Two Janus cannabinoids that are both CB$_2$ agonists and CB$_1$ antagonists

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Running title: Two Janus cannabinoids

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Text pages: 30
Number of tables: 1
Number of figures: 7

Abstract: 237 words
Introduction: 499 words
Discussion: 871 words

Non-standard abbreviations:
bovine serum albumen (BSA), excitatory postsynaptic currents (EPSCs),
depolarization induced suppression of excitation (DSE), picocoulombs (pC),
HEPES buffered saline (HBS), human embryonic kidney (HEK), Hertz (Hz),
homogeneous time-resolved fluorescence (HTRF), inositol phosphate (IP),
Phospholipase C (PLC), Tris buffered saline (TBS)

Recommended section: Neuropharmacology
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. Though the cannabinoid signaling system has therapeutic potential, a persistent hurdle has remained the development of receptor-selective ligands. Because CB₁ and CB₂ are involved in diverse processes, it would be advantageous develop ligands that differentially engaging CB₁ and CB₂. We now report that GW405833 and AM1710, described as selective CB₂ agonists, can antagonize CB₁ receptor signaling. In autaptic hippocampal neurons, GW405833 and AM1710 both interfere with CB₁-mediated depolarization induced suppression of excitation (DSE), with GW405833 being more potent. In addition, in CB₁-expressing HEK293 cells, GW504833 non-competitively antagonizes adenylyl cyclase activity, ERK1/2 phosphorylation, PIP₂ signaling and CB₁ internalization by CP55940. In contrast, AM1710 behaved as a low potency competitive antagonist/inverse agonist in these signaling pathways. GW405833 interactions with the CB₁/arrestin signaling were complex: it 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-dependency. In summary we have 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 physiological 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 have previously reported that JWH015, 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 also blocks CB₁ signaling at relatively low concentrations. Thus, 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 not merely to 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, which is a peripherally restricted CB₁ antagonist, and 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 have examined the activity of the CB₂ agonists, GW405833 and AM1710, towards CB₁ receptors in autaptic hippocampal neurons as well as in...
several additional signaling assays using CB1-expressing HEK293 or 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 Ki of 3.92 ± 1.58 nM (Valenzano et al., 2005). While at CB1 receptors, GW405833 was a low affinity ligand with a Ki of 4772 ± 1676 nM (~1200 fold more selective for CB2 receptors) (CHO-K1 cells stably expressing human CB1 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; Deng et al., 2012; Wilkerson et al., 2012; Rahn et al., 2014; Deng et al., 2015). AM1710 displayed high affinity for CB2 receptors (HEK cells stably expressing human CB2 receptors) with a Ki of 6.7 nM (Khanolkar et al., 2007) and an EC50 of 11 nM (E_max 48 ± 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 Ki of 360 nM (95% confidence intervals, 330-390) (Khanolkar et al., 2007) (~30 fold more selective for CB2 receptors). We now report that in addition to CB2 agonism, GW405833 and AM1710 also serve as antagonists at CB1 receptors, albeit with distinct pharmacological properties.

METHODS

Hippocampal culture preparation. All procedures used in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and approved by the Animal Care Committee of Indiana University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. 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) DMEM containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than three hours after removal from 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 (in mM) 119 NaCl, 5 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 30 glucose, and 20 HEPES. Continuous flow of solution through the bath chamber (~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 used on the same day.

Recording pipettes of 1.8-3 MΩ were filled with (in mM) 121.5 K-Gluconate, 17.5 KCl, 9 NaCl, 1 MgCl₂, 10 HEPES, 0.2 EGTA, 2 MgATP, and 0.5 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 ms 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 pC). 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 10-20 second 0.5 Hz baseline, DSE was evoked by depolarizing to 0 mV for 50 msec, 100 msec, 300 msec, 500 msec, 1 sec, 3 sec and 10 sec, 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) are normalized to one. DSE inhibition values are presented as fractions of 1, i.e. a 50% inhibition from the baseline response is 0.50 ± standard error of the mean. 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 are obtained to determine pharmacological properties of endogenous 2-AG signaling by depolarizing neurons for progressively longer durations (50 msec, 100 msec, 300 msec, 500 msec, 1 sec, 3 sec and 10 sec). The data are fitted with a nonlinear regression, allowing calculation of an ED$_{50}$, the effective dose or duration of depolarization at which a 50% inhibition is achieved. Statistical significance in these curves is based on non-overlapping 95% confidence intervals.

**On-cell Western for receptor internalization**

The internalization of the receptor was measured using an on-cell Western assay (Daigle et al., 2008). Briefly, HA-CB$_1$ expressing human embryonic kidney (HEK) cells were grown to 95% confluence in DMEM + 10% FBS + 0.5% Pen/Strep (Daigle et al., 2008). Cells were washed once with HEPES buffered saline (HBS)/bovine serum albumen (BSA) (BSA @ 0.08 mg/ml) 200μL per well. Drugs in HBS/BSA were applied at the indicated concentrations to cells and were incubated for indicated amount of time at 37°C. Cells were then fixed with 4% PFA for 20 minutes and washed 4 times (200μl per well) with tris-buffered saline (TBS). Blocking buffer (Odyssey Blocking buffer, LI-COR, Inc. Lincoln, NE) was
applied at 100 μl per well for 1h at room temperature. Anti-HA antibody, (mouse monoclonal, 1:500, Covance, Princeton, NJ) diluted in 50:50 Odyssey Blocking Buffer and PBS, was then applied for one hour at room temperature. Following this, the plate was washed 4 times (200 μl per well) with TBS. Secondary antibody diluted (anti-mouse 680 antibody 1:800, LI-COR, Inc.) in 50:50 blocking buffer and PBS, was then applied for one hour at room temperature. Following this, the plate was washed 4 times (200 μl per well) with TBS. The plate was imaged using an Odyssey scanner (700 nm channel, 5.0 intensity, LI-COR, Inc.). Receptor internalization (expressed in % of basal surface levels) was calculated by dividing average integrated intensities of the drug treated wells by average integrated intensities of vehicle-treated wells. (Binding of HA antibody to wildtype HEK cells was <10% of transfected cells.) All assays were performed in triplicate, unless otherwise mentioned.

pERK1/2 assay

ERK1/2 activation was measured using an in cell western assay (Wong, 2004; Atwood et al., 2012). HA-CB1 expressing human embryonic kidney (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 following day, media was replaced by 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% PFA for 20 mins followed by treatment with ice-cold methanol with the plate maintained at -20°C for an additional 15 min. Plates were then washed with TBS/0.1% Triton X-100 for 25 mins (5x 5 min washes). The final wash solution was then replaced by Odyssey blocking buffer (150 μL) and incubated further for 90 min with gentle shaking at room temperature. Blocking solution was then removed and replaced with blocking solution containing anti-phospho-ERK1/2 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
min (5x 5 min 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 again washed 5 times with TBS/0.05% Tween-20 solution. The plates were patted dry and scanned (700nm channel, 5.5 intensity) using a LI-COR Odyssey scanner. ERK1/2 activation (expressed in %) was calculated by dividing average integrated intensities of the drug treated wells by 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 otherwise mentioned.

Adenylyl cyclase assay

Adenylyl cyclase assays were optimized using Perkin Elmer’s LANCE® ultra cAMP kit (Catalog # TRF0262, Perkin Elmer, Boston, MA) as per the manufacturer’s instruction. All assays were performed at room temperature using 384-optiplates (Catalog# 6007299, Perkin Elmer). Briefly, HA-CB1 human embryonic kidney (HEK) cells were detached from ~60% confluent plates/dish using versene. Cells were then resuspended gently in 1X stimulation buffer (1X Hank’s Balanced Salt Solution (HBSS), 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4, made fresh on the day of experiment) and further incubated for 1 hour at 37 °C, 5% CO₂ and humidified air. Cells were then transferred to a 384-optiplate (500 cells/µl, 10µl) and stimulated with drugs/compounds (made in stimulation buffer, 5µL, 4x concentration, 1µM final concentration) and forskolin (made in stimulation buffer, 5µL, 4x concentration, 1µM final concentration) as appropriate for 5 mins at room temperature. Cells were then lysed by addition of 10µl Eu-cAMP tracer working solution (4X, made fresh in 1X lysis buffer supplied with the kit; under subdued light conditions) and 10 µl Ulight™ anti-cAMP working solution (4X, made fresh in 1X lysis buffer) and further incubated for 1 hour at room temperature. Plates were then read with the TR FRET mode on an Enspire plate reader (Perkin Elmer).
Arrestin recruitment assay

Arrestin recruitment assays were performed using the PathHunter® CHO-K1 CNR1 (CHO-mCB₁, catalog # 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 (β gal) 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 gal enzyme, which then acts on substrate to emit light that can be measured on a luminescence plate reader. Cells were thawed, grown and maintained in PathHunter AssayComplete™ (catalog # 92-0018GF2, DiscoveRx) media. All assays were performed in poly-D-lysine coated 96 well plates. About 20,000 cells/well were plated and grown overnight at 37 °C, 5% CO₂ in humidified air. The next day, media was replaced with 90 μl of HBS/BSA (BSA, 0.2 mg/ml) and cells were challenged with 10 μl of drugs/compounds (10X concentration) in HBS/BSA and incubated for 90 mins at 37°C, 5% CO₂, humidified air. For time course assays, cells were pretreated with GW405833 for the time described in the text, followed by CP55940 + GW405833 treatment and cell lysis. Reactions were terminated by addition of Pathhunter™ detection reagent (DiscoveRx) and the plate was further incubated for 60 mins at room temperature. Complementation reactions were monitored by chemiluminiscence using an Enspire multiplate reader.

IP1 assay

Accumulation of myo-Inositol Phosphate 1(IP1), a downstream metabolite of IP3, was measured by using IP-One HTRF®kit (Catalog # 62, IPAPEB, Cisbio, Bedford, MA). Functional coupling of CB₁ receptor to G₄ G protein leads to Phospholipase Cβ (PLC) 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 (LiCl) and IP1 levels can be easily
quantified using an HTRF (homogeneous time-resolved fluorescence) assay. HA-CB1 human embryonic kidney (HEK) cells were detached from ~60% confluent plates/dish using versene. Cells (10 µl, 5000 cells) were resuspended in 1X stimulation buffer (containing LiCl, supplied with the kit) and were incubated for 1 hour at 37 °C, 5% CO2 and humidified air and then transferred to a 384-optiplate, followed by stimulation with drugs/compounds made in DMSO/ethanol as appropriate, for 10 mins. Cells were then lysed with 5µl of IP1-d2 (made fresh in lysis buffer, supplied with the kit) followed by addition of 5µl 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 triplicates and were repeated at least two times, unless otherwise mentioned.

**Schild analysis**

Schild plots were generated for internalization assays by employing the method of Schild (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 (DR) 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(DR-1) was then plotted against the logarithm of antagonist concentration using linear regression (GraphPad Prism,4.0) 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 was from Sigma-Aldrich (St. Louis, MO). CP55940 was obtained through the NIDA Drug Supply Program (Bethesda, MD). AM1710 was prepared in the laboratory of Dr. Alex Makriyannis (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 (DSE (Straiker and Mackie, 2005)). This can be quantified by stimulating the neuron with a series of successively longer depolarizations (50ms, 100ms, 300ms, 500ms, 1sec, 3 sec, 10 sec) resulting in progressively greater inhibition of neurotransmission (Straiker et al., 2011a; Straiker et al., 2011b). This yields a “depolarization-response curve” that permits the characterization of some pharmacological properties of cannabinoid signaling, including the calculation of an effective-dose (depolarization) 50 (ED₅₀), corresponding in this case to the duration of depolarization that results in 50% of the maximal inhibition.

We found that while GW405833 did not directly inhibit neurotransmission (Fig. 1A-B, relative EPSC charge after 10μM GW405833: 1.02 ± 0.02, n=4), it did interfere with CB₁-mediated DSE in a concentration-dependent manner with an IC₅₀ of 2.6μM (Fig. 1C-D). Similarly, AM1710 did not directly inhibit neurotransmission (Fig. 2A-B, relative EPSC charge after 10μM 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. 2C-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-Western assay (Daigle et al., 2008).
GW405833 (10μM) does not alter CB₁ receptor surface levels over a 2-hour period (Fig. 3A; surface levels at 120 mins (%): 102 ± 15; n=16, p>0.05 by 1 way ANOVA with Dunnet’s post hoc test vs. baseline), suggesting GW405833 is not an inverse agonist for CB₁ receptor trafficking. The CB₁ inverse agonist, SR141716, incubated with the cells for two hours served as a positive control for externalization (Atwood et al., 2012), increasing CB₁ surface levels by ~20% after 120 minutes of incubation (Fig. 3A). However, GW405833 antagonized internalization induced by two-hour treatment with CP55940. GW405833, at concentrations of 100nM and above, antagonizes internalization induced by 5nM CP55940 (Fig. 3B; GW405833 (10μM) + CP55940 (5nM) receptor surface level 91 ± 7% vs. CP55940 (5nM) receptor surface level 73 ± 3%, n=24; *, p<0.05, 2 way ANOVA with Bonferroni post hoc test). Moreover, 10μM GW405833 prevents internalization by a two hour treatment with 100nM CP55940 (Fig. 3C; Cell surface receptors at 120mins (% of control values): CP55940(100nM): 63 ± 4%; CP55940 (100nM) + GW405833 (10μM): 97 ± 6.5%, n=24, **, p<0.01, 2 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 Figure 3E and Table 1, the response profile is consistent with non-competitive 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 (10μM) slightly internalized CB₁ receptors after a 2-hour period (Fig. 4A; surface levels at 120 mins (%): 93 ± 1.5; n=16, p=0.02 by Student’s t-test vs. baseline), suggesting AM1710 is a modestly efficacious agonist or for CB₁ receptor internalization. Furthermore, in contrast to
GW405833, 10μM AM1710 does not significantly alter the time course of internalization during a two-hour treatment with 100nM CP55940 (Fig. 4B; Cell surface levels at 120mins (% of baseline): CP55940 (100nM): 62 ± 8%; CP55940 (100nM) + AM1710 (10μM): 65 ± 6.7% 17; n= 24, p>0.05, 2 way ANOVA). In examining the effects of a range of AM1710 concentrations on CP55940-induced internalization, we found that AM1710 only modestly right-shifted the CP55940 response curve, even at 10μM. 20μM and 30μM 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. 4C-D), sufficient to conduct a Schild analysis. As shown in Figure 4D and Table 1, the Schild analysis is consistent with a low affinity (KB ~10 μM), competitive antagonism.

GW405833 and AM1710 attenuate inhibition of forskolin-stimulated cAMP accumulation by CP55940

We next examined if GW405833 affected forskolin-stimulated cAMP accumulation or its inhibition by CB1 agonists in HEK cells stably transfected with rCB1. As expected, CP55940 inhibits cAMP accumulation in a concentration-dependent manner (Fig 5A; EC50: 9.5 nM, E_max(% basal): 45.6 ± 8.3). While GW405833 had no effect on its own, at 1μM it completely blocks adenylyl cyclase inhibition by CP55940 at CP55940 concentrations up to at least 1μM (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; Student’s t tests for 1 μM concentration) (E_max (%basal): CP+GW405833(300 nM) 23.2 ±8.1; CP+GW405833(500 nM) 13 ± 0.7) with no significant change in the potency (Fig 5A; EC50(95%CI): CP--9.5 nM (2.1-19); CP+GW405833(300 nM)--12 nM (3.4-18.3); CP55940+GW405833(500 nM)--15 nM(4.5-23.6). Classically, reduction in E_max, with no change in potency, indicates a non-competitive 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; E_max: 117 ± 5%; p<0.01 at 1 µM AM1710). AM1710 decreased the potency, but not the efficacy (Student’s t test at 1 µM concentration), of CP55940 inhibition of adenylyl cyclase at 10 and 20 µM (Fig. 5B; EC₅₀ (95%CI): CP: 6.7 nM(2.3-10.1); CP+AM1710 (10µM): 23.5 nM (18.8-33.3); CP+AM1710 (20µM): 57.3 nM (45.1-77.4)). The decrease in potency with no effects on efficacy indicate competitive mode of inhibition. Thus, AM1710 and CP55940 bind to the same site on CB1 receptors leading to decreased potency of CP55940 towards 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 has no effect on its own, but at 1µM it completely blocks the effects of CP55940 at CP55940 concentrations up to at least 1µM (Fig. 5C). Increasing concentrations of GW405833 (300 nM, 500 nM and 1 µM) inhibited CP55940 induced pERK1/2 activation (E_max(%basal), (95%CI): CP55940--72% (65-73.4); CP+GW405833(300 nM) --62 (58.5-63.1); CP+GW405833(500 nM) --45 (42.4-48.2). However, GW405833 did not affect the potency of CP55940 in ERK1/2 activation (EC₅₀(95%CI): CP55940--9.6 nM (3.9-17.5); CP+GW4059833 (300 nM)--13.1 nM (7.1-21.6); CP+GW405833(500 nM) 15.8 nM(8.3-27.8).

AM1710 also had no effect on its own on pERK1/2 levels at 10µM, but AM1710 at 10µM and 20µM progressively reduced CP55940 activation of ERK1/2 (Fig. 5D). AM170 shifted the CP55940 concentration response curve to the right indicating reduction in the potency of CP55940 for ERK1/2 activation in presence of AM1710(10 µM and 20 µM)(EC₅₀(95%CI): CP55940--4.7 nM (3.6-8.9); CP+AM1710(10 µM)--34 nM(27.7-37.1); CP+AM1710(20 µM)--47 nM(42.4-57.8).
Interestingly, increasing concentrations of AM1710 decreased the efficacy of CP55940 for ERK1/2 activation ($E_{\text{max}}$(95%CI): CP55950--54.3 (50.1-59.4); CP+AM1710(10 µM)--45.1(42.2-47.6); CP+AM1710(20µM)--26(19.9-32.3). This mixed behavior (reduction in potency and $E_{\text{max}}$ of CP55940 in the presence of AM1710) indicates mix modes of inhibition by AM1710 in this assay.

**GW405833 time-dependently alters CP55940 recruitment of arrestin while AM1710 does so at relatively lower potency.**

Activation of GPCRs often recruits beta-arrestins to the cell membrane. As expected, a 90 minute treatment with CP55940 potently and efficaciously recruits arrestin in CHO-mCB1 cells in a concentration-dependent fashion (Fig. 6A) ($EC_{50}$(95%CI) 4.3 nM(2.8-6.1), $E_{\text{max}}$(% control)(95%CI 248 (233-257). Surprisingly, a 90 minute treatment with GW405833 modestly recruits arrestin on its own in a concentration-dependent fashion (Fig. 6A, $EC_{50}$ (95% CI): 0.25nM (0.08-0.82); p<0.05, 1 way ANOVA with Bonferroni post hoc test; $E_{\text{max}}$(%control)(95%CI), 46 (43-48)). However, in contrast to other signaling pathways examined, a 5 minute pretreatment with GW405833 (1μM) did not inhibit CP55940 recruitment of arrestin to CB1 (Fig. 6A)($EC_{50}$(95%CI) 9.1 nM(3.5-16.8); $E_{\text{max}}$(%control)(95%CI), 263 (249-270). We also tested a 5 minute pretreatment with 5µM and 10µM GW405833, finding that they, too, were without effect (data not shown). Interestingly, the effect of GW405833 on CP55940-mediated arrestin recruitment was time-dependent. Following a twenty 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 mins of GW405833 pretreatment, CP55940 recruitment of arrestin was inhibited by GW504833 pretreatment (Fig. 6B). The potentiation was statistically significant at 20 mins as were the inhibitions relative to CP55940 alone at 60 and 90 minutes (p<0.05, 1 way ANOVA with Bonferroni post hoc test). Importantly, arrestin recruitment by GW405833 alone (Fig. 6A) appeared time-dependent, as
it was not observed following 60 minutes of GW405833 treatment (Fig. 6B; p>0.05, 1 way ANOVA).

To rule 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 where it modestly potentiated WIN55212-2 mediated recruitment of arrestin after 20 minutes of treatment (p<0.05, 1 way ANOVA with Bonferroni post hoc test) and then antagonized arrestin recruitment with longer treatments (90 min; p<0.0001, 1 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 dualsteric ligand and sequentially binds to two different sites (sites distinct from the orthosteric binding site)-the first site potentiates CB₁ agonist-mediated arrestin recruitment, while the second site inhibits recruitment (e.g. (Grundmann et al., 2016)).

AM1710 also modestly recruited arrestin on its own (Fig. 6D; Eₘₐₓ (% control ): 138 ± 1; p<0.05, t-test vs. baseline), but in contrast to GW405833, five minute pretreatment with AM1710 (10µM and 20µM) reduced the extent of CP55940-mediated arrestin recruitment (Fig. 6D, p<0.01 for 10µM; p<0.005 for 20µM), 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 Gq 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 CB_1 agonist WIN55212-2 increased IP_1 levels by ~50% (Fig. 7A). Pretreatment for 5 mins with 10 \( \mu \text{M} \) GW405833 fully blocked the WIN55212 increase in IP_1 (Fig. 7A), while GW405833 had no effect on its own. High concentrations of AM1710 alone modestly reduced IP-1 accumulation (Fig. 7B; \( E_{\text{max}} \) (% basal): 81 \( \pm \) 2, p<0.05, t-test vs. baseline) and, as with GW405833, AM1710 attenuated the increase in IP_1 elicited by WIN55212, doing so fully at 20\( \mu \text{M} \) (Fig. 7B: \( E_{\text{max}} \) (% basal) at 20\( \mu \text{M} \): 81 \( \pm \) 4, p<0.05, t-test vs. WIN55212-2).

**DISCUSSION**

Our chief finding is that GW405833 and AM1710 are not only CB_2 agonists as previously reported but also interact with CB_1 receptors with important functional consequences. These structurally distinct compounds have differential properties at CB_1; most notably our data suggest that GW405833 is a non-competitive antagonist while 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\( \mu \text{M} \) concentrations to produce a statistically significant effect. Consistent with this observation was a KB of 10\( \mu \text{M} \) in the Schild analysis of internalization. The non-competitive inhibition of CB_1 signaling by GW405833 is consistent between the several systems used: the autaptic neurons that utilize the endogenous cannabinoid, 2-AG, and 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 while 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 where 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, where CB₂ agonists and CB₁ antagonists have both been shown to be beneficial in various preclinical models (Costa et al., 2005; Pernia-Andrade et al., 2009; Comelli et al., 2010; Gutierrez et al., 2011). It has also been suggested that in a model of neuropathic nociception the inclusion of CB₁ antagonist properties would be advantageous (Rahn et al., 2008). Of course, an important question is whether the non-competitive 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 as a low affinity (KB ~10μM) competitive ligand CB₁. In contrast, the action of GW405833 appears non-competitive 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 GPR55 and to enhance the signaling of the putative GPR55 ligand lysophosphatidylinositol (Anavi-Goffer et al., 2012), though 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 completely blocking the effect of CP55940 at 1 μM. However, it was not as potent for the inhibition of DSE in autaptic neurons, with the relatively high IC50 of 2.6μM. This may indicate that the interaction of GW405833 with CB1 depends on the local environment (e.g., neurons versus over expression) 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 dependency 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 post-treatment with GW405833 (Fig 3C). This time-dependence of GW405833 effects 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 10μM. However that concentration response data was collected with a 5-minute pre-treatment of GW405833 followed by co-treatment 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 in other experiments brief treatments were
sufficient to inhibit CB₁ signaling, this raised the question of why the time-dependence was limited to arrestin recruitment.

In summary, we have found that the CB₂ agonist GW405833 acts broadly as a medium-potency, non-competitive CB₁ antagonist. AM1710 is a low potency, low affinity ligand with mixed pathway-dependent low efficacy agonist/inverse agonist properties at CB₁. Interestingly while AM1710 appears to act competitively, GW405833 acts as a non-competitive 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.
Author contributions:
Participated in research design: Straiker, Mackie
Conducted experiments: Straiker, Dhopeshwarkar, Murataeva.
Contributed new reagents or analytic tools: Makriyannis
Performed data analysis: Straiker, Dhopeshwarkar, Murataeva.
Wrote or contributed to the writing of the manuscript: Straiker, Murataeva, Dhopeshwarkar, Mackie, Makriyannis.
References


Footnote:

This work was supported by the National Institutes of Health [Grants DA009158 (AM&KM), DA011322 (KM), DA021696 (KM), EY24625 (AS)].
Legend for Figures

Figure 1. GW405833 antagonizes CB1 signaling in autaptic hippocampal neurons.

A) Sample time course shows that treatment with 10μM GW405833 does not inhibit EPSCs. B) Summary of data showing lack of direct inhibition of neurotransmission by GW405833 at 10μM. C) GW405833 inhibits CB1-dependent depolarization-induced suppression of excitation in a concentration-dependent fashion (DSE, red triangles). Inhibition resulting from 3-second depolarization without drug is also shown (black square). D) Sample DSE time courses before and with treatment of 3μM GW405833. Right panels show EPSC traces at corresponding time points just before depolarization (1) and immediately after depolarization (2). Top traces are control and bottom traces are after treatment with 3μM GW405833. Axes: 2nA, 30msec

Figure 2. AM1710 antagonizes CB1 signaling in autaptic hippocampal neurons.

A) Sample time course shows that treatment with 10μM AM1710 does not inhibit EPSCs. B) Summary of data showing lack of direct inhibition of neurotransmission by AM1710 at 10μM. C) AM1710 inhibits CB1-dependent depolarization-induced suppression of excitation in a concentration-dependent fashion (DSE, black circles). Inhibition resulting from 3-second depolarization without drug is also shown (black square). D) Sample DSE time courses before
and with treatment of 10μM AM1710. Right panels show EPSC traces at corresponding time points just before depolarization (1) and immediately after depolarization (2). Top traces are control and bottom traces are after treatment with 10μM AM1710. Axes: 2nA, 50msec

Figure 3. GW405833 does not internalize CB₁ receptors but non-competitively inhibits CP55940-mediated CB₁ internalization.

