

AB-CHMINACA, AB-PINACA, and FUBIMINA: Affinity and Potency of Novel Synthetic Cannabinoids in Producing Δ^9 -Tetrahydcannabinol-Like Effects in Mice

Jenny L. Wiley, Julie A. Marusich, Timothy W. Lefever, Kateland R. Antonazzo,

Michael T. Wallgren, Ricardo A. Cortes, Purvi R. Patel,

Megan Grabenauer, Katherine N. Moore, and Brian F. Thomas

RTI International
3040 Cornwallis Road
Research Triangle Park, NC 27709-2194 U.S.A.

Running title: Affinity and Potency of Novel Synthetic Cannabinoids

To whom correspondence should be addressed:

Jenny L. Wiley, Ph.D.
RTI International
3040 Cornwallis Road
Research Triangle Park, NC 27709-2194
Phone: 919-541-7276
Fax: 919-541-6499
Email: jwiley@rti.org
(in vivo work)

Brian F. Thomas, Ph.D.
RTI International
3040 Cornwallis Road
Research Triangle Park, NC 27709-2194
Phone: 919-541-6552
Fax: 919-541-6499
Email: bft@rti.org
(in vitro work)

Text pages: 26
Tables: 4
Figures: 7
References: 31

Word counts
Abstract: 219
Introduction: 638
Discussion: 1144

Abbreviations:

AB-CHMINACA = N-[1-amino-3-methyl-oxobutan-2-yl]-1-[cyclohexylmethyl]-1H-indazole-3-carboxamide; AB-PINACA = N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide; CP47,497 = [rel-5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol]; CP55,940 = (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; FUBIMINA = (1-(5-fluoropentyl)-1H-benzo[d]imidazol-2-yl)(naphthalen-1-yl)methanone; GTP = guanosine-5'-triphosphate; JWH-018 = [1-pentyl-3-(1-naphthoyl)indole]; MPE = maximum possible (antinociceptive) effect; Δ^9 -THC = Δ^9 -tetrahydrocannabinol; WIN55,212-2 = ([(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl)-methanone, monomethanesulfonate)

Recommended section: Neuropharmacology

Abstract

Diversion of synthetic cannabinoids for abuse began in the early 2000s. Despite legislation banning compounds currently on the drug market, illicit manufacturers continue to release new compounds for recreational use. This study examined new synthetic cannabinoids AB-CHMINACA, AB-PINACA, and FUBIMINA, with the hypothesis that these compounds, like those before them, would be highly susceptible to abuse. Cannabinoids were examined in vitro for binding and activation of CB₁ receptors, and in vivo for pharmacological effects in mice and in Δ^9 -THC discrimination. AB-CHMINACA, AB-PINACA and FUBIMINA bound to and activated CB₁ and CB₂ receptors, and produced locomotor suppression, antinociception, hypothermia, and catalepsy. Further, these compounds, along with JWH-018, CP47,497, and WIN55,212-2, substituted for Δ^9 -THC in Δ^9 -THC discrimination. Rank order of potency correlated with CB₁ receptor binding affinity, and all three compounds were full agonists in [³⁵S]GTP γ S binding, as compared to the partial agonist Δ^9 -THC. Indeed, AB-CHMINACA and AB-PINACA exhibited higher efficacy than most known full agonists of the CB₁ receptor. Preliminary analysis of urinary metabolites of the compounds revealed the expected hydroxylation. AB-PINACA and AB-CHMINACA are of potential interest as research tools due to their unique chemical structures and high CB₁ receptor efficacies. Further studies on these chemicals is likely to include research on understanding cannabinoid receptors and other components of the endocannabinoid system that underlie the abuse of synthetic cannabinoids.

Introduction

In the 1960s, Raphael Mechoulam's isolation and elucidation of the chemical structure of Δ^9 -tetrahydrocannabinol (Δ^9 -THC) (Gaoni and Mechoulam, 1964), the primary psychoactive substituent in *Cannabis sativa*, initiated a concerted effort directed at manipulation of its chemical structure, with one goal being to produce a compound with medicinal effects and no psychoactive properties. Synthesis of phytocannabinoid analogs was followed by development of bicyclic cannabinoids (e.g., CP55,940) and aminoalkylindoles (e.g., WIN55,212-2). The effort was renewed with the discovery and initial characterization of the endocannabinoid system (Devane et al., 1988), which added the structural templates of arachidonic acid derivative agonists (anandamide and 2-arachidonoylglycerol) (Devane et al., 1992; Hanus et al., 2001) and a pyrazole antagonist, rimonabant (Rinaldi-Carmona et al., 1994). Structure-activity relationship studies focused on delineation of the ways in which these diverse chemical structures could bind to the two identified cannabinoid receptors (CB₁ and CB₂) and differentiation of features which might enhance selectivity for the CB₂ cannabinoid receptor. CB₁ receptor mediation of the marijuana-like psychoactive effects of cannabinoids was confirmed during this time (Wiley et al., 1995b), and the high correlation between binding affinity and potency for producing these psychoactive effects in mice was noted (Compton et al., 1993). The systematic synthesis of cannabinoids for use as research tools to probe the structure and functioning of the cannabinoid receptors or for use as lead candidates in medication development efforts continued to produce a multitude of novel synthetic cannabinoids (e.g., see Manera et al., 2008). After the initial publication of this medicinal chemistry research, much of it lay dormant, with the exception of occasional retrieval by scientists.

