Two Janus Cannabinoids That Are Both CB₂ Agonists and CB₁ Antagonists

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ABSTRACT

The cannabinoid signaling system includes two G protein–coupled receptors, CB₁ and CB₂. 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 CB₁ and CB₂ are involved in diverse processes, it would be advantageous to develop ligands that differentially engage CB₁ and CB₂. We now report that GW405833 [1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-3-[2-(4-morpholinyl)ethyl]-1*H*-indole] and AM1710 [1-hydroxy-9-methoxy-3-(2-methyloctan-2-yl)benzo[*c*]chromen-6-one], described as selective CB₂ agonists, can antagonize CB₁ receptor signaling. In autaptic hippocampal neurons, GW405833 and AM1710 both interfered with CB₁-mediated depolarization-induced suppression of excitation, with GW405833 being more potent. In addition, in CB₁-expressing human embryonic kidney 293 cells, GW405833

noncompetitively antagonized adenylyl cyclase activity, extracellular signal-regulated kinase 1/2 phosphorylation, phosphatidylinositol 4,5-bisphosphate signaling, and CB₁ internalization by CP55940 (2-[(1*R*,2*R*,5*R*)-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 CB₁/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 CB₁ receptor, with no evident time dependence. In summary, we determined that GW405833 and AM1710 are not only CB₂ agonists but also CB₁ antagonists, with distinctive and complex signaling properties. Thus, experiments using these compounds must take into account their potential activity at CB₁ 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, CB₁ and CB₂, 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 CB₁ or CB₂. The widespread distribution of these

receptors, particularly of CB₁, 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 CB₂ agonists in some preclinical pain models may be due to their concurrent activation of CB₁ 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 CB₂ agonist, is also a potent and efficacious CB₁ agonist (Murataeva et al., 2012). In the same study, we noted that the CB₂ antagonist AM630 [6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone] also blocks CB₁ 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

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ABBREVIATIONS: 95% CI, 95% confidence interval; AM1710, 1-hydroxy-9-methoxy-3-(2-methyloctan-2-yl)benzo[*c*]chromen-6-one; AM630, [6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone; ANOVA, analysis of variance; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CP55940, 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol; DR, dose ratio; DSE, depolarization-induced suppression of excitation; EPSC, excitatory postsynaptic current; ERK1/2, extracellular signal-regulated kinase 1/2; GW405833, 1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-3-[2-(4-morpholinyl)ethyl]-1*H*-indole; HA, hemagglutinin; HBS, HEPES-buffered saline; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; IP, inositol phosphate; JWH015, 1-propyl-2-methyl-3-(1-naphthoyl)indole; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; rCB₁, ratCB₁; SR141716, 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; TBS, Tris-buffered saline; URB447, [4-amino-1-[(4-chlorophenyl)methyl]-2-methyl-5-phenylpyrrol-3-yl]-phenylmethanone; WIN55,212-2, (1*R*)-2-methyl-11-[(morpholin-4-yl)methyl]-3-(naphthalene-1-carbonyl)-9-oxa-1-azatricyclo[6.3.1.0^{4,12}]dodeca-2,4(12),5,7-tetraene.

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]-1*H*-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 (CHOK1 cells stably expressing human CB₂), with a K_i of 3.92 ± 1.58 nM (Valenzano et al., 2005). While at CB₁ receptors, GW405833 was a low-affinity ligand, with a K_i of 4772 ± 1676 nM, and was approximately 1200-fold more selective for CB₂ receptors (Valenzano et al., 2005). Similarly, the structurally distinct AM1710 has been used in several publications 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 (Dhopeswarkar 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.

Materials and Methods

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 (Furshpan 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 CaCl₂, 1.5 mM MgCl₂, 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 MgCl₂, 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 $0.50 \pm$ 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)-CB₁-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 μ l/well HEPES-buffered saline (HBS)/bovine serum albumin (BSA; 0.08 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 μ l/well) with Tris-buffered saline (TBS). Odyssey blocking buffer (LI-COR Inc., Lincoln, NE) was applied at

100 μ l/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 μ l/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 μ l/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.

