Two Janus Cannabinoids That Are Both CB$_2$ Agonists and CB$_1$ Antagonists

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Received July 13, 2016; accepted December 5, 2016

ABSTRACT

The cannabinoid signaling system includes two G protein–coupled receptors, CB$_1$ and CB$_2$. These receptors are widely distributed throughout the body and have each been implicated in many physiologically important processes. Although the cannabinoid signaling system has therapeutic potential, the development of receptor-selective ligands remains a persistent hurdle. Because CB$_1$ and CB$_2$ are involved in diverse processes, it would be advantageous to develop ligands that differentially engage CB$_1$ and CB$_2$. We now report that GW405833 [1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-3-[2-(4-morpholinyl)ethyl]-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone$^\text{c}$ and AM1710 [1-hydroxy-9-methoxy-3-(2-methyloctan-2-yl)phenol] noncompetitively antagonized adenylyl cyclase activity, extracellular signal–regulated kinase 1/2 phosphorylation, phosphatidylinositol 4,5-bisphosphate signaling, and CB$_1$ internalization by CP55940 (2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol). In contrast, AM1710 behaved as a low-potency competitive antagonist/inverse agonist in these signaling pathways. GW405833 interactions with CB$_2$/arrestin signaling were complex: GW405833 differentially modulated arrestin recruitment in a time-dependent fashion, with an initial modest potentiation at 20 minutes followed by antagonism starting at 1 hour. AM1710 acted as a low-efﬁcacy agonist in arrestin signaling at the CB$_1$ receptor, with no evident time dependence. In summary, we determined that GW405833 and AM1710 are not only CB$_2$ agonists but also CB$_1$ antagonists, with distinctive and complex signaling properties. Thus, experiments using these compounds must take into account their potential activity at CB$_1$ receptors.

Introduction

Cannabinoid receptors are part of an endogenous signaling system that is found throughout much of the body (Herkenham et al., 1990). The two canonical cannabinoid receptors, CB$_1$ and CB$_2$, were identified in the early 1990s (Matsuda et al., 1990; Munro et al., 1993). Cannabinoids have since been implicated in several major physiologic processes (Corcoran et al., 2015; Di Marzo et al., 2015; Alexander, 2016) and cannabinoid receptors remain a promising pharmacological target. However, a persistent hurdle has been the development of ligands that are selective for CB$_1$ or CB$_2$. The widespread distribution of these receptors, particularly of CB$_1$, raises the specter of signiﬁcant off-target actions, particularly if a given drug can engage both receptors. For example, it has been speculated that the analgesic activity of CB$_2$ agonists in some preclinical pain models may be due to their concurrent activation of CB$_1$ receptors (Manley et al., 2011). We previously reported that JWH015 [1-propyl-2-methyl-3-(1-naphthoyl)indole], a compound widely used as a selective CB$_2$ agonist, is also a potent and efﬁcacious CB$_1$ agonist (Murataeva et al., 2012). In the same study, we noted that the CB$_2$ antagonist AM630 (6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl)-(4-methoxyphenyl)methanone also blocks CB$_1$ signaling at relatively low concentrations. The identiﬁcation and careful characterization of cannabinoid receptor ligands is therefore an important task facing the cannabinoid ﬁeld.

When confronted with two related receptors (e.g., activated by the same endogenous ligands), there are times when it is...
advantageous to not merely selectively activate one receptor, but to actively block signaling of the other receptor. Compounds with this dual quality are rare and represent an important resource. To date, the only well characterized cannabinoid receptor ligand reported to have this profile is URB447 ([4-amino-1-[(4-chlorophenyl)methyl]-2-methyl-5-phenylpyrrol-3-yl]-phenylmethanone), which is a peripherally restricted CB1 antagonist and a CB2 agonist (LoVerme et al., 2009). Even if such a compound has limited efficacy or potency, it may serve as a lead compound to allow chemists to develop novel variants. To further explore dual-action cannabinoid ligands, we examined the activity of the CB2 agonists, GW405833 [1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-3-[2-(4-morpholinyl)ethyl]-1H-indole] and AM1710 [1-hydroxy-9-methoxy-3-(2-methyloctan-2-yl)benzo[c]chromen-6-one], toward CB1 receptors in autaptic hippocampal neurons as well as in several additional signaling assays using CB1-expressing human embryonic kidney (HEK) 293 cells or Chinese hamster ovary (CHO) cells. GW405833 is a compound that was developed as a CB2 agonist several years ago and has been used as a CB2-selective agonist in nearly 20 publications (e.g., Clayton et al., 2002; LaBuda et al., 2005; Valenzano et al., 2005; Whiteside et al., 2005). In radio-ligand binding assays, GW405833 showed high binding affinity for CB2 receptors (CHO-K1 cells stably expressing human CB2), with a $K_i$ of $3.92 \pm 1.58$ nM (Valenzano et al., 2005). While at CB1 receptors, GW405833 was a low-affinity ligand, with a $K_i$ of $4772 \pm 1676$ nM, and was approximately 1200-fold more selective for CB2 receptors (Valenzano et al., 2005). Similarly, the structurally distinct AM1710 has been used as a CB2 agonist mostly relating to pain research (Khanolkar et al., 2007; Rahn et al., 2011, 2014; Deng et al., 2012, 2015; Wilkerson et al., 2012). AM1710 displayed high affinity for CB2 receptors (HEK cells stably expressing human CB2 receptors), with a $K_i$ of 6.7 nM (Khanolkar et al., 2007) and an EC$_{50}$ of 11 nM (E$_{max}$ of 48% $\pm$ 0.3%) to inhibit cAMP accumulation (Dhopeshwarkar and Mackie, 2016). The affinity of AM1710 for rat CB1 receptors (tested in rat brain synaptosomal membranes) was lower, with a $K_i$ of 360 nM [95% confidence interval (95% CI), 330–390] (Khanolkar et al., 2007), and was approximately 30-fold more selective for CB2 receptors. We now report that in addition to acting as CB2 agonists, GW405833 and AM1710 also serve as antagonists at CB1 receptors, albeit with distinct pharmacological properties.

