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Cannabinoids in Disguise: Δ^9 -Tetrahydrocannabinol-Like Effects of Tetramethylcyclopropyl Ketone Indoles

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Abstract

Synthetic indole-derived cannabinoids have become commonly used recreational drugs and continue to be abused despite their adverse consequences. As compounds that were identified early in the epidemic (e.g., naphthoylindoles) have become legally banned, new compounds have appeared on the drug market. Two tetramethylcyclopropyl ketone indoles, UR-144 [(1-pentyl-1H-indol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone] and XLR-11 [(1-(5-fluoropentyl)-1H-indol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone], recently have been identified in confiscated products. These compounds are structurally related to a series of CB₂-selective compounds explored by Abbott Labs. The purpose of the present study was to evaluate the extent to which UR-144 and XLR-11 shared cannabinoid effects with Δ^9 -tetrahydrocannabinol (Δ^9 -THC). Indices of in vitro and in vivo activity at cannabinoid receptors were assessed. Similar to other psychoactive cannabinoid agonists, XLR-11 and UR-144 showed low nanomolar (< 30) affinity for CB₁ and CB₂ receptors, activated these receptors as full agonists, and produced dose-dependent effects that were blocked by rimonabant in mice, including antinociception, hypothermia, catalepsy and suppression of locomotor activity. The potency of both compounds was several-fold greater than Δ^9 -THC. XLR-11 and UR-144 also substituted for Δ^9 -THC in a Δ^9 -THC discrimination procedure in mice, effects that were attenuated by rimonabant. Analysis of urine from mice treated with the compounds revealed that both were extensively metabolized, with predominant urinary excretion as glucuronide conjugates. Together, these results demonstrate that UR-144 and XLR-11 share a pharmacological profile of in vitro and in vivo effects with Δ^9 -THC and other abused indole-derived cannabinoids and would be predicted to produce Δ^9 -THC-like subjective effects in humans.

1. Introduction

Originally developed for research purposes, synthetic cannabinoids began to appear as drugs of abuse in Europe and the U.S. during the mid-2000s (EMCDDA, 2009). These chemicals are synthesized in clandestine labs, sprayed on dried plant material, and packaged in foil packets with product names such as “Spice,” “K2,” “herbal incense,” or “Scooby Snax.” Usually labeled “not for human consumption,” the products are nevertheless typically smoked in order to achieve a marijuana-like intoxication, although most of the synthetic

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cannabinoids that have been identified from product samples are structurally distinct from the tetrahydrocannabinols contained in marijuana (Cox et al., 2012; Denooz et al., 2013; Logan et al., 2012). Further, anecdotal evidence suggest that they may be more toxic, with tachycardia, anxiety and psychoses sometimes reported (Forrester et al., 2012; Gunderson et al., 2012), as well as recently reported cases of acute kidney failure (Bhanushali et al., 2013; Center for Disease Control and Prevention, 2013). Usage also seems to have increased dramatically over the last few years. For example, in 2010, the number of calls to the American Association of Poison Control Centers regarding synthetic cannabinoids totaled 2,906, with calls coming from 48 different states (Wells and Ott, 2011). This number increased to 6,968 and 5,202 calls in 2011 and 2012, respectively (American Association of Poison Control Centers, 2013). In comparison, approximately four times fewer calls were made to Texas Poison Control centers concerning marijuana versus synthetic cannabinoids in 2010 (Forrester, et al., 2012).

To date, the most prevalent synthetic cannabinoids identified in herbal incense products can be classified into seven structural groups: naphthoylindoles (e.g., JWH-018, JWH-073, JWH-081, AM-2201), naphthylmethylinindoles (JWH-185, JWH-199), naphthoylpyrroles (JWH-369, JWH-370), naphthylmethylinenes (JWH-176), phenylacetylindoles (JWH-250, RCS-4), cyclohexylphenols (CP47,497), and tetrahydrocannabinols (HU-210) (EMCDDA, 2009). Initials generally refer to the lab in which the chemical was originally synthesized: JWH (John W. Huffman, Clemson University, Clemson, SC; Huffman et al., 1994), AM (Alexandros Makriyannis, Northeastern University, Boston, MA; Järbe et al., 2011), CP (Pfizer, Inc., Groton, CT; Little et al., 1988), HU (Hebrew University, Jerusalem, Israel; Mechoulam et al., 1988), and RCS (unconfirmed derivation; Kavanagh et al., 2012). With the exception of novel compounds that were synthesized outside of the auspices of recognized research laboratories (e.g., RCS compounds), binding affinities for CB₁ (brain) and CB₂ (peripheral) cannabinoid receptors have been published for most compounds in all categories (Manera et al., 2008); however, extant *in vivo* research on synthetic cannabinoids of abuse is relatively sparse, with the majority of work on naphthoylindoles (Ginsburg et al., 2012; Hrubá et al., 2012; Järbe, et al., 2011; Wiebelhaus et al., 2012; Wiley et al., 1998). The results of this limited research suggest that the potency of a synthetic cannabinoid for producing cannabimimetic effects is related to its affinity for the CB₁ receptor (Wiley et al., 2012; Wiley, et al., 1998), although there are exceptions (e.g., JWH-415; Wiley et al., 2012). In compounds with a naphthoyl substituent (e.g., JWH-018; Figure 1), tolerance for structural diversity of the non-naphthoyl substituent has been observed, with low nanomolar (< 100) CB₁ affinity and cannabimimetic activity in mice having been reported for compounds with indole, pyrrole and indene substituents (Huffman and Padgett, 2005; Wiley, et al., 1998). Indole-derived cannabinoids with substitutions for the prototypic naphthoyl have been less explored (although see research on phenylacetylindoles: Wiley, et al., 2012); however, increased legal restriction has resulted in exploitation of new structural motifs from the scientific literature or *de novo* inventions. For example, XLR-11, one of the newest synthetic cannabinoids identified in products (Uchiyama et al., 2013), appears to have been developed solely for recreational use. It is a derivative of a series of 3-(tetramethylcyclopropylmethanoyl)indole compounds, which includes UR-144 (another compound identified in products; Kavanagh et al., 2013; Uchiyama, et al., 2013), A-796,260, and A-834,735, but it is not listed in the scientific or patent literature along with these related compounds (Frost et al., 2010; Frost et al., 2008). UR-144 and XLR-11 were recently placed in Schedule I of the Controlled Substance Act (Drug Enforcement Administration, 2013). As more compounds are banned, manufacturers must find alternative compounds in an attempt to provide products that are still legal, resulting in a myriad of unusual chemicals that have never been evaluated scientifically (Shanks et al., 2012; Uchiyama, et al., 2013).

