Published in final edited form as:

Eur J Pharmacol. 2014 March 15; 727: 35–42. doi:10.1016/j.ejphar.2014.01.041.

The cannabinoid agonist HU-210: pseudo-irreversible discriminative stimulus effects in rhesus monkeys

Lenka Hrubá and Lance R. McMahon

Department of Pharmacology, The University of Texas Health Science Center at San Antonio

SUMMARY

Synthetic cannabinoid abuse and case reports of adverse effects have raised concerns about the pharmacologic mechanisms underlying *in vivo* effects. Here, a synthetic cannabinoid identified in abused products (HU-210) was compared to the effects of 9 -THC and two other synthetic cannabinoid agonists used extensively in pre-clinical studies (CP 55,940 and WIN 55,212-2). One group of monkeys discriminated 9 -THC (0.1 mg/kg *i.v.*); a separate group received chronic 9 -THC (1 mg/kg/12 h *s.c.*) and discriminated rimonabant (1 mg/kg *i.v.*). CP 55,940, HU-210, 9 -THC, and WIN 55,212-2 produced 9 -THC lever responding. HU-210 had a long duration (*i.e.*, 1–2 days), whereas that of the other cannabinoids was 5 h or less. Rimobabant (1 mg/kg) produced surmountable antagonism; single dose-apparent affinity estimates determined in the presence of 9 -THC, CP 55,940, and WIN 55,212-2 did not differ from each other. In contrast, rimobabant (1 mg/kg) produced a smaller rightward shift in the HU-210 dose-effect function. In 9 -THC treated monkeys, the relative potency of CP 55,940, 9 -THC, and WIN 55,212-2 to attenuate the discriminative stimulus effects of rimobabant was the same as that evidenced in the 9 -THC discrimination, whereas HU-210 was unexpectedly more potent in attenuating the effects of rimobabant. In conclusion, the same receptor subtype mediates the discriminative stimulus effects of 9 -THC, CP 55,940 and WIN 55,212-2. The limited effectiveness of rimobabant to either prevent or reverse the effects of HU-210 appears to be due to very slow dissociation or pseudo-irreversible binding of HU-210 at cannabinoid receptors.

Keywords

apparent affinity; cannabinoid; drug discrimination; HU-210; pseudo-irreversible

1. Introduction

The goal of this study was to compare the *in vivo* pharmacology of the synthetic cannabinoid HU-210, recently detected in Spice herbal blends, to 9 -tetrahydrocannabinol

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Correspondence: Lance R. McMahon, Ph.D., Department of Pharmacology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900, Telephone: (210) 567 0143, Fax: (210) 567 4303, mcmahonl@uthscsa.edu.

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(Δ^9 -THC) and other synthetic cannabinoids including CP 55940 and WIN 55212-2. Δ^9 -THC is the natural product in Cannabis that exerts psychopharmacological effects that are primarily responsible for the widespread use of Cannabis. Δ^9 -THC is an agonist at two G-protein coupled receptor subtypes designated CB₁ and CB₂. CB₁ receptors are abundant in the hippocampus, basal ganglia, cortex, amygdala and cerebellum (Herkenham et al., 1991; Gifford et al., 1999), and are also present in adipose tissue, skeletal muscle and liver (Lindborg et al., 2010; Wu et al., 2011). CB₂ receptors are expressed mainly in the immune system. Δ^9 -THC is a tricyclic terpenoid derivative bearing a benzopyran moiety. Two other CB₁ and CB₂ receptor agonists of synthetic origin include CP 55,940, a bicyclic analog of Δ^9 -THC lacking the pyran ring, and WIN 55212-2, an aminoalkylindole (Palmer et al., 2002 for review). CP 55940 and WIN 55212-2 have been used extensively as reference compounds in pre-clinical studies involving relatively novel cannabinoids.

HU-210 is one synthetic cannabinoid that has been added to non-Cannabis plant material and marketed under a variety of trade names such as Spice or K2 in the United Kingdom, apparently in an attempt to circumvent laws banning Cannabis (Fattore and Fratta, 2011). HU-210 belongs to the same chemical class as Δ^9 -THC and was initially synthesized in the laboratory of Raphael Mechoulam at the Hebrew University. HU-210 is a high-affinity CB₁ and CB₂ receptor agonist (Burkey et al., 2002; Howlett et al., 2002) and was reported to be highly potent in producing effects associated with cannabinoid agonism in rats (12.5–100 μ g/kg i.p.) and pigeons (12.5–50 μ g/kg, s.c.), including discriminative stimulus effects, decreased locomotor activity, rearing, and grooming, long-lasting hypothermia of a greater magnitude than that produced by Δ^9 -THC, increased vocalization and circling, and sedative effects (Järbe et al., 1989; Ovadia et al., 1995; Ferrari et al., 1999). CB₁ receptors appear to mediate the in vivo effects of HU-210 as evidenced by attenuation of those effects by CB₁ receptor antagonists (e.g., rimonabant; Bosier et al., 2010; Janoyan et al., 2002) as well as in CB₁ receptor knockout mice (Zimmer et al., 1999).