**Hippocampal Culture Preparation**

All procedures used in this study were carried out in accordance with and conform to the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the U.S. National Institutes of Health and were approved by the Animal Care Committee of Indiana University. Mouse hippocampal neurons isolated from the CA1-CA3 region were cultured on microislands as described previously (Purspan et al., 1976; Bekkers and Stevens, 1991). Neurons were obtained from mice (C57Bl/6, unknown sex, postnatal day 0–2) and plated onto a feeder layer of hippocampal astrocytes that had been laid down previously (Levison and McCarthy, 1991). Cultures were grown in high-glucose (20 mM) Dulbecco’s modified Eagle’s medium containing 10% horse serum, without mitotic inhibitors, and were used for recordings after 8 days in culture and for no more than 3 hours after removal from the culture medium.

**Electrophysiology**

When a single neuron is grown on a small island of permissive substrate, it forms synapses—or “autapses”—onto itself. All experiments were performed on isolated autaptic neurons. Whole-cell voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained 119 mM NaCl, 5 mM KCl, 2.5 mM CaCl$_2$, 1.5 mM MgCl$_2$, 30 mM glucose, and 20 mM HEPES. Continuous flow of solution through the bath chamber (approximately 2 ml/min) ensured rapid drug application and clearance. Drugs were typically prepared as stocks and then diluted into extracellular solution at their final concentration and were used on the same day.

Recording pipettes of 1.8–3 MΩ were filled with 121.5 mM K gluconate, 17.5 mM KCl, 9 mM NaCl, 1 mM MgCl$_2$, 10 mM HEPES, 0.2 mM EGTA, 2 mM MgATP, and 0.5 mM LiGTP. Access resistance and holding current were monitored and only cells with both stable access resistance and holding current were included for data analysis.

**Conventional Stimulus Protocol.** The membrane potential was held at −70 mV and excitatory postsynaptic currents (EPSCs) were evoked every 20 seconds by triggering an unclamped action current with a 1.0-millisecond depolarizing step. The resultant evoked waveform consisted of a brief stimulus artifact and a large downward spike representing inward sodium currents, followed by the slower EPSC. The size of the recorded EPSCs was calculated by integrating the evoked current to yield a charge value (in picocoulombs). Calculating the charge value in this manner yields an indirect measure of the amount of neurotransmitter released while minimizing the effects of cable distortion on currents generated far from the site of the recording electrode (the soma). Data were acquired at a sampling rate of 5 kHz.

**DSE Stimuli.** After establishing a 10- to 20-second 0.5-Hz baseline, depolarization-induced suppression of excitation (DSE) was evoked by depolarization to 0 mV for 50 milliseconds, 100 milliseconds, 300 milliseconds, 500 milliseconds, 1 second, 3 seconds, and 10 seconds, followed in each case by resumption of a 0.5-Hz stimulus protocol for 20–80 seconds, allowing EPSCs to recover to baseline values. This approach allowed us to determine the sensitivity of the synapses to DSE induction. To allow comparison, baseline values (prior to the DSE stimulus) were normalized to 1. DSE inhibition values are presented as fractions of 1 (i.e., a 50% inhibition from the baseline response is 0.50 ± S.E.M.). The x-axis of DSE depolarization response curves are log-scale seconds of the duration of the depolarization used to elicit DSE.

Depolarization response curves were obtained to determine pharmacological properties of endogenous 2-arachidonoylglycerol signaling by depolarizing neurons for progressively longer durations (50 milliseconds, 100 milliseconds, 300 milliseconds, 500 milliseconds, 1 second, 3 seconds, and 10 seconds). The data were fitted with nonlinear regression, allowing calculation of the effective dose or duration of depolarization at which a 50% inhibition was achieved (ED$_{50}$). Statistical significance in these curves was based on non-overlapping 95% CIs.

**On-Cell Western Assay for Receptor Internalization**

The internalization of the receptor was measured using an on-cell Western assay (Daigle et al., 2008). Briefly, hemagglutinin (HA)-CB$_1$-expressing HEK cells were grown to 95% confluence in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 0.5% penicillin/streptomycin (Daigle et al., 2008). Cells were washed once with 200 μl/well HEPES-buffered saline (HBS)/bovine serum albumin (BSA; 0.8 mg/ml). Drugs in HBS/BSA were applied at the indicated concentrations to cells and were incubated for the indicated amount of time at 37°C. Cells were then fixed with 4% paraformaldehyde for 20 minutes and washed four times (200 μl/well) with Tris-buffered saline (TBS). Odyssey blocking buffer (LI-COR Inc., Lincoln, NE) was applied at

**Materials and Methods**

**Hippocampal Culture Preparation**

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Phosphorylated Extracellular Signal-Regulated Kinase 1/2 Assay