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 ice-cold methanol with the plate 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 μ l) 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, 5.5) using a LI-COR Odyssey scanner. ERK1/2 activation (expressed in percentages) was calculated by dividing the average integrated intensities of the drug-treated wells by the average integrated intensities of vehicle-treated wells. No primary antibody wells were used to determine nonspecific binding of the secondary antibody. 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 $\times$ stimulation buffer (1 $\times$ 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 transferred to a 384-well OptiPlate (500 cells/ μ l, 10 μ l) and stimulated with drugs/compounds (made in stimulation buffer, 5 μ l, 4 $\times$ concentration, 1 μ M final concentration) and forskolin (made in stimulation buffer, 5 μ l, 4 $\times$ concentration, 1 μ M final concentration) as appropriate for 5 minutes at room temperature. Cells were then lysed by addition of 10 μ l Eu-cAMP tracer working solution (4 $\times$, made fresh in 1 $\times$ lysis buffer supplied with the kit; under subdued light conditions)

and 10 μ l ULight anti-cAMP working solution (4 $\times$, made fresh in 1 $\times$ 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 μ l HBS/BSA (BSA, 0.2 mg/ml), an additional 10 μ l of HBS/BSA containing a 10X concentration of drugs/compounds (10 $\times$ 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-[(1*R*,2*R*,5*R*)-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 G_q G protein leads to phospholipase C β activation and initiation of the inositol phosphate (IP) cascade. Accumulated IP₃ is quickly dephosphorylated to IP₂ and then IP₁. This assay takes advantage of the fact that accumulated IP₁ is protected from further degradation by the addition of lithium chloride and IP₁ 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 μ l, 5000 cells) were resuspended in 1 $\times$ 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 μ l IP₁-d₂ (made fresh in lysis buffer, supplied with the kit), followed by addition of 5 μ l Ab-cryptate (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

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 [(11*R*)-2-methyl-11-[(morpholin-4-yl)methyl]-3-(naphthalene-1-carbonyl)-9-oxa-1-azatricyclo[6.3.1.0^{4,12}]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 μ M 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 μ M (Fig. 1, C and D). Similarly, AM1710 did not directly inhibit neurotransmission (Fig. 2, A and B) (relative EPSC charge after 10 μ M 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. GW405833 antagonizes CB₁ signaling in autaptic hippocampal neurons. (A) Sample time course shows that treatment with 10 μ M GW405833 does not inhibit EPSCs. (B) Summary of data showing lack of direct inhibition of neurotransmission by GW405833 at 10 μ M. (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 μ M 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 μ M GW405833. Axes: 2 nA, 30 milliseconds. GW40, GW405833; pC, picocoulomb.

Fig. 2. AM1710 antagonizes CB₁ signaling in autaptic hippocampal neurons. (A) Sample time course shows that treatment with 10 μ M AM1710 does not inhibit EPSCs. (B) Summary of data showing lack of direct inhibition of neurotransmission by AM1710 at 10 μ M. (C) AM1710 inhibits CB₁-dependent DSE in a concentration-dependent fashion (red circles). Inhibition resulting from 3-second depolarization without drug is also shown (black square). (D) Sample DSE time courses before and with 10 μ M AM1710 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 10 μ M AM1710. Axes: 2 nA, 50 milliseconds. pC, picocoulomb.

Western assay (Daigle et al., 2008). GW405833 (10 μ M) did not alter CB₁ receptor surface levels over a 2-hour period (Fig. 3A), with surface levels of $102\% \pm 15\%$ 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 ≥ 100 nM, antagonized internalization induced by 5 nM CP55940 (Fig. 3B), with receptor surface level values of $91\% \pm 7\%$ for GW405833 (10 μ M) plus CP55940 (5 nM) and $73\% \pm 3\%$ for CP55940 (5 nM) ($n = 24$, $P < 0.05$, two-way ANOVA with Bonferroni post hoc test). Moreover, 10 μ M GW405833 prevented internalization by a 2-hour treatment with 100 nM CP55940 (Fig. 3C), with cell surface receptor values (% of control) of $63\% \pm 4\%$ for CP55940 (100 nM) and $97\% \pm 6.5\%$ for CP55940 (100 nM) plus GW405833 (10 μ M) 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 μ M) slightly internalized CB₁ receptors after a 2-hour period (Fig. 4A) (surface levels of $93\% \pm 1.5\%$ at 120 minutes; $n = 16$, $P = 0.02$, t test versus baseline), suggesting that AM1710 is a modestly efficacious agonist for CB₁ receptor internalization. Furthermore, in contrast with GW405833, 10 μ M 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 $62\% \pm 8\%$ for CP55940 (100 nM), and $65\% \pm 6.7\%$ for CP55940 (100 nM) plus AM1710 (10 μ M) 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 μ M. In addition, 20 μ M and 30 μ M AM1710 more substantially shifted the dose-response curve for CP55940 (Fig. 4C).

