used to explore other possible receptor binding modes of ORG27569. Glide was used to generate a grid based on the centroid of select residues in the binding site (from the manual dock). Standard precision (SP) was selected for the docking setup. Recently, ORG27569 has been observed to act as an inverse agonist in G protein-mediated pathways (9, 14); thus, we hypothesized that ORG27569 may interact with K3.28 192—a residue that we have previously reported to be critical to SR141716A’s inverse agonism (26-28). The geometry of ORG27569’s global minimum energy conformation (as well as the receptor’s topography near K3.28 192) suggested that its piperidine nitrogen would be the most likely hydrogen bond acceptor to interact with K3.28 192. Therefore, the formation of a hydrogen bond between ORG27569’s piperidine nitrogen and K3.28 192 was the only constraint used for the automatic docking of ORG27569. The 26 lowest-energy conformations (≤ 0.67 kcal/mol above global min) of ORG27569 were docked using Glide; the Glide dock with the best geometry between ORG27569’s piperidine nitrogen and K3.28 192 was selected for additional calculations.

Docking of PHR015 in the presence of CP55,940—The geometry of the global minimum energy conformers of ORG27569 and PHR015 are quite similar (see Figure 6C). Therefore, we manually docked PHR015 using the ORG27569 selected Glide output as a guide. The primary difference between the ORG27569 and PHR015 docks is that PHR015 did not form an electrostatic interaction with K3.28 192. The model was then minimized using a 3-stage minimization protocol (described below).

Docking of ORG27569 alone in the R (inactive) conformation of wild-type and F3.25 189 A CB₁—As previously mentioned, ORG27569 acts as an inverse agonist (i.e. reduces basal signaling) of the G protein-mediated pathway when applied alone. This suggests that ORG27569, when applied alone, preferentially binds to an inactive (R) conformation of CB₁. However, before docking ORG27569 in our previously published model of CB₁ in its inactive conformation (27), the helices and loops were pulled apart 2 Å in the x-y plane (a plane that would be parallel to the plane of a lipid bilayer). This was necessary because the binding region of the inactive model of CB₁ was more compact than the active model, and thus pulling the helices apart allowed a better exploration of possible ORG27569 binding modes. The F3.25 189 A inactive model was constructed by mutating F3.25 189 to an alanine after the helices had been pulled apart. Performing the mutation at this point (after pulling the model apart, but before subsequent calculations) provided the model the conformational freedom to respond to the structural consequences of the mutation.

In addition, in recent functional studies from the Farrens lab it was observed that ORG27569 did not act as an inverse agonist (when applied alone) when the N and C terminus were truncated (24). Therefore, the N (S88-N112) and C (S414-L471) termini were modeled using Modeller (as described above). Structures with a low value of the Modeller objective function were chosen. In running Modeller on the C terminus, only the unstructured regions were explored—residues A440-M461 were modeled as an alpha helix that would be parallel to a lipid bilayer (i.e. the same plane as Helix 8); this is consistent with NMR results that suggests the existence of a 'helix 9' in the C terminus (31).
Glide was then used to dock the 26 lowest-energy conformations (≤ 0.67 kcal/mol above global min) of ORG27569, in both the wild-type R and F3.25198 A R models, exactly as described above. The minimization protocol used to minimize these inactive models is described below, with one alteration: only stages 2 and 3 were performed (stage 1 was omitted, as the TMHs needed to first pull together, before allowing the loops/termini to relax).

**Receptor model energy minimization protocol**—The energy of the ligand(s)/CB1 complex, including loop regions, was minimized using the OPLS 2005 force field in Macromodel 9.9 (Schrödinger, LLC, New York, NY, 2011). An 8.0-Å extended nonbonded cutoff (updated every 10 steps), a 20.0-Å electrostatic cutoff, and a 4.0-Å hydrogen bond cutoff were used in each stage of the calculation. The minimization was performed in three stages. In the first stage of the calculation, the ligand(s) and TMH bundle were frozen, but the loops were allowed to relax. The generalized Born/surface area continuum solvation model for water as implemented in Macromodel was used. This stage of the calculation consisted of a Polak–Ribier conjugate gradient minimization in 1000-step increments until the bundle reached the 0.05 kJ/mol gradient. In the second stage, a harmonic constraint was placed on all the TMH backbone torsions (φ, ψ, and ω), with this constraint gradually reduced to zero in 500-step increments (using a total of 2500 steps to reach zero). In addition, a 500 kcal/mol harmonic constraint was placed on the backbone torsions of the loops. No constraints were placed on the ligands during this stage. The minimization consisted of a conjugate gradient minimization using a distance-dependent dielectric, performed in 1000-step increments until the bundle reached the 0.05 kJ/mol gradient. The third stage was performed exactly like the first stage; this was done to allow the loops to adjust to changes that occurred in the TMH region (during the second stage of the minimization).

**Assessment of pairwise interaction and total energies**—Interaction energies between the allosteric modulator and the CB1,R**-CP55,940, wild-type CB1,R, and F3.25198 A CB1,R complexes were calculated using Macromodel, as previously described (25). Specifically, after defining the atoms of the allosteric modulator as one group (group 1) and the atoms corresponding to a residue that lines the binding site in the final ligand/CB1,R** complex as another group (group 2), Macromodel was used to output the pair-wise interaction energy (Coulombic and van der Waals) for a given pair.

**Molecular dynamics simulation of ORG27569 docked in the CB1,R**-CP55,940 complex, and of CP55,940 alone at CB1,R**—The minimized model of ORG27569 docked in CB1,R** (in the presence of CP55,940), as well as the model of CP55,940 (alone) at CB1,R**, was further studied using molecular dynamics. The OPLS2005 force field was utilized with a distance dependent dielectric (coefficient of 2.0 to match the docking studies). The extended nonbonded treatment was employed, as in the minimization procedure discussed above. The dynamics module of Macromodel 9.1 was invoked, using stochastic dynamics at 300K with the use of SHAKE constraints for bonds to hydrogen allowing a 1.5 fs time step. The models were first minimized for 500 steps with restraints on all the heavy atoms, using a large force constant of 4184 kJ/mol. The molecular dynamics was then initialized to 300K, and an initial 100 ps of molecular dynamics was run. Subsequently, these restraints were slowly released for the side chain heavy atoms (4184–0.05 kJ/mol halving in each step for a total of 16 steps), at each step 150 ps of dynamics was performed. Finally, a 22.5 ns molecular dynamics simulation was conducted. Because the goal of this simulation was to explore the dynamic behavior of both ORG27569 and CP55,940 in the binding pocket (as well as CP55,940 alone in its binding site), in these simulations only the amino acid side chains and ligands were free to move. Macromodel was used to calculate the docking energy of ORG27569 and CP55,940, as a function of simulation time, with a resolution of 1 ns.

**RESULTS**

**Chemistry**—A synthesis of a novel cyclohexyl analog of ORG27569, 5-chloro-N-[2-(4-cyclohexylphenyl)ethyl]-3-ethyl-1H-indole-2-carboxamide (PHR015, 9), wherein the piperidine ring is replaced with a cyclohexyl ring was developed for this study. The synthesis of the analog was not known and that of the parent ORG27569 had not yet been reported (32). Thus, commercially available 1-bromo-4-cyclohexylbenzene (1) was metallated via a metal-
halogen exchange with n-butyl lithium and the metallated intermediate captured with dimethylformamide (DMF) to afford the corresponding aldehyde (2) following the patent procedure (17) in 91% yield after chromatographic purification. Condensation of 2 with nitromethane (18) provided the nitrostyrene 3 in 43% yield. The 1H NMR spectrum exhibited trans-coupled vinyl protons. Reduction (19) of the nitrostyrene 3 with lithium aluminum hydride gave the amine 4 in 90% unpurified yield. The electrospray mass spectrum (ES MS) showed the expected M+1 molecular ion.

The indole portion of the molecule was prepared from commercially available ethyl 5-chloroindole-2-carboxylate 5 in essentially the method recently reported (20,32). Thus, starting by acylating 5 with acetyl chloride catalyzed by diethylaluminum chloride to provide the 3-acetyl-indole 6 in 56% yield after chromatographic purification (20). Selective reduction of the keto carbonyl of 6 with triethylsilane in the presence of carbonyl of the ester 8 in 90% yield. The 1H NMR and ES MS supported the expected structures of 5 - 7.