Activation of extracellular signal-regulated kinase 1/2 (ERK1/2) was measured using an in-cell Western assay (Wong, 2004; Atwood et al., 2012). HA-CB1–expressing HEK cells were seeded onto poly-D-lysine–coated 96-well plates (75,000 cells/well) and grown overnight at 37°C in 5% CO₂, humidified air. The next day, the media were replaced with HBS/BSA (0.2 mg/ml) and cells were challenged with drugs/compounds for 5 minutes at 37°C in 5% CO₂, humidified air. After drug incubation, plates were emptied and quickly fixed with ice-cold 4% paraformaldehyde for 20 minutes followed by treatment with 10% sucrose maintained at −20°C for an additional 15 minutes. Plates were then washed with TBS–0.1% Triton X-100 for 25 minutes (five 5-minute washes). The final wash solution was then replaced with Odyssey blocking buffer (150 μl) and further incubated for 90 minutes with gentle shaking at room temperature. Blocking solution was then removed and replaced with blocking solution containing anti–phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) antibody (1:150; Cell Signaling Technology, Danvers, MA) and was gently shaken overnight at 4°C. The next day, plates were washed with TBS containing 0.05% Tween 20 for 25 minutes (five 5-minute washes). Secondary antibody—donkey anti-rabbit conjugated with IR800 dye (Rockland, Limerick, PA), prepared in blocking solution—was added and gently shaken for 1 hour at room temperature. The plates were then washed again five times with TBS–0.05% Tween 20 solution. The plates were patted dry and scanned (channel, 700 nm; intensity, 5.0; LI-COR, Inc.). Receptor internalization (expressed as the percent of basal surface levels) was calculated by dividing the average integrated intensities of the drug-treated wells by the average integrated intensities of vehicle-treated wells. (Binding of HA antibody to wild-type HEK cells was <10% of transfected cells.) All assays were performed in triplicate, unless mentioned otherwise.

Adenylyl Cyclase Assay

Adenylyl cyclase assays were optimized using the LANCE Ultra cAMP kit (PerkinElmer, Boston, MA) per the manufacturer’s instructions. All assays were performed at room temperature using 384-well OptiPlates (PerkinElmer). Briefly, HA-CB1 HEK cells were detached from approximately 60% confluent plates/dish using versene. Cells were then resuspended gently in 1× stimulation buffer (1× Hank’s balanced salt solution, 5 mM HEPES, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.1% BSA, pH 7.4, made fresh on the day of the experiment) and were further incubated for 1 hour at 37°C in 5% CO₂, humidified air. Cells were then resuspended in a 384-well OptiPlate (500 cells/μl, 10 μl) and stimulated with drugs/compounds (made in stimulation buffer, 5 μl, 4× concentration, 1 μM final concentration) and forskolin (made in stimulation buffer, 5 μl, 4× concentration, 1 μM final concentration) as appropriate for 5 minutes at room temperature. Cells were then lysed by addition of 10 μl Eu-cAMP tracer working solution (4×, made fresh in 1× lysis buffer supplied with the kit; under subdued light conditions) and 10 μl ULight anti-cAMP working solution (4×, made fresh in 1× lysis buffer) and further incubated for 1 hour at room temperature. Plates were then read with the time-resolved fluorescence energy transfer mode on an Enspire plate reader (PerkinElmer).

Arrestin Recruitment Assay

Arrestin recruitment assays were performed using the PathHunter CHO-K1 CNR1 assay (CHO-mouseCB1, catalog no. 93-0959C2; DiscoverX, Fremont, CA). The assay principle is based on enzyme fragment complementation technology. In this engineered cell line, a deletion mutant of β-galactosidase is fused with arrestin and a smaller fragment of the enzyme (ProLink) is fused to the C-terminal domain of the cannabinoid receptor. The activation of the cannabinoid receptor leads to arrestin recruitment and formation of an active β-galactosidase enzyme, which then acts on substrate to emit light that can be measured on a luminescence plate reader. Cells were thawed and grown and maintained in PathHunter AssayComplete media (catalog no. 92-0018GF2; DiscoverX).

All assays were performed in poly-D-lysine–coated 96-well plates. Approximately 20,000 cells/well were plated and grown overnight at 37°C in 5% CO₂, humidified air. The next day, media was replaced with 90 μl HBS/BSA (0.2 mg/ml), an additional 10 μl of HBS/BSA containing a 10X concentration of drugs/compounds (10× concentration) was added and then incubated for 90 minutes at 37°C in 5% CO₂, humidified air. For time course assays, cells were pretreated with GW405833 for the time described in the text, followed by CP55940 (2-(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl)-5-(2-methyloctan-2-yl)phenol) plus GW405833 treatment and cell lysis. Reactions were terminated by the addition of PathHunter detection reagent (DiscoverX) and the plate was further incubated for 60 minutes at room temperature. Complementation reactions were monitored by chemiluminescence using an Enspire multiplate reader.

Inositol Phosphate 1 Assay

Accumulation of myo-inositol phosphate 1 (IP₁), a downstream metabolite of IP₃, was measured by using a IP-One homogeneous time-resolved fluorescence (HTRF) kit (catalog no. 62, IPAPEB; Cisbio, Bedford, MA). Functional coupling of the CB1 receptor to Gα,G protein leads to phospholipase Cβ activation and initiation of the inositol phosphate (IP) cascade. Accumulated IP₁ is quickly dephosphorylated to IP₂ and then IP₃. This assay takes advantage of the fact that accumulated IP₁ is protected from further degradation by the addition of lithium chloride and IP₃ levels can be easily quantified using a HTRF assay. HA-CB1 HEK cells were detached from approximately 60% confluent plates/dish using versene. Cells (10 μl, 5000 cells) were resuspended in 1× stimulation buffer (containing lithium chloride, supplied with the kit) and were incubated for 1 hour at 37°C in 5% CO₂, humidified air and then transferred to a 384-well OptiPlate, followed by stimulation with drugs/compounds made in dimethylsulfoxide/ethanol as appropriate, for 10 minutes. Cells were then lysed with 5 μl IP₁-Ab-cryptate (made fresh in lysis buffer, supplied with the kit), followed by addition of 5 μl Ab-acceptor (made fresh in lysis buffer). Plates were incubated further for 90 minutes at room temperature and then read in HTRF mode on an Enspire plate reader. All cell-based assay experiments were performed in triplicate and were repeated at least two times, unless mentioned otherwise.