Fig. 3. GW405833 does not internalize CB₁ receptors but noncompetitively inhibits CP55940-mediated CB₁ internalization. (A) Data from the on-cell Western assay show that GW405833 (10 μM) does not affect CB₁ surface levels. The CB₁ inverse agonist SR141716 (1 μM) reliably increases cell surface receptors and is included for comparison. (B) GW405833 diminishes CP55940-mediated CB₁ internalization at higher concentrations. **P* < 0.05, one-way ANOVA with Dunnett post hoc test versus CP55940 (5 nM). (C) Cotreatment with 10 μM GW405833 and 100 nM CP55940 prevents CP55940-mediated internalization. ***P* < 0.01, two-way ANOVA with Bonferroni post hoc for drug conditions at different time points. (D) CP55940 dose-response curves with increasing concentrations of GW405833 that were used to prepare a Schild plot (summarized in Table 1). (E) The Schild plot for GW405833 antagonism of CP55940 is consistent with noncompetitive antagonism [slope (value ± S.E.M.) ≠ 1]. Receptor internalization (expressed in % basal) was calculated by dividing the average integrated intensities of the drug-treated wells by the average integrated intensities of vehicle-treated wells (see the *Materials and Methods*). All assays were performed in triplicate, unless mentioned otherwise. EC₅₀ and/or *E*_{max} values were obtained by fitting the dose-response curve using nonlinear regression with GraphPad Prism 4.0 software. A Schild plot was generated from the data plotted in Fig. 3D. Briefly, full concentration-response curves were obtained for CP55940 in the presence and absence of GW405833 at 0.5 μM, 1 μM, and 10 μM concentrations. Dose ratios (DRs) were obtained by dividing the EC₅₀ of CP55940 obtained in the presence of various concentrations of GW405833 by the EC₅₀ of CP55940 alone. Log(dose ratio-1) was plotted against antagonist concentrations on a logarithmic scale using linear regression (GraphPad Prism) to yield the Schild slope. CP/CP55, CP55940; GW40, GW405833; SR1, SR141716.

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 (*K_B* of approximately 10 μM), competitive antagonism.

TABLE 1

Schild analysis for CB₁ internalization is consistent with noncompetitive antagonism for GW405833 and competitive antagonism for AM1710. Data are presented with S.E.M. unless indicated otherwise.

Antagonist	Schild Slope	Hill Slope	<i>K_B</i>	<i>pA</i> ₂	<i>R</i> ²
			μM		
GW405833	0.22 ± 0.02	ND	ND	ND	0.98
AM1710	0.93 ± 0.11	ND	10	5	0.95

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, *K_B*, and *pA*₂. 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.

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 *E*_{max} (% 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 *E*_{max} (*P* < 0.01, *t* tests for 1 μM concentration) (Fig. 5A), with *E*_{max} (% basal) values of 23.2 ± 8.1 for CP55940 plus GW405833 (300 nM) and 13 ± 0.7 for

Fig. 4. AM1710 does not internalize CB₁ receptors but competitively inhibits CP55940-mediated CB₁ internalization. (A) Data from the on-cell Western assay show that AM1710 (10 μ M) modestly internalized CB₁ receptors after 120 minutes of treatment. The CB₁ inverse agonist SR141716 (1 μ M) significantly increases cell surface receptors and is included for comparison. (B) In contrast with GW405833, cotreatment with 10 μ M AM1710 and 100 nM CP55940 has little effect on CP55940-mediated internalization. (C) High concentrations of AM1710 decrease the potency of CP55940-mediated internalization, albeit at a higher concentration than GW405833. (D) The Schild plot for AM1710 antagonism of CP55940-induced internalization is consistent with competitive antagonism (slope $\approx$ 1). Receptor internalization (expressed in % basal) was calculated by dividing the average integrated intensities of the drug-treated wells by the average integrated intensities of vehicle-treated wells (see the *Materials and Methods*). All assays were performed in triplicate, unless mentioned otherwise. EC₅₀ and/or *E*_{max} values were obtained by fitting the dose-response curve using nonlinear regression with GraphPad Prism 4.0 software. The Schild plot was generated from internalization assay experiments (C). Briefly, full concentration-response curves were obtained for CP55940 in the presence and absence of AM1710 at 1 μ M, 3 μ M, 10 μ M, 20 μ M, and 30 μ M concentrations. Dose ratios were obtained by dividing the EC₅₀ of CP55940 obtained in the presence of various concentrations of AM1710 by the EC₅₀ of CP55940 alone. Log(dose ratio-1) was plotted against antagonist concentrations on a logarithmic scale using linear regression (GraphPad Prism 4.0) to yield the Schild slope. All experiments were performed in triplicate and repeated at least twice, unless mentioned otherwise. CP, CP55940; DR-1, dose ratio - 1; SR1, SR141716.

