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

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Received March 11, 2019; accepted August 17, 2019

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 $\alpha_{i/o}$ 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 adenylate 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).

This work was supported by the National Institutes of Health (NIH) National Institutes on Drug Abuse [Grant P01DA009158] (A.M. and L.M.B.) and Grant R37DA023142 (A.M.). R.B.L. is supported by a postdoctoral fellowship from the Canadian Institutes of Health Research.

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<https://doi.org/10.1124/mol.119.116483>.

[§] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: CB₁, cannabinoid receptor 1; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; HA, hemagglutinin; THC, Δ^9 -tetrahydrocannabinol.

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)-4-methyl-3-(piperidin-1-ylcarbamoyl)-1H-pyrazol-5-yl)phenyl)but-3-yn-1-yl nitrate], AM6542 [5-(4-(but-3-en-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 $\geq 95\%$) using methods as described previously (Makriyannis et al., 2011; Makriyannis and Vemuri, 2014, 2017; Hua et al., 2016). CP55,940, SR141716A (Tocris, Bristol, UK), Δ^9 -tetrahydrocannabinol (THC), and JWH-018 (Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO in PBS and diluted to final solvent concentrations of 1%. Compounds were added directly to the cell culture media at the times and concentrations indicated.

Molecular Docking. Prediction of ligand binding to CB₁ was done as described previously (Hua et al., 2016) using the Schrodinger Suite 2015-4, Protein Preparation Wizard, LigPrep, and Glide 6.9 programs (Friesner et al., 2004, 2006; Halgren et al., 2004; Schrödinger, 2015).

Protein Stability Assay. Protein thermostability was tested by a microscale fluorescent thermal stability assay as described previously (Hua et al., 2016). Further, protein homogeneity was checked by analytical size-exclusion chromatography using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA) as described previously (Hua et al., 2016).

Cell Culture. The 3 $\times$ HA (hemagglutinin)-tagged hCB₁ cDNA was obtained from cDNA.org and subcloned into a murine stem cell virus vector for cell line transduction (pMSCV-puro; Clontech). Chinese hamster ovary cells (CHO-K1) were obtained from American Type Culture Collection (Manassas, VA; ID no. CCL-61). Stable CHO cell lines were generated after antibiotic (puromycin) selection. CHO cells expressing hCB₁ in the PathHunter β arrestin GPCR assay platform (CHO-hCB₁ Dx) were purchased from DiscoverX (Freemont, CA). Cells were maintained as described previously (Janero et al., 2015; Hua et al., 2016). Cell lines were negative for mycoplasma.

CISBIO cAMP Homogenous Time-Resolved Fluorescence. Inhibition of forskolin-stimulated cAMP accumulation was determined using the CISBIO cAMP Homogenous Time-Resolved Fluorescence HiRange assay according to the manufacturer's instructions (Cisbio Assays, Bedford, MA). Forskolin stimulates adenylyl cyclase directly to elevate cAMP levels, activation of CB₁ leads to a decrease in cAMP from G $\alpha_{i/o}$ -mediated inhibition of cAMP. We have presented the data as stimulation of CB₁, which is measured as an inhibition of forskolin-stimulated cAMP accumulation. For the assay, 3 $\times$ HA-hCB₁ CHO cells (Hua et al., 2016) were dissociated from cell culture dishes with 0.05% trypsin-EDTA and centrifuged at 2000g. The cell pellet was resuspended in Opti-MEM containing 1% fetal bovine serum, and cells were counted and standardized to 1 $\times$ 10⁶ cells/ml. Five thousand cells/well (5 μ l) were transferred to a 384-well plate, which was incubated for 3 hours at 37°C before the addition of 25 μ M RO-20-1724 (a phosphodiesterase inhibitor to prevent cAMP degradation) and 20 μ M forskolin (to stimulate directly adenylyl cyclase and elevate cAMP levels) (Sigma-Aldrich); vehicle, antagonists, and agonists were then added at the times and concentrations indicated. Cells were incubated with cAMP-d2 antibody and cryptate solution in lysis buffer

Fig. 1. Chemical structures of SR141716A, AM6538 (nitrate-substituted), AM4112 (hydroxyl-substituted), and AM6542 (ene-yne eliminated).

for 60 minutes at room temperature (Cisbio Assays). The fluorescence ratio of 665/620 emission channels was used to assess the levels of cAMP using a Perkin-Elmer EnVision plate reader (Waltham, MA) (Hua et al., 2016). 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.

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 hourCB₁ CHO cells were treated at the time(s) and concentrations indicated and as described previously (Hua et al., 2016). Chemiluminescent signal was measured as described previously (Hua et al., 2016). β arrestin 2 recruitment was determined in cells incubated with compound vehicle (1% DMSO in PBS) such that 0% corresponds to cells incubated with vehicle and 100% corresponds to maximal β arrestin 2 recruitment by the CB₁ agonist 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 by 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. 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.

The maximal fold CB₁ activation was determined for each agonist over vehicle response within each experiment and set as 100% stimulation. The average fold inhibition of forskolin-stimulated cAMP accumulation (with 95% confidence interval) for each agonist was CP55,940: 2.3 (1.8–2.7) ($n = 11$); JWH-018: 2.1 (1.2–3.1) ($n = 5$); and THC: 1.7 (1.2–2.1) ($n = 6$). The maximal fold over vehicle responses for β arrestin 2 recruitment (with 95% confidence interval) were CP55,940 8.3 (6.5–10) ($n = 12$); JWH-018 6.2 (3.6–8.8) ($n = 5$); and THC 5.4 (4.5–6.2) ($n = 7$). We did not observe significant inverse agonism following antagonist pretreatment and washing as shown in Supplemental Fig. S1D, where 6-hour treatment of 3×HA-hCB₁ CHO cells with 1 μ M antagonist did not differ from vehicle treatment. Therefore, we shared the $E_{\max}$ and $E_{\min}$ in the allosteric modulation analysis for both the cAMP and the β arrestin 2 assay. Agonist concentration-response curves were fit to a nonlinear regression (three-parameter) model to determine EC_{50} and $E_{\max}$ in Prism v.6.0e (GraphPad Software Inc., San Diego, CA). Concentration-response curves for competition data were fit to a global nonlinear regression model of competitive antagonism (eq. 1; Prism) (Hall and Langmead, 2010). To best-fit data to eq. 1, pEC_{50} , pA_2 , $E_{\min}$, $E_{\max}$, and Hill slope were shared for all data sets.

$$E = E_{\min} + \frac{E_{\max} - E_{\min}}{1 + \left(\frac{10^{\log EC_{50}} \left(1 + \left(\frac{[B]}{10^{-pA_2}} \right) \right)}{[A]} \right)^n} \quad (1)$$

The functional off-rate (ΔpA_2) is estimated by graphing individual pA_2 values (unitless, logarithmic) determined using eq. 1 against time of antagonist pretreatment (Kenakin et al., 2006; Tautermann, 2016). ΔpA_2 is then calculated as the absolute slope value for the linear regression through these pA_2 values, which were analyzed by two-way ANOVA followed by Dunnett's post-hoc test. ΔpA_2 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

AM6538, AM4112, and AM6542 are competitive antagonists of hCB₁. Inhibition of forskolin-stimulated cAMP accumulation in 3xHA-hCB₁ Chinese hamster ovary (CHO) cells cotreated with forskolin (cAMP assays only) and hCB₁ agonists and antagonists for 30 minutes and β arrestin2 recruitment in hCB₁ DiscoverX CHO cells cotreated with hCB₁ agonists and antagonist for 90 minutes. Data are presented as mean with 95% confidence interval. For antagonism of cAMP inhibition: $n = 5$ (THC with AM4112, THC with AM6542), $n = 3$ for all other treatments. For antagonism of β arrestin 2 recruitment, $n = 4$ (JWH-018 with AM6542), $n = 3$ for all other treatments; experiments performed in duplicate. Data were fit to a competitive nonlinear regression model using Prism 6.0. Concentration-response curves are available in Supplemental Figs. S1–S3. pA_2 values for inhibition of forskolin-stimulated cAMP accumulation with THC excluded 1 and 10 μ M AM4112 and AM6542 in global nonlinear regression analysis.

Antagonist pA_2 values for inhibition of forskolin-stimulated cAMP accumulation by hCB₁ agonists.

Antagonist	CP55,940	THC	JWH-018
SR141716A	9.5 (9.3–9.9) ^a	9.2 (8.7–9.8) ^a	8.6 (7.3–9.8)
AM6538	9.0 (8.6–9.2) ^a	9.4 (9.1–9.9) ^a	8.1 (5.8–11)
AM4112	8.7 (5.5–12)	9.2 (8.7–9.6)	9.3 (9.0–9.7)
AM6542	8.2 (7.2–9.2)	8.2 (6.7–9.6)	8.8 (8.1–9.6)

Antagonist pA_2 values for β arrestin 2 recruitment by hCB₁ agonists.

Antagonist	CP55,940	THC	JWH-018
SR141716A	9.5 (9.3–9.9) ^a	8.7 (6.2–11) ^a	7.8 (7.5–8.0)
AM6538	9.4 (9.1–9.6) ^a	7.8 (7.1–8.8) ^a	7.8 (7.6–8.0)
AM4112	8.6 (7.1–9.9)	8.8 (6.5–11)	8.4 (7.3–9.5)
AM6542	9.1 (8.5–9.6)	8.9 (6.5–11)	8.2 (7.3–9.1)

^aData are from Hua et al. (2016) wherein SR141716A and AM6538 are originally described as competitive antagonists of CP55,940 and THC.

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). The role of this nitrate group is to serve as a polar group that could be replaced by a suitable nucleophile (e.g., thiol, cysteine) or form a tight, near-irreversible series of hydrogen bond interactions (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 Tyr275^{5,39} and π - π interaction with Trp279^{5,43}. 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

Fig. 3. 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 μ M 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 pEC₅₀ 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; $^{***}P < 0.001$ 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×HA-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

Fig. 4. AM6538 antagonism of CP55,940-dependent β arrestin 2 recruitment is resistant to wash out. hCB₁ CHO cells were pretreated with vehicle (1% DMSO in PBS), 1 μ M 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 μ M CP55,940. (E) Summary of pEC₅₀ 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. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ 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.

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 μ M SR141716A (A–C), AM6538 (D–F), 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 SR141716A 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.

cAMP accumulation) and Fig. 4E (β arrestin 2 recruitment). 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

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 pA_2 . 3xHA-hCB₁ CHO cells were pre-treated with 1 nM–10 μ M SR141716A, AM6538, AM4112, or AM6542 for 1, 3, or 6 hour followed by treatment with 0.03 nM–10 μ M CP55,940 for 30 minute in the presence of forskolin. pA_2 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. $^{\dagger}P < 0.05$; $^{\dagger\dagger\dagger}P < 0.001$ AM6538; $^*P < 0.05$ AM4112 and AM6542 compared with SR141716A within time point for pA_2 , as determined by two-way ANOVA followed by Dunnett's post-hoc analysis; $^{\#}P < 0.05$, AM6538 compared with SR141716A for ΔpA_2 , as determined by one-way ANOVA followed by Tukey's post-hoc analysis.

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

We next measured the change in pA_2 by SR141716A, AM6538, AM4112, and AM6542 by graphing pA_2 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 pA_2 values (ΔpA_2) (Copeland et al., 2006; Tummino and Copeland, 2008). The ΔpA_2 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 ΔpA_2 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).

Fig. 7. AM6538 treatment decreases $E_{\max}$. 3xHA-hCB₁ CHO cells were co-treated with forskolin (for cAMP assays), 0.03 nM–10 μ M JWH-018 $\pm$ 1 nM–10 μ M SR141716A, AM6538, AM4112, or AM6542 for (A) 30 minute and inhibition of forskolin-stimulated cAMP accumulation was quantified, or (B) 90 minute and β arrestin2 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 β arrestin2 recruitment $n = 3$ (SR141716A, 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.

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 and quantifying changes in $E_{\max}$ (Supplemental Fig. S2 and S3). JWH-018 is ideal for studying insurmountable antagonism in this system because it is a potent full agonist of hCB₁ with an $E_{\max}$ that is observed over a wide concentration-response range. SR141716A treatment does not change $E_{\max}$ (Fig. 7), as expected for a reversible antagonist. AM6538 treatment led to a reduction in $E_{\max}$ (Fig. 7), consistent with AM6538 being an irreversible antagonist. AM4112 and AM6542 do not change $E_{\max}$ observed with JWH-018 (Fig. 7). From these data, we conclude AM6538 is an irreversible hCB₁ antagonist that produces a demonstrable reduction in $E_{\max}$ consistent with receptor depletion (Kenakin et al., 2006).

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 SR141716A.

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 orthosteric antagonists of

Fig. 8. AM6538 antagonism of CB₁-mediated behaviors is more-persistent than SR141716A. Male C57BL/6J mice were treated SR141716A (3 mg/kg *i.p.*), AM6538 (3 mg/kg *i.p.*), or vehicle (1:1:8 DMSO:Tween80:dH₂O) on day 0 and challenged with CP55,940 (1 mg/kg *i.p.*). Responsiveness in the tail withdrawal assay (A), body temperature (B), and catalepsy (C) were recorded as described in the *Materials and Methods*. Data are presented as mean with S.D., *n* = 6 for each treatment group. ***P* < 0.01; ****P* < 0.001 SR141716A compared with vehicle treatment within day; ^^*P* < 0.01; ^^^*P* < 0.001 AM6538 compared with vehicle treatment within day as determined by two-way ANOVA followed by Tukey's post-hoc analysis.

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 ΔpA_2 of $0.45 \pm 0.10 \text{ hour}^{-1}$, which is in agreement with the previously published dissociation rate (K_{off}) of [³H]SR141716A (Rinaldi-Carmona et al., 1995). AM6538 displays a change in pA_2 of $0.05 \pm 0.08 \text{ hour}^{-1}$, which is not different from 0. The change in ΔpA_2 for AM4112 ($0.18 \pm 0.05 \text{ hour}^{-1}$) and AM6542 ($0.26 \pm 0.05 \text{ hour}^{-1}$) 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 ΔpA_2 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 ΔpA_2 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 ΔpA_2 presented here represents the first such estimation of change in pA_2 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 E_{max} . 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 E_{max} 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 mediation 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 possible previously.

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.

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