Schild Analysis

Schild plots were generated for internalization assays by employing the Schild method (Schild, 1947; Arunlakshana and Schild, 1959; Wyllie and Chen, 2007). Briefly, full concentration-response curves were obtained for CP55940 in the presence and absence of various concentrations of GW405833 or AM1710 (Figs. 3D and 4C). Next, dose ratios were calculated by dividing the half maximal effect obtained by CP55940 in the presence of a particular antagonist concentration by

100 μl/well for 1 hour at room temperature. Anti-HA antibody (mouse monoclonal, 1:500; Covance, Princeton, NJ), diluted in 50:50 Odyssey blocking buffer and phosphate-buffered saline, was then applied for 1 hour at room temperature. Afterward, the plate was washed four times with TBS (200 μl/well). Secondary antibody (anti-mouse 680 antibody, 1:800; LI-COR, Inc.) diluted in 50:50 blocking buffer and phosphate-buffered saline was then applied for 1 hour at room temperature. The plate was then washed four times with TBS (200 μl/well). The plate was imaged using an Odyssey scanner (channel, 700 nm; intensity, 5.0; LI-COR, Inc.). Receptor internalization (expressed as the percent of basal surface levels) was calculated by dividing the average integrated intensities of the drug-treated wells by the average integrated intensities of vehicle-treated wells. (Binding of HA antibody to wild-type HEK cells was <10% of transfected cells.) All assays were performed in triplicate, unless mentioned otherwise.
the half maximal effect obtained with CP55940 in the absence of antagonist. Log(dose ratio-1) was then plotted against the logarithm of antagonist concentration using linear regression (GraphPad Prism 4.0 software; GraphPad Inc., La Jolla, CA) to yield the Schild slope. A slope of 1 indicates a competitive mode of inhibition of CP55940 by a particular antagonist.

Drugs

GW405833 was obtained from Tocris Bioscience (Bristol, UK). WIN55,212-2 [(1R)-2-methyl-11-[(morpholin-4-yl)methyl]-3-(naphthalene-1-carbonyl)-9-oxa-1-azatricyclo[6.3.1.04,12]dodeca-2,4(12),5,7-tetraene] was from Sigma-Aldrich (St. Louis, MO). CP55940 was obtained through the National Institute on Drug Abuse Drug Supply Program (National Institutes of Health, Bethesda, MD). AM1710 was prepared in the laboratory of Dr. Alex Makriyannis (Department of Pharmaceutical Sciences, Center for Drug Discovery, Northeastern University, Boston, MA) (Khanolkar et al., 2007).

Results

GW405833 and AM1710 Differentially Antagonize CB1 Signaling in Autaptic Hippocampal Neurons

We first tested the effects of GW405833 and AM1710 on CB1-dependent signaling in autaptic hippocampal neurons. Depolarization of excitatory autaptic hippocampal neurons elicits a form of retrograde inhibition termed “depolarization induced suppression of excitation” or DSE (Straiker and Mackie, 2005). This can be quantified by stimulating the neuron with a series of successively longer depolarizations (50 milliseconds, 100 milliseconds, 300 milliseconds, 500 milliseconds, 1 second, 3 seconds, and 10 seconds), resulting in progressively greater inhibition of neurotransmission (Straiker et al., 2011, 2012). This yields a depolarization response curve that permits the characterization of some pharmacological properties of cannabinoid signaling, including the calculation of a median effective dose (ED50), corresponding in this case to the duration of depolarization that results in 50% of the maximal inhibition.

We found that although GW405833 did not directly inhibit neurotransmission (Fig. 1, A and B) (relative EPSC charge after 10 μM GW405833, 1.02 ± 0.02; n = 4), it did interfere with CB1-mediated DSE in a concentration-dependent manner, with an IC50 of 2.6 μM (Fig. 1, C and D). Similarly, AM1710 did not directly inhibit neurotransmission (Fig. 2, A and B) (relative EPSC charge after 10 μM AM1710, 1.01 ± 0.02; n = 5). However, like GW405833, AM1710 also attenuated DSE but with less efficacy and lower potency. This low potency did not allow for the calculation of an IC50 (Fig. 2, C and D).

GW405833 Does Not Internalize CB1 Receptors But Antagonizes CP55940-Induced Internalization in a Concentration-Dependent Manner

We next explored the action of GW405833 on rCB1 receptor internalization in CB1-expressing HEK293 cells in an on-cell

![Fig. 1](image-url)