Cannabinoid discrimination procedures in rhesus monkeys have been used previously to demonstrate that synthetic cannabinoids detected in Spice products, including JWH-018 and JWH-073, exert discriminative stimulus effects by acting at the same receptors as those mediating the effects of Δ^9 -THC. In rhesus monkeys discriminating Δ^9 -THC, both JWH-018 and JWH-073 substituted for Δ^9 -THC (Ginsberg et al., 2012). Schild analysis with rimonabant was used to compare the receptor site(s) of action of Δ^9 -THC, JWH-018, and JWH-073. Rimonabant appeared to be a simple, competitive, and reversible antagonist of each agonist, as evidenced by slopes of Schild plots that were not significantly different from unity. The apparent affinity (pA₂) or potency of rimonabant did not differ among agonists, suggesting that the same receptors mediated the discriminative stimulus effects of Δ^9 -THC, JWH-018, and JWH-073. Moreover, each of these agonists attenuated rimonabant-induced Δ^9 -THC withdrawal in a second drug discrimination assay with a relative potency similar to that for producing discriminative stimulus effects in the Δ^9 -THC discrimination assay. Collectively, these drug discrimination data strongly suggest that Δ^9 -THC, JWH-018, and JWH-073 act at the same receptor to produce subjective effects.

Here, the effects of HU-210, Δ^9 -THC, CP 55940, and WIN 55212-2 were compared in drug discrimination assays in rhesus monkeys that have documented utility for conducting

quantitative analysis of drug interactions. One group of monkeys discriminated 9 -THC (0.1 mg/kg i.v.) from vehicle. A second group of monkeys discriminated rimonabant (1 mg/kg i.v.) while receiving 1 mg/kg of 9 -THC s.c. every 12 h. The 9 -THC discrimination assay was used to examine the potency and time course of HU-210, CP 55,940 and WIN 55,212-2, as well as antagonism of their effects by rimonabant. 9 -THC treated monkeys discriminating rimonabant were used to examine the capacity of rimonabant to reverse the effects of 9 -THC, CP 55,940, WIN 55,212-2, and HU-210. CP 55,940 and WIN 55,212-2 were shown to attenuate the rimonabant discriminative stimulus previously (Stewart and McMahon, 2010), although potency was only determined in the presence of a single dose of rimonabant. Here, the potency of agonists was determined from the relationship between agonist dose and magnitude of rightward shift in the rimonabant dose-effect function as described (Ginsburg et al., 2012). The results strongly suggest that HU-210 binds pseudo-irreversibly to CB₁ receptors, defined as a marked decrease in the rate of offset of receptor binding (Kenakin, 2009), which in turn interferes with the binding of rimonabant.

2. Material and Methods

2.1 Subjects

Two female and two male adult rhesus monkeys (*Macaca mulatta*) discriminated 9 -THC from vehicle and two female and two male adult rhesus monkeys discriminated rimonabant during chronic 9 -THC (1 mg/kg s.c. 12 h) treatment. Monkeys were housed individually on a 14-h light/10-h dark schedule. They were maintained at 95% free-feeding weight (range 6.0–11.3 kg) with a diet consisting of primate chow (High Protein Monkey Diet; Harlan Teklad, Madison, WI), fresh fruit, and peanuts; water was provided in the home cage. The monkeys had received cannabinoids and noncannabinoids in previous studies (Hrubá et al., 2012; Ginsburg et al., 2012). Monkeys were maintained in accordance with the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio and the *Guide for the Care and Use of Laboratory Animals* (2011).

2.2 Surgery

Monkeys were anesthetized with ketamine (10 mg/kg i.m.) followed by isoflurane (1.5–3.0% inhaled via facemask). A catheter (heparin-coated polyurethane; o.d. = 1.68 mm; i.d. = 1.02 mm; Instech Laboratories, Plymouth Meeting, PA) was inserted into a subclavian or femoral vein and secured to the vessel with suture silk (coated vicryl; Ethicon Inc., Somerville, NJ). The catheter extended from the vessel to the midscapular region of the back and was attached to a vascular access port located s.c. (Mida-cbas-c50; Instech Laboratories).

2.3 Apparatus

Monkeys were seated in chairs (Model R001; Primate Products, Miami, FL) and were placed in ventilated, sound-attenuating chambers equipped with two levers; a light was positioned above each lever. Feet were placed in shoes containing brass electrodes to which a brief electric stimulus (3 mA, 250 ms) could be delivered from an A/C generator. The chambers were connected to a computer with an interface (MED Associates, St. Albans,

VT); experimental events were controlled and recorded with Med-PC software (MED Associates).