Then, in the 2000s, several of the previously reported compounds were identified in confiscated "herbal incense" labeled as "Spice" (Vardakou et al., 2010). Largely due to the rapid

proliferation of information through the internet, the use of JWH-018 and other research chemicals for their intoxicating effects was spreading throughout the world. Legal bans of the cannabinoids contained in early products resulted in the emergence of additional compounds not yet illegal, creating a type of “whack a mole” situation between drug control agencies and illicit manufacturers. For example, prevalence of JWH-018 faded as it was subsequently replaced by AM-2201 and then, by tetramethylcyclopropyl ketone indoles (XLR-11 and UR-144). Most recently, two indazole carboxamide cannabinoids, AB-CHMINACA and AB-PINACA, have achieved prominence, resulting in their temporary placement into Schedule I (i.e., compounds with high abuse potential and no accepted medical use) by the U.S. Drug Enforcement Agency (U.S. Drug Enforcement Administration, 2015). An associated problem with the rapid proliferation of synthetic cannabinoids is detection and identification of their metabolites in biological fluids. This forensic information is often helpful for detection of use for the purposes of medical treatment, employee screening, or legal prosecution.

The purpose of the present study was to examine the *in vitro* and *in vivo* pharmacology of AB-CHMINACA and AB-PINACA (Figure 1). FUBIMINA, an analog of a previously identified synthetic cannabinoid of abuse, AM-2201, was also evaluated (Figure 1). Assessment centered on assays used to predict the abuse liability of cannabinoids (Wiley and Martin, 2009), including binding and activation of CB₁ receptors, pharmacological equivalence with Δ^9 -THC in a battery of four tests in mice, and Δ^9 -THC discrimination in mice. In the Δ^9 -THC discrimination procedure, results are also presented for synthetic cannabinoids from different chemical classes, including the prototypic indole-derived synthetic cannabinoid JWH-018, a bicyclic cannabinoid CP47,497, and the aminoalkylindole WIN55,212-2. These representative compounds are from different chemical classes of cannabinoids which have been well-

characterized previously (Compton et al., 1992a; Compton et al., 1992b; Wiley et al., 1998). They were tested here to provide a basis for comparison with the structurally innovative compounds shown in Figure 1. To assist in the development of forensic markers and to examine metabolic transformations, preliminary analysis of urinary metabolites of the three novel synthetic cannabinoids also was undertaken.

Methods

Animals

Adult drug naïve male ICR mice (31-34g; Harlan, Frederick, MD) and C57/Bl6J mice (20-25g; Jackson Laboratories, Bar Harbor, ME) were used in the tetrad battery and drug discrimination experiments, respectively. All mice were housed singly in polycarbonate mouse cages in a temperature-controlled (20-22°C) environment with a 12-hour light-dark cycle (lights on at 6 a.m.). Water was freely available in the home cage. Whereas separate mice, with unlimited access to food, were used for testing each compound dose in the tetrad battery, mice in the drug discrimination experiments were maintained at 85-90% of free-feeding body weights by restricting daily ration of standard rodent chow and were tested repeatedly. At the start of this project, some of these mice had already been trained to discriminate Δ^9 -THC from vehicle; however, others were trained to discriminate Δ^9 -THC *de novo*. 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) and were approved by our Institutional Animal Care and Use Committee.

Apparatus

For the tetrad test battery in mice, measurement of spontaneous activity occurred in Plexiglas locomotor activity chambers (47 cm x 25.5 cm x 22 cm). Beam breaks (4 X 8 beam array) were recorded by San Diego Instruments Photobeam Activity System software (San Diego, CA) on a computer located in the experimental room. A standard tail flick device for rodents (Stoelting, Dale, IL) was used to assess antinociception. 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 = 28 cm) attached to a metal stand.