Coupling the amine 4 with the acid 8, mediated by dicyclohexylcarbodiimide and hydroxybenztriazole, provided the target amide 9 in 17% yield after purification by chromatography and trituration. The compound was > 98% pure by HPLC analysis and the structure was supported by 1H NMR and HRMS.

Generation of CB1 receptor mutants—
Since previous studies have demonstrated that in ligand displacement assays, ORG27569 can displace the CB1 antagonist/inverse agonist, SR141716A, mutation studies focused on residues shown to be important for SR141716A binding: K3.28192A, F3.36200A, W6.48356A, and W5.43279A (21,22,27,28), as well as an aromatic binding pocket residue shown not to be important for SR141716A binding, F3.25189A (21,22).

[^35]S[GTPγS binding assays: functional analysis of ORG27569 at wild-type and mutant CB1 receptors—[^35]S[GTPγS binding assays were used to measure the stimulation of GTPγS binding at wild-type and mutant CB1 receptors upon addition of CP55,940, in the presence (or absence) of ORG27569;[^35]S[GTPγS binding assays were also used to measure the ability of ORG27569 to act as an inverse agonist at wild-type and mutant CB1 receptors. In wild-type cells, ORG27569 (1 µM) abolished the effect of CP55940, such that there was a reduction in the basal[^35]S[GTPγS binding (see Figure 2A). In line with this, ORG27569 behaved as an inverse agonist in WT cells, displaying a level of inverse efficacy in line with that of SR141716A (see Figure 2B). K3.28192A—CP55,940 has an EC50 value of 225nM (95% CL: 55 - 923) when applied alone at the K3.28192A mutant; this EC50 is significantly larger than wild-type (1.3nM; 95% CL: 0.3 - 5) and suggests that this mutation does influence CP55,940’s activity. This result is consistent with CB1 K3.28192A mutation studies that showed a significant loss of binding affinity and efficacy for the classical cannabinoid, HU-210, the non-classical cannabinoid, CP55,940, and the endogenous cannabinoid, anandamide (33). In cells expressing the K3.28192A mutant, ORG27569 lost the ability to completely antagonize the efficacy of CP55,940 (see Figure 2C). Thus, 1 µM ORG27569 produced a complete antagonism in WT cells (see Figure 2A), but only an inhibition of 41% at the same concentration in cells expressing the K3.28192A mutant (see Table 1, Figure 2C).

Figure 2D, shows that for WT CB1, ORG27569 at concentrations of 0.1 µM, 1 µM and 10 µM produced a statistically significant decrease in basal[^35]S[GTPγS binding, rendering it an inverse agonist. Figure 2D also suggests a trend towards inverse agonism for ORG27569 at the K3.28192A mutant, however the decrease in basal[^35]S[GTPγS binding even at the highest concentration of 10 µM did not reach statistical significance. We were not able to test higher concentrations of ORG27569 due to solubility issues. Regardless, these results clearly illustrate that ORG27569’s inverse agonism is greatly attenuated at the K3.28192A mutant. Altogether, these results suggest that K3.28192A is part of ORG27569’s binding site; in addition, these results suggest that an interaction with K3.28192 may be important (though not unequivocally required) for ORG27569’s inverse agonism.

F3.36200A and W6.48356A—ORG27569 completely antagonized the efficacy of CP55,940 at both the F3.36200A and W6.48356A mutant (see Table 1, Figure 3A, 3C). If ORG27569 interacted...
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significantly with either of these residues, one would expect that these mutations would reduce the ability of ORG27569 to antagonize the efficacy of CP55,940. Also like wild-type, ORG27569 acted as an inverse agonist when applied alone (at either of these mutants), as well as in the presence of CP55,940 (see Figure 3B, 3D and Figure 3A, 3C, respectively). Therefore, these results suggest that neither F3.36200 nor W6.48356 is part of ORG27569’s binding site.

W5.43279A—ORG27569 was unable to antagonize the efficacy of CP55,940 at the W5.43279A mutant at a concentration that abolished the effect of CP55940 in WT cells (see Table 1, Figure 4A). If ORG27569 interacted significantly with W5.43279, one would expect that the W5.43279A mutation would reduce the ability of ORG27569 to antagonize the efficacy of CP55,940—but not completely eliminate its ability to antagonize CP55,940. Also unlike wild-type, ORG27569 was unable to act as an inverse agonist when applied alone at the W5.43279A mutant (see Figure 4B), nor in the presence of CP55,940 (see Figure 4A). W5.43279 is a large, central binding pocket residue in the TMH3-4-5 region of CB1. We have shown previously that while the W5.43279A mutation does not affect CP55,940 binding or signaling, it has a profound effect on CP55,940’s signaling or act as an inverse agonist. Our computational results suggested that ORG27569’s piperidine ring nitrogen was the most likely hydrogen bond acceptor to interact with K3.28192. Therefore, we synthesized and characterized (using [35S]GTPγS binding assays, at wild-type and K3.28192A CB1) an analog of ORG27569 (PHR015, see Figure 1), in which ORG27569’s piperidine ring has been replaced with a cyclohexyl ring, eliminating the hydrogen bond accepting capability in this ring.

PHR015 was unable to completely antagonize the efficacy of CP55,940 at wild-type CB1 (see Table 1, Figure 5A) and results for PHR015 at the K3.28192A mutant were quite similar (see Table 1, Figure 5B). If there is a direct interaction between the ORG27569 piperidine nitrogen and K3.28192, then one would expect significant effects if the ligand is “mutated”, i.e.
ORG27569 → PHR015 and tested at WT CB1 or the amino acid is mutated, WT CB1 → K3.28192A and tested with ORG27569.

As previously discussed, PHR015 was unable to completely antagonize the efficacy of CP55,940 at wild-type CB1, nor was ORG27569 able to completely antagonize the efficacy of CP55940 at the K3.28192A mutant. More importantly, these two E_max’s are not statistically different from each other [E_max= 31% (9-53) for PHR015, E_max= 41% (17-66) for ORG27569]. The decrease in efficacy due to the deletion of ligand functionality may result from a loss in binding energy, whereas a decrease in efficacy due to receptor residue substitution may come from conformational contributions. These losses may have similar magnitudes, even if the deleted groups do not directly interact with each other (34). Therefore, key to the determination of whether deletions have occurred between two groups that interact indirectly or directly is the effect produced by simultaneous deletion of both groups (i.e., PHR015 at CB1 K3.28192A). If the modified groups do not interact directly with each other in the WT state then the effect of the two simultaneous changes will be additive. If the interaction is a direct one, deletion of ligand functionality plus mutation of the amino acid will not be additive, but should be comparable to either of the single changes. PHR015 decreased the E_max of CP55940 to an extent at the K3.28192A mutant (E_max= 15% (-10-39) see Table 1 and Figure 5B) that is not statistically significantly different from the single change cases listed above. Therefore, these results suggest that a direct interaction occurs between the ORG27569 piperidine nitrogen and residue K3.28192 in WT CB1.

The same conclusion can be reached by considering the effect on [35S]GTPγS binding produced by ORG27569 vs. PHR015 at WT CB1 or the K3.28192A mutant when each compound is applied alone. Figure 2D shows that at concentrations of 0.1 µM, 1 µM and 10 µM, ORG27569 has a statistically significant inverse effect on basal [35S]GTPγS signaling. In contrast, although ORG27569 at the K3.28192A mutant showed a trend towards an inverse effect on basal [35S]GTPγS binding at higher concentrations, this effect did not achieve statistical significance (Figure 2D). Figure 5C shows that PHR015 has no dose dependent effect upon basal [35S]GTPγS signaling at WT CB1 when applied alone (see Figure 5C). Therefore, using the logic of the mutant cycle as described previously, key to the determination of whether deletions have occurred between two groups that interact indirectly or directly is the effect produced by simultaneous deletion of both groups (i.e., PHR015 at CB1 K3.28192A). Figure 5C shows that PHR015 also has no dose dependent effect on [35S]GTPγS binding at the K3.28192A mutant when applied alone. This result suggests a direct interaction between the piperidine nitrogen of ORG27569 and K3.28192 in WT CB1 is important for ORG27569 to act as an inverse agonist.

**Modeling studies: conformational analysis**—Figure 6A illustrates the global minimum energy conformer (hereafter ‘global min’) of ORG27569 (orange), superimposed on its final docked conformer (lime green); it is clear from this figure that the docked conformation of ORG27569 is quite similar to its global min. The energetic cost of ORG27569 adopting its final docked conformation was calculated to be 0.72 kcal/mol.