GW405833 antagonizes CB1 signaling in autaptic hippocampal neurons. (A) Sample time course shows that treatment with 10 μM GW405833 does not inhibit EPSCs. (B) Summary of data showing lack of direct inhibition of neurotransmission by GW405833 at 10 μM. (C) GW405833 inhibits CB1-dependent DSE in a concentration-dependent fashion (red triangles). Inhibition resulting from 3-second depolarization without drug is also shown (black square). (D) Sample DSE time courses before and with 3 μM GW405833 treatment. Right panels show EPSC traces at corresponding time points just before depolarization (1) and immediately after depolarization (2). Top traces are the control and bottom traces are after treatment with 3 μM GW405833. Axes: 2 nA, 30 milliseconds. GW40, GW405833; pC, picocoulomb.
GW405833 (10 μM) did not alter CB1 receptor surface levels over a 2-hour period (Fig. 3A), with surface levels of 102% ± 15% at 120 minutes (n = 16, P > 0.05, one-way analysis of variance (ANOVA) with Dunnett post hoc test versus baseline), suggesting that GW405833 is not an inverse agonist for CB1 receptor trafficking. The CB1 inverse agonist, SR141716 \[5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide\], incubated with the cells for 2 hours served as a positive control for externalization (Atwood et al., 2012), increasing CB1 surface levels by approximately 20% after 120 minutes of incubation (Fig. 3A). However, GW405833 antagonized internalization induced by 2-hour treatment with CP55940. GW405833, at concentrations $\geq 100$ nM, antagonized internalization induced by 5 nM CP55940 (Fig. 3B), with receptor surface level values of 91% ± 7% for GW405833 (10 μM) plus CP55940 (5 nM) and 73% ± 3% for CP55940 (5 nM) (n = 24, P < 0.05, two-way ANOVA with Bonferroni post hoc test). Moreover, 10 μM GW405833 prevented internalization by a 2-hour treatment with 100 nM CP55940 (Fig. 3C), with cell surface levels (% baseline) of 63% ± 4% for CP55940 (100 nM) and 97% ± 6.5% for CP55940 (100 nM) plus GW405833 (10 μM) at 120 minutes (n = 24, P < 0.01, two-way ANOVA with Bonferroni post hoc test). Taking together the DSE and internalization data, it appears that GW405833 is an efficacious and moderately potent antagonist at rodent CB1 receptors.

Western assay (Daigle et al., 2008). GW405833 (10 μM) did not alter CB1 receptor surface levels over a 2-hour period (Fig. 3A), with surface levels of 102% ± 15% at 120 minutes (n = 16, P > 0.05, one-way analysis of variance (ANOVA) with Dunnett post hoc test versus baseline), suggesting that GW405833 is not an inverse agonist for CB1 receptor trafficking. The CB1 inverse agonist, SR141716 \[5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide\], incubated with the cells for 2 hours served as a positive control for externalization (Atwood et al., 2012), increasing CB1 surface levels by approximately 20% after 120 minutes of incubation (Fig. 3A). However, GW405833 antagonized internalization induced by 2-hour treatment with CP55940. GW405833, at concentrations $\geq 100$ nM, antagonized internalization induced by 5 nM CP55940 (Fig. 3B), with receptor surface level values of 91% ± 7% for GW405833 (10 μM) plus CP55940 (5 nM) and 73% ± 3% for CP55940 (5 nM) (n = 24, P < 0.05, two-way ANOVA with Bonferroni post hoc test). Moreover, 10 μM GW405833 prevented internalization by a 2-hour treatment with 100 nM CP55940 (Fig. 3C), with cell surface levels (% baseline) of 63% ± 4% for CP55940 (100 nM) and 97% ± 6.5% for CP55940 (100 nM) plus GW405833 (10 μM) at 120 minutes (n = 24, P < 0.01, two-way ANOVA with Bonferroni post hoc test). Taking together the DSE and internalization data, it appears that GW405833 is an efficacious and moderately potent antagonist at rodent CB1 receptors.

To explore the nature of the antagonism between GW405833 and CP55940 at CB1, we tested internalization responses for a range of GW405833 and CP55940 concentrations (Fig. 3D), sufficient to conduct a Schild analysis. As shown in Fig. 3E and Table 1, the response profile is consistent with noncompetitive antagonism.

AM1710 Does Not Internalize CB1 Receptors But Antagonizes CP55940-Induced Internalization in a Concentration-Dependent Manner

Using the same model system, we tested the effect of AM1710 in CB1 receptor internalization. AM1710 (10 μM) slightly internalized CB1 receptors after a 2-hour period (Fig. 4A) (surface levels of 93% ± 1.5% at 120 minutes; n = 16, P = 0.02, t test versus baseline), suggesting that AM1710 is a modestly efficacious agonist for CB1 receptor trafficking. Furthermore, in contrast with GW405833, 10 μM AM1710 did not significantly alter the time course of internalization during a 2-hour treatment with 100 nM CP55940 (Fig. 4B), with cell surface levels (% baseline) of 62% ± 8% for CP55940 (100 nM), and 65% ± 6.7% for CP55940 (100 nM) plus AM1710 (10 μM) at 120 minutes (n = 24, P > 0.05, two-way ANOVA). In examining the effects of a range of AM1710 concentrations on CP55940-induced internalization, we found that AM1710 only modestly shifted the CP55940-response curve to the right, even at 10 μM. In addition, 20 μM and 30 μM AM1710 more substantially shifted the dose-response curve for CP55940 (Fig. 4C).
To explore the nature of the antagonism between AM1710 and CP55940 at CB₁, we tested this receptor’s internalization responses for a range of AM1710 and CP55940 concentrations (Fig. 4, C and D), sufficient to conduct a Schild analysis. As shown in Fig. 4D and Table 1, the Schild analysis is consistent with a low-affinity (Kᵦ) of approximately 10 µM, competitive antagonism.

**GW405833 and AM1710 Attenuate Inhibition of Forskolin-Stimulated cAMP Accumulation by CP55940**

We next examined whether GW405833 affected forskolin-stimulated cAMP accumulation or its inhibition by CB₁ agonists in HEK cells stably transfected with rCB₁. As expected, CP55940 inhibited cAMP accumulation in a concentration-dependent manner (Fig. 5A), with an EC₅₀ of 9.5 nM and an Eₘₐₓ (% basal) of 45.6 ± 8.3. Although GW405833 had no effect on its own, at 1 µM it completely blocked adenylyl cyclase inhibition by CP55940 at CP55940 concentrations up to at least 1 µM (Fig. 5A). Increasing concentrations of GW405833 (300 nM, 500 nM, and 1 µM) attenuated CP55940-induced inhibition of forskolin-stimulated adenylyl cyclase (Fig. 5A). GW405833 treatment reduced the Eₘₐₓ (P < 0.01, t tests for 1 µM concentration) (Fig. 5A), with Eₘₐₓ (% basal) values of 23.2 ± 8.1 for CP55940 plus GW405833 (300 nM) and 13 ± 7.0 for

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**TABLE 1**

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<th>Antagonist</th>
<th>Schild Slope</th>
<th>Hill Slope</th>
<th>Kᵦ (µM)</th>
<th>pA₂</th>
<th>R²</th>
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<td>GW405833</td>
<td>0.22 ± 0.02</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>AM1710</td>
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<td>10</td>
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Schild plots were generated from the internalization experiments. Briefly, full concentration-response curves were obtained for CP55940 in the presence and absence of increasing concentrations of antagonist. Dose ratios were obtained by dividing the EC₅₀ of CP55940 obtained in the presence of various concentrations of antagonist by the EC₅₀ of CP55940 alone. Log(dose ratio-1) values were plotted against antagonist concentrations on a logarithmic scale using linear regression (GraphPad Prism 4.0) to yield the Schild slope, Kᵦ, and pA₂. The Schild analysis of the concentration-response curves for CP55940 with various concentrations of putative CB₁ antagonists GW405833 and AM1710 yielded profiles that are consistent with noncompetitive and competitive CB₁ antagonism, respectively. ND, not detected.
CP55940 plus GW405833 (500 nM) with no significant change in the potency. EC50 values were 9.5 nM (95% CI, 2.1–19) for CP55940, 12 nM (95% CI, 3.4–18.3) for CP55940 plus GW405833 (300 nM), and 15 nM (95% CI, 4.5–23.6) for CP55940 plus GW405833 (500 nM). Classically, a reduction in Emax, with no change in potency, indicates a noncompetitive inhibition. Thus, GW405833 likely binds to a site on CB1 that is topographically distinct from that of CP55940.

AM1710 modestly potentiated cAMP accumulation on its own (Fig. 5B), with an Emax of 117% ± 5% (P < 0.01 at 1 μM AM1710). AM1710 decreased the potency, but not the efficacy (t test at 1 μM concentration), of CP55940 inhibition of adenylyl cyclase at 10 and 20 μM (Fig. 5B), with EC50 values of 6.7 nM (95% CI, 2.3–10.1) for CP55940, 23.5 nM (95% CI, 18.8–33.3) for CP55940 plus AM1710 (10 μM), and 57.3 nM (95% CI, 45.1–77.4) for CP55940 plus AM1710 (20 μM). The decrease in potency with no effects on efficacy indicates a competitive mode of inhibition. Thus, AM1710 and CP55940 bind to the same site on CB1 receptors, leading to decreased potency of CP55940 toward CB1 receptors in the presence of AM1710.

Both GW405833 and AM1710 Attenuate CP55940 Activation of pERK1/2

Turning to pERK1/2 activation, again using HEK293 cells stably transfected with rCB1, we confirmed that CP55940 activates pERK1/2 in a concentration-dependent manner (Fig. 5C). As with cAMP experiments, GW405833 had no effect on its own, but at 1 μM it completely blocked the effects of CP55940 at CP55940 concentrations up to at least 1 μM (Fig. 5C). Increasing concentrations of GW405833 (300 nM, 500 nM, and 1 μM) inhibited CP55940-induced pERK1/2 activation, with Emax (% basal) values of 72% (95% CI, 65–73.4) for CP55940, 62% (95% CI, 58.5–63.1) for CP55940 plus GW405833 (300 nM), and 45% (95% CI, 42.4–48.2) for CP55940 plus GW405833 (500 nM). However, GW405833 did not affect the potency of CP55940 in ERK1/2 activation, with EC50 values of 9.6 nM (95% CI, 3.9–17.5) for CP55940, 13.1 nM (95% CI, 7.1–21.6) for CP55940 plus GW405833 (300 nM), and 15.8 nM (95% CI, 8.3–27.8) for CP55940 plus GW405833 (500 nM).

AM1710 also had no effect on pERK1/2 levels on its own at 10 μM, but AM1710 at 10 μM and 20 μM progressively reduced CP55940 activation of ERK1/2 (Fig. 5D). AM1710...
shifted the CP55940 concentration-response curve to the right, indicating a reduction in the potency of CP55940 for ERK1/2 activation in the presence of AM1710 (10 \( \mu M \) and 20 \( \mu M \)), with EC\(_{50} \) values of 4.7 nM (95% CI, 3.6–8.9) for CP55940, 34 nM (95% CI, 27.7–37.1) for CP55940 plus AM1710 (10 \( \mu M \)), and 47 nM (95% CI, 42.4–57.8) for CP55940 plus AM1710 (20 \( \mu M \)). Interestingly, increasing concentrations of AM1710 decreased the efficacy of CP55940 for ERK1/2 activation, with E\(_{\text{max}} \) values of 54.3 (95% CI, 50.1–59.4) for CP55940, 45.1 (95% CI, 42.2–47.6) for CP55940 plus AM1710 (10 \( \mu M \)), and 26 (95% CI, 19.9–32.3) for CP55940 plus AM1710 (20 \( \mu M \)). This mixed behavior (reduction in potency and E\(_{\text{max}} \) of CP55940 in the presence of AM1710) indicates mixed modes of inhibition by AM1710 in this assay.