Mice in the drug discrimination experiment were trained and tested in mouse operant chambers (Coulbourn Instruments, Whitehall, PA), housed within light- and sound-attenuating cubicles. Each chamber contained two retractable response levers or nose poke apertures, with stimulus lights located over each lever/aperture, and a separate 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/apertures. Illumination of lights, delivery of food pellets, and recording of lever presses or nose pokes were controlled by a computer-based system (Coulbourn Instruments, Graphic State Software, v 3.03, Whitehall, PA).

Experimental Procedures

All in vitro and in vivo experimental procedures were similar to those described in our previous publication (Wiley et al., 2013), in which we described results of tests with two tetramethylcyclopropyl ketone indoles, XLR-11 and UR-144, that are also classified as synthetic cannabinoids of abuse.

Receptor Binding

Transfected cell membrane preparations with human CB₁ (hCB₁) and human CB₂ (hCB₂) receptors (Perkin Elmer, Waltham, MA) isolated from a HEK-293 expression system were used for cannabinoid binding assays, as previously described (Zhang et al., 2010). Binding was initiated with the addition of 40 fmol of cell membrane proteins to polypropylene assay tubes containing 0.62 nM [³H]CP55,940 (ca. 130 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. Nonspecific binding was determined by the inclusion of 10 μM unlabeled CP55,940. All cannabinoid agonists were prepared from a 10 mM ethanol stock by suspension in buffer A. 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. Liquid scintillation spectrometry was used to measure radioactivity after incubating the plates in cocktail for at least 30 min. Assays were done in duplicate, and results represent combined data from three independent displacement curves.

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.

Mouse Tetrad

Each mouse was tested in a tetrad of tests, in which cannabinoid agonists produce a profile of in vivo effects (Martin et al., 1991): suppression of locomotor activity, decreased rectal temperature, antinociception, and catalepsy. Prior to injection, baseline values were obtained for rectal temperature and in the tail flick test in each mouse. In the latter procedure, the mouse's tail was placed under an intense light (radiant heat) and the latency to remove it (in s) was recorded. In order to minimize tail damage, a 10-s maximal latency was employed. After baseline measurements were taken, mice were injected intraperitoneally (i.p.) with vehicle or drug 30 min before being placed into individual activity chambers for a 10-min session. Immediately upon removal from the chambers, tail-flick latency and rectal temperature were measured again followed by placement on the elevated ring apparatus at 50 min post-injection. The amount of time the animals remained motionless on the ring during a 5 min period was recorded. If a mouse fell off the ring during the catalepsy test, it was immediately placed back on and timing was continued for up to 9 falls. After the 10th fall, the test was terminated for the mouse.

At least one week after completion of all agonist tests, combinations of vehicle or 3 mg/kg rimonabant and active doses of each compound (56 mg/kg Δ^9 -THC, 3 mg/kg AB-CHMINACA, and 30 mg/kg AB-PINACA) were re-tested in a subset of the same mice. Procedural details were identical to those described above, with the exception that mice received an i.p. injection of vehicle or rimonabant 10 min prior to i.p. injection of the agonist test compound.

Because FUBIMINA was not active at doses up to 100 mg/kg, i.p., a probe dose of 56 mg/kg (and vehicle) was administered i.v. to separate groups of mice. Evaluation in the tetrad

tests proceeded as described above, with the exception that mice were placed into the locomotor chambers five min after injection and placed on the ring apparatus, 25 min post-injection. Subsequently, the effect of 3 mg/kg rimonabant (i.v.) in combination with the 56 mg/kg dose (i.v.) of FUBIMINA was assessed. Rimonabant was injected 10 min prior to FUBIMINA or vehicle.

Drug Discrimination

Two groups of adult male mice were trained to discriminate Δ^9 -THC in standard operant chambers, as described previously (Vann et al., 2009). Mice were trained to press one of two levers following i.p. administration of 5.6 mg/kg Δ^9 -THC and to press the other lever after i.p. vehicle injection. Ten consecutive responses on the correct (injection-appropriate) lever resulted in delivery of a food pellet [i.e., fixed ratio 10 (FR10)] whereas responses on the incorrect lever reset the ratio requirement on the correct lever. A double alternation sequence of Δ^9 -THC and vehicle (e.g., drug, drug, vehicle, vehicle) was instituted. Fifteen 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) $\geq 80\%$ of the total responding occurred on the correct lever, and (3) response rate was ≥ 0.17 responses/s.