Figure 6B illustrates the global min of PHR015 (mauve), superimposed on its final docked conformer (lavender); it is clear from this figure that the docked conformation of PHR015 is quite similar to its global min. The energetic cost of PHR015 adopting its final docked conformation was calculated to be 0.44 kcal/mol.

Finally, Figure 6C illustrates the global min of ORG27569 (orange), superimposed on the global min of PHR015 (mauve); it is clear from this figure that the global mins of ORG27569 and PHR015 are quite similar. These results indicate that ORG27569 and PHR015 may assume similar conformations, suggesting that both ligands may bind at a similar region of CB1.

**Creation of the CB1R** **complex: docking** ORG27569 (in the presence of CP55,940) in the CB1R model—The CB1R** model represents a receptor state that is promoted by ORG27569 in the presence of CP55,940. As such, it exists only in complex with ORG27569 and CP55,940. This state is one that would promote the binding of agonist, yet not signal via G protein-mediated pathways. The CB1R** complex model was derived from our previously published activated state CB1R model (25), and differs from the R** model primarily on the extracellular side of the receptor.
in the conformations of the EC-2 and EC-3 loops and the EC end of TMH6. The conformations of these components are restricted by the presence of ORG27569 and CP55,940.

Before docking ORG27569, the global min of CP55,940 (with respect to its ring systems) was docked at its previously identified binding site (35)—the TMH1-2-3-7 region of CB1 (i.e. the ‘classical/endogenous cannabinoid’ binding site). In general, CP55,940 is oriented so that its three hydroxyl groups are near the extracellular face of the receptor; in contrast, its hydrophobic dimethylheptyl tail is positioned lower, within the transmembrane core. Most interestingly, CP55,940 forms an important hydrogen bond with K3.28\(^{192}\); this is consistent with mutation studies that indicate CP55,940 has a reduced binding affinity at the K3.28\(^{192}\)A mutant (33). For additional information regarding CP55,940’s binding site at the CB1 receptor, see reference (35).

Figure 7 illustrates ORG27569 (lime green) docked at its binding site, in the presence of CP55,940 (cyan). Residues that contribute 5.5% (or more) of ORG27569’s total interaction energy with the CB1 receptor-CP55,940 complex are shown in orange; K3.28\(^{192}\) is shown in bright orange. ORG57569’s net interaction energy with the receptor-CP55,940 complex is -56.22 kcal/mol. The binding site of ORG27569 was identified by Glide docking studies to be in the TMH3-6-7 region of CB1 (see Figure 7); this is consistent with our recently published mutation results that suggest that ORG27569 does not bind at the W5.43\(^{279}\)A mutant CB1 receptor (14).

Ligands that bind at the TMH3-4-5-6 region of the CB1 receptor have little to no binding affinity at the W5.43\(^{279}\)A mutant — this is likely due to a gross conformational change that occurs in this region of the receptor upon removal of this large, central residue (21,22).

Figure 8 illustrates that ORG27569’s binding site overlaps with our previously identified binding site for SR141716A (22,26,27); this is consistent with the results of equilibrium binding studies that suggest that ORG27569 displaces SR141716A (13). However, ORG27569’s binding site is more extracellular than SR141716A’s binding site. These results are consistent with the results of our mutation studies that indicate that ORG27569’s ability to influence CP55,940’s binding and signaling is unaffected at the F3.36\(^{200}\)A and W6.48\(^{356}\)A mutants (see Table 1, Figures 3A, 3C)—suggesting that ORG27569 does not bind low enough in the receptor to directly interact with F3.36\(^{200}\) or W6.48\(^{356}\). In contrast, we have previously reported that these residues are part of the SR141716A binding site (22).

The results of our pairwise interaction energy calculations suggest that ORG27569’s most important interaction is with residue F7.35\(^{379}\); this is likely because ORG27569 forms several aromatic stacking interactions with F7.35\(^{379}\) (see Figure 7). F7.35\(^{379}\) forms an off-parallel aromatic stack with ORG27569’s indole ring (both rings A and B, see Figure 1); the ring centroid to centroid distances are 6.02 Å and 4.75 Å, and the angles between the ring planes are 5.71° and 9.42° (for rings A and B, respectively). F7.35\(^{379}\) also forms an aromatic T-stack interaction with ORG27569’s phenyl ring (ring C); the ring centroid to centroid distance is 5.60 Å and the angle between the ring planes is 64.60° (see Figures 1, 7). Together, these aromatic interactions help position ORG27569 in the receptor, placing a significant amount of ORG27569’s steric bulk against the extracellular (EC) end of TMH6. These results are consistent with recently published structural studies from the Farrens lab that suggest that ORG27569 antagonizes CP55,940’s efficacy (in part) by preventing a necessary conformational change of TMH6 during receptor activation (24).

ORG27569’s indole ring (both rings A and B) also forms an aromatic T-stack interaction with F268 (an EC-2 loop residue, see Figure 7); the ring centroid to centroid distances are 5.63 Å and 4.95 Å, and the angles between the ring planes are 66.70° and 69.79° (for rings A and B, respectively). The importance of the interaction between ORG27569 and F268 is discussed later (see ORG27569 Prevents Extracellular (EC) Loop Conformational Changes Critical for Signal Transduction).

Additionally, ORG27569’s piperidine nitrogen forms an important hydrogen bond with K3.28\(^{192}\); the N-N distance is 3.00 Å and N-H...N angle is 124.5°. This interaction is almost as important to ORG27569’s interaction energy as F7.35\(^{379}\) (-6.32 kcal/mol and -7.30 kcal/mol, respectively; see Figure 7). The predicted importance of this residue’s interaction with
ORG27569 is consistent with our mutation results that suggest that ORG27569 has a significantly reduced ability to antagonize CP55,940’s efficacy at the K3.28192A mutant (see Table 1, Figure 2A, 2C). We also hypothesized that this interaction may be important for ORG27569’s ability to act as an inverse agonist; this is because we have previously shown that K3.28192 was required for the inverse agonism of SR14716A (21,26,27). Our mutation results are consistent with this hypothesis; at concentrations up to 10 µM, ORG27569 was unable to significantly act as an inverse agonist at the K3.28192A mutant (see Figure 2D).

Finally, ORG27569 has significant van der Waals interactions (in order of importance) with CP55,940, I6.54362, M371, and Y6.57365; ORG27569 also forms significant Columbic and van der Waals interactions with D6.58366 (see Figure 7). Interestingly, with the exception of CP55,940, all of these residues are found on TMH6 or the EC-3 loop; again, this is consistent with results from the Farrens lab that suggest that ORG27569 antagonizes CP55,940’s efficacy (in part) by preventing a necessary conformational change of TMH6 during receptor activation (24).

Docking of PHR015 (in the presence of CP55,940) in the CB1R** complex—The chemical structures of ORG27569 and PHR015 are extremely similar (the only difference between them is that ORG27569 has a piperidine ring and PHR015 has a cyclohexyl ring, see Figure 1); therefore, it is not surprising that the global min of these two structures are nearly identical (see Figure 6C). Thus, PHR015 was docked in the same general region (TMH3-6-7) of the receptor as ORG27569 (see Figure 9).

Figure 9 illustrates PHR015 (lavender) docked at its binding site, in the presence of CP55,940 (cyan); residues that contribute 5.5% (or more) of PHR015’s total interaction energy with the CB1R** receptor-CP55,940 complex are shown in orange; K3.28192 is shown in mauve (and does NOT significantly contribute to PHR015’s total interaction energy). PHR015’s net interaction energy with the CB1R** receptor-CP55,940 complex is -49.69 kcal/mol.

The results of our total interaction energy calculations suggest that PHR015 has a significantly reduced net interaction energy with the receptor-CP55,940 complex compared to ORG27569 (-49.69 kcal/mol and -56.22 kcal/mol, respectively). The results of our pairwise interaction energy calculations suggest that this difference in net interaction energy is due almost exclusively to each ligand’s respective ability/ inability to form a hydrogen bond with K3.28192 (i.e. PHR015 has a weaker net interaction energy than ORG27569, because its cyclohexyl ring cannot form a hydrogen bond with K3.28192; see Figures 7, 9). These observations are consistent with the results of the [35S]GTPγS binding assays; PHR015 was not able to completely antagonize the efficacy of CP55,940 (see Table 1, Figure 5A).