GW405833 Time-Dependently Alters CP55940 Recruitment of Arrestin, Whereas AM1710 Does So at Relatively Lower Potency

Activation of G protein–coupled receptors often recruits \( \beta \)-arrestins to the cell membrane. As expected, a 90-minute treatment with CP55940 potently and efficaciously recruited arrestin in CHO-mouseCB\(_1 \) cells in a concentration-dependent fashion (Fig. 6A), with an EC\(_{50} \) of 4.3 nM (95% CI, 2.8–6.1) and an E\(_{\text{max}} \) (% control) of 248 (95% CI, 233–257). Surprisingly, a 90-minute treatment with GW405833 modestly recruited arrestin on its own in a concentration-dependent fashion (Fig. 6A), with an EC\(_{50} \) of 0.25 nM (95% CI, 0.08–0.82; \( P < 0.05 \), one-way ANOVA with Bonferroni post hoc test) and an E\(_{\text{max}} \) (% control) of 46 (95% CI, 43–48). However, in contrast with other signaling pathways examined, a 5-minute pretreatment with GW405833 (1 \( \mu M \)) did not inhibit CP55940 recruitment of arrestin to CB\(_1 \) (Fig. 6A), with an EC\(_{50} \) of 9.1 nM (95% CI, 3.5–16.8) and an E\(_{\text{max}} \) (% control) of 263 (95% CI, 249–270). We also tested a 5-minute pretreatment with 5 \( \mu M \) and 10 \( \mu M \) GW405833, finding that they too were without effect (data not shown). Interestingly, the effect of GW405833 on CP55940-mediated arrestin recruitment was time dependent. After a 20-minute pretreatment with GW405833, arrestin recruitment by CP55940 was enhanced (Fig. 6B). However, for longer GW405833 pretreatments, CP55940 recruitment was similar to recruitment without GW405833, and ultimately, starting at 60 minutes of GW405833 pretreatment, CP55940 recruitment of arrestin was inhibited by GW405833 pretreatment (Fig. 6B). The potentiation was statistically significant at 20 minutes, as were the inhibitions relative to CP55940 alone at 60 and 90 minutes (\( P < 0.05 \), one-way ANOVA with Bonferroni post hoc test). Importantly, arrestin recruitment by GW405833 alone (Fig. 6A) appeared to be time dependent, because it was not observed.
after 60 minutes of GW405833 treatment (Fig. 6B; \( P < 0.05 \), one-way ANOVA).

To explore the possibility of a chemical interaction between CP55940 and GW405833 that may lead to a complex arrestin signaling profile, similar experiments were performed using the cannabinoid receptor agonist, WIN55212-2 (Fig. 6C). Again, GW405833 displayed a profile in which it modestly potentiated WIN55212-2–mediated recruitment of arrestin after 20 minutes of treatment (\( P < 0.05 \), one-way ANOVA with Bonferroni post hoc test) and then antagonized arrestin recruitment with longer treatments (90 minutes; \( P < 0.0001 \), one-way ANOVA with Bonferroni post hoc test). Thus, it appears that the biphasic stimulation/inhibition seen with GW405833 generalizes to structurally dissimilar cannabinoid receptor agonists and is not secondary to a chemical interaction between GW405833 and CP55940. One possibility is that GW405833...
favors multiple/different conformations of the receptor at different time points. Another possibility is that GW405833 is a dual-steric ligand and sequentially binds to two different sites (sites distinct from the orthosteric binding site); the first site potentiates CB1 agonist-mediated arrestin recruitment, whereas the second site inhibits recruitment (e.g., Grundmann et al., 2016).

AM1710 also modestly recruited arrestin on its own (Fig. 6D), with an $E_{\text{max}}$ (% control) of 138 ± 1 ($P < 0.05$, t test versus baseline). However, in contrast with GW405833, 5-minute pretreatment with AM1710 (10 $\mu$M and 20 $\mu$M) reduced the extent of CP55940-mediated arrestin recruitment (Fig. 6D) ($P < 0.01$ for 10 $\mu$M; $P < 0.005$ for 20 $\mu$M), without significantly affecting CP55940 potency.

**GW405833 and AM1710 Attenuate WIN55212-2–Induced Increases in IP$_1$ Levels**

We have previously shown that certain CB1 agonists, especially aminoalkylindoles such as WIN55212-2, can engage CB1 to activate G$q$ signaling to increase intracellular calcium via activation of phospholipase C and release of IP$_3$ (Lauckner et al., 2005). Therefore, we tested whether GW405833 and AM1710 affected G$q$ signaling in rCB1-expressing HEK cells. The CB1 agonist WIN55212-2 increased IP$_1$ levels by approximately 50% (Fig. 7A). Pretreatment for 5 minutes with 10 $\mu$M GW405833 fully blocked the WIN55212-2 increase in IP$_1$ (Fig. 7A), whereas GW405833 had no effect on its own. High concentrations of GW405833 –200 $\mu$M (Fig. 7A) reduced the extent of CP55940-mediated arrestin recruitment (Fig. 6D) ($P < 0.01$ for 10 $\mu$M; $P < 0.005$ for 20 $\mu$M), without significantly affecting CP55940 potency.