2.4 Drug Discrimination Training

Four monkeys discriminated 9 -THC (0.1 mg/kg i.v.) from vehicle (1 part absolute ethanol, 1 part Emulphor-620, and 18 parts saline) while responding under a fixed ratio 5 (FR5) schedule of stimulus-shock termination. Four other monkeys received 1 mg/kg 9 -THC administered s.c. twice daily (at 6:15 AM and 6:15 PM) and discriminated rimonabant (1 mg/kg i.v.) from vehicle starting at 12:15 PM under an FR5 schedule of stimulus-shock termination. Experimental sessions were divided into multiple, consecutive 10-min cycles. Each cycle began with a 5-min timeout and responding on a lever during the timeout had no programmed consequence. The timeout was followed by a 5-min schedule of stimulus-shock termination, the beginning of which was signaled by illumination of red lights. Five consecutive responses on the correct lever extinguished the red lights, prevented delivery of an electric stimulus, and initiated a 30-s timeout. Otherwise, an electric stimulus was delivered every 40 s in monkeys discriminating 9 -THC and 10 s in monkeys discriminating rimonabant. Responding on the incorrect lever reset the response requirement on the correct lever. Determination of correct levers varied among monkeys (e.g. left lever associated with the training dose; right lever associated with vehicle) and remained the same for that monkey for the duration of the study.

Training sessions consisted of a minimum of three and a maximum of six cycles. Drug training consisted of administration of 9 -THC (0.1 mg/kg i.v.) or rimonabant (1 mg/kg i.v.) in the respective discrimination assays within the first min of three cycles; sham (dull pressure applied to the skin overlying the vascular access port) was administered within the first min of the second and third cycle. Vehicle training involved administration of vehicle within the first min of a cycle followed by vehicle or sham in subsequent cycles for a maximum of six cycles. Zero to three vehicle-training cycles immediately preceded three 9 -THC training cycles. Completion of the FR on the correct lever was required for reinforcement during each training cycle. Monkeys had previously satisfied the criteria for testing, i.e., at least 80% of the total responses occurred on the correct lever and fewer than five responses occurred on the incorrect lever before completion of the first FR on the correct lever within a cycle for all cycles during five consecutive or six of seven training sessions. Tests were conducted after performance satisfied the test criteria for consecutive training sessions including both vehicle and drug training sessions. The order of training with drug or vehicle was non-systematic.

2.5 Drug Discrimination Testing

During test sessions, five consecutive responses on either lever postponed the shock schedule. In monkeys discriminating 9 -THC, dose-effect functions for cannabinoid agonists were determined by administering vehicle in the first cycle followed by doses increasing by 0.5 log unit in subsequent cycles. The dose-effect function included ineffective doses (i.e., doses producing responses predominantly on the vehicle lever) up to doses that produced greater than 80% of responses on the 9 -THC lever. To establish a time course, sessions were conducted at various times after administration of CP 55,940 (0.01

mg/kg), HU-210 (0.01 mg/kg), Δ^9 -THC (0.1 mg/kg), and WIN 55,212-2 (0.1 mg/kg). For WIN 55,212-2, CP 55,940 and Δ^9 -THC experimental session were conducted at 10 min, 30 min and 1 h increments thereafter. For HU-210, sessions were conducted at 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h. In monkeys discriminating Δ^9 -THC, rimonabant was studied by administering 1 mg/kg i.v. in the first cycle followed by cumulative doses of WIN 55,212-2, CP 55,940 and HU-210 in subsequent cycles. In Δ^9 -THC-treated monkeys, CP 55,940 (0.1 and 0.32 mg/kg) and HU-210 (0.032 and 0.1 mg/kg) were studied in combination with rimonabant by administering a dose of an agonist at the beginning of the first cycle followed by doses of rimonabant increasing by 0.25 and 0.5 log unit in subsequent cycles. Because WIN 55,212-2 had a short duration of action (i.e. less than 1 h; see Results), the combined and separate effects of WIN 55,212-2 (1.0 and 3.2 mg/kg) and rimonabant were examined by administering a dose of each during separate tests consisting of a single cycle. Rimonabant was studied from ineffective doses up to doses that produced greater than 80% of responses on the rimonabant lever or up to a dose of 5.6 mg/kg, whichever occurred first. Due to limitations in the solubility of rimonabant in the vehicle and volume of drug used for i.v. administration, 5.6 mg/kg was the largest dose studied. The order of testing with drugs was non-systematic.

2.6 Drugs

Δ^9 -tetrahydrocannabinol (Δ^9 -THC; 100 mg/ml in absolute ethanol) and rimonabant (The Research Technology Branch, National Institute on Drug Abuse, Rockville, MD, USA), WIN 55,212-2 ((*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; Tocris, Ellisville, MO, USA), CP 55,940 ((1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; Tocris, Ellisville, MO, USA) and HU-210 (3-(1,1'-dimethylheptyl)-6*aR*,7,10,10*aR*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b,d*]pyran-9-methanol, Cayman chemical company, Ann Arbor, Michigan, USA) were dissolved in a mixture of 1 part absolute ethanol, 1 part Emulphor-620 (Rhodia Inc., Cranbury, NJ), and 18 parts physiologic saline and were administered intravenously in a volume of 0.1 to 1 ml/kg. Doses were expressed as the weight of the forms listed above in milligrams per kilogram of body weight.

2.7 Data analyses

Discrimination data were expressed as a percentage of responses on the drug lever out of the total number of responses on both the drug and vehicle levers. Rate of responding on both levers (i.e., drug and vehicle) was calculated as responses per s excluding responses during timeouts. Rate of responding during a test was expressed as the percentage of the control response rate for individual animals. The control was defined as the average response rate for all cycles during the five previous vehicle training sessions excluding sessions during which the test criteria were not satisfied. Discrimination and rate data were averaged among subjects, separately per training drug and were plotted as a function of dose and time.

To estimate the ED₅₀ value or dose producing 50% responding on the drug lever, individual dose-response data were analyzed with linear regression (Prism version 5.0 for Windows; GraphPad Software Inc., San Diego, CA). The analyses included doses spanning the linear

portion of the dose-response curve, and a common, best-fitting slope was used for further analyses except where noted (Kenakin, 1997). Doses corresponding to the 50% level of effect (ED_{50} value), potency ratios, and their 95% confidence limits were calculated by parallel line analyses of data from individual subjects (Tallarida, 2000). Potencies were considered significantly different when the 95% confidence limits of the potency ratio did not include 1.

Time course data were converted to area under the curve per animal, and differences among drugs were analyzed with repeated measures one way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests with Prism. To compare the magnitude of shift in dose response curve alone and in combination with 1 mg/kg rimonabant, a dose ratio (i.e., ED_{50} value determined in the presence of test drug alone divided by ED_{50} value determined in the presence of test drug in combination with rimonabant) was calculated for individual subjects and significant differences were assessed with one way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison test with Prism.

In 9 -THC-treated monkeys discriminating rimonabant, the potencies of WIN 55,212-2, CP 55,940 and HU-210 were calculated by expressing the mean shift in the rimonabant dose-response curve (i.e., ED_{50} value of rimonabant determined in the presence of agonist divided by the ED_{50} value of rimonabant alone) as a function of dose for individual monkeys. Linear regression of the individual data was used to estimate the dose of agonist producing a 10-fold rightward shift in the rimonabant dose-response curve. The effects of drugs on response rate were examined with one way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests with Prism.

3. Results

3.1 Effects of HU-210, CP 55,940, 9 -THC and WIN 55,212-2 in monkeys discriminating 9 -THC

All cannabinoid agonists dose-dependently increased mean responding on the 9 -THC lever (Fig. 1, top left). Maximum responding on the drug lever was 97 % at 0.01 mg/kg of CP 55,940, 99% at 0.01 mg/kg of HU-210, 100% at 0.1 mg/kg of 9 -THC and 97% at 0.1 mg/kg of WIN 55,212-2. Vehicle produced 0% responding on the 9 -THC lever (Fig. 1, top left, VEH). The slopes of the four dose-response curves were not significantly different from each other ($F_{3,31} = 0.77$; $p = 0.52$). The ED_{50} values and 95% confidence limits calculated from the common slope are shown in Table 1. CP 55,940 and HU-210 were 10.4- and 7.1-fold more potent than 9 -THC, respectively and WIN 55,212-2 was equipotent to 9 -THC. At the doses tested, the cannabinoid agonists did not significantly modify response rate (Fig. 1, bottom left).

When discriminative stimulus effects were examined over time, HU-210 was found to have a significantly longer duration of action as compared with WIN 55,212-2, CP 55,940 and 9 -THC ($F_{3,10} = 4814$; $P < 0.0001$) (Fig. 1, top right). On the other hand, WIN 55,212-2 had a shorter duration of action (i.e., less than 1 h) than CP 55,940 and 9 -THC. Monkeys switched from high levels of responding on the 9 -THC lever to predominantly vehicle-lever responding at 3 h for CP 55,940, 5 h for 9 -THC and 48 h for HU-210. The

cannabinoid agonists did not significantly modify response rate as a function of time (Fig. 1, bottom right).

Rimonabant (1.0 mg/kg) alone produced 0% responding on the 9 -THC lever and antagonized the discriminative stimulus effects of all cannabinoid agonists (Fig. 2, left). A dose of 1 mg/kg rimonabant increased the ED₅₀ value 12.5-fold for CP 55,940, 3.8-fold for HU-210, 12.5-fold for 9 -THC and 14.1-fold for WIN 55,212-2 (Table 1). Tukey's post hoc test demonstrated that the mean dose ratio of HU-210 was significantly less than those of CP 55,940, 9 -THC and WIN 55,212-2 ($F_{3,12} = 5.51$; $P < 0.05$). Rate of responding was not significantly altered by CP 55,940, HU-210, 9 -THC and WIN 55,212-2 in combination with rimonabant (1 mg/kg) (Fig. 2, right).

3.2 Effects of HU-210, CP 55,940 and WIN 55,212-2 in 9 -THC-treated monkeys discriminating rimonabant

Rimonabant dose-dependently increased drug-lever responding, with 0.1 mg/kg producing no more than 8% drug-lever responding and larger doses producing 82% or greater drug-lever responding (Fig. 3 left, closed circle). The ED₅₀ value was 0.23 mg/kg when rimonabant was administered as cumulative doses and 0.18 mg/kg when rimonabant was administered in a single dose per test session (Table 2).

Vehicle produced no more than 1% responding on the rimonabant lever and CP 55,940 (0.1 and 0.32 mg/kg), HU-210 (0.032 and 0.1 mg/kg) and WIN 55,212-2 (1 and 3.2 mg/kg) produced a maximum of 5% responding on the rimonabant lever.

When monkeys received an i.v. dose of WIN 55,212-2, CP 55,940 or HU-210, 6 h after the dose (1 mg/kg) of 9 -THC administered every 12 h, the rimonabant discriminative stimulus was attenuated. Doses of 1 and 3.2 mg/kg WIN 55,212-2 increased the ED₅₀ value of rimonabant by 3.7 and 9.7-fold, respectively (Table 2). CP 55,940 (0.1 and 0.32 mg/kg) increased the ED₅₀ value of rimonabant by 3.4- and 8.5-fold, respectively and HU-210 (0.032 and 0.1 mg/kg) increased the ED₅₀ value of rimonabant by 5.4- and 17.5-fold, respectively. Data for 9 -THC shown in Table 2 are from a previous study (Ginsburg et al., 2012).

Rimonabant alone did not change response rates (Fig. 3, right). One way analysis of variance showed that 0.1 mg/kg HU-210 and 3.2 mg/kg WIN 55,212-2 decreased response rate ($P < 0.05$). Both 0.1 and 0.32 mg/kg CP 55,940 significantly decreased response rate (maximum was 27% of control) ($P < 0.0001$). Rimonabant antagonized the rate-decreasing effects of each dose of cannabinoid agonist.

The magnitude of shift in the rimonabant dose-response curve expressed as a function of dose of cannabinoid agonist is shown in Figure 4. The slopes of these functions were significantly different from each other ($F_{3,22} = 5.00$; $P < 0.01$). The slope of HU-210 was greater (i.e., steeper) than the slope of CP 55,940, WIN 55,212-2 or 9 -THC, which were not different from each other. The individual slopes were used to estimate the dose of each agonist producing a 10-fold rightward shift in the rimonabant dose-response curve; the values were 3.4 mg/kg for WIN 55,212-2, 0.32 mg/kg for CP 55,940 and 0.044 mg/kg for

HU-210. The value calculated previously for Δ^9 -THC was 3.6 mg/kg (Ginsburg et al., 2012). For CP 55,940, WIN 55,212-2, and Δ^9 -THC, the potency for preventing the effects of rimonabant was not different from the potency in substituting for the Δ^9 -THC discriminative stimulus. Specifically, Δ^9 -THC and WIN 55,212-2 were equipotent and relatively less potent than CP 55,940. On the other hand, HU-210 prevented the effects of rimonabant at relatively small dose and was more potent than CP 55,940.

4. Discussion

HU-210, CP 55,940 and WIN 55,212-2 substituted for the discriminative stimulus effects of Δ^9 -THC. HU-210 and CP 55,940 were equipotent and both were more potent than Δ^9 -THC and WIN 55,212-2, which in turn were equipotent. The duration of action of intravenous HU-210 was much longer (at least 24 h) than that of Δ^9 -THC (less than 5h), CP 55,940 (less than 3 h) and WIN 55,212-2 (less than 1 h). Rimonabant (1 mg/kg) antagonized the discriminative stimulus effects and rate-decreasing effects of the agonists. However, rimonabant (1 mg/kg) produced a significantly smaller increase in the ED₅₀ value of HU-210 as compared with the other cannabinoid agonists. When rimonabant was studied for its capacity to reverse the effects of the agonists in Δ^9 -THC-treated monkeys, HU-210 was demonstrated to be unexpectedly more potent against rimonabant as compared with CP 55,940, WIN 55,212-2 and Δ^9 -THC. Collectively, these data suggest that HU-210 binds pseudo-irreversibly to cannabinoid receptors.

HU-210 shared discriminative stimulus effects with Δ^9 -THC in monkeys (current study) and in rats trained to discriminate the novel cannabinoid agonist BAY 59-3074 (De Vry and Jentsch, 2004). The order of potency obtained in the BAY 59-3074 discrimination assay was similar to that obtained here: CP 55,940 > HU-210 > BAY 59-3074 = WIN 55,212-2 = Δ^9 -THC. Cross-substitution among drugs is necessary but not sufficient evidence that drugs have common actions at a receptor(s). To determine the extent to which cannabinoid agonists were acting at the same receptor type, agonist dose-response functions were re-determined in the presence of the cannabinoid antagonist rimonabant (1 mg/kg) and the magnitude of shift was quantified. The magnitude of rightward shift and associated pK_B values of rimonabant were not significantly different for Δ^9 -THC, CP 55,940, and WIN 55,212-2. In contrast, rimonabant (1 mg/kg) produced a smaller rightward shift in the HU-210 dose-response function for discriminative stimulus effects. These results provide strong evidence that HU-210 interacts with cannabinoid receptors differently than other cannabinoid agonists.

Differences in the magnitude of antagonism at a fixed dose of rimonabant could reflect differential binding of the agonists to multiple subtypes of receptor; however, this is an unlikely explanation of the current results. The pharmacological effects of Cannabis and other cannabinoid agonists are mediated via two known G-protein-coupled receptor subtypes: CB₁ receptors that are found predominantly in the central nervous system and CB₂ receptors that are expressed by immune cells in the periphery and whose presence in the CNS remains controversial (Pertwee, 2006). In recent years, novel cannabinoid receptor subtypes have been identified, such as the orphan G-protein coupled receptor GPR55 (Ryberg et al., 2007), peroxisome proliferator-activated receptors (Panlilio et al., 2013), and

ion channel receptors such as TRPV1 (Di Marzo and De Petrocellis, 2012), and each of these receptors is reported to be differentially stimulated by not only plant-derived and synthetic cannabinoids, but also endogenous cannabinoids. Δ^9 -THC, HU-210, and CP 55,940 are GPR55 agonists, as evidenced by stimulation of G proteins in vitro, whereas WIN 55212-2 displays no activity at GPR55 (Ryberg et al., 2007). From this profile, antagonism of WIN 55212-2 might be expected to differ from the other drugs. The available binding and functional data for cannabinoids at currently identified receptor subtypes do not clearly differentiate HU-210 from the other cannabinoids tested here. Of the various cannabinoid agonists tested in rhesus monkeys discriminating Δ^9 -THC (McMahon, 2006; McMahon, 2009; Ginsburg et al., 2012), Schild analysis and single-dose apparent affinity estimates with rimonabant strongly suggest that discriminative stimulus effects are mediated by the CB₁ receptor subtype.

Even though a single receptor subtype appears to mediate the discriminative stimulus effects of cannabinoids, cannabinoids are proposed to bind at different domains of the CB₁ receptor and/or to produce conformational changes that could alter the affinity and or efficacy of other cannabinoids (Thomas et al., 1998). Mutation of the CB₁ receptor demonstrated that Lys 192 was critical for binding of HU-210 or anandamide, but was not important for binding of WIN 55,212-2 (Song and Bonner, 1996). A proposed functional consequence of differential CB₁ receptor binding is activation of different signal transduction pathways coupled to CB₁ receptors. CB₁ receptor agonism results in activation of the pertussis toxin-sensitive G-protein subtypes G_i and G_o (Howlett, 1995; Bidaut-Russell et al., 1990). When measuring CB₁ receptor-mediated activation of G_i, HU-210 and WIN 55,212-2 produced maximal activation, whereas Δ^9 -THC produced significantly less G_i activation. In contrast, only HU-210 produced maximal CB₁ receptor stimulation of G_o, whereas WIN 55,212-2 and Δ^9 -THC stimulated the same pathway 60% and 75%, respectively, of the response obtained with HU-210 (Glass and Northup, 1999). The relationship between different binding domains, functional selectivity at CB₁ receptors, and behavioral effects remains unknown and it is not clear to what extent this relationship impacts receptor antagonism.

HU-210 had a much longer duration of action than CP 55,940, Δ^9 -THC and WIN 55,212-2, i.e., at least 24 h after a single intravenous injection. The duration of effect following intravenous Δ^9 -THC in the current study (4 h) is similar to that following inhalation of combusted Cannabis. HU-210 had a similar long duration (24 h) when measuring decreases in distance travelled, velocity, and vertical activity (rearing), as well as increases in immobility in rats (Bosier et al., 2010). Intracerebroventricular injection of HU-210 also produced a long-lasting (12-18 h) hypothermic effect in rats and caused a greater decrease in body temperature than Δ^9 -THC (Ovadia et al., 1995), perhaps indicating that HU-210 has higher CB₁ receptor agonist efficacy than Δ^9 -THC (Little et al., 1989; Howlett et al., 1995). The long duration following direct application into the brain ventricular system suggests that metabolism to other cannabinoid agonists does not account for the long duration of action of HU-210. Collectively, these data suggest that inhalation of combusted HU-210 results in effects that endure for at least one day.