We have previously shown that K3.28192 is required for the inverse agonism of SR14716A (21,26,27) and we have shown here that ORG27569 also acts as an inverse agonist and interacts with K3.28192. Our docking studies indicate that while PHR015 occupies the same binding pocket location as ORG27569, removal of the piperidine nitrogen renders PHR015 incapable of interacting with K3.28192. This would suggest that PHR015 should not be an inverse agonist. Consistent with this hypothesis, in [35S]GTPγS binding assays (see Figure 5C), PHR015 did not act as an inverse agonist. Together, these results for ORG27569 and PHR015 strongly suggest that an interaction between ORG27569’s piperidine nitrogen and K3.28192 is important for the ability of ORG27569 to bind at the CB1 receptor, and that this interaction is important for ORG27569 to act as an inverse agonist.

Analogous to ORG27569, the results of our pairwise interaction energy calculations suggest that PHR015’s most important interaction is with residue F7.35379; this is likely because PHR015 forms several aromatic stacks with F7.35379 (see Figure 9). F7.35379 forms an off-set parallel aromatic stack with PHR015’s indole ring (both rings A and ring B, see Figures 1, 9); the ring centroid-ring centroid distances are 5.56 Å and 4.24 Å, and the angles between the ring planes are 7.50° and 8.26° (for rings A and B, respectively). F7.35379 also forms an aromatic T-stack interaction with PHR015’s phenyl ring (ring C); the ring centroid-ring centroid distance is 6.00 Å and the angle between the ring planes is 61.36° (see Figure 1). Together, these aromatic interactions help position PHR015 in the receptor, placing a significant amount of PHR015’s steric...
bulk against TMH6. These results suggest that (like ORG27569) PHR015 antagonizes CP55,940’s efficacy (in part) by preventing a necessary conformational change of TMH6.

Like ORG27569, PHR015’s indole ring (both rings A and B) also forms an aromatic T-stack interaction with F268 (a EC-2 loop residue) (see Figure 9); the ring centroid-ring centroid distances are 6.14 Å and 5.68 Å, and the angles between the ring planes are 86.46° and 87.54° (for rings A and B, respectively). The importance of the interaction between PHR015 and F268 is discussed later (see Figure 9).

Finally, PHR015 has significant van der Waals interactions (in order of importance) with I6.54, CP55,940, I375, and Y6.57. PHR015 also forms significant Coulombic and van der Waals interactions with D6.58 and K7.32 (see Figure 9). Interestingly, with the exception of CP55,940 and K7.32, all of these residues are found on TMH6 or the EC-3 loop; again, these results suggest that (like ORG27569) PHR015 antagonizes CP55,940’s efficacy (in part) by preventing a necessary conformational change of TMH6.

ORG27569 prevents extracellular loop conformational changes critical for signal transduction—We have previously reported that two extracellular (EC) loop conformational changes must occur in order for the CB₁ receptor to signal via G protein-mediated pathways (25). Specifically, our modeling results, based on mutation studies from the Kendall lab (36,37), suggested that the EC-2 loop moves down towards the transmembrane core upon receptor activation (see Figure 10A). Figure 10A illustrates that this EC-2 loop conformational change places F268 in close proximity to CP55,940 (25). In addition, we also reported that upon receptor activation an important ionic interaction forms between TMH2 and the EC-3 loop (specifically, an interaction forms between residues D2.63 and K373); this ionic interaction is necessary for signal transduction and promotes a conformation of the EC-3 loop that is pulled over the top (extracellular face) of the receptor (see Figure 11A) (25).

As described in Methods, low energy EC loop conformational changes were added to our model of ORG27569 docked at the CB₁R** receptor (in the presence of CP55,940). Importantly, none of the EC-2 loop Modeller output conformations placed the EC-2 loop near the transmembrane core, nor F268 in close proximity to CP55,940. Figure 10B clearly illustrates why these results were observed; ORG27569’s indole ring sterically blocks the EC-2 loop from moving towards the transmembrane core. Specifically, F268 has formed an aromatic T-stack interaction with ORG27569’s indole ring, preventing the EC-2 loop from adopting its active conformation.

Likewise, none of the EC-3 loop Modeller output conformations had the D2.63 and K373 interaction formed, nor did any of the output structures have the EC-3 loop pulled over the top (extracellular face) of the receptor. Figure 11B clearly illustrates why these results were observed; ORG27569’s indole ring sterically blocks the EC-3 loop from pulling across the top of the receptor. Consequently, the presence of ORG27569’s steric bulk makes it essentially impossible for the interaction between D2.63 and K373 to form. Together, these results suggest that ORG27569 antagonizes the efficacy of CP55,940 by sterically blocking the necessary conformational changes of the EC-2 and EC-3 loops during activation (in addition to sterically blocking conformational changes of TMH6, as previously described).

CP55,940/Receptor Pairwise and Total Interaction Energies—The results of recently published binding assays illustrate that ORG27569 does not significantly affect CP55,940’s K_d, but does significantly increase the measured B_max (in a concentration-dependent manner) (14). This suggests that ORG27569 may increase the equilibrium binding of CP55,940 by shifting the population of receptors from an inactive conformation (R) to an intermediate conformation (R**), and that ORG27569 is NOT increasing the binding affinity of CP55,940. To test if our models agreed with these results, CP55,940’s total interaction energies were calculated for: CP55,940-alone at the CB₁R* receptor, CP55,940 (in the presence of ORG27569) in the CB₁R** complex, and CP55,940 (in the presence of PHR015) in the CB₁R** complex. CP55,940’s net interaction energy (includes conformational expense) was calculated to be -53.37, -53.38, and -
51.87 kcal/mol for CP55,940 alone in the receptor, and in the presence of ORG27569 and PHR015, respectively). These results suggest that neither the presence of ORG27569 or PHR015 resulted in a significant change in the net interaction energy between CP55,940 and the receptor/allosteric modulator complex. Together, these results indicate that our computational models are consistent with the results of the equilibrium binding assays.

Molecular dynamics simulation of ORG27569 docked in the CB1R*-CP55,940 complex, and of CP55,940 alone at CB1R*—In order to elucidate the dynamic behavior of ORG27569 and CP55,940 with the receptor (as well as each other), a 22.5 ns molecular dynamics simulation was run on the final CB1R*-CP55,940-ORG27569 complex (shown in Figure 7). Specifically, the simulations were run for three reasons: (1) to observe if the interaction (as proposed in the static model) between ORG27569 and K3.28192 persists in a dynamic simulation; (2) to observe if the interaction (as proposed in the static model) between CP55,940 and K3.28192 persists in a dynamic simulation; (3) to observe how the interaction energy of ORG27569 and CP55,940 evolve throughout the simulation.

As predicted by the static model (of CB1R**-CP55,940-ORG27569 complex), the interaction between ORG27569’s piperidine nitrogen and K3.28192 persisted throughout the entire simulation. The average distance between ORG27569’s piperidine nitrogen and K3.28192’s terminal nitrogen was 3.19 Å; furthermore, this distance did not significantly deviate from the average over the course of the trajectory. Together, the static and dynamic models are consistent with our experimental results that suggest that ORG27569’s piperidine nitrogen forms an important and persistent interaction with K3.28192.

Interestingly, the interaction between CP55,940 (in the presence of ORG27569) and K3.28192 did not persist throughout the simulation. This interaction breaks early in the simulation and does not reform. The average distance between CP55,940’s alkyl-hydroxyl (southern aliphatic hydroxyl, SAH) oxygen and K3.28192’s terminal nitrogen was 6.80 Å; the average distance between CP55,940’s phenolic oxygen and K3.28192’s terminal nitrogen was 5.83 Å. To determine if the loss of this interaction was due to the presence of ORG27569, a molecular dynamics simulation was run of our static model of CP55,940-alone docked at CB1R* (employing the same methodology used for the CB1R**-CP55,940-ORG27569 complex). In this simulation, the interaction between CP55,940 and K3.28192 persisted throughout the entire simulation. The average distance between CP55,940’s SAH oxygen and K3.28192’s terminal nitrogen was 3.41 Å (note—the hydrogen bond between CP55,940’s SAH and K3.28192 breaks briefly between 15-18 ns (observed as a sharp increase in distance); however, this interaction quickly reforms and persists for the rest of the simulation). The average distance between CP55,940’s phenolic oxygen and K3.28192’s terminal nitrogen was 3.12 Å. Furthermore, these distances did not significantly deviate from their averages over the course of the trajectory. This result is consistent with our experimental results that suggest that when CP55,940 is applied alone, it forms a significant interaction with K3.28192. Together, these results suggest that ORG27569 may weaken the interaction between CP55,940 and K3.28192, resulting in a more transient interaction between CP55,940 and K3.28192. Intriguingly, the loss of the interaction between CP55,940 and K3.28192 did not decrease CP55,40’s total interaction energy with the model, as described below.

To determine if the presence of ORG27569 affects the interaction energy of CP55,940 in a dynamic system, we calculated the interaction energy of CP55,940 in the CB1R*-CP55,940-ORG27569 complex as a function of time. As predicted by the static model, CP55,940’s interaction energy did not significantly change over the course of the trajectory; CP55,940’s average interaction energy (over the course of the trajectory) was -54.62 kcal/mol. Furthermore, the energy did not significantly deviate from the average over the course of the trajectory. This was surprising considering CP55,940’s loss of an interaction with K3.28192. However, after inspecting the simulation, the reason became clear—the tremendous flexibility of CP55,940’s SAH substituent allows it to easily and consistently find other hydrogen bond partners to compensate.

In addition, the interaction energy of CP55,940-alone in CB1R* was calculated over the
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course of its trajectory; CP55,940’s average interaction energy (over the course of the trajectory) was -53.19 kcal/mol. Consistently, CP55,940’s interaction energy did not significantly change over the course of the trajectory, nor was it significantly different than CP55,940’s interaction energy in the CB\textsubscript{1}R\textsuperscript{−−}-CP55,940-ORG27569 complex. Finally, the interaction energy of ORG27569 (in the CB\textsubscript{1}R\textsuperscript{−−}-CP55,940-ORG27569 complex) was calculated as a function of time; ORG27569’s interaction energy did not significantly change over the course of the trajectory. Together, the results of our static and dynamic models are consistent with our experimental results that suggest that ORG27569 does not influence CP55,940’s binding affinity for the CB\textsubscript{1} receptor.

Docking of ORG27569 (alone) in the wild-type and F3.25\textsuperscript{189} A CB\textsubscript{1}R (inactive) models—As previously discussed, our experimental results suggest that when applied alone at wild-type CB\textsubscript{1}, ORG27569 acts as an inverse agonist (in G protein-mediated pathways). In addition, the results of our mutant cycle suggest that this inverse agonism is related to the formation of an interaction between ORG27569’s piperidine nitrogen and K3.28\textsuperscript{192}. However, we observed that ORG27569 was also unable to act as an inverse agonist at the F3.25\textsuperscript{189} A mutant, whereas it was still able to antagonize CP55,940’s efficacy at this mutant. These results may suggest that ORG27569 does not influence CP55,940’s binding affinity for the CB\textsubscript{1} receptor.

Figure 12 illustrates ORG27569 (lime green) docked alone at its binding site in the wild-type CB\textsubscript{1}R (inactive) model. Residues that contribute 5.5\% (or more) of ORG27569’s total interaction energy with the CB\textsubscript{1}R model are shown in orange; K3.28\textsuperscript{192} is shown in bright orange. ORG57569’s net interaction energy with the receptor is -52.06 kcal/mol. ORG27569’s binding site in CB\textsubscript{1}R is quite similar to its binding site in the CB\textsubscript{1}R\textsuperscript{−−}-CP55,940-ORG27569 complex. The binding site of ORG27569 was identified to be in the TMH3-6-7 region of CB\textsubscript{1} (see Figure 12); this is consistent with our recently published mutation results that suggest that ORG27569 does not bind at the W5.43\textsuperscript{279} A mutant CB\textsubscript{1} receptor (14). Ligands that bind at the TMH3-4-5-6 region of the CB\textsubscript{1} receptor have little to no binding affinity at the W5.43\textsuperscript{279} A mutant—this is likely due to a gross conformational change that occurs in this region of the receptor upon removal of this large, central residue (21,22).

As in the CB\textsubscript{1}R\textsuperscript{−−}-CP55,940 model, ORG27569’s binding site (when docked alone) is more extracellular than SR141716A’s binding site. These results are consistent with the results of our mutation studies that illustrate that ORG27569’s ability to act as an inverse agonist is unaffected at the F3.36\textsuperscript{200} A and W6.48\textsuperscript{356} A mutants (see Table 1, Figures 3B, and 3D)—suggesting that ORG27569 does not bind low enough in the receptor to directly interact with F3.36\textsuperscript{200} or W6.48\textsuperscript{356}. In contrast, we have previously reported that these residues are part of the SR141716A binding site (22).

The results of our pairwise interaction energy calculations suggest that, when docked alone, ORG27569’s most important interaction is with residue D6.58\textsuperscript{366}. In addition, as in the CB\textsubscript{1}R\textsuperscript{−−} complex, ORG27569’s piperidine nitrogen forms an important hydrogen bond with K3.28\textsuperscript{192} (see Figure 7 and Figure 12, respectively). The N-N distance is 2.90 Å and N-H...N angle is 152.8°. This interaction is almost as important to ORG27569’s interaction energy as D6.58\textsuperscript{366} (-7.43 kcal/mol and -9.39 kcal/mol, respectively; see Figure 12). We also hypothesized that this interaction may be important for ORG27569’s ability to act as an inverse agonist; this is because we have previously shown that K3.28\textsuperscript{192} was required for the inverse agonism of SR14716A (21,26,27). Our mutation results are consistent with this hypothesis; at concentrations up to 10 µM, ORG27569 was unable to significantly act as an inverse agonist at the K3.28\textsuperscript{192} A mutant (see Figure 2D).

In addition, ORG27569 forms aromatic interactions with three residues. First, ORG27569’s indole ring (both rings A and B) also forms an aromatic T-stack interaction with F268 (an EC-2 loop residue; see Figure 12); the ring centroid to centroid distances are 5.25 Å and 4.86 Å, and the angles between the ring planes are 84.28° and 95.60° (for rings A and B, respectively). Second, ORG27569’s indole ring...
(ring A) forms an aromatic T-stack interaction with F108 (an N terminus residue); the ring centroid to centroid distance is 4.97 Å, and the angle between the ring planes is 128.48°.

Finally, unlike in the CB₁R⁺⁺ model, Figure 12 illustrates that in the CB₁R model ORG27569’s indole ring (ring A, see Figure 1); forms a significant aromatic stack with F3.25¹⁸⁹; the ring centroid to centroid distance is 5.51 Å, and the angle between the ring planes is 43.71°. F3.25¹⁸⁹ also forms an aromatic T-stack interaction with ORG27569’s phenyl ring (ring C); the ring centroid to centroid distance is 5.36 Å and the angle between the ring planes is 119.72° (see Figure 12). While this aromatic stack does significantly contribute to ORG27569’s interaction energy with the CB₁R model, it may serve a more important structural role. Specifically, this aromatic interaction between ORG27569 and F3.25¹⁸⁹ may help sterically orient ORG27569 in the receptor so that it can form a hydrogen bond with K3.28¹⁹².

To test this hypothesis, we also used Glide to dock ORG27569 at the F3.25¹⁸⁹A mutant CB₁R model, using the exact same protocol as used for the wild-type model. Figure 13 illustrates ORG27569 (lime green) docked alone at its binding site in the F3.25¹⁸⁹A mutant CB₁R model. Residues that contribute 5.5% (or more) of ORG27569’s total interaction energy with the CB₁R receptor are shown in orange; K3.28¹⁹² is shown in mauve (and does form a modest hydrophobic interaction with ORG27569, though it does not form a hydrogen bond with ORG27569’s piperidine nitrogen). ORG57569’s net interaction energy with the receptor is –37.17 kcal/mol. Figure 13 illustrates that ORG27569’s binding site at the F3.25¹⁸⁹A mutant is similar to wild-type. Nonetheless, there is a profound difference; without the steric bulk provided by F3.25¹⁸⁹, ORG27569 positions its indole ring much closer the backbone of TMH3. This orientation greatly damages ORG27569’s ability to form several aromatic interactions that form in the wild-type model, resulting in a significantly reduced interaction energy compared to the wild-type model. However, ORG27569 does form an aromatic T-stack interaction with F268; the ring centroid to centroid distance is 5.74 Å and the angle between the ring planes is 89.73°. In addition, this orientation also makes it sterically impossible for ORG27569’s piperidine nitrogen to form a significant interaction with K3.28¹⁹². This lack of an interaction with K3.28¹⁹² is consistent with our mutation results that suggest ORG27569 cannot act as an inverse agonist at the F3.25¹⁸⁹A mutant receptor. Altogether, our computational and experimental results suggest that ORG27569 forms a significant interaction with F3.25¹⁸⁹ when applied alone (that it doesn’t necessarily form in the presence of CP55,940), and that this interaction is important in sterically orienting ORG27569 so that its piperidine nitrogen can form a hydrogen bond with K3.28¹⁹².

**DISCUSSION**

**Identification of ORG27569’s binding site at the CB₁ receptor**—In the present study, we have used computational methods together with mutagenesis, synthesis, and pharmacological studies to identify an allosteric binding site for ORG27569 at the CB₁ receptor and to probe its relationship to G protein signaling effects. Our results suggest that ORG27569 binds in the TMH3-6-7 region of the CB₁ receptor. In this region, ORG27569’s binding site overlaps with our previously reported binding site for SR141716A (but not CP55,940) (21,26,27)—consistent with the results of equilibrium binding assays that illustrate that SR141716A is displaced by ORG27569 (13). This allosteric site is also consistent with our results that suggest that, at the W5.43²⁷⁹A mutant, ORG27569 cannot antagonize the efficacy of CP55,940, nor act as an inverse agonist. To our knowledge, all cannabinoids that bind in the TMH3-4-5-6 region have little or no affinity (nor efficacy) at the W5.43²⁷⁹A mutant, suggesting that this mutation may cause a gross conformational change in this region of the receptor (21,22). Finally, our results suggest that ORG27569 forms a distinct interaction with F3.25¹⁸⁹ when applied alone (that it does not necessarily form when applied with CP55,940). More importantly, we observed that this interaction is important for sterically orienting ORG27569 so that its piperidine nitrogen can form a hydrogen bond with K3.28¹⁹².

An interaction between ORG27569’s piperidine nitrogen and K3.28¹⁹² is important for ORG27569’s potent antagonism of CP55,940 and important for its inverse agonism—We report
here that ORG27569 acts as an inverse agonist at CB1 when applied alone by reducing basal activity. Our previous combined mutation cycle/modeling studies have identified the binding site of the CB1 antagonist, SR141716A (21,22,26). These studies identified an interaction between the carboxamide oxygen of SR141716A and K3.28192 that is possible only in the inactive state of CB1. It is this interaction that gives SR141716A a higher affinity for the inactive state of CB1, thereby rendering it an inverse agonist. At a K3.28192A mutant, SR141716A acts as a neutral antagonist. Analogs that lack the carboxamide oxygen such as VCHSR and PIMSR are neutral antagonists at wild-type CB1 (26,27).

Similarly, we report here the results of a mutant cycle performed to study the model-proposed interaction between ORG27569’s piperidine nitrogen and K3.28192. We observed that ORG27569’s ability to antagonize CP55,940 was reduced at the K3.28192A mutant (see Figure 2C). This result suggests that an interaction with K3.28192 is important for ORG27569’s ability to antagonize CP55,940 efficacy. PHR015 was designed to test if the piperidine nitrogen was the interaction site for K3.28192 and results reported here are consistent with this hypothesis. Specifically, PHR015 did not antagonize CP55,940 at WT CB1 (see Figure 5A). Importantly, PHR015’s ability to antagonize CP55,940 was unaffected by the K3.28192A mutation (see Figure 5). These results strongly suggest that an interaction between ORG27569’s piperidine nitrogen and K3.28192 is important for ORG27569’s ability to antagonize CP55,940; this is because removal of either ORG27569’s piperidine nitrogen, or removal of K3.28192, reduced ORG27569’s ability to antagonize CP55,940, but the effects of removing both the piperidine nitrogen and K3.28192 were not additive.

Likewise the same mutant cycle was performed to explore whether an interaction with K3.28192 is important for ORG27569’s ability to reduce basal signaling. While ORG27569 is an inverse agonist at WT CB1, at the K3.28192A mutant, ORG27569 shows a trend toward inverse agonism at high concentrations but the effect did not reach statistical significance (see Figure 2D). Further, PHR015 did not act as an inverse agonist at wild-type CB1 (see Figure 5C), nor at the K3.28192A mutant. Using the same logic of the mutant cycle (as just described), these results suggest that an interaction between ORG27569’s piperidine nitrogen and K3.28192 may be important (though not unequivocally required) for ORG27569 to act as an inverse agonist. Thus, there is a parallel between SR141716A and ORG27569 action, suggesting that ORG27569 is both a negative allosteric modulator in the presence of CP55,940, but also an inverse agonist when applied alone.

The binding site of ORG27569 is more extracellular than the binding site of SR141716A—Our results suggest that ORG27569’s binding site overlaps with the SR141716A binding site, but extends more extracellularly than that of SR141716A; specifically, ORG27569 does not bind low enough in the receptor to significantly interact with F3.36200 or W6.48356. In contrast, we have reported that both of these residues are part of the binding site of SR141716A (21,22). Therefore, these results support our hypothesis that ORG27569’s binding site is more extracellular than the binding site of SR141716A.

ORG27569 does not sterically block CP55,940 from exiting the CB1 receptor—It is not surprising that our results suggest that ORG27569 binds at a partially extracellular (EC) region of the CB1 receptor, considering that many allosteric binding sites of Class A GPCRs have also been found in EC regions (4). This is perhaps best exemplified by the muscarinic family of receptors, in which a common allosteric binding site has been reported in all five muscarinic receptor subtypes; this site is located in an EC region (38). It has been hypothesized that by binding more extracellularly than orthosteric agonists, allosteric modulators may sterically hinder an orthosteric agonist from exiting the receptor (39-41).

However, unlike many GPCRs that bind hydrophilic ligands that enter the receptor via the receptor’s extracellular surface, the cannabinoid receptors bind hydrophobic, lipid-derived ligands—this fundamental difference in hydrophobicity may suggest that these hydrophobic ligands enter by an alternate route. For example, we have previously reported that sn-2-arachidonoylglycerol (2-AG) enters the CB2 receptor (via TMH6-7) from the lipid bilayer (42). Likewise, Hanson et al. have reported that ligands
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may enter the sphingosine 1-phosphate receptor (another lipid-binding GPCR) between TMH1 and TMH7, also from the lipid bilayer (43). These observations may suggest that an allosteric ligand that binds in an extracellular region of a GPCR may not block the entry or exit of orthosteric ligands that enter/exit from the lipid bilayer.

Consistent with this hypothesis, we have recently reported that ORG27569 does not significantly decrease CP55,940’s dissociation rate (k_{off}), nor does ORG27569 significantly affect CP55,940’s equilibrium binding constant (14). The results of our docking studies are consistent with consistent with the results of the dissociation experiments. Specifically, our models suggest that ORG27569 docks in an extracellular region of CB₁. By docking in an extracellular region, ORG27569 would not be able to hinder the entry/exit of orthosteric ligands that enter/exit via the lipid bilayer.

In addition, because ORG27569 does not significantly impact CP55,94’s K_d, this can be taken as evidence that ORG27569 does not sterically block CP55,940 form exiting the CB₁ receptor. This is because if ORG27569 sterically blocked CP55,940 from exiting, this would be observed as a significant change in CP55,940’s K_d (i.e. if ORG27569 blocked CP55,940 from exiting, CP55,940’s K_d would increase). The results of our energy calculations performed on both the static models and dynamic simulations are consistent with these observations. Specifically, CP55,940 was found to have a similar total interaction energy, regardless of whether it was docked alone in the receptor, or in the presence of ORG27569 (or PHR015). Altogether, these results suggest that ORG27569 does not sterically block CP55,940’s exit from the CB₁ receptor.

ORG27569 sterically blocks extracellular (EC) loop movements and interactions critical to signal transduction—Recently, the relationship between where an allosteric modulator binds and its mechanism of action has enjoyed intense interest (4,44,45). For example, the EC-2 and EC-3 loops have been reported to be important to the binding and efficacy of allosteric modulators at the adenosine A₁ receptor (46). In the muscarinic field, not only have the EC loops been implicated in the binding and efficacy of allosteric modulators, but have also been reported to play a role in receptor subtype specificity (i.e. sequence differences in the EC loop regions help determine allosteric modulator-receptor subtype preference) (47). This correlation between allosteric modulator binding/efficacy and the EC region of GPCRs is not surprising, given the fundamental importance of the EC loops to signal transduction (48).

Our results suggest that upon receptor activation, the EC-2 loop moves down towards the transmembrane core; this movement places F268 in close proximity to CP55,940. In addition, we have presented evidence that suggests ORG27569 may sterically block this movement, specifically by the formation of an aromatic interaction between ORG27569’s indole ring and F268. This correlation between a conformational change in the EC-2 loop and receptor activation has been observed in numerous GPCRs. For example, the results of NMR studies have suggested that upon activation, rhodopsin’s EC-2 loop undergoes a necessary conformational change that is coupled to the breaking of the intracellular ‘ionic lock’ (i.e. an ionic interaction between R3.50 and D/E6.30 that promotes an inactive
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GPCR conformation) (49). Additionally, the results of recent mutation studies of the angiotensin II type 1 receptor suggest that the EC-2 loop undergoes ligand-specific conformational changes that are required for receptor activation (50,51). Finally, circular dichroism and steady-state fluorescence studies have been used to illustrate that the EC-2 loop, of the serotonin 5-HT₄(a) receptor, adopts specific loop conformations that are determined by the receptor’s activation state (52).

We have recently reported that the EC-3 loop and TMH2 form an ionic interaction (specifically, K373 and D2.63₁₇₆) that is necessary for G protein-mediated signaling of CB₁ (25). Our computational results suggest that ORG27569 may sterically block the EC-3 loop from being able to reach across the top (extracellular face) of the receptor, preventing this interaction from forming. In analogy to the EC-2 loop, there appears to be a strong correlation between conformational changes of the EC-3 loop and GPCR activation. For example, mutation studies of the C-X-C chemokine receptor type 4 (i.e. CXCR4) suggest that an interaction between the EC-3 loop and the N-terminus may form an ‘activation microswitch’; additionally, these results suggest that the conformation of the EC-3 loop may influence both basal and agonist-induced G protein-mediated signaling (53). In addition, mutation studies of the δ-opioid receptor suggest that its EC-3 loop may form a tight hairpin structure and that the disruption of this conformation may trigger receptor activation (54).

ORG27569 may sterically prevent TMH6 from undergoing an important conformational change—The hallmark of Class A GPCR activation by an agonist is the “tripping” of the toggle switch within the binding pocket that allows TMH6 to flex in the CWXP hinge region and straighten. This straightening breaks the “ionic lock” between R3.50 and D/E6.30 at the intracellular end of the receptor. The result is the formation of an intracellular opening of the receptor, exposing residues that can interact with the C-terminus of the G protein’s Ga subunit (55).

Our docking studies suggest that ORG27569 interacts with several residues on TMH6-7 and the EC-3 loop. These interactions position ORG27569 so that it packs tightly against TMH6; these results may suggest that ORG27569 antagonizes the efficacy of CP55,940 by preventing TMH6 from undergoing a necessary conformational change. These results are consistent with structural studies from the Farrens lab that suggest ORG27569 prevents an agonist-induced, conformational change at the intracellular end of TMH6 (24). Indeed, there is a significant amount of evidence in the literature that indicates that the extracellular end of TMH6 moves towards the transmembrane core, as the intracellular end of TMH6 moves away from the receptor (56).

Therefore, ORG27569 may antagonize the efficacy of CP55,940 by preventing TMH6 from undergoing necessary conformational changes.

Non-G-Protein Mediated Signaling by ORG27569—In addition to its effects on G-protein mediated signaling, ORG27569 has recently been reported to also signal via the ERK pathway when applied alone or in the presence of an orthosteric agonist (9,14). This signaling has been reported to be mediated specifically by β-arrestin 1 (57). In the present work, we have confined our study to how ORG27569 exerts its effects on G protein-mediated signaling only. Future studies will explore conformational changes induced by ORG27569 in other receptor regions that may correlate with β-arrestin mediated ERK signaling.
References:


(SR141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB1 receptor. *Mol Pharmacol* 62, 1274-1287


47. Huang, X. P., and Ellis, J. (2007) Mutational disruption of a conserved disulfide bond in muscarinic acetylcholine receptors attenuates positive homotropic cooperativity between multiple allosteric sites and has subtype-dependent effects on the affinities of muscarinic allosteric ligands. Mol Pharmacol 71, 759-768
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FIGURE LEGENDS

FIGURE 1. Compounds evaluated in this study. Atom numbers are used in description of conformational analysis. Ring letters are used in the description of aromatic interactions.

FIGURE 2. The effect of ORG27569 CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding, as well as the effect on basal levels of $[^{35}\text{S}]$GTP$\gamma$S binding, in wild-type and hCB$_1$R cells expressing the K3.28$^{192}$A mutant. (A) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding when applied alone (DMSO) or in the presence of ORG27569 (1 µM). The effect of CP55940 is expressed as a percentage of the E$_{\text{max}}$ of CP55940 in the presence of vehicle. (B) The effect of ORG27569 and SR141617A on basal levels of $[^{35}\text{S}]$GTP$\gamma$S binding in wild-type (WT) cells. (C) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding when applied alone (DMSO) or in the presence of ORG27569 (1 µM) in K3.28$^{192}$A mutant hCB$_1$R cells. The effect of CP55940 is expressed as a percentage of the E$_{\text{max}}$ of CP55940 in the presence of vehicle. (D) The effect of ORG27569 on basal levels of $[^{35}\text{S}]$GTP$\gamma$S binding in WT and K3.28$^{192}$A mutant hCB$_1$R cells is illustrated here. Data was analyzed via one sample t-test compared to zero where ** p ≤ 0.01; *** p ≤ 0.001.

FIGURE 3. (A) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding in F3.36$^{200}$A mutant hCB$_1$R cells, when applied alone (DMSO) or in the presence of ORG27569 (1 µM) (B) effect of ORG27569 alone on $[^{35}\text{S}]$GTP$\gamma$S basal binding in WT and F3.36$^{200}$A hCB1 cells; (C) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding in W6.48$^{356}$A mutant hCB$_1$R cells, when applied alone (DMSO) or in the presence of ORG27569 (1 µM) (D) effect of ORG27569 alone on basal $[^{35}\text{S}]$GTP$\gamma$S binding in WT and W6.48$^{356}$A hCB1 cells. Symbols represent mean values ±S.E.M. from three to six experiments carried out in duplicate. Effect of CP55940 is expressed as a percentage of the E$_{\text{max}}$ of CP55940 in the presence of vehicle.

FIGURE 4. (A) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding in W5.43$^{279}$A mutant hCB$_1$R cells, when applied alone (DMSO) or in the presence of ORG27569 (1 µM) (B) effect of ORG27569 alone on $[^{35}\text{S}]$GTP$\gamma$S basal binding in WT and W5.43$^{279}$A hCB1 cells; (C) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding in F3.25$^{192}$A mutant hCB$_1$R cells, when applied alone (DMSO) or in the presence of ORG27569 (1 µM) (D) effect of ORG27569 alone on basal $[^{35}\text{S}]$GTP$\gamma$S binding in WT and F3.25$^{192}$A cells. Symbols represent mean values ±S.E.M. from three to six experiments carried out in duplicate. Effect of CP55940 is expressed as a percentage of the E$_{\text{max}}$ of CP55940 in the presence of vehicle.

FIGURE 5. Characterization of PHR015 (A) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding when applied alone (DMSO) or in the presence of PHR015 (1 µM) (B) CP55,940-induced $[^{35}\text{S}]$GTP$\gamma$S binding when applied alone (DMSO) or in the presence of PHR015 (1 µM) in K3.28$^{192}$A mutant cells (C) effect of PHR015 alone on basal $[^{35}\text{S}]$GTP$\gamma$S binding in WT and K3.28$^{192}$A mutant hCB$_1$R cells. Symbols represent mean values ±S.E.M. from three to six experiments carried out in duplicate. Effect of CP55940 is expressed as a percentage of the E$_{\text{max}}$ of CP55940 in the presence of vehicle.

FIGURE 6. Comparison of the global minimum energy conformers (global min) and docked conformers of ORG27569 and PHR015. (A) The final docked conformer of ORG27569 (lime green) is shown overlaid on its global min (orange); the energy expense of ORG27569 to adopt its docked conformation was calculated to be 0.72 kcal/mol. (B) The final docked conformer of PHR015 (lavender) is shown overlaid on its global min (mauve); the energy expense of PHR015 to adopt its docked conformation was calculated to be 0.44 kcal/mol. (C) The global min of PHR015 (mauve) is shown overlaid with the global min of ORG27569 (orange).