A) Data from on cell western assay shows that GW405833 (10μM) does not affect CB₁ surface levels. The CB₁ inverse agonist SR141716 (1μM) 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’s post hoc test vs. CP55940 (5nM). C) Co-treatment with 10μM GW405833 and 100nM CP55940 prevents CP55940-mediated internalization. **, p<0.01, two way ANOVA with Bonferroni post hoc for drug conditions at different time points. D) Panel shows CP55940 dose response curves with increasing concentrations of GW405833 that were used to prepare a Schild plot (summarized in Table 1). E) Schild plot for GW405833 antagonism of CP55940 is consistent with non-competitive antagonism (slope (value ± SEM) ≠ 1). Receptor internalization (expressed in % basal) was calculated by dividing average integrated intensities of the drug treated wells by average integrated intensities of vehicle-treated wells (see Methods). All assays were performed in triplicate, unless otherwise mentioned. EC₅₀ and/or Eₘₐₓ values were obtained by fitting the dose response curve using nonlinear regression with
GraphPad Prism 4.0. 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 (DR) were obtained by dividing EC₅₀ of CP55940 obtained in the presence of various concentrations of GW405833 to the EC₅₀ of CP55940 alone. Log(DR-1) was plotted against antagonist concentrations on a logarithmic scale using linear regression (GraphPad Prism) to yield the Schild slope.

**Figure 4. AM1710 does not internalize CB₁ receptors but competitively inhibits CP55940-mediated CB₁ internalization.**

A) Data from on cell western assay shows that AM1710 (10μM) modestly internalized CB₁ receptors after 120 minutes of treatment. The CB₁ inverse agonist SR141716 (1μM) significantly increases cell surface receptors and is included for comparison. B) In contrast to GW405833, co-treatment with 10μM AM1710 and 100nM 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) Schild plot for AM1710 antagonism of CP55940-induced internalization is consistent with competitive antagonism (slope=1). Receptor internalization (expressed in % basal) was calculated by dividing average integrated intensities of the drug treated wells by average integrated intensities of vehicle-treated wells (see Methods). All assays were performed in triplicate,
unless otherwise mentioned. EC₅₀ and/or Eₘₐₓ values were obtained by fitting the dose response curve using nonlinear regression with GraphPad Prism 4.0. The Schild plot was generated from internalization assay experiments (Fig 4C). Briefly, full concentration response curves were obtained for CP55940 in the presence and absence of AM1710 at 1, 3, 10, 20 and 30 μM concentrations. Dose ratios (DR) were obtained by dividing the EC₅₀ of CP55940 obtained in the presence of various concentrations of AM1710 to the EC₅₀ of CP55940 alone. Log(DR-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 triplicates and repeated at least twice, unless otherwise mentioned.

Figure 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 μM completely blocks the action of CP55940. B) AM1710 slightly potentiates cAMP accumulation on its own (p<0.01, Student’s t-test at 1 μM) 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 μM completely blocks the action of CP55940. D)
For pERK1/2 activation, AM1710 alone does not affect pERK1/2 levels, however 10μM and 20μM AM1710 reduce phosphorylation of ERK1/2 by CP55940 (p<0.01, Student’s t-test at 1 μM AM1710). pERK1/2 levels (expressed in %) were calculated by dividing average integrated intensities of the drug treated wells by average integrated intensities of vehicle-treated wells (see Methods). All experiments were performed in triplicates and repeated at least twice, unless otherwise mentioned. EC_{50} and/or E_{max} values were obtained by fitting the dose response curve using nonlinear regression with GraphPad Prism 4.0.

Figure 6. GW405833 and AM1710 differentially alter arrestin recruitment by CP55940. A) Treatment with GW405833 (90 min followed by cell lysis) modestly increases arrestin recruitment in CHO-mCB1 cells but does not alter CP55940-induced arrestin recruitment (cells were treated with vehicle or 1 μM GW405833 for 5 min followed by 1 hour of vehicle or GW40833 + CP55940) (p<0.01; Student’s t-test at 1 μM). B) GW405833 time-dependently alters CP55940-dependent arrestin-recruitment to CB1 receptors. Brief GW405833 pretreatment (20 min) followed by coapplication of GW405833 with CP55940 enhances CP55940-mediated arrestin recruitment, while pretreatment with GW405833 for an hour or more antagonizes CP55940-mediated recruitment (p<0.05, 1 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 min), followed by coapplication of GW405833 with WIN55212-2 enhances WIN55212-2 mediated arrestin recruitment, while
pretreatment for 90 min antagonized WIN55212-2 mediated arrestin recruitment. (p<0.05, 1 way ANOVA with Bonferroni post hoc test) D) AM1710 modestly increases arrestin recruitment on its own and attenuates CP55940-induced arrestin recruitment at 10μM and 20μM. *, p<0.05, ***, p<0.001, one way ANOVA with Bonferroni post hoc test. All experiments were performed in triplicates and repeated at least twice, unless otherwise mentioned. EC₅₀ and/or Eₘₐₓ values were obtained by fitting the dose response curve using nonlinear regression with GraphPad Prism 4.0.