Substitution tests began after the mice met acquisition criteria. These 15-min tests usually occurred on Tuesdays and Fridays and were interspersed with training sessions on other weekdays. During test sessions, 10 consecutive responses on either lever delivered reinforcement. To be tested in the experiment, mice must have met the same 3 criteria as for acquisition on the preceding day and during the previous training session with the alternate training compound (training drug or vehicle). A dose-effect curve was determined with Δ^9 -THC

in both groups of mice. The first group of mice was subsequently tested with several cannabinoid tricyclic dibenzopyran analogs (data not shown) and with two indole-derived synthetic cannabinoids (Wiley et al., 2013). In the present study, the first group of mice were tested with JWH-018, WIN55,212-2, and CP47,497, and a second dose-effect curve was determined Δ^9 -THC.

The second group of mice was tested with several noncannabinoid compounds (data not shown). Subsequently, their response requirement was changed from lever presses to nose pokes. This change was necessitated by transition of all of the lab's mouse operant equipment to nose poke apertures and was not specifically related to this study. Additional mice were trained *de novo* on the nose poke response (using the acquisition procedure described above) and were combined with this second group of mice. With the exception of the actual response (nose poke vs. lever press), all other procedural details remained the same (e.g., FR10, food reinforcement, testing criteria). After acquisition of the nose poke response, a dose-effect curve with Δ^9 -THC was determined (i.e., for *de novo* mice) or re-determined (i.e., for transition mice) followed by dose-effect curve determinations with AB-PINACA, AB-CHMINACA, and FUBIMINA.

Metabolite Analysis

Twelve mice (n=4 per drug) were given i.p. injections of 3 mg/kg AB-PINACA, 3 mg/kg AB-CHMINACA, or 100 mg/kg FUBIMINA. Immediately following injections the mice were placed into metabolism cages and urine was collected over a 24 hour period. Urine from mice dosed with the same compound was pooled for analysis. Samples were extracted using a salting out liquid-liquid extraction (SALLE) method prior to analysis. Acetonitrile (200 μ L) was added to 100 μ L of urine, then the samples were vortexed and 50 μ L of 5 M ammonium acetate was

added as a salting out agent. Samples were vortexed and centrifuged at 10,000 rcf for 5 min. The top aqueous layer was removed and dried down at 40 °C and reconstituted with 50 µL of mobile phase A.

Samples were analyzed on a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Waters Synapt G2 HDMS quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, MA). The mass spectrometer was operated under resolution mode, positive electrospray ionization, source temperature of 150 °C, desolvation temperature of 500 °C, desolvation gas at 1,000 L/hr, capillary voltage at 2.99 kV, sampling cone at 35 V, and extraction cone at 4.3 V. The mass spectrometer was externally calibrated from 50 - 1000 m/z using a sodium formate solution. Leucine enkephalin was used as a lockmass to correct for mass shifts during acquisition. Full scan data was collected in both low (4 eV) and high (15 to 40 eV ramp) collision energies nearly simultaneously for every m/z using MS^E acquisition mode (Bateman et al., 2002).

Samples were separated on an Acquity BEH C18 column (1.7 µm 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 mobile phase composition for AB-PINACA and AB-CHMINACA was held at 90% A for 1.5 min, decreased to 55% A over 15 min, decreased to 5% A over 3 min and held at 90% A for 2.9 min for column re-equilibration. For FUBIMINA the gradient was held at 90% A for .5 min, decreased to 85% A over 1 min to 35% A over 15 min, then to 5% A over 3 min and held at 90% A for 2.9 min for column re-equilibration. For EG-18, The gradient was held at 90% A for 0.5 min, decreased to 65% A over 1 min and 35% A over 13

min, then decreased to 5% A over 5 min and held at 90% A for 2.9 min for column re-equilibration. All mobile phase composition changes were done linearly.

Drugs and Chemicals

Δ^9 -THC, JWH-018 [1-pentyl-3-(1-naphthoyl)indole], CP47,497 [rel-5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol] and rimonabant (the prototypic CB₁ receptor antagonist/inverse agonist) were obtained from the National Institute on Drug Abuse (NIDA, Bethesda, MD) through the NIDA Drug Supply Program. WIN55,212-2 [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate) was purchased commercially (Cayman Chemical, Ann Arbor, MI). AB-PINACA [N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide], AB-CHMINACA [N-[1-amino-3-methyl-oxobutan-2-yl]-1-[cyclohexylmethyl]-1H-indazole-3-carboxamide], and FUBIMINA [(1-(5-fluoropentyl)-1H-benzo[d]imidazol-2-yl)(naphthalen-1-yl)methanone] were provided to RