Probing the CB1 Cannabinoid Receptor Binding Pocket with AM6538, a High-Affinity Irreversible Antagonist

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ABSTRACT

Cannabinoid receptor 1 (CB1) is a potential therapeutic target for the treatment of pain, obesity and obesity-related metabolic disorders, and addiction. The crystal structure of human CB1 has been determined in complex with the stabilizing antagonist AM6538. In the present study, we characterize AM6538 as a tight-binding/irreversible antagonist of CB1, as well as two derivatives of AM6538 (AM4112 and AM6542) as slowly dissociating CB1 antagonists across binding simulations and cellular signaling assays. The long-lasting nature of AM6538 was explored in vivo wherein AM6538 continues to block CP55,940-mediated behaviors in mice up to 5 days after a single injection. In contrast, the effects of SR141716A abate in mice 2 days after injection. These studies demonstrate the functional outcome of CB1 antagonist modification and open the path for development of long-lasting CB1 antagonists.

Introduction

Cannabinoid receptor 1 (CB1) is the most abundant G protein–coupled receptor (GPCR) in the human central nervous system, as well as being expressed in peripheral tissues (Marsicano and Kuner, 2008). CB1 is known to signal through inhibitory G proteins and interacts with β arrestins (Mackie, 2006). CB1 in the central nervous system is predominantly localized to axon terminals (Castillo et al., 2012). Activation of CB1 inhibits the release of neurotransmitters from the presynaptic neuron via inhibition of Ca2+ channels and the activation of inward-rectifying K+ channels. In addition, the CB1 inhibits adenylyl cyclase production of cAMP and increases the phosphorylation of kinases associated with cell survival, such as extracellular signal–regulated kinase (Howlett et al., 2004; Bosier et al., 2010; Flores-Otero et al., 2014). Through these effects in neurons, the CB1 regulates locomotion, mood, reward, nociception, and appetite (Castillo et al., 2012; Lutz et al., 2015). Consequently, agonists of CB1 have been investigated as potential treatments for dyskinesia, depression, pain, and cachexia (Lutz et al., 2015). Antagonists of CB1 have been investigated as potential treatments for addiction and mental illness and for the suppression of appetite (Black et al., 2011; Mazier et al., 2015; Rubino et al., 2015; Schindler et al., 2016).

The CB1-selective antagonist SR141716A (rimonabant) was originally approved by the European Medical Agency as an adjunct treatment of obesity; however, it was withdrawn from use because of reports of dysphoria, depression, and suicidal ideation (Rinaldi-Carmona et al., 1994; Janero and Makriyannis, 2009; Fong and Heymsfield, 2009). This experience aside, the inhibition of CB1 remains a potential therapeutic target for the treatment of obesity-related metabolic disorders and addiction if more tolerable compounds can be developed (Janero and Makriyannis, 2009).

AM6538 is a structural analog of SR141716A that was developed as a high-affinity CB1 antagonist capable of stabilizing CB1 and facilitated the formation of high-quality crystals that were used to solve the crystal structure (Hua et al., 2016). This structure, along with a confirming structure of the receptor bound to taranabant (Shao et al., 2016), another CB1 antagonist structurally unrelated to SR141716A, provides templates for understanding the antagonist binding pocket. These crystal structures have enhanced our understanding of the key structural components involved in the antagonist-bound receptor and allow for further probing of the binding pocket to refine therapeutics (Hua et al., 2016).
In this study, we characterize AM6538 as a competitive, irreversible antagonist of CB1, in binding simulations, cell culture, and in vivo. We also compare two additional structurally related antagonists, AM4112 and AM6542, to elucidate the relationship between these structural modifications and observed residence time at the CB1 receptor. The observations provide functional evidence for irreversible and slowly dissociating CB1 antagonists that produce persistent pharmacodynamic effects that are attributable to structural features of the antagonists.

Materials and Methods

Compounds and Chemistry. AM6538 [4-(4-(1-(2,4-dichlorophenyl)-3-methyl-3-(piperidin-1-ylcarbamoyl)-1H-pyrazol-5-yl)phenyl)but-3-yn-1-yl]nitrate, AM6542 [5-(4-(but-3-ene-1-yn-1-yl)phenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide], and AM4112 [1-(2,4-dichlorophenyl)-5-(4-(4-hydroxybut-1-yn-1-yl)phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide] were synthesized (purity ≥95%) using methods as described previously (Makriyannis et al., 2011; Makriyannis and Vemuri, 2014, 2017; Hua et al., 2016). CP55,940, SR141716A (Tocris, Bristol, UK), (Makriyannis et al., 2011; Makriyannis and Vemuri, 2014, 2017; Hua et al., 2016). CP55,940, SR141716A (Tocris, Bristol, UK), AM6538 (nitrate-substituted), AM4112 (hydroxy-substituted), and AM6542 (ene-yne eliminated).