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 *E*_{max}, 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 *E*_{max} of 117% $\pm$ 5% (*P* < 0.01 at 1 μ M AM1710). AM1710 decreased the potency, but not the efficacy (*t* test at 1 μ M concentration), of CP55940 inhibition of adenylyl cyclase at 10 and 20 μ M (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 μ M), and 57.3 nM (95% CI, 45.1–77.4) for CP55940 plus AM1710 (20 μ M). 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 μ M it completely blocked the effects of CP55940 at CP55940 concentrations up to at least 1 μ M (Fig. 5C). Increasing concentrations of GW405833 (300 nM, 500 nM, and 1 μ M) inhibited CP55940-induced pERK1/2 activation, with *E*_{max} (% 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 μ M, but AM1710 at 10 μ M and 20 μ M progressively reduced CP55940 activation of ERK1/2 (Fig. 5D). AM1710

Fig. 5. GW405833 and AM1710 differentially modulate CP55940 inhibition of cAMP production and activation of pERK1/2. (A) CP55940 diminishes cAMP accumulation induced by forskolin. GW405833 has no effect on its own but increasing concentrations progressively attenuate CP55940 inhibition of cAMP accumulation and 1 μ M completely blocks the action of CP55940. (B) AM1710 slightly potentiates cAMP accumulation on its own ($P < 0.01$, t test at 1 μ M) and modestly decreases the potency of CP55940. (C) CP55940 increases pERK1/2 activation. GW405833 alone does not affect pERK1/2 levels but increasing concentrations progressively attenuate CP55940 stimulation of pERK1/2 accumulation and 1 μ M completely blocks the action of CP55940. (D) For pERK1/2 activation, AM1710 alone does not affect pERK1/2 levels; however, 10 μ M and 20 μ M AM1710 reduce phosphorylation of ERK1/2 by CP55940 ($P < 0.01$, t test at 1 μ M AM1710). pERK1/2 levels (expressed in %) were calculated by dividing the average integrated intensities of the drug-treated wells by the average integrated intensities of vehicle-treated wells (see the *Materials and Methods*). All experiments were performed in triplicate and repeated at least twice, unless mentioned otherwise. EC_{50} and/or E_{max} values were obtained by fitting the dose-response curve using non-linear regression with GraphPad Prism 4.0 software. CP55940.

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 μ M and 20 μ 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 μ M), and 47 nM (95% CI, 42.4–57.8) for CP55940 plus AM1710 (20 μ M). Interestingly, increasing concentrations of AM1710 decreased the efficacy of CP55940 for ERK1/2 activation, with E_{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 μ M), and 26 (95% CI, 19.9–32.3) for CP55940 plus AM1710 (20 μ M). This mixed behavior (reduction in potency and E_{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 β -arrestins to the cell membrane. As expected, a 90-minute treatment with CP55940 potently and efficaciously recruited arrestin in CHO-mouseCB₁ 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_{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_{max} (% control) of 46 (95% CI, 43–48). However, in contrast with other signaling pathways examined, a 5-minute pretreatment with GW405833 (1 μ M) did not inhibit CP55940 recruitment of arrestin to CB₁ (Fig. 6A), with an EC_{50} of 9.1 nM (95% CI, 3.5–16.8) and an E_{max} (% control) of 263 (95% CI, 249–270). We also tested a 5-minute pretreatment with 5 μ M and 10 μ 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