**Discussion**

Our chief finding is that GW405833 and AM1710 are not only CB2 agonists as previously reported, but they also interact with CB1 receptors with important functional consequences. These structurally distinct compounds have differential properties at CB1; most notably, our data suggest that GW405833 is a noncompetitive antagonist, whereas AM1710 is a competitive antagonist/inverse agonist at the orthosteric site for G protein signaling and a low-efficacy agonist for arrestin recruitment and internalization. AM1710 was generally less potent than GW405833, sometimes requiring 20 $\mu$M concentrations to produce a statistically significant effect. Consistent with this observation was a $K_I$ of 10 $\mu$M in the Schild analysis of internalization. The noncompetitive inhibition of CB1 signaling by GW405833 is consistent between the systems used: 1) the autaptic neurons that use the endogenous cannabinoid, 2-arachidonoylglycerol, and 2) the cell-based plate assays using transfected cells and synthetic cannabinoids. In contrast, AM1710 showed signs of pathway selectivity, with internalization and arrestin data suggesting that AM1710 is a low-potency, low-efficacy agonist for these pathways, whereas the cyclase and IP$_1$ data are more consistent with AM1710 being a moderate-affinity inverse agonist at these pathways. Thus, the structure of AM1710 may offer an entry point for the development of arrestin-biased CB1 agonists.

These dual agonist/antagonist properties make GW405833 and AM1710 rare additions to the pharmacological toolkit available to the cannabinoid field. The only other published compound with this profile is URB447 (LoVerme et al., 2009). A compound with this profile is particularly valuable in a multidimensional system in which both CB1 and CB2 receptors are present and can potentially mediate opposing functions. For example, in the immune system where both CB1 and CB2 receptors have been found to be active, GW405833 may offer a single-drug option to dissect out the contributions of each receptor system to immune function. Another example is treatment of chronic pain, in which CB2 agonists and CB1 antagonists have both been shown to be beneficial in various preclinical models (Costa et al., 2005; Pernía-Andrade et al., 2009; Comelli et al., 2010; Gutierrez et al., 2011). It has also been suggested that the inclusion of CB1 antagonist properties in a model of neuropathic nociception would be advantageous.

**Fig. 7.** GW405833 and AM1710 block WIN55212-2 elevation of IP$_1$ levels. (A) WIN55212-2 increased IP$_1$ levels in a concentration-dependent manner, an effect that was fully blocked by 5-minute pretreatment with 10 $\mu$M GW405833. GW405833 had no effect on its own. (B) AM1710 had no effect on IP$_1$ levels but did concentration-dependently block WIN55212-2–induced IP$_1$ accumulation. IP$_1$ levels were determined as described in the Materials and Methods ($P < 0.01$, t test comparing all values to IP$_1$ accumulation after 1 $\mu$M WIN55212-2). All experiments were performed in triplicate and repeated at least twice, unless mentioned otherwise.
Our Schild analysis suggests that AM1710 is a low-affinity ($K_B$ of approximately 10 $\mu$M) competitive ligand at CB$_1$. In contrast, the action of GW405833 appears to be noncompetitive in nature, suggesting that GW405833 does not bind to the orthosteric site of CB$_1$, which is consistent with ligand binding studies (e.g., Valenzano et al., 2005). A plausible mechanism is that GW405833 acts at an allosteric site on CB$_1$, in that case making it a negative allosteric modulator of CB$_1$. Our results do not, however, rule out indirect action via some other receptor or signaling pathway. For example, GW405833 has also been reported to serve as a partial agonist at G protein–coupled receptor 55 and to enhance the signaling of the putative G protein–coupled receptor 55 ligand lysophosphatidylinositol (Anavi-Goffer et al., 2012), although these particular examples are unlikely in the systems studied here since GW405833 generally had little effect on its own.

GW405833 was, however, broadly efficacious, acting as an antagonist in every assay examined (albeit with a time dependence when inhibiting arrestin recruitment).

The interactions of GW405833 with CB$_1$–mediated arrestin recruitment are quite intriguing. Brief treatment with GW405833 modestly enhanced arrestin recruitment to the CB$_1$ receptor both in the presence and absence of CP55940. A longer treatment with GW405833 further enhanced arrestin recruitment by CP55940. However, by 1 hour, this enhancement by GW405833 shifted to a pronounced inhibition. The net inhibitory effect is consistent with the inhibitory actions seen for other signaling pathways. Transitory stimulation of arrestin signaling is also consistent with the observation that the inhibition of CP55940–mediated CB$_1$ internalization was only evident at 30 minutes after treatment with GW405833 (Fig. 3C). This time dependence of the effects of GW405833 on arrestin recruitment was notable for several reasons. Based on our initial experiments, we would have concluded that GW405833 had no effect on arrestin recruitment by CP55940 even at 10 $\mu$M. However, those concentration–response data were collected with a 5-minute pretreatment with GW405833 followed by cotreatment with CP55940. Our results underscore the importance of considering the time course of drug actions even in relatively simple model systems (Klein Herenbrink et al., 2016). Separately, given that brief treatments were sufficient to inhibit CB$_1$ signaling in other experiments, this raised the question of why the time dependence was limited to arrestin recruitment.

In summary, we found that the CB$_2$ agonist GW405833 acts broadly as a medium-potency, noncompetitive CB$_1$ antagonist. AM1710 is a low-potency, low-affinity ligand with mixed pathway-dependent, low-agonist activity/inverse agonist properties at CB$_1$. Interestingly, although AM1710 appears to act competitively, GW405833 acts as a noncompetitive antagonist. The unusual pharmacological profile of either compound may prove therapeutically advantageous in certain instances. These compounds may also serve as the starting point for the development of molecules with more favorable efficacy and potency at either of the receptors while retaining duality of action.


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