The interaction between two drugs can depend on the order of drug administration. An antagonist can be administered first to examine prevention of the effects of agonists, or it

can be administered second to examine reversal of the effects of agonists. By comparing the potency of an antagonist administered before or after agonists, it is possible to gain insight into the relative rate of agonist dissociation from receptors. Buprenorphine differs from other μ opioid agonists in this regard. Whereas the potency of naltrexone to antagonize the effects of morphine does not depend on the order of drug administration, the potency of naltrexone to antagonize the effects of buprenorphine does. Naltrexone is much less potent and effective at reversing the behavioral effects of buprenorphine than at preventing the effects of buprenorphine (Cowan et al., 1977; France et al., 1984). These data provide evidence of insurmountable agonism in vivo that might be due to irreversible drug-receptor interactions. In the current study, the impact of administering agonist first followed by rimonabant was examined in a novel way: the relative potency of agonists in substituting for the 9 -THC discriminative stimulus was compared to the relative potency of the agonists in attenuating (i.e., preventing) the discriminative stimulus effects of rimonabant. The latter was determined by examining the relationship between agonist dose and the magnitude of rightward shift in the rimonabant dose-response function. Whereas the relative potency of CP 55,940, 9 -THC, and WIN 55212-2 was the same in substituting for 9 -THC and reversing the effects of rimonabant, HU-210 was unexpectedly more potent in reversing the effects of rimonabant, i.e., rimonabant was less potent at reversing the effects of HU-210. Collectively, these data suggest that HU-210 dissociates more slowly than other agonists from cannabinoid receptors and interferes with the binding of rimonabant to those receptors.

A Schild slope that is not different from unity is consistent with a simple, competitive, and reversible interaction (Arunlakshana and Schild, 1959). The current results predict that the Schild slope for rimonabant in combination with HU-210 is different from unity. However, confirming this prediction under the present experimental conditions is impractical given the long duration of action of HU-210, the disruptive effects observed in monkeys for up to one week after the doses tested here, and the need for studies with even larger doses of HU-210 to construct the Schild plot. For an agonist dissociating slowly from a receptors, the Schild slope is predicted to be less than unity or shallow as evidenced by smaller than expected rightward shifts of the HU-210 dose-response function per increment in rimonabant dose. However, the converse is evident when HU-210 is studied for its capacity to attenuate the effects of rimonabant, i.e., see the relatively steep slope for HU-210 in Figure 4. At the same increment in agonist dose (i.e., 0.5 log), HU-210 produces a larger rightward shift in the rimonabant dose-response function as compared with the shifts produced by 9 -THC, CP 55,40, and WIN 55212-2. The relatively large shift in the rimonabant dose-response function in the presence of HU-210 is consistent with pseudo-irreversible binding of HU-210 to CB₁ receptors.

Herbal blends containing synthetic cannabinoids, marketed under various names such as Spice or K2, have become increasingly popular alternatives to Cannabis. The mixture of synthetic cannabinoids identified in these products has been changing and expanding to include a wide range of structurally diverse cannabinoids. HU-210, one of the cannabinoids found in these products, could be of particular concern given its long duration of action and apparent slow rate of dissociation from cannabinoid receptors. An irreversible drug-receptor interaction involves a covalent chemical bond such that the rate of offset or dissociation from receptors is 0 (Kenakin, 2009). Even though dissociation of some drugs from receptors

is greater than 0, the rate can be slow enough to be essentially irreversible or pseudo-irreversible. The pseudo-irreversible profile of behavioral effects demonstrated here for HU-210 appears to be somewhat novel among available cannabinoids including Δ^9 -THC. This could pose serious concerns with respect to impairment and the dependence that develops from long-term, daily use of Spice products containing HU-210.

Acknowledgments

The authors are grateful to A. Zaki and D. Schulze for technical assistance. This work was supported by the National Institutes of Health National Institute on Drug Abuse [DA19222].

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Fig. 1.

Effects of CP 55,940, HU-210, Δ^9 -THC and WIN 55,212-2, as a function of dose (left) and time (right) in rhesus monkeys discriminating Δ^9 -THC. Abscissae: vehicle (VEH) or dose in milligram per kilogram of body weight (left) and time (right). Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the Δ^9 -THC lever (top) and mean ($\pm$ S.E.M) response rate expressed as a percentage of the control rate (bottom).

Fig. 2.