Figure 7. GW405833 and AM1710 block WIN55212-2 elevation of IP1 levels
A) WIN55212 increased IP1 levels in a concentration-dependent manner, an effect that was fully blocked by 5 min pretreatment with 10μM 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 Methods. (p<0.01; Student’s t-test comparing all values to IP1 accumulation following 1 μM WIN55212-2). All experiments were performed in triplicates and repeated at least twice, unless otherwise mentioned.
### Table 1. Schild analysis for CB₁ internalization is consistent with non-competitive antagonism for GW405833 and competitive antagonism for AM1710.

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 (DR) were obtained by dividing the EC₅₀ of CP55940 obtained in the presence of various concentrations of antagonist to the EC₅₀ of CP55940 alone. log(DR-1) were plotted against antagonist concentrations on logarithmic scale using linear regression (GraphPad Prism 4.0) to yield Schild slope, KB, and pA₂. The Schild analysis of the concentration response curves for CP55940 with various concentrations of putative CB₁ antagonists GW405833 and AM1710 yield profiles that are consistent with non-competitive and competitive CB₁ antagonism, respectively.

<table>
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<tr>
<th>Antagonist</th>
<th>Schild slope (values ± SEM)</th>
<th>Hill Slope</th>
<th>KB</th>
<th>pA₂</th>
<th>R²</th>
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<td>GW405833</td>
<td>0.22 ± 0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.98</td>
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<tr>
<td>AM1710</td>
<td>0.93 ± 0.11</td>
<td>ND</td>
<td>10 µM</td>
<td>5</td>
<td>0.95</td>
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</table>
Figure 2

A) AM1710 (10 μM)

B) Relative EPSC Charge (norm)

C) Relative EPSC Charge (norm) vs log[AM1710]

D) Relative EPSC Charge (norm) vs time (sec)

- DSE (3 sec)
- DSE w/ AM1710 (10 μM)
Figure 3

**A**
Graph showing CB₁ surface levels (% basal level) over time (min) for GW40 10μM (black line) and SR1 1μM (red square).

**B**
Graph showing CB₁ surface levels (% basal level) as a function of log [drug] for CP55 5nM + GW40 (black line), GW40 (black dashed line), CP55 (red square).

**C**
Graph showing CB₁ surface levels (% basal level) over time (min) for GW40 10μM + CP 100nM (black line) and CP 100nM (red square).

**D**
Graph showing CB₁ surface levels (% basal level) as a function of log [CP55940] for CP55940 (black circle), CP+GW40 (0.5 μM) (black triangle), CP+GW40 (1 μM) (red square), CP+GW40 (10 μM) (green triangle).

**E**
Graph showing Log(DR-1) as a function of Log[GW405833]M with a slope of 0.22 ± 0.02.
Figure 6

(A) Graph showing the relationship between Log[Drug] M and Arrestin recruitment (% control) for CP55940, CP+GW405833 (1 μM), and GW405833.

(B) Bar graph comparing Arrestin recruitment (% control) for different treatments: Control, CP only (1 hour), GW (5 min)+CP (1 hour), GW (20 min)+CP (1 hour), GW (40 min)+CP (1 hour), GW (60 min)+CP (1 hour), GW (90 min)+CP (1 hour), GW405833 only (1 hour). Significance levels are indicated by asterisks and "ns".

(C) Bar graph showing Arrestin recruitment (% control) for WIN only (1 hour), GW (5 min)+WIN (1 hour), GW (20 min)+WIN (1 hour), GW (40 min)+WIN (1 hour), GW (60 min)+WIN (1 hour), GW (90 min)+WIN (1 hour), WIN (1 hour). Significance levels are indicated by asterisks and "ns".

(D) Graph showing the relationship between Log[Drug] M and Arrestin recruitment (% control) for CP55940, CP+AM1710 (10 μM), CP+AM1710 (20 μM), and AM1710.
Figure 7

A

WIN55212-2
WIN+GW405833 (10 μM)
GW405833

IP-1 accumulation (% Basal)

B

WIN55212-2
WIN+AM1710 (10 μM)
WIN+AM1710 (20 μM)
AM1710

IP-1 accumulation (% Basal)

Log[Drug] M

Log[Drug] M