The purpose of the present study was to examine the in vitro and in vivo pharmacology of UR-144 and XLR-11, two 3-tetramethylcyclopropyl ketone indole-derived cannabinoids (Figure 1) that have been identified with increasing frequency in recently confiscated products (Terrence Boos, U.S. Drug Enforcement Agency, personal communication). Emphasis was placed on assessment in assays that have been used to predict the abuse liability of cannabinoids, including binding and activation of CB₁ receptors, pharmacological equivalence with Δ^9 -tetrahydrocannabinol (Δ^9 -THC) in a battery of tests in mice, and Δ^9 -THC drug discrimination. In addition, preliminary analysis of urinary metabolites was undertaken to aid in development of forensic markers and assessment of metabolic transformations, chemical exposures, biochemical responses, and their sequelae.

2. Methods

2.1 Subjects

Male ICR mice (25–32g), obtained from Harlan Laboratories (Indianapolis, IN) and housed singly in polycarbonate mouse cages, were used for assessment of locomotor suppression, antinociception, hypothermia, and catalepsy. These mice had free access to food when in their home cages. Separate mice were used for testing each dose of each compound (Δ^9 -THC, XLR-11, and UR-144) in this battery of procedures. Some of these mice were later reused to evaluate rimonabant antagonism of the cannabimimetic effects of the compounds in the tetrad. Singly housed male C57/B16J inbred mice (20–25 g) [Jackson Laboratories, Bar Harbor, ME] were used in the drug discrimination experiments. These mice were maintained at 85–90% of free-feeding body weights by restricting daily ration of standard rodent chow. At the start of this investigation, these mice had already been trained to discriminate Δ^9 -THC from vehicle and had been tested with other cannabinoid compounds as part of another (unpublished) study. All animals were maintained in a temperature-controlled (20–22°C) environment with a 12-hour light-dark cycle (lights on at 6 a.m.) and received water ad libitum. The in vivo studies reported in this manuscript were carried out in accordance with guidelines published in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

2.2 Apparatus

Mouse operant chambers (Coulbourn Instruments, Whitehall, PA), housed within light- and sound-attenuating cubicles, were used for behavioral training and testing in the drug discrimination studies. Each chamber contained two retractable response levers, with stimulus lights over each lever, and a house light. A food dispenser delivered 20-mg food pellets (Bioserv Inc., Frenchtown, NJ) into a food cup (with a light) centered between the two levers. Illumination of lights, delivery of food pellets, and recording of lever presses were controlled by a computer-based system (Coulbourn Instruments, Graphic State Software, v 3.03, Whitehall, PA).

For the tetrad test battery in mice, measurement of spontaneous activity occurred in standard Plexiglas locomotor activity chambers (33cm x 51cm x 23 cm). Beam breaks were recorded by San Diego Instruments Photobeam Activity System software (model SDI: V-71215, San Diego, CA) on a computer located in the experimental room. The apparatus contained two 4-beam infrared arrays that measured horizontal movement. A glass beaker filled with water heated to 55°C was used for the warm water tail withdrawal procedure. A digital thermometer (Physitemp Instruments, Inc., Clifton, NJ) was used to measure rectal temperature. The ring immobility device consisted of an elevated metal ring (diameter = 5.5 cm, height = 16 cm) attached to a metal stand.

2.3 Experimental Procedures

2.3.1 Receptor Binding—The cannabinoid receptor binding assay involves human CB₁ (hCB₁) and human CB₂ (hCB₂) membrane preparations purchased from Perkin Elmer (Waltham, MA) isolated from a HEK-293 expression system. The methods used for performing binding assays in these transfected cell membrane preparations were similar to those previously described (Zhang et al., 2010). Binding was initiated with the addition of 40 fmol of cell membrane proteins to assay tubes containing [³H]CP55,940 (ca. 130 Ci/mmol) or [³H]-rimonabant (ca. 26.8 Ci/mmol), a test compound (for displacement studies), and a sufficient quantity of buffer A (50 mM Tris•HCl, 1 mM EDTA, 3 mM MgCl₂, 5 mg/mL BSA, pH 7.4) to bring the total incubation volume to 0.5 mL. All assays were performed in polypropylene test tubes. In the displacement assays, the concentrations of [³H]CP55,940 and [³H]rimonabant were 7.2 nM and 2 nM, respectively. Nonspecific binding was determined by the inclusion of 10 μM unlabeled CP55,940, or rimonabant. All cannabinoid analogs were prepared by suspension in buffer A from a 10 mM ethanol stock. Following incubation at 30°C for 1 h, binding was terminated by vacuum filtration through GF/C glass fiber filter plates (Perkin Elmer), pretreated in 0.1% (w/v) PEI for at least 1 h, in a 96-well sampling manifold (Brandel, Gaithersburg, MD). Reaction vessels were washed three times with ~2 mL of ice cold buffer B (50 mM Tris•HCl, 1 mg/mL BSA). The filter plates were air-dried and sealed on the bottom. Liquid scintillate was added to the wells and the top sealed. After incubating the plates in cocktail for at least 30 min, the radioactivity present was determined by liquid scintillation spectrometry. Assays were done in duplicate, and results represent combined data from three independent displacement curves.

2.3.2 Agonist-Stimulated [³⁵S]GTPγS Binding—G-protein coupled signal transduction (GTPγ[³⁵S]) assays of test compounds were conducted in an incubation mixture consisting of a test compound (0.25 nM–20 μM), GDP (20 μM), GTPγ[³⁵S] (100 pM), and the hCB₁ and hCB₂ membrane preparations described above (40 fmol) in a total volume of 0.45 mL of assay buffer (50 mM TRIS-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 3 mM MgCl₂, 0.5% (w/v) BSA). Nonspecific binding was determined in the presence of 100 μM unlabeled GTPγS, and basal binding was determined in the absence of drug. Duplicate samples were incubated for 1 h at 30 °C, and the bound complex was filtered from the reaction mixture as described above and counted in a liquid scintillation counter.

2.3.3 Mouse Tetrad—Each mouse was tested in a battery of four tests, in which cannabinoid agonists produce a characteristic profile of in vivo effects (Martin et al., 1991): suppression of locomotor activity, antinociception, decreased rectal temperature and ring immobility. Prior to injection, rectal temperature and baseline latency in the warm water tail withdrawal test were measured in the mice. The latter procedure involved immersing the mouse's tail into a water bath maintained at 55 °C and recording latency (in s) for tail removal. Control latencies were 1–3 s. A 10 s maximal latency was used in order to avoid damage to the mouse's tail. After measurement of temperature and baseline tail withdrawal latency, mice were injected intravenously (i.v.) in the tail vein with vehicle or drug. Five min later they were placed into individual activity chambers for 10 min. Spontaneous activity was measured as the total number of beam interruptions during the entire session. Tail-withdrawal latency was measured again at 20 min post-injection. Rectal temperature was measured again at 30 min after injection. At 40 min post-injection, the mice were placed on the elevated ring apparatus, and the amount of time the animals remained motionless during a 5 min period was recorded.

After completion of all agonism tests, rimonabant antagonism was evaluated in the same set of mice. There was a nine day washout period between agonist and antagonism tests. A dose of each compound that was active in the first test (10 mg/kg Δ⁹-THC, 3 mg/kg XLR-11, and

3 mg/kg UR-144) was re-tested in combination with vehicle or 3 mg/kg rimonabant. Procedural details were identical to those described above for agonist tests, with the exception that mice received an i.v. injection of vehicle or rimonabant in the tail vein 10 min prior to i.v. injection of Δ^9 -THC, XLR-11, or UR-144.

2.3.4 Drug Discrimination—Training in the mouse discrimination procedure was similar to that described previously (Vann et al., 2009). Briefly, each mouse was placed in a standard operant conditioning chamber with two response levers. Mice were trained to respond on one of the two levers following intraperitoneal (i.p.) administration of 5.6 mg/kg Δ^9 -THC and to respond on the other lever following i.p. vehicle injection according to a fixed ratio 10 (FR10) schedule of food reinforcement, under which 10 consecutive responses on the correct (injection-appropriate) lever resulted in delivery of a food pellet. Responses on the incorrect lever reset the ratio requirement on the correct lever. Daily injections were administered on a double alternation sequence of Δ^9 -THC and vehicle (e.g., drug, drug, vehicle, vehicle). Daily 15 min training sessions were held Monday-Friday until the mice consistently met three criteria: (1) the first completed FR10 was on the correct lever, (2) 80% of the total responding occurred on the correct lever, and (3) response rate must have been 0.17 responses/s. When the criteria were met, acquisition of the discrimination was established and substitution testing began. Mice had already acquired the discrimination prior to the start of this study and had been tested with several compounds (see below).

Stimulus substitution tests were typically conducted on Tuesdays and Fridays during 15 min test sessions, with maintenance of training continuing on intervening days. During test sessions, 10 consecutive responses on either lever delivered reinforcement. If a mouse pressed the other lever prior to completing 10 responses on a lever, the ratio requirement on the original lever was reset. To be tested in the experiment, mice must have completed the first FR10 on the correct lever, 80% of the total responding must have occurred on the correct lever, and response rate must have been 0.17 responses/s during the prior day's training session. In addition, the mouse must have met these same criteria during previous training sessions with the alternate training compound (training drug or vehicle). A dose-effect curve had been determined with Δ^9 -THC (see results) in each mouse, followed by tests with several cannabinoid tricyclic dibenzopyran analogs (data not shown). In the present study, doses of Δ^9 -THC, XLR-11, and UR-144 were administered i.p. at a volume of 10 ml/kg 30 min prior to the test session.

Subsequent to completion of substitution tests with each compound, rimonabant antagonism of the effects of 5.6 mg/kg XLR-11 and UR-144 was assessed. For antagonist tests, 3 mg/kg rimonabant was injected i.p. 10 min prior to i.p. injection of XLR-11 or UR-144. The subsequent testing protocol was identical to that described above for substitution tests.

2.3.5 Metabolite Analysis—Three mice administered UR-144, three with XLR-11, and two with vehicle were randomly selected from the mice tested in the tetrad tests. Immediately following the in vivo tests, these mice were placed in metabolism cages and urine was collected over a 24 hr period. Urine from animals dosed with the same compound was pooled for liquid chromatography mass spectrometry (LC-MS) analysis. A modified salting-out assisted liquid-liquid extraction (SALLE) method was used for sample clean-up prior to analysis (Yanes and Lovett, 2012; Zhang et al., 2009). Acetonitrile (200 μ L) was added to 100 μ L of urine. The samples were vortexed and 50 μ L of 10 M ammonium acetate was added as a salting out agent. Samples were vortexed and centrifuged at 13,000 rcf for 5 minutes. The top layer was removed to a new vial and to this, 100 μ L of water with 0.1% formic acid was added. The samples were vortexed and filtered using 0.22 μ m spin filters (Agilent Technologies, Santa Clara, CA) at 10,000 rcf for 5 min.

LC-MS data were acquired using a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Synapt G2 HDMS quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, MA). The mass spectrometer was operated under the following conditions: resolution mode, positive electrospray ionization, capillary voltage at 3.0 kV, source temperature of 150 °C, desolvation temperature of 500 °C, desolvation gas at 1000 L/hr, sampling cone at 35 V, and extraction cone at 4.3 V. The mass spectrometer was externally calibrated from 50 to 1000 m/z using a sodium formate solution and mass shifts during acquisition were corrected for using leucine enkephalin as a lockmass. All data were acquired using a MS^E method that collects low energy and high energy data simultaneously (Bateman et al., 2002). Low energy data were acquired with a trap collision energy (CE) of 4 eV and high energy fragment data were acquired with a trap CE ramp from 15 to 40 eV.