DiscoveRx β Arrestin 2 Recruitment. β arrestin 2 recruitment was determined using the PathHunter assay (cat. no. 93-0200C2; DiscoveRx) according to the manufacturer’s instructions. The inhibition of forskolin-stimulated cAMP accumulation was determined in cells incubated with vehicle in the presence of forskolin. That is, 0% inhibition of cAMP accumulation corresponds to vehicle + forskolin, and 100% corresponds to maximal inhibition of cAMP by the CB1 ligand used.

Animals and Behavioral Experiments. Male C57BL/6J mice (4–6 months of age) sourced from Jackson Laboratories were used for these studies and had ad libitum access to food and water. Compounds administered intraperitoneally were prepared in DMSO and Tween-80 in deionized water (1:1:8). Mouse weight was recorded daily, and all procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals with approval from The Scripps Research Institute Animal Care and Use Committee.

Assessment of In Vivo Cannabinoid Effects. Catalepsy was assessed in the bar-holding assay 5 minutes after drug administration (Ignatowska-Jankowska et al., 2015; Grim et al., 2017). Mice were placed such that their forepaws clasped a 0.7-cm ring clamp positioned 4.5 cm above the surface of the testing space. The length of time the ring was held was recorded (seconds). The trial was ended if the mouse turned its head or body or made three consecutive escape attempts (Ignatowska-Jankowska et al., 2015; Grim et al., 2017). Body temperature was measured by rectal thermometer 15 minutes after drug administration. Antinociceptive effects were assessed in the warm water (52°C) tail-flick test 20 minutes after drug administration. Response was defined as the removal of the tail from the warm water, with a threshold time of 20 seconds.

Baseline measurements of catalepsy, temperature, and antinociception were taken at the beginning of the study in untreated animals. After baseline measurements, mice were injected with 3 mg/kg SR141716A, 3 mg/kg AM6538, or vehicle. The ability of SR141716A or AM6538 to antagonize CB1-dependent catalepsy, hypothermia, and antinociception was then challenged with 1 mg/kg CP55,940 at 1 hour, 2, 5, and 7 days after treatment with SR141716A or AM6538. In all cases, animals were only used once, and experiments were performed with the approval of the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Data Analysis and Statistics. Data are presented as the mean with S.D., or 95% confidence interval, of at least three independent experiments conducted in duplicate. Significance was determined by one- or two-way ANOVA followed by Tukey’s or Dunnett’s post-hoc analysis, as indicated. P < 0.05 was considered significant.

Fig. 1. Chemical structures of SR141716A, AM6538 (nitrate-substituted), AM4112 (hydroxy-substituted), and AM6542 (ene-yne eliminated).
The functional off-rate (ΔP_{A2}) is estimated by graphing individual P_{A2} values (unitless, logarithmic) determined using eq. 1 against time of antagonist pretreatment (Kenakin et al., 2006; Tautermann, 2016). ΔP_{A2} is then calculated as the absolute slope value for the linear regression through these P_{A2} values, which were analyzed by two-way ANOVA followed by Dunnett’s post-hoc test. E_{max} values were analyzed by one-way ANOVA followed by Tukey’s post-hoc test. E_{max} was obtained by normalizing data to percent maximal JWH-018 stimulation and fitting concentration-response curves to the four-parameter concentration-response model (Prism). Bias was calculated for THC and JWH-018 using CP55,940 as the reference agonist using the operational model (Black and Leff, 1983; Stahl et al., 2015) in Prism (Supplemental Fig. S1C).

**Results**

**AM6538, AM4112, and AM6542** are structural analogs of the well known CB₁ antagonist SR141716A. Structure-Table 1

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>CP55,940</th>
<th>THC</th>
<th>JWH-018</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM6538</td>
<td>9.0 (8.6–9.2)^a</td>
<td>9.4 (9.1–9.9)^a</td>
<td>8.1 (5.8–11)</td>
</tr>
<tr>
<td>AM4112</td>
<td>8.7 (5.5–12)</td>
<td>9.2 (8.7–9.6)</td>
<td>9.3 (9.0–9.7)</td>
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<td>AM6542</td>
<td>8.2 (7.2–9.2)</td>
<td>8.2 (6.7–9.6)</td>
<td>8.8 (8.1–9.6)</td>
</tr>
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Antagonist P_{A2} values for inhibition of forskolin-stimulated cAMP accumulation by hCB₁ agonists.