Fig. 6. GW405833 and AM1710 differentially alter arrestin recruitment by CP55940. (A) Treatment with GW405833 (90 minutes followed by cell lysis) modestly increases arrestin recruitment in CHO-mouseCB₁ cells but does not alter CP55940-induced arrestin recruitment (cells were treated with vehicle or 1 μM GW405833 for 5 minutes, followed by 1 hour of vehicle or GW405833 plus CP55940) ($P < 0.01$, t test at 1 μM). (B) GW405833 time-dependently alters CP55940-dependent arrestin recruitment to CB₁ receptors. Brief GW405833 pretreatment (20 minutes) followed by coapplication of GW405833 with CP55940 enhances CP55940-mediated arrestin recruitment, whereas pretreatment with GW405833 for an hour or more antagonizes CP55940-mediated recruitment ($P < 0.05$, one-way ANOVA with Bonferroni post hoc test). (C) GW405833 alters WIN55212-2-dependent arrestin recruitment to CB₁ receptors in a time-dependent fashion. Brief GW405833 pretreatment (20 minutes) followed by coapplication of GW405833 with WIN55212-2 enhances WIN55212-2-mediated arrestin recruitment, whereas pretreatment for 90 minutes antagonizes WIN55212-2-mediated arrestin recruitment ($P < 0.05$, one-way ANOVA with Bonferroni post hoc test). (D) AM1710 modestly increases arrestin recruitment on its own and attenuates CP55940-induced arrestin recruitment at 10 μM and 20 μM. * $P < 0.05$; *** $P < 0.001$, one-way ANOVA with Bonferroni post hoc test. All experiments were performed in triplicate and repeated at least twice, unless mentioned otherwise. EC₅₀ and/or E_{max} values were obtained by fitting the dose-response curve using nonlinear regression with GraphPad Prism 4.0 software. CP, CP55940; GW, GW405833 ns, not significant; WIN, WIN55212-2.

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

GW405833 and AM1710 Attenuate WIN55212-2-Induced Increases in IP₁ 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 IP₃ (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 IP₁ levels by approximately 50% (Fig. 7A). Pretreatment for 5 minutes with 10 μ M GW405833 fully blocked the WIN55212-2 increase in IP₁ (Fig. 7A), whereas GW405833 had no effect on its own. High concentrations of AM1710 alone modestly reduced IP₁ accumulation (Fig. 7B), with an $E_{\max}$ (% basal) of 81 ± 2 ($P < 0.05$, t test versus baseline). As with GW405833, AM1710 attenuated the increase in IP₁ elicited by WIN55212-2, doing so fully at 20 μ M (Fig. 7B), with an $E_{\max}$ (% basal) of 81 ± 4 at 20 μ M ($P < 0.05$, t test versus WIN55212-2).

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 μ M concentrations to produce a statistically significant effect. Consistent with this observation was a K_B of 10 μ 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 IP₁ 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 IP₁ levels. (A) WIN55212-2 increased IP₁ levels in a concentration-dependent manner, an effect that was fully blocked by 5-minute pretreatment with 10 μ M GW405833. GW405833 had no effect on its own. (B) AM1710 had no effect on IP₁ levels but did concentration-dependently block WIN55212-2-induced IP₁ accumulation. IP₁ levels were determined as described in the *Materials and Methods* ($P < 0.01$, t test comparing all values to IP₁ accumulation after 1 μ M WIN55212-2). All experiments were performed in triplicate and repeated at least twice, unless mentioned otherwise.

(Rahn et al., 2008). Of course, an important question is whether the noncompetitive antagonism of CB₁ receptors by GW405833 has the psychiatric liabilities associated with potent CB₁ inverse agonists such as SR141716/rimonabant.

Our Schild analysis suggests that AM1710 is a low-affinity (K_B of approximately 10 μ M) competitive ligand at CB₁. In contrast, the action of GW405833 appears to be noncompetitive in nature, suggesting that GW405833 does not bind to the orthosteric site of CB₁, 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 CB₁, in that case making it a negative allosteric modulator of CB₁. 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 often a potent and efficacious antagonist; in several instances, it completely blocked the effect of CP55940 at 1 μ M. However, it was not as potent for the inhibition of DSE in autaptic neurons, with the relatively high IC₅₀ of 2.6 μ M. This may indicate that the interaction of GW405833 with CB₁ depends on the local environment (e.g., neurons versus overexpression) or the nature and/or efficiency of receptor effector coupling in the various expression systems. 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 CB₁-mediated arrestin recruitment are quite intriguing. Brief treatment with GW405833 modestly enhanced arrestin recruitment to the CB₁ 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 CB₁ 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 μ 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 CB₁ signaling in other experiments, this raised the question of why the time dependence was limited to arrestin recruitment.

In summary, we found that the CB₂ agonist GW405833 acts broadly as a medium-potency, noncompetitive CB₁ antagonist. AM1710 is a low-potency, low-affinity ligand with mixed pathway-dependent, low-efficacy agonist/inverse agonist properties at CB₁. 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.

Authorship Contributions

Participated in research design: Straiker, Mackie.
Conducted experiments: Dhopeswarkar, Murataeva, Straiker.
Contributed new reagents or analytic tools: Makriyannis.
Performed data analysis: Dhopeswarkar, Murataeva, Straiker.
Wrote or contributed to the writing of the manuscript: Dhopeswarkar, Murataeva, Makriyannis, Straiker, Mackie.

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