Liquid chromatography was carried out using an Acquity BEH C18 column (1.7 μm X 2.1 X 50 mm) connected to a Vanguard BEH C18 pre-column (1.7 μm X 2.1 X 5 mm) and held at 30 °C. Injection volume was 10 μL. A gradient elution with a flow rate of 500 μL/min was used with mobile phase A consisting of water with 0.1% formic acid and mobile phase B consisting of acetonitrile with 0.1% formic acid. The gradient was held at 90% A for 0.5 min and decreased to 5% A linearly over 10 min, increased to 90% A in 0.1 min and held at 90% A for 2.9 min for column re-equilibration. During the first minute, the liquid chromatography eluate was diverted to waste and mass spectrometry data was not acquired.

2.4 Drugs and Chemicals

All compounds tested in vivo were obtained from the National Institute on Drug Abuse (NIDA, Bethesda, MD) through the NIDA Drug Supply Program. Δ⁹-THC, XLR-11 [(1-(5-fluoropentyl)-1H-indol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone], UR-144 [(1-pentyl-1H-indol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone], and rimonabant (the prototypic CB₁ receptor antagonist/inverse agonist) were dissolved in a vehicle of 7.8% Polysorbate 80 N.F. (VWR, Marietta, GA), and 92.2% sterile saline USP (Butler Schein, Dublin, OH).

Guanosine 5' diphosphate and bovine serum albumin, were purchased from Sigma Chemical Company (St. Louis, MO). GTPγS was purchased from Roche Diagnostics (Branchburg, NJ). [³⁵S]GTPγS (1150–1300 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). Scintillation fluid (MicroScint 20) was purchased from Perkin Elmer. Adenosine deaminase was purchased from Sigma Chemical Company. Acetonitrile was HPLC grade and purchased from EMD Millipore Chemicals (Billerica, MA). Water for LC-MS analysis was from an in-house purification system. Ammonium acetate and formic acid were purchased from Sigma-Aldrich (St. Louis, Missouri). UR-144 and XLR-11 reference standards were obtained from Cayman Chemical (Ann Arbor, MI).

2.5 Data analysis

2.5.1 Binding data analysis—Specific binding was calculated by subtracting nonspecific binding from total binding for each concentration of displacing ligand. Displacement data were analyzed by unweighted nonlinear regression analysis of specific receptor binding data. For displacement studies, curve-fitting and IC₅₀ calculation were done with GraphPad Prism (GraphPad Software, Inc., Version 5, San Diego, CA), which fits the data to one and two-site models and compares the two fits statistically. K_i values were estimated from IC₅₀ values using the Cheng-Prusoff equation.

Data for [³⁵S]GTPγS binding experiments are reported as mean and standard error of at least three replicates. Specific binding was calculated by subtracting nonspecific binding from

total binding and dividing by the total basal binding minus nonspecific binding. Data were plotted and analyzed with GraphPad Prism. Non-specific binding was subtracted from each sample. Net stimulated [^{35}S]GTP γ S binding was defined as agonist-stimulated minus basal [^{35}S]GTP γ S binding, and percent stimulation was defined as (net-stimulated/basal [^{35}S]GTP γ S binding) $\times$ 100%. Nonlinear iterative regression analyses of agonist concentration-effect curves were performed with GraphPad Prism. Significance was defined as $p < 0.05$.

2.5.2 Mouse Tetrad—Rectal temperature values were expressed as the difference between control temperature (before injection) and temperature following drug administration ($\Delta^\circ\text{C}$). Spontaneous activity was measured as total number of photocell beam interruptions during the 10-min session. For the purpose of potency calculation, it was expressed as % inhibition of activity of the vehicle group. Antinociception was expressed as the percent maximum possible effect (MPE) using a 10-s maximum test latency as follows: $[(\text{test-control})/(\text{10-control})]\times 100$. During assessment for catalepsy, the total amount of time (in s) that the mouse remained motionless on the ring apparatus (except for breathing and whisker movement) was measured and was used as an indication of catalepsy-like behavior. This value was divided by 300 s and multiplied by 100 to obtain a percent immobility. For compounds that produced one or more cannabinoid effects, ED_{50} was calculated separately using least-squares linear regression on the linear part of the dose-effect curve for each measure in the mouse tetrad, plotted against $\log_{10}$ transformation of the dose. ED_{50} was defined as the dose at which half maximal effect occurred. Based on data obtained from numerous previous studies with cannabinoids, maximal cannabinoid effect in each procedure was estimated as follows: 100% inhibition of spontaneous activity, 100% MPE in the tail withdrawal, -6°C change in rectal temperature, and 100% ring immobility. Separate between-subjects ANOVAs were also used to analyze each measure for agonist tests with each of the compounds. Significant differences from control (vehicle) were further analyzed with Tukey post hoc tests ($\alpha = 0.05$) as necessary. Factorial ANOVAs (rimonabant dose $\times$ compound dose) were used to analyze results of antagonist tests. Significant main effects and interactions were further analyzed with Tukey post hoc tests ($\alpha = 0.05$) as necessary. ANOVAs and post hoc tests for the tetrad and for drug discrimination data were conducted with Number Crunchers Statistical Software (2004, NCSS, Keyville, Utah).

2.5.3 Drug Discrimination—For each session, percentage of responses on the drug lever and response rate (responses/s) were calculated. Full substitution was defined as = 80% responding on the drug-associated lever (Vann, et al., 2009). ED_{50} values were calculated on the linear part of the drug lever selection dose-response curve for each drug using least squares linear regression analysis, followed by calculation of 95% CI. Since mice that responded less than 10 times during a test session did not respond on either lever a sufficient number of times to earn a reinforcer, their data were excluded from analysis of drug lever selection, but response rate data were included. Response-rate data were analyzed using repeated-measures ANOVA across dose. Repeated measures ANOVAs were also used to compare percentage of drug lever responding data under three conditions: (1) vehicle condition, (2) 5.6 mg/kg of each compound alone and (3) in combination with 3 mg/kg rimonabant. In cases of missing data (i.e., two mice did not respond at 5.6 mg/kg XLR-11 dose), a mean substitution was used to maintain equal n 's across conditions. Significant ANOVAs were further analyzed with Tukey post hoc tests ($\alpha = 0.05$) to specify differences between means.

2.5.4 Metabolite Identification—LC-MS data was analyzed using Waters MassLynx 4.1 with the aid of the MetaboLynx application manager. Automated data processing with MetaboLynx was supplemented by manual interrogation of the data using mass defect

filtering and precursor ion searching techniques (Grabauer et al., 2012). Presence of potential metabolites was determined by exact mass match to predicted elemental compositions in the low energy data function. Further refinement of the site of modification was determined by presence of characteristic fragment ions (Figure 2) present in the high energy function at the same retention time.

3. Results

3.1 CB₁ Receptor Binding and Agonist-Stimulated [³⁵S]GTPγS Binding

XLR-11 and UR-144 displaced [³H]CP55,940 with nM affinity at the CB₁ receptor binding site (Table 1). Although apparent K_is for the tetramethylcyclopropyl derivatives were over 20-fold greater than the positive control CP55,940 (K_i = 1 nM), they were at least 2-fold lower than that obtained with Δ⁹-THC. Noticeably, XLR-11 and UR-144 also displaced the radiolabel agonist [³H]CP55,940 much more readily than they were able to affect the displacement of the radiolabeled antagonist [³H]rimonabant from the CB₁ receptor (Table 1), with apparent K_is 9- to 13-fold higher for antagonist versus agonist displacement. As has been seen with other alkylindoles, the two test compounds also showed nM affinity for the CB₂ receptor (Table 1), with XLR-11 having an apparent K_i of 2.13 ± 0.62 nM, and UR-144 having an apparent K_i of 4.51 ± 1.69 nM. XLR-11 and UR-144 also stimulated GTPγ³⁵S turnover through both the hCB₁ receptor and the hCB₂ receptor at nM concentrations, which is indicative of compounds that can act as agonists at these G-protein coupled receptor sites (Figure 3, panels A and B, respectively). Mean EC₅₀s (± SEM) for stimulation of the CB₁ receptor were 25 (14.4) nM for CP55,940, 159 (38) nM for XLR-11, and 98 (20.4) nM for UR-144. For CB₂ receptors, EC₅₀s were 23 (6.8) nM for CP55,940, 145 (73.8) nM for XLR-11, and 334 (170) nM for UR-144. All three compounds were full agonists at both receptors.

3.2 Mouse tetrad effects

Figure 4 shows the effects of Δ⁹-THC (positive control) and the two test compounds (XLR-11 and UR-144) on tetrad tests in mice. Δ⁹-THC, XLR-11, and UR-144 exhibited the complete profile of cannabinoid effects in the tetrad tests in mice, with each compound producing dose-dependent suppression of spontaneous activity (Figure 4, panel A), antinociception (Figure 4, panel B), hypothermia (Figure 4, panel C), and ring immobility (Figure 4, panel D). Across the four tests, potencies ranged from 3 to 15 μmol/kg for Δ⁹-THC, from 0.6 to 3.3 μmol/kg for XLR-11, and from 0.6 to 2.6 μmol/kg for UR-144 (Table 1). Hence, XLR-11 was 4- to 17-fold more potent than Δ⁹-THC across measures whereas UR-144 was 3- to 15-fold more potent than Δ⁹-THC. Further, the 95% CI for XLR-11 and UR-144 did not overlap with those of Δ⁹-THC for any of the measures. Consistent with their similar CB₁ receptor binding affinities, the two test compounds were approximately equipotent with each other across all four measures. With the exception of the effect of UR-144 on spontaneous activity, one or more doses of each compound significantly affected each measure (compared to vehicle, the negative control) [THC: F(4,25)=3.8, p<0.05; F(4,25)=62.3, p<0.05; F(4,25)=63.3, p<0.05; and F(4,25)=34.3, p<0.05 for locomotion, antinociception, temperature and ring immobility, respectively] [XLR-11: F(4,25)=5.7, p<0.05; F(4,25)=15.6, p<0.05; F(4,25)=29.0, p<0.05; and F(4,24)=54.9, p<0.05 for locomotion, antinociception, temperature and ring immobility, respectively] [UR-144: F(4,24)=21.0, p<0.05; F(4,24)=12.0, p<0.05; and F(4,24)=20.5, p<0.05 for antinociception, temperature and ring immobility, respectively]. Although the ANOVA was significant for UR-144 [F(4,24)=3.7, p<0.05], the effect for individual doses (compared to vehicle) did not reach statistical significance in the post-hoc follow-up.

Figure 5 shows the effects of Δ^9 -THC (10 mg/kg), XLR-11 (3 mg/kg), and UR-144 (3 mg/kg) in combination with vehicle and with rimonabant (3 mg/kg) in the tetrad tests in mice. When tested with vehicle, all three compounds (Δ^9 -THC, XLR-11, and UR-144) significantly suppressed locomotor activity (Figure 5, panel A) and produced antinociception (Figure 5, panel B), hypothermia (Figure 5, panel C), and catalepsy (Figure 5, panel D), as compared to the vehicle/vehicle condition. These results provide replication of results obtained by testing each substance alone. Rimonabant significantly attenuated the antinociceptive effects of all three compounds [interaction: $F(3,40)=16.8$, $p<0.05$], the hypothermic effects of XLR-11 and UR-144 [interaction: $F(3,40)=7.5$, $p<0.05$], and the cataleptic effects of Δ^9 -THC and UR-144 [interaction: $F(3,39)=3.3$, $p<0.05$]. In each case, comparisons are to the respective compound plus vehicle condition. Since the interaction between pre-treatment condition (rimonabant vs. vehicle) and treatment condition (vehicle, Δ^9 -THC, XLR-11 or UR-144) was not significant for the locomotor activity measure, post hoc analysis is allowed only for the significant main effects. This analysis revealed that each of the compounds (THC, XLR-11 and UR-144) decreased overall locomotor activity compared to vehicle [main effect of treatment: $F(3,40)=7.3$, $p<0.05$]. Further, rimonabant produced an overall increase in locomotion (vs. vehicle) [main effect of pre-treatment: $F(1,40)=4.9$, $p<0.05$], but this effect was not specific for any of the treatment conditions [no significant interaction: $F(3,40)=0.6$, $p>0.05$].

3.3 Drug Discrimination in Mice

Before the start of this investigation, mice had been trained to discriminate 5.6 mg/kg Δ^9 -THC from vehicle and a Δ^9 -THC dose-effect curve had been determined. Δ^9 -THC (positive control) dose-dependently substituted for the training dose, with an ED_{50} of 5.4 $\mu\text{mol/kg}$ (95% CI: 4.5–6.4 $\mu\text{mol/kg}$) [Figure 6, panel A]. None of the Δ^9 -THC doses decreased response rates compared to rates obtained after vehicle injection (Figure 6, panel B). During control tests with vehicle (negative control) and 5.6 mg/kg Δ^9 -THC, mice responded predominantly on the vehicle- and Δ^9 -THC-associated lever, respectively (Figure 6, left side of panel A). XLR-11 also fully and dose-dependently substituted for Δ^9 -THC (Figure 6, panel A). It was 1.5-fold more potent than Δ^9 -THC, with an ED_{50} of 3.5 $\mu\text{mol/kg}$ (95% CI: 2.5–4.9 $\mu\text{mol/kg}$). Full substitution occurred at a dose (3 mg/kg) that did not alter response rates (Figure 6, panel B). Similar to XLR-11, UR-144 fully and dose-dependently substituted for Δ^9 -THC (Figure 6, panel A), with an ED_{50} of 7.4 $\mu\text{mol/kg}$ (95% CI: 6.3–8.7 $\mu\text{mol/kg}$). Full substitution occurred at a dose (5.6 mg/kg) that did not decrease response rates (Figure 6, panel B). Rimonabant (3 mg/kg) significantly antagonized substitution of 5.6 mg/kg doses of XLR-11 and UR-144 [$F(2,20)=51.9$, $p<0.05$ and $F(2,16)=63.7$, $p<0.05$, respectively]. Although response rates following the agonist-antagonist combination were not significantly affected for XLR-11, they were significantly decreased (compared to vehicle) for UR-144 [$F(2,16)=15.5$, $p<0.05$].

3.4 Metabolite Identification

3.4.1 UR-144—In urine from mice administered UR-144, the primary metabolites appeared to be mono-hydroxylated glucuronides. At least three distinct monohydroxylated metabolites were observed, along with their corresponding glucuronide conjugates. Hydroxylation occurred on the tetramethylcyclopropyl group, confirmed by a monohydroxylated metabolite with a fragment ion for the unmodified indole side of the molecule (m/z 214, see Figure 2), as well as on either the indole moiety or the N-pentyl side chain, confirmed by the presence of a monohydroxylated metabolite with an unmodified tetramethylcyclopropyl group (m/z 125). Two distinct dihydroxylated metabolites were found, each with one hydroxylation on the indole side of the molecule and one hydroxylation on the tetramethylcyclopropyl group. A fragment at 160 m/z was found for one of the dihydroxylated metabolites, confirming that a hydroxyl group is located on the

indole moiety. Three additional dihydroxylated metabolites were found, but fragment data was insufficient to further localize the modification sites. Dihydroxylation followed by conjugation with glucuronic acid was observed at several retention times (RTs). In all cases, a fragment at 406 m/z was observed, indicating that glucuronic acid conjugation occurred on the hydroxyl group on the indole side of the molecule.

The exact masses for a carboxylated and monohydroxylated metabolite and its corresponding glucuronide conjugate were found at several RTs. Fragments present at 186 m/z and 230 m/z indicate that the carboxylation occurs on the tetramethylcyclopropyl side of the molecule, possibly involving a ring opening to accommodate the carboxylation. Several metabolites with exact masses consistent with either carboxylated or desaturated dihydroxylated metabolites along with their corresponding glucuronide conjugates were found. The exact identity of these metabolites cannot be confirmed without further analysis including derivatization or running concurrent standards for retention time match.

3.4.2 XLR-11—The primary urinary metabolites identified after XLR-11 administration were also mono-hydroxylated glucuronides. Again, three distinct monohydroxylated metabolites were identified, along with their corresponding glucuronide conjugates. For one of the monohydroxylated metabolites, hydroxylation was confirmed on the tetramethylcyclopropyl group due to the presence of a fragment for the unmodified indole side of the molecule (m/z 232). Two distinct dihydroxylated metabolites were identified along with their corresponding glucuronide conjugates. One of the metabolites was confirmed to contain one hydroxyl group on the tetramethylcyclopropyl group and one hydroxyl group on the 5-fluoropentyl chain. This finding was confirmed by the presence a fragment at m/z 248, indicating monohydroxylation on the indole side of the molecule, and a fragment at m/z 144 fragment, indicating an unmodified indole moiety.

The data also indicate the presence of carboxylated and desaturated dihydroxylated metabolites. Fragmentation data show that the indole side of the molecule is unmodified, thus these modifications occur on the tetramethylcyclopropyl group. The conjugated metabolites were also present. Further analysis is needed to confirm the presence of these two metabolites and to further localize the modification sites. XLR-11 underwent defluorination. Both defluorination followed by hydroxylation and defluorination followed by carboxylation were observed. These metabolites had the same RTs as the corresponding hydroxylated and carboxylated metabolites of UR-144.

4. Discussion

The preponderance of evidence presented here supports the hypothesis that XLR-11 and UR-144 are CB₁ and CB₂ receptor agonists and that their profile of in vivo effects in mice resembles that of Δ^9 -THC (present study; Martin, et al., 1991), as well as other psychoactive synthetic cannabinoid agonists, including CP55,940 (Compton et al., 1992; Little, et al., 1988), WIN 55,212-2 (Compton et al., 1992), and indole-derived cannabinoids such as JWH-018 (Wiley, et al., 2012; Wiley, et al., 1998). In vitro, XLR-11 and UR-144 bind to CB₁ and CB₂ receptors, with lower affinity for the latter. This finding is not surprising, given that these compounds are structurally related to a series of indol-3-ylcycloalkyl ketones that were originally synthesized by Abbott Laboratories as part of their effort to develop cannabinoids with selectivity for CB₂ receptors (Frost, et al., 2010; Frost, et al., 2008). Indeed, much of the original research on indole-derived synthetic cannabinoids focused on development of CB₂-selective ligands (Huffman and Padgett, 2005; Huffman et al., 2005) and many of the compounds that have been discovered in forensic samples have low nanomolar affinity for CB₂ receptors (e.g., JWH-018, JWH-081, JWH-073, AM-2201) (Aung et al., 2000; Cox, et al., 2012), as does Δ^9 -THC (Showalter et al., 1996). Despite their

selectivity for CB₂ receptors, however, XLR-11 and UR-144 also have low nanomolar affinity (< 30 nM) for CB₁ receptors in an agonist displacement assay and activate both types of cannabinoid receptors (as measured by [³⁵S]GTPγS binding). Similar to bicyclic cannabinoids such as CP55,940 (present study; Breivogel and Childers, 2000) and to other indole-derived cannabinoids (Atwood et al., 2010; Atwood et al., 2011; Huffman et al., 2005), XLR-11 and UR-144 were full agonists at CB₁ and CB₂ receptors. In contrast, Δ⁹-THC (and other tetrahydrocannabinol cannabinoids) are weak partial agonists at these receptors (Griffin et al., 1999). Finally, XLR-11 and UR-144 displace [³H]CP55,940 at much lower concentrations than they displace [³H]rimonabant, as has been reported for other CB₁ receptor agonists and partial agonists (Table 1; Thomas et al., 1998). In contrast, rimonabant and its structural analogs show better displacement of the latter radioligand. These findings suggest that the structural properties of XLR-11 and UR-144 are more closely related to those of other cannabinoid agonists than to that of rimonabant.

In mice, XLR-11 and UR-144 produced a profile of pharmacological effects that was shared by Δ⁹-THC, including suppression of locomotor activity, antinociception, hypothermia and catalepsy (present study; Martin, et al., 1991). Although non-cannabinoid compounds may share one or more of these in vivo effects and a few may produce the complete Δ⁹-THC-like pharmacological profile (Wiley and Martin, 2003), two findings distinguish results with XLR-11 and UR-144 from these non-cannabinoids. First, previous research has demonstrated a strong association between CB₁ binding affinity and potency in the tetrad tests (Compton et al., 1993). Consistent with this idea, both synthetic cannabinoids bind to CB₁ receptors with lower affinities than Δ⁹-THC and are several-fold more potent in all four tests. Second, with the exception of the effects of XLR-11 in the ring immobility test, the cannabinoid effects of XLR-11 and UR-144 in the tetrad battery were blocked by prior administration of rimonabant, an effect which has not been observed with non-cannabinoids (Wiley and Martin, 2003). The antagonism results further suggest CB₁ receptor mediation of the tetrad effects of these two synthetic cannabinoids.

CB₁ receptor mediation of the Δ⁹-THC-like discriminative stimulus effects of XLR-11 and UR-144 is also implicated. In mice trained to discriminate Δ⁹-THC from vehicle, both compounds substituted fully and dose-dependently for Δ⁹-THC, effects that were blocked by rimonabant. Δ⁹-THC discrimination is the most pharmacologically selective animal model available to predict marijuana-like subjective effects of novel drugs in humans (reviewed in Balster and Prescott, 1992). Its pharmacological selectivity has been empirically demonstrated in rats (Barrett et al., 1995), mice (McMahon et al., 2008; Vann, et al., 2009), nonhuman primates (Wiley et al., 1995), and in humans (Lile et al., 2009). The present results are also consistent with the results of a number of previous drug discrimination studies in which indole-derived synthetic cannabinoids with low nanomolar CB₁ receptor affinities substituted for Δ⁹-THC or CP55,940 in animals trained to discriminate one of these two cannabinoid agonists from vehicle (Ginsburg, et al., 2012; Järbe, et al., 2011; Vann, et al., 2009; Wiley, et al., 1995; Wiley, et al., 1998). Together, these results suggest that XLR-11 and UR-144 would share the Δ⁹-THC-like subjective effects that human users of other indole-derived synthetic cannabinoids have reported (Gunderson, et al., 2012), although experienced marijuana users tended to prefer natural cannabis over synthetic cannabinoids (Winstock and Barratt, 2013).

Consistent with the idea that UR-144 and XLR-11 have abuse potential similar to other cannabinoids, detection of one or both of these compounds in purchased products has been reported in several countries (Center for Disease Control and Prevention, 2013; Sobolevsky et al., 2012; Uchiyama, et al., 2013), suggesting a global market for these synthetic cannabinoids. In the present study, preliminary analysis of urinary metabolites was undertaken to aid in future development of forensic markers and assessment of metabolic

transformations. Both UR-144 and XLR-11 were extensively metabolized, and no intact UR-144 or XLR-11 was found in urine from mice administered either compound. In both cases, metabolites were predominantly excreted in urine as glucuronide conjugates, with monohydroxylation followed by conjugation with glucuronic acid being the most abundant metabolite found. This metabolic pathway is in agreement with published studies on metabolism of other synthetic cannabinoids (Hutter et al., 2012; Sobolevsky, et al., 2012), as well as with a report on urinary metabolites of UR-144 in human users (Sobolevsky, et al., 2012). Monohydroxylation occurs on the tetramethylcyclopropyl group, and on either the indole moiety or the alkyl chain. Dihydroxylated metabolites were also seen consisting of monohydroxylation of both the tetramethylcyclopropyl group and the indole side of the molecule. In addition to more common metabolic transformations, XLR-11 underwent defluorination (conversion to UR-144), resulting in several metabolites common to animals administered both compounds. A possible consequence of this conversion is that the duration of psychoactivity experienced with XLR-11 is longer than for UR-144. Unlike many other synthetic cannabinoids (Sobolevsky, et al., 2012; Wintermeyer et al., 2010), however, no dihydrodiol formation was observed. After confirmation of the identity of these metabolites, synthesis and testing will be necessary to determine whether they are psychoactive and/or contribute to the pharmacological profile observed with these tetramethylcyclopropyl ketone indoles.