**Antagonist** CP55,940 THC JWH-018

<table>
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<tr>
<th>Antagonist</th>
<th>CP55,940</th>
<th>THC</th>
<th>JWH-018</th>
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<td>SR141716A</td>
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<td>9.2 (8.7–9.8)^a</td>
<td>8.6 (7.3–9.8)</td>
</tr>
<tr>
<td>AM6538</td>
<td>9.0 (8.6–9.2)^a</td>
<td>9.4 (9.1–9.9)^a</td>
<td>8.1 (5.8–11)</td>
</tr>
<tr>
<td>AM4112</td>
<td>8.7 (5.5–12)</td>
<td>9.2 (8.7–9.6)</td>
<td>9.3 (9.0–9.7)</td>
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<tr>
<td>AM6542</td>
<td>8.2 (7.2–9.2)</td>
<td>8.2 (6.7–9.6)</td>
<td>8.8 (8.1–9.6)</td>
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Antagonist P_{A2} values for β arrestin 2 recruitment by hCB₁ agonists.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>CP55,940</th>
<th>THC</th>
<th>JWH-018</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR141716A</td>
<td>9.5 (9.3–9.9)^a</td>
<td>8.7 (6.2–11)^a</td>
<td>7.8 (7.5–8.0)</td>
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<tr>
<td>AM6538</td>
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<tr>
<td>AM4112</td>
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<td>8.8 (6.5–11)</td>
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<td>AM6542</td>
<td>9.1 (8.5–9.6)</td>
<td>8.9 (6.5–11)</td>
<td>8.2 (7.3–9.1)</td>
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*aData are from Hua et al. (2016) wherein SR141716A and AM6538 are originally described as competitive antagonists of CP55,940 and THC.

**Fig. 2.** Molecular docking and stability assay of the three ligands. (A) Docking poses of AM6542 (yellow sticks) and AM4112 (magenta sticks) and comparisons with AM6538 (green sticks) bound CB₁ crystal structure. (B) Analytical size exclusion chromatography (aSEC) results of different ligands show various CB₁ protein homogeneity and yield. (C) N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) fluorescent dye ramping assay indicates the effects of different ligands on CB₁ protein thermostability. (D) Structural comparison of different CB₁ antagonists with CPM fluorescent dye binding. (E) Additional structural comparison of different ligands with CPM fluorescent dye binding.
activity relationship studies indicated that replacement of the chloro group at the para position of the 5-phenyl ring in SR141716A with an acetylenic chain did not result in loss of affinity for CB₁. This four-carbon acetylenic chain, bearing the nitrate group (ONO₂) on the ω position in AM6538, remains a key structure feature for its high CB₁ affinity and for its CB₁ stabilization ability (Fig. 1) (Hua et al., 2016). An indication of this property was reported with AM6538 binding to CB₁ being wash-resistant in radioligand competition assays (Hua et al., 2016). Based on structure-activity relationship studies, AM4112, containing a hydroxyl substitution on the ω carbon, and AM6542 the ene-yne eliminated form were also synthesized (Fig. 1). Compared with the docking poses of AM6542 and AM4112, the nitrate group of AM6538 forms a hydrogen bond with Tyr2755.39 and π–π interaction with Trp2795.42. These additional interactions make AM6538 bind more tightly to the CB₁ (Fig. 2A). In addition, AM6538 could further improve the protein yield, homogeneity, and thermostability of CB₁ compared with the other two ligands (Fig. 2, B and C).