Effects of CP 55,940, HU-210, Δ^9 -THC and WIN 55,212-2 in rhesus monkeys discriminating Δ^9 -THC: antagonism by rimonabant. Abscissae: dose in milligram per kilogram of body weight or vehicle (VEH). Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the Δ^9 -THC lever (left) and mean ($\pm$ S.E.M.) response rate expressed as a percentage of the control rate (right). The control dose-response curves for CP 55,940, HU-210, Δ^9 -THC and WIN 55,212-2 are replotted from Fig.1.

Fig. 3.

Effects of CP 55,940, HU-210 and WIN 55,212-2 in 9 -THC treated rhesus monkeys discriminating rimonabant. Abscissae: dose of rimonabant in milligram per kilogram of body weight or vehicle (VEH). Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the rimonabant lever (left) and mean ($\pm$ S.E.M.) response rate expressed as a percentage of the control rate (right).

Fig. 4. Magnitude of rightward shift in the rimonabant dose-response function expressed as a function of CP 55,940, HU-210, WIN 55,212-2 and Δ^9 -THC dose. Abscissae: dose in milligram per kilogram of body weight. Ordinate: mean ($\pm$ S.E.M.) rightward shift in the rimonabant dose-response function, calculated as the rimonabant ED_{50} value after pretreatment with a cannabinoid agonist divided by the control rimonabant ED_{50} value.

Table 1

ED₅₀ values in milligram per kilogram and 95% confidence limits (CL) for ⁹-THC, CP 55,940, HU-210, and WIN 55,212-2, alone and in combination with rimonabant (1 mg/kg i.v.), in rhesus monkeys discriminating ⁹-THC (0.1 mg/kg i.v.). Potency ratios and 95% CLs are the ED₅₀ values of the agonist in combination with rimonabant divided by the ED₅₀ value of the agonist alone.

Drug alone	ED ₅₀ Value (95% CL)	Potency Ratio (95%CL)
⁹-THC	0.036 (0.022-0.059)	
CP 55,940	0.003 (0.003-0.004)	10.4 (7.0-15.5) vs. ⁹ -THC ^a
HU-210	0.005 (0.003-0.007)	7.1 (4.1-12.4) vs. ⁹ -THC ^a
WIN 55,212-2	0.051 (0.030-0.087)	0.7 (0.4-1.3) vs. ⁹ -THC
Drug in combination with rimonabant		
⁹-THC	0.448 (0.32-0.62) ^b	12.5 (2.2-72) ^c
CP 55,940	0.043 (0.03-0.061) ^b	12.5 (9.2-17.0) ^c
HU-210	0.019 (0.018-0.02) ^b	3.8 (2.7-5.4)
WIN 55,212-2	0.724 (0.45-1.16) ^b	14.1 (1.5-136.3) ^c

^aSignificantly more potent than ⁹-THC.

^bSignificantly different from the respective controls (i.e., ⁹-THC alone, CP 55,940 alone, HU-210 alone, and WIN 55,212-2 alone).

^cSignificantly different from HU-210 in combination with 1 mg/kg rimonabant.

Table 2

ED₅₀ values in milligram per kilogram and 95% CLs for rimonabant, alone and in combination with CP 55,940, HU-210, WIN 55,212-2, and ⁹-THC, in ⁹-THC (1 mg/kg/12 h)-treated rhesus monkeys discriminating rimonabant (1 mg/kg i.v.). Potency ratios and 95% CLs are the ED₅₀ values of rimonabant in combination with the agonist divided by the ED₅₀ value of rimonabant alone.

Drug	ED ₅₀ Value (95% CL)	Potency Ratio (95%CL)
Rimonabant (cumulative dosing)	0.23 (0.16-0.32)	
	0.78 (0.46-1.35) ^a	3.4 (2.2-5.5)
+ CP 55,940 (0.1 mg/kg)	1.94 (1.23-3.07) ^a	8.5 (5.5-13.1)
+ CP 55,940 (0.32 mg/kg)	1.15 (0.88-1.49) ^a	5.4 (3.7-7.9)
+ HU-210 (0.032 mg/kg)	3.71 (2.50-5.50) ^a	17.5 (11.1-27.7)
+ HU-210 (0.1 mg/kg)	0.18 (0.16-0.20)	
Rimonabant (single bolus dosing)	0.66 (0.50-0.87) ^a	3.7 (3.0-4.6)
	1.74 (1.67-1.82) ^a	9.7 (9.4-10.1)
+ WIN 55,212-2 (1.0 mg/kg)	0.20 (0.14-0.29)	
+ WIN 55,212-2 (3.2 mg/kg)	0.59 (0.53-0.66) ^a	2.9 (1.9-4.4)
Rimonabant (cumulative dosing)^b	1.70 (0.84-3.5) ^a	8.5 (4.5-16)
+ ⁹ -THC (1.0 mg/kg) ^b		
+ ⁹ -THC (3.2 mg/kg) ^b		

^aSignificantly different from rimonabant alone.

^bData from Ginsburg et al. (2012)