In summary, despite the structural differences of XLR-11 and UR-144 as compared to Δ^9 -THC and other classical and bicyclic cannabinoids, results presented here demonstrate that these two compounds produce a profile of in vitro and in vivo pharmacological effects that clearly demarcate them as psychoactive cannabinoids. Similar to other abused indole-derived cannabinoids such as JWH-018 and AM-2201, XLR-11 and UR-144 bind to CB₁ and CB₂ receptors with low nanomolar (< 30 nM) affinity and activate these receptors. Further, rimonabant attenuated cannabimimetic effects of these compounds in the mouse tetrad test battery and in a Δ^9 -THC discrimination procedure in mice. Based upon the observed preclinical profile, XLR-11 and UR-144 would be predicted to produce Δ^9 -THC-like subjective effects in humans, a prediction which is consistent with their detection in products labeled as “herbal incense.” Unfortunately, these compounds represent only the latest example of diversion of the knowledge gained from legitimate scientific research for synthesis of cannabinoids targeted for recreational use. As more compounds are banned, the battle between profit-seeking manufacturers and enforcement agencies continues, fueled by the rapid distribution of information through the internet.

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Highlights

- The Δ^9 -THC-like effects of XLR-11 and UR-144 were studied in mice.
- UR-144 and XLR-11 bind to both CB₁ and CB₂ receptors with nM affinity.
- They also produced cannabimimetic in vivo effects in mice.
- UR-144 and XLR-11 substituted for THC in mouse drug discrimination.
- They would be predicted to produce THC-like subjective effects in humans.

Figure 1.
Chemical structures of UR-144, XLR-11, and JWH-018.

Figure 2.

Structure of UR-144 showing characteristic fragment masses for monohydroxylated metabolites. The presence of the fragment at 144 m/z confirms modification on the alkyl chain or the tetramethylcyclopropyl group. The presence of the 160 m/z fragment confirms monohydroxylation on the indole moiety.

Figure 3. Effects of CP55,940 (squares), XLR-11 (circles), and UR-144 (triangles) on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ turnover in hCB₁ and hCB₂ receptors (panels A and B, respectively) expressed in HEK-293 cells. Each concentration-effect curve represents the mean (± SEM) of 3–5 repetitions.

Figure 4.

Effects of Δ^9 -THC (squares), XLR-11 (circles), and UR-144 (triangles) on spontaneous activity (panel A), antinociception (panel B), rectal temperature (panel C), and catalepsy (panel D). Values represent the mean ($\pm$ SEM) of 5–6 mice per group. Asterisks (*) indicate a significant difference for a compound ($p < 0.05$), relative to its respective vehicle condition (at the left side of each panel).

Figure 5.

Effects of vehicle, Δ^9 -THC (10 mg/kg), XLR-11 (3 mg/kg), and UR-144 (3 mg/kg) tested in combination with vehicle (unfilled bars) or rimonabant (filled bars) on spontaneous activity (panel A), antinociception (panel B), rectal temperature (panel C), and catalepsy (panel D). Values represent the mean ($\pm$ SEM) of 5–6 mice per group. Dollar signs (\$) indicate significant main effects ($p < 0.05$) of compound dose and rimonabant dose, as compared to vehicle. Asterisks (*) and number symbols (#) indicate a significant interaction (with post-hoc confirmation of difference) between compound ($p < 0.05$), relative to the compound plus vehicle condition or the vehicle/vehicle condition (left side of each panel), respectively.

Figure 6. Effects of Δ^9 -THC (squares), XLR-11 (circles), and UR-144 (triangles) on percentage of Δ^9 -THC-lever responding (**panel A**) and response rates (**panel B**) in mice trained to discriminate 5.6 mg/kg Δ^9 -THC from vehicle. Open symbols represent the effects of antagonist tests with rimonabant (3 mg/kg) and 5.6 mg/kg XLR-11 (circles) and UR-144 (triangles). Points above V and T represent the results of control tests with vehicle and 5.6 mg/kg Δ^9 -THC conducted before each dose-effect determination. For each dose-effect curve determination, values represent the mean ($\pm$ SEM) of 9–12 mice, except for the 10 mg/kg dose of UR-144 where $n = 5$ and 7 for percentage of Δ^9 -THC-lever responding and response rates, respectively, and 10 mg/kg XLR-11 where $n = 2$ and 6 for percentage of Δ^9 -THC-lever responding and response rates, respectively. For agonist tests, asterisk (*) indicates a significant difference in response rates ($p < 0.05$) relative to the vehicle condition. For antagonist tests, number sign (#) indicates a significant difference ($p < 0.05$) of the compound plus rimonabant, relative to the compound tested alone.

Table 1
Cannabinoid receptor binding and *in vivo* effects of XLR-11, UR-144, and controls

Compound (MW) ^a	Receptor Affinities ^b K_i in nM			Effects in Mice ^c ED ₅₀ in μ mol/kg			
	[³ H]CP55	CB ₁	CB ₂	SA	%MPE	RT	RI
Δ^9 -THC (314)	67 <i>d</i> (3)	764 <i>d</i> (76)	36 <i>e</i> (10)	15 (4.8 – 41.9)	12 (9.3 – 16.8)	4 (2.8 – 6.5)	3 (1.9 – 5.2)
CP55,940	1 (0.06)	31 <i>d</i> (8)	0.7 <i>e</i> (0.02)	NT	NT	NT	NT
Rimonabant	6 <i>d</i> (0.2)	1.8 (0.07)	702 <i>e</i> (62)	NT	NT	NT	NT
SR144528	NT	NT	7.7 (3.0)	NT	NT	NT	NT
XLR-11 (329.45)	24 (4.6)	234 (34)	2.1 (0.6)	0.9 (0.36 – 1.64)	3.3 (2.22 – 4.77)	0.6 (0.58 – 0.91)	0.6 (0.57 – 0.64)
UR-144 (311.46)	29 (0.9)	368 (85)	4.5 (1.7)	1.0 (0.55 – 2.25)	2.6 (1.83 – 4.05)	0.6 (0.51 – 0.74)	1.0 (0.64 – 1.66)

^a MW = molecular weight shown in parentheses for compounds tested *in vivo*.

^b SEM values for receptor affinities are shown in parentheses.

^c SA = spontaneous activity, %MPE = % maximum possible antinociceptive effect, RT = rectal temperature, RI = ring immobility, NT = not tested. Values shown are ED₅₀s (95% confidence intervals in parentheses). All doses are expressed as μ mol/kg.

^d (Wiley, et al., 2012)

^e (Showalter, et al., 1996)