AM6538, AM4112, and AM6542 are Competitive Antagonists of hCB₁. AM6538 is a competitive inhibitor of CP55,940- and THC-dependent inhibition of forskolin-stimulated cAMP accumulation and β arrestin 2 recruitment (Hua et al., 2016). Here the competitive antagonism of AM6538, and two derivative compounds, AM4112 and AM6542, were used to challenge three distinct chemotypes of CB₁ agonists: CP55,940 (full agonist, classic synthetic cannabinoid), THC (partial agonist, phytocannabinoid), and JWH-018 (potent full agonist, aminoalkylindole cannabinoid) (Atwood et al., 2010) (Supplemental Fig. S1). Like SR141716A, AM6538 antagonism of CP55,940-dependent inhibition of forskolin-stimulated cAMP accumulation is resistant to wash out. 3HA-hCB₁ CHO cells were pretreated with vehicle (1% DMSO in PBS), 1 μM SR141716A (A), AM6538 (B), AM4112 (C), or AM6542 (D) for 6 hours and then washed one, three, or five times with PBS, followed by 30-minute treatment with increasing concentrations of CP55,940. Antagonist pretreatment had no significant effect on forskolin-stimulated cAMP accumulation (Supplemental Fig. S1D). (E) Summary of pEC_{50} values for data presented in (A–D). Data are presented as mean with S.D., n = 3 experiments/treatment performed in duplicate. Data were normalized to the vehicle (+ forskolin; 0%) and maximum stimulation obtained with CP55,940 (100%) in each experiment and are fit to a nonlinear regression model using Prism 6.0. ***P < 0.001 AM6538; **P < 0.01 AM4112; ^^^P < 0.001 AM6542 compared with SR141716A within wash number, as determined by two-way ANOVA followed by Dunnett’s post-hoc analysis.
and AM6538 (Hua et al., 2016), AM4112 and AM6542 are competitive antagonists of CP55,940 and THC (Supplemental Fig. S2 and S3). In addition, SR141716A, AM6538, AM4112, and AM6542 are competitive antagonists of JWH-018 (Supplemental Fig. S2 and S3). The antagonists tested do not differ in pA2 (Table 1).

**Antagonism of hCB1 by AM6538 Is Wash-Resistant.** We tested the persistent functional antagonism of SR141716A, AM6538, AM4112, and AM6542 on inhibition of forskolin-stimulated cAMP accumulation (Fig. 3) and β arrestin 2 recruitment (Fig. 4). For inhibition of forskolin-stimulated cAMP accumulation, 3×10^4 hCB1 CHO cells were treated with antagonists for 6 hours and then washed one, three, or five times with PBS, followed by 30-minute treatment with CP55,940 in the presence of forskolin (Fig. 3). Six hours of antagonist pretreatment did not affect forskolin-stimulated cAMP accumulation compared with vehicle pretreatment (Supplemental Fig. S1D). For β arrestin 2 recruitment, CHO hCB1 cells were treated with antagonists for 6 hours and then washed one, three, or five times with PBS, followed by 30-minute treatment with CP55,940 (Fig. 4). In both assays, CP55,940 agonism is readily restored in cells pretreated with SR141716A after successive washes (Figs. 3A and 4A), whereas AM6538 blocks agonist stimulation despite repeated washes (Figs. 3B and 4B). Antagonism by AM4112, the hydroxyl-substituted derivative of AM6538, can be partially reversed with washes, but restoration of the full agonism of CP55,940 is not achieved (Figs. 3C and 4C). Antagonism by AM6542, the ene-yne–eliminated derivative of AM6538, is fully reversible after repeated washes (Figs. 3D and 4D). The change in agonist potency after repeated washing of cells is summarized in Fig. 3E (inhibition of forskolin-stimulated cAMP accumulation) and Fig. 4 (β arrestin 2 recruitment).

![Graphs showing wash-resistant antagonism](image-url)

**Fig. 4.** AM6538 antagonism of CP55,940-dependent β arrestin 2 recruitment is resistant to wash out. 3×10^4 CHO cells were treated with vehicle (1% DMSO in PBS), 1 μM SR141716A (A), AM6538 (B), AM4112 (C), or AM6542 (D) for 6 hours and then washed one, three, or five times with PBS, followed by 90-minute treatment with 0.1 nM–10 μM CP55,940. (E) Summary of pEC_{50} values for data presented in (A–D). Data are presented as mean with S.D., n = 3 experiments/treatment performed in duplicate. Data were normalized to the vehicle (0%) and maximum stimulation obtained with CP55,940 (100%) in each experiment and are fit to a nonlinear regression model using Prism 6.0. †P < 0.05; ††P < 0.01 AM6538; *P < 0.05; **P < 0.01; ***P < 0.001 AM4112; ^P < 0.05; ^^P < 0.01 AM6542 compared with SR141716A within wash number, as determined by two-way ANOVA followed by Dunnett’s post-hoc analysis.
We previously showed that AM6538 is tightly bound to CB1 based on its persistent prevention of [3H]CP55,940 binding in hCB1-HEK293 cell membranes after repeated washes (Hua et al., 2016). The data presented here demonstrate that this irreversible antagonism of hCB1 by AM6538 is persistent in the intact cellular systems. Moreover, we observed a rank order of wash resistance with AM4112 that is more resistant than AM6542, which, in turn, is more wash resistant than SR141716A. Thus, modifications of the ω carbon of the cAMP accumulation) and Fig. 4E (β arrestin 2 recruitment).

Fig. 5. AM6538, AM4112, and AM6542 are persistent antagonists of CP55,940-dependent inhibition of forskolin-stimulated cAMP accumulation. 3xHA-hCB1 CHO cells were pre-treated with 1 nM–10 μM SR141716A (A-C A-C), AM6538 (D-F D), AM4112 (G-I), or AM6542 (J-L) for 1 hour (A, D, G, J), 3 hours (B, E, H, K), or 6 hours (C, F, I, L), followed by treatment with increasing concentrations of CP55,940 for 30 minute in the presence of forskolin. Data are presented as mean with S.D.; n = 7 for SR14171A 1 hour, n = 3 for all other treatment groups; experiments performed in duplicate. Data were normalized to the vehicle (0%) and maximum CP55,940 (100%) inhibition of forskolin-stimulated cAMP accumulation within each experiment and are fit to a competitive nonlinear regression model (eq. 1) using Prism 6.0. Schild regression analysis presented in Fig. 6.
observed pA2 values (antagonist off-rate was estimated as the rate of change for against time (Figs. 5 and 6). From this, the functional derived from fitting data to a model of competitive antagonism and Copeland, 2008). The experiment (than for AM6538, which did not change within the time of the (Tautermann, 2016).

Therefore, AM4112 and AM6542 are slowly dissociating reversible hCB1 antagonists compared with their parent compound, SR141716A. These data confirm our hypothesis that AM6538 is an irreversible, competitive hCB1 antagonist (Tautermann, 2016).

molecule determine the overall functional affinity of the ligand for securing an inactive state of the receptor.

We next measured the change in pA2 by SR141716A, AM6538, AM4112, and AM6542 by graphing pA2 values derived from fitting data to a model of competitive antagonism against time (Figs. 5 and 6). From this, the functional antagonist off-rate was estimated as the rate of change for observed pA2 values (ΔpA2) (Copeland et al., 2006; Tummino and Copeland, 2008). The ΔpA2 for SR141716A was greater than for AM6538, which did not change within the time of the experiment (P < 0.05 one-way ANOVA) (Fig. 6). The ΔpA2 values for AM4112 and AM6542 were intermediate between SR141716A and AM6538 and not different from either (Fig. 6). Therefore, AM4112 and AM6542 are slowly dissociating reversible hCB1 antagonists compared with their parent compound, SR141716A. These data confirm our hypothesis that AM6538 is an irreversible, competitive hCB1 antagonist (Tautermann, 2016).

AM6538 Treatment Results in CB1 Ablation. We determined whether AM6538 treatment produces hCB1 depletion by cotreating cells with JWH-018 and SR141716A, AM6538, AM4112, or AM6542 for 1, 3, or 6 hour followed by treatment with 0.03 nM–10 μM CP55,940 for 30 minute in the presence of forskolin. pA2 values are presented as a function of time. Data are presented as mean with S.D.; n = 7 for SR14171A 1 hour, n = 3 for all other treatment groups; performed in duplicate. †P < 0.05; ††P < 0.001 AM6538; †††P < 0.001 AM4112 and AM6542 compared with SR141716A within time point for pA2, as determined by two-way ANOVA followed by Dunnett’s post-hoc analysis; #P < 0.05, AM6538 compared with SR141716A for ΔpA2, as determined by one-way ANOVA followed by Tukey’s post-hoc analysis.

AM6538 is a Persistent CB1 Antagonist In Vivo. We sought to determine whether the irreversible nature of AM6538 at CB1 could be observed in vivo by testing how long it could block agonist-induced effects in typical mouse cannabinoid response assays: antinociception (warm water tail immersion), hypothermia (rectal-probe thermometer), and catalepsy (bar test). Vehicle pretreatment, administered 1 hour before the initial CP55,940 challenge, served as a control for both day by day effects. C57BL/6J mice were treated with vehicle, SR141716A (3 mg/kg, i.p.) or AM6538 (3 mg/kg, i.p.) and then challenged with CP55,940 (1 mg/kg, i.p.) 1 hour, 2 days, 5 days, and 7 days after antagonist treatment (Fig. 8). SR141716A effectively blocked CP55,940-induced effects up to 2 days; however, AM6538 retained efficacy through 5 days as CP55,940 effects were restored on day 7 after initial antagonist dosing (Fig. 8). Thus, the duration of action of AM6538 to block cannabimetic effects of CP55,940 persist beyond those of SR14176A.