FIGURE 7. Dock of ORG27569 (lime green) at the CP55,940 (cyan)-CB$_1$R$^{**}$ complex. The view is from the lipid bilayer looking toward TMH3. TMH2 and the EC-1 loop have been omitted for clarity. Residues
that contribute $\geq 5.5\%$ of ORG27569’s total interaction energy are shown in orange. K3.28$^{192}$ contributes 11.09\% of ORG27569’s total interaction energy and is shown in bright orange; K3.28$^{192}$ is shown forming a hydrogen bond (yellow dashes) with both ORG27569 and CP55,940.

**FIGURE 8.** Overlay of ORG27569 (lime green) and SR141716A (purple) in the CB$_1$R$^{\ast\ast}$ model. (A) SR141716A (contoured at its VdW radii) occupies the TMH3-4-5-6 aromatic microdomain of CB$_1$, extending its piperidine ring into the TMH2/7 region. (B) SR141716A and ORG27569 are illustrated here in their CB$_1$ binding sites, docks superimposed. As indicated by the red circle, there is severe steric overlap between these ligands. This steric overlap may explain why ORG27569 displaces SR141716A in equilibrium binding assays. Here it is also clear that the binding site of ORG27569 extends higher in the TMH bundle towards EC loops than the binding site of SR141716A.

**FIGURE 9.** Dock of PHR015 (lavender) at the CP55,940 (cyan)-CB$_1$R$^{\ast\ast}$ complex. The view is from the lipid bilayer towards TMH3 with TMH2 and the EC-1 loop omitted for clarity. Residues that contribute $\geq 5.5\%$ of ORG27569’s total interaction energy are shown in orange. K3.28$^{192}$ (mauve) forms two hydrogen bonds (yellow dashes) with CP55,940, but does not interact with PHR015.

**FIGURE 10.** Extracellular loop-2 (EC-2) conformations of various states of the CB$_1$ receptor. (A) EC-2 loop conformation of the CB$_1$ receptor in its inactive (R; purple) and active (R$^{\ast}$; orange) state conformations. CP55,940 is shown in cyan contoured at its VdW radii. The EC-2 loop residue, F268 contoured at its VdW radii is shown in purple for R and orange in R$^{\ast}$. This residue is not in close proximity to CP55,940 in the CB$_1$R state, but is in close proximity to CP55,940 in the activated state. This image illustrates that the EC-2 loop moves down, towards the transmembrane core upon activation. (B) EC-2 loop conformation in the presence of ORG27569 docked at the CP55,940-CB$_1$ complex. ORG27569 is shown in lime green; CP55,940 is shown in cyan; the EC-2 loop and F268 are shown in green. This image illustrates that ORG27569 is sterically preventing the EC-2 loop from adopting its active conformation. Here, F268 has formed an aromatic T-stack with the ORG27569 indole ring. This steric block of the EC-2 loop may explain how ORG27569 antagonizes the efficacy of CP55,940.

**FIGURE 11.** Extracellular loop-3 (EC-3 loop) conformations (and interactions) of various states of the CB$_1$ receptor. The view is from lipid looking towards TMH3 with TMH1, the EC-1 loop, and the EC-2 loop omitted for clarity. (A) EC-3 loop conformations of the CB$_1$ receptor in its $R^{\ast}$ conformation. CP55,940 is shown in cyan, contoured at its VdW radii. D2.63$^{176}$ and K373 (orange tube) are shown forming a hydrogen bond (yellow dashes). We have recently reported (25) that, upon activation, this interaction promotes an EC-3 loop conformation that is pulled across the extracellular face of the CB$_1$ receptor. (B) EC-3 loop conformation of the CP55,940 (cyan, VdW)-CB$_1$ complex, in the presence of ORG27569 (lime green, VdW). D2.63$^{176}$ and K373 are shown here in orange tube. This image shows ORG27569 sterically blocking D2.63$^{176}$ and K373 from forming a hydrogen bond, preventing this interaction from promoting an active conformation of the EC-3 loop. This steric hindrance of the D2.63$^{176}$ and K373 (and indeed, the EC-3 loop in general) may provide an additional reason for ORG27569 antagonism of CP55,940 efficacy.

**FIGURE 12.** Dock of ORG27569 (lime green) at the WT CB$_1$R (inactive) model. The view is from the lipid bilayer looking toward TMH6-7. The extracellular (EC) ends of TMH6-7 and the EC-3 loop have been omitted for clarity. Residues that contribute $\geq 5.5\%$ of ORG27569’s total interaction energy are shown in orange. This includes F3.25$^{189}$, K3.28$^{192}$ is shown in bright orange; K3.28$^{192}$ is shown forming a hydrogen bond (yellow dashes) with ORG27569.

**FIGURE 13.** Dock of ORG27569 (lime green) at the F3.25$^{189}$A mutant CB$_1$R (inactive) model. The view is from the lipid bilayer looking toward TMH6-7. The EC ends of TMH6-7 and the EC-3 loop have been omitted for clarity. The net result of the F3.25$^{189}$A mutation is a change in the WT CB$_1$ binding position
of ORG27569 (compare to Figure 12). Residues that contribute ≥ 5.5% of ORG27569’s total interaction energy are shown in orange. K3.28\textsuperscript{192} is shown in mauve (and does NOT form a hydrogen bond with ORG27569 due to the relocation of ORG27569 in this mutant.
Table 1.
CP55,940-induced $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in wild-type and mutant hCB$_1$R cells, when applied alone (DMSO) or in the presence of an allosteric modulator (1 µM)

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<tr>
<th>Cell Line</th>
<th>DMSO/Allosteric</th>
<th>$E_{\text{max}}^a$ (%) (95% CL)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>DMSO</td>
<td>100 (72 - 122)</td>
</tr>
<tr>
<td></td>
<td>ORG27569</td>
<td>Inverse effect$^\ddagger$</td>
</tr>
<tr>
<td></td>
<td>PHR015</td>
<td>31 (9 - 53)$^\dagger$</td>
</tr>
<tr>
<td>K3.28$^{192}$A</td>
<td>DMSO</td>
<td>100 (75 - 125)</td>
</tr>
<tr>
<td></td>
<td>ORG27569</td>
<td>41 (17 - 66)$^\dagger$</td>
</tr>
<tr>
<td></td>
<td>PHR015</td>
<td>15 (-10 - 39)$^\dagger$</td>
</tr>
<tr>
<td>F3.36$^{209}$A</td>
<td>DMSO</td>
<td>100 (56 - 144)</td>
</tr>
<tr>
<td></td>
<td>ORG27569</td>
<td>Inverse effect$^\ddagger$</td>
</tr>
<tr>
<td>W5.43$^{279}$A</td>
<td>DMSO</td>
<td>100 (73 - 127)</td>
</tr>
<tr>
<td></td>
<td>ORG27569</td>
<td>103 (45 - 160)</td>
</tr>
<tr>
<td>W6.48$^{356}$A</td>
<td>DMSO</td>
<td>100 (56 - 144)</td>
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<tr>
<td></td>
<td>ORG27569</td>
<td>Inverse effect$^\ddagger$</td>
</tr>
<tr>
<td>F3.25$^{189}$A</td>
<td>DMSO</td>
<td>100 (78 - 123)</td>
</tr>
<tr>
<td></td>
<td>ORG27569</td>
<td>19 (-10 - 48)$^\dagger$</td>
</tr>
</tbody>
</table>

$^a$ Maximal agonist effect expressed as a % of the maximum effect of CP55940 in the presence of vehicle, determined using nonlinear regression analysis. Values represent the mean with 95% CI of four to six experiments.

$^\dagger$ Significantly different (non-overlapping confidence limits) from the DMSO vehicle.

$^\ddagger$ Decrease in the basal binding indicative of inverse effect.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.
Figure 12.
Figure 13.
The CB₁ Receptor's Allosteric Binding Site and Mechanism

Scheme 1.

1. n-BuLi, THF, -78 °C; 2. DMF, -78 °C - 0 °C; b) CH₃NO₂, NH₂OAc, 100 °C; c) LiAlH₄, THF, reflux; d) Et₂AlCl, THF, 0 °C
2. AcCl, 0 °C - r.t.; e) Et₃SiH, TFA, r.t.; f) NaOH aq., 1,4-Dioxane, reflux; g) DCC, HOBT, CH₂Cl₂, r.t.

Scheme 1.
Allosteric Modulation of a Cannabinoid G Protein-Coupled Receptor: Binding Site Elucidation and Relationship to G Protein Signaling


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