Discussion

In the present study, we provide the functional characterization of the irreversible, high-affinity CB1 antagonist AM6538 and its derivative compounds, AM4112 and AM6542. AM6538, AM4112, and AM6542 all exhibit the characteristics of competitive orothostic antagonists of

![Fig. 6. AM6538 is an irreversible antagonist hCB1. Data presented in Fig. 5 were expressed as % maximal CP55,940 inhibition of forskolin-stimulated cAMP accumulation and fit to a nonlinear regression model (eq. 1, Prism 6.0) to determine pA2. 3xHA-hCB1 CHO cells were pre-treated with 1 nM–10 μM SR141716A, AM6538, AM4112, or AM6542 for 3, 5, or 6 hour followed by treatment with 0.03 nM–10 μM CP55,940 for 30 minute in the presence of forskolin. pA2 values are presented as a function of time. Data are presented as mean with S.D.; n = 7 for SR14171A 1 hour, n = 3 for all other treatment groups; performed in duplicate. †P < 0.05; ††P < 0.001 AM6538; †††P < 0.001 AM4112 and AM6542 compared with SR141716A within time point for pA2, as determined by two-way ANOVA followed by Dunnett’s post-hoc analysis; #P < 0.05, AM6538 compared with SR141716A for ΔpA2, as determined by one-way ANOVA followed by Tukey’s post-hoc analysis.](https://example.com/fig6)

![Fig. 7. AM6538 treatment decreases E_max. 3xHA-hCB1 CHO cells were co-treated with forskolin (for cAMP assays), 0.03 nM–10 μM JWH-018 ± 1 nM–10 μM SR141716A, AM6538, AM4112, or AM6542 for (A) 30 minute and inhibition of forskolin-stimulated cAMP accumulation was quantified, or (B) 90 minute and βarrestin2 recruitment was quantified. Concentration-response data were fit to a nonlinear regression model in Prism 6.0 to determine E_max. Data are presented as mean with S.D.; for inhibition of forskolin-stimulated cAMP accumulation n = 3 (AM6538, AM4112, AM6542) n = 4 (SR141716A); for βarrestin2 recruitment n = 3 (SR14176A, AM6538, AM4112), n = 4 (AM6542) experiments/treatment performed in duplicate. Concentration-response curves available in Supplemental Figs. S2 and S3. **P < 0.01 AM6538 compared with SR141716A within antagonist dose, as determined by two-way ANOVA followed by Tukey’s post-hoc analysis.](https://example.com/fig7)
hCB1 in CHO cells stably expressing the receptor. Unlike SR141716A, antagonism of hCB1 by AM6538, AM4112, and AM6542 is resistant to washing in both inhibition of forskolin-stimulated cAMP accumulation and β arrestin 2 recruitment assays. When antagonism of hCB1 is quantified as a function of time, SR141716A displays a ΔpA2 of 0.45 ± 0.10 hour⁻¹, which is in agreement with the previously published dissociation rate (Koff) of [3H]SR141716A (Rinaldi-Carmona et al., 1995). AM6538 displays a change in pA2 of 0.05 ± 0.08 hour⁻¹, which is not different from 0. The change in ΔpA2 for AM4112 (0.18 ± 0.05 hour⁻¹) and AM6542 (0.26 ± 0.05 hour⁻¹) is faster than that of AM6538 and slower than that of SR141716A, but it is not statistically different from either. AM6538 has been reported to be a wash-resistant competitive antagonist of CB1 that inhibited in vivo antinociception and drug discrimination in a dose-dependent manner (Hua et al., 2016; Paronis et al., 2018). The present comprehensive cell culture analyses and the studies of Hua et al. (2016) and Paronis et al. (2018) support our hypothesis that AM6538 acts as an irreversible antagonist of hCB1 in functional studies of receptor action. In the present study, the ΔpA2 was determined through quantification of biologic activity—namely, the inhibition of forskolin-stimulated cAMP accumulation. The approach is limited because antagonist affinity cannot be empirically determined in functional studies; the ΔpA2 represents a change that is determined by measuring downstream effectors and not the direct dissociation of antagonist from receptor (Copeland et al., 2006; Kenakin et al., 2006; Tummino and Copeland, 2008). The major advantage of this approach is that the biologic effect(s) caused by antagonism are often directly attributed to the compounds’ residence time (Copeland et al., 2006; Tummino and Copeland, 2008), and here antagonism of biologic function is expressed as a function of time. Although this approach has been used previously for enzyme antagonists (Tummino and Copeland, 2008), the functional estimate of CB1 antagonist pA2 presented here represents the first such estimation of change in pA2 over time at a GPCR (Kenakin et al., 2006; Tautermann, 2016). This approach should prove useful at other GPCR systems because there is currently an unmet need to determine the functional kinetics of ligand-receptor interaction (Tautermann, 2016).

As an irreversible antagonist, AM6538 selectively depleted the free receptor population of hCB1 in the cell system, as demonstrated by the concentration-dependent decrease in Emax. In all cases, JWH-018 could stimulate signaling (inhibition of forskolin-stimulated cAMP accumulation or β arrestin 2 recruitment), even at the highest concentration of AM6538 used. These data suggest a large hCB1 reserve in the CHO cells being used (Kenakin et al., 2006; Colquhoun, 2007). If the cell system had little or no receptor reserve, then high receptor occupancy by AM6538 would result in a rapid loss of hCB1 agonist-dependent maximum response (Kenakin et al., 2006; Colquhoun, 2007). In vivo studies in mice revealed that AM6538 prevented CP55,940-dependent effects at 1 hour, 2 days, and 5 days after treatment, whereas antagonism of CP55,940 by SR141716A dissipated 2 days after treatment. Similarly, Paronis et al. (2018) observed that 3 or 10 mg/kg AM6538 antagonized THC-, WIN55,212-2-, or AM4054-dependent antinociception and drug discrimination in mice for up to 7 days and reduced the Emax for these effects. AM6538 inhibition of CP55,940 may have worn off by 7 days because AM6538 was excreted. Alternatively, newly synthesized CB1 may have supplanted the receptor population antagonized by AM6538, resulting in the resumption of CP55,940 sensitivity (Howlett et al., 2000).
CB1 antagonists, including SR141716A, are suppressors of appetite and addictive behaviors (Fong and Heymsfield, 2009); however, SR141716A was discontinued in the clinic because its use resulted in dysphoria, depression, and suicidal ideation (Fong and Heymsfield, 2009). It is possible that tight-binding CB1 antagonists with slower off-rates, such as AM4112 and AM6542, may allow for long-lasting inhibition of CB1 that is beneficial compared with more rapidly dissociating antagonists (Cusack et al., 2015). Slowly dissociating antagonists of the protease-activated receptor 1 (Chackalamannil et al., 2008), muscarinic M3 (Moulton and Fryer, 2011), and neurokinin 1 receptors (Lindström et al., 2007) receptors are known to be more efficacious antagonists because of their longer duration of action, reduced dosing frequency, and receptor subtype selectivity. Whether the CB1 antagonists described here are of greater clinical utility than SR141716A remains unknown. Because the crystal structure of CB1 is now known (Hua et al., 2016), we can determine the structure-activity relationship for CB1 ligands to an extent that was not previously possible and develop safer, more effective CB1 antagonists. For this development, AM6538, AM4112, and AM6542 represent compounds whose profiles as competitive antagonists may be useful.

Conclusions

AM6538 is a functionally irreversible antagonist of CB1 in vitro and in vivo. Previous attempts to develop irreversibly binding CB1 antagonists have produced compounds with poor CB1 selectivity (Fernando and Pertwee, 1997) or compounds that bound CB1 but were not functional antagonists (Howlett et al., 2000). AM6538 may prove useful in studying CB1 abundance and turnover in vitro and tolerance and tissue-specific medication of CB1-evoked effects in vivo (Howlett et al., 2000). AM6538, AM4112, and AM6542 may be useful tools for determining the kinetic effects of CB1 blockade in vivo. The ability to deplete CB1 and correlate that depletion with changes in CB1-mediated signal transduction and behaviors (Howlett et al., 2000) will allow a much more thorough understanding of how and where cannabinoid-dependent effects occur than has been previously possible.

Authorship Contributions

Participated in research design: Laprairie, Stahl, Ho, Grim, Makriyannis, Bohn.

Conducted experiments: Laprairie.

Contributed new reagents or analytical tools: Vemuri, Korde, Ho, Wu, Stevens, Hua, Liu, Makriyannis.

Performed data analysis: Laprairie, Stahl, Bohn.

Wrote or contributed to the writing of the manuscript: Laprairie, Vemuri, Stahl, Korde, Ho, Grim, Makriyannis, Bohn.

References


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Probing the CB1 cannabinoid receptor binding pocket with AM6538, a high-affinity irreversible antagonist.

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Supplemental Data
Figure S1. Modulation of hCB₁-dependent signaling. (A) 3xHA-hCB₁ CHO cells were treated with forskolin and vehicle or 0.03 nM – 10 µM CP55,940, THC, or JWH-018 for 30 min and inhibition of forskolin-stimulated cAMP accumulation was quantified. Data were normalized by setting vehicle treatment as 0% and CP55,940 E_max as 100% within assays performed on the same plate, mean with SD plotted. Measures derived from 3 parameter non-linear regression (Prism 6.0) for each individual assay were averaged (with 95% confidence intervals): CP55,940 (n=11): EC₅₀ 4.8 (1.1 – 8.5) nM, E_max 100%; JWH-018 (n=5): EC₅₀ 1.4 (0.04 – 3.2) nM, E_max 91 (80 – 101)%; THC (n=6): EC₅₀ 51 (6.7 – 110) nM, E_max 54 (42 – 67%). (B) hCB₁ CHO PathHunter (DiscoveRx) cells were treated with vehicle or agonist for 90 min and β-arrestin2 recruitment was quantified following substrate incubation. Data were normalized by setting vehicle treatment as 0% and CP55,940 E_max as 100% within assays performed on the same plate. Data are presented as the mean with SD. Measures derived from 3 parameter non-linear regression (Prism 6.0) for each individual assay were averaged (with 95% confidence interval) to provide: CP55,940 (n=12): EC₅₀ 18 (11 – 26) nM, E_max 100%; JWH-018(n=5): EC₅₀ 1.4 (0.71 – 2.1) nM; E_max 90 (79 – 101)%, (n=7): EC₅₀ 70 (8.9 – 150) nM, E_max 48 (43 – 54%). (C) ΔΔLogR values estimated using the operational model and displayed as mean with 95% confidence interval from the data in A and B. 

Data related to figure 3: (D) 3xHA-hCB₁ CHO cells were treated with vehicle (1% DMSO in PBS) or 1 µM antagonist for 6 h with 5 washes, followed by forskolin stimulation (20 µM, 30 min). The 6 hour antagonist treatment did not affect cAMP accumulation compared to vehicle without antagonist. Data are mean with SD; n = 6 (basal and vehicle), 3 (SR141716A, AM6538, AM4112, AM6542).
Inhibition of forskolin-stimulated cAMP accumulation: antagonist competition at of hCB1 by SR141716A, AM6538, AM4112, and AM6542. 3xHA-hCB1 CHO cells were co-treated with 20 µM forskolin and vehicle or 0.03 nM – 10 µM CP55,940, THC, or JHW-018 ± SR141716A, AM6538, AM4112, or AM6542 for 30 min and inhibition of forskolin-stimulated cAMP accumulation was quantified. Figures A, B, E, and F were published in the supplemental information from Hua et al., 2016 and are shown here for comparison. Concentration-response data were globally fit to a competitive nonlinear regression model (eq. 1) in Prism 6.0. Data were normalized by setting the vehicle treatment as 0% and maximum stimulation obtained with CP55,940 as 100%; mean with SD; n = 5 (THC with AM4112, THC with AM6542), n = 3 for all other treatments; experiments performed in duplicate. pA2 values for inhibition of forskolin-stimulated cAMP accumulation with THC excluded 1 and 10 µM AM4112 and AM6542 in global nonlinear regression analysis.
**Figure S3.** β-arrestin2 recruitment: antagonist competition at of hCB1 by SR141716A, AM6538, AM4112, and AM6542. hCB1 CHO DiscoveRx cells were treated with 0.03 nM – 10 µM CP55,940, THC, or JHW-018 ± SR141716A, AM6538, AM4112, or AM6542 for 90 min and β-arrestin2 recruitment was quantified. Concentration-response data were globally fit to a competitive nonlinear regression model (eq. 1) in Prism 6.0. Figures A, B, E, and F were published in the supplemental information from *Hua et al., 2016* and are shown here for comparison. Data were normalized to the vehicle (0%) and maximum stimulation obtained with CP55,940 (100%); mean with SD; n = 4 (JWH-018 with AM6542); n = 3 for all other treatments; experiments performed in duplicate. pA2 parameters are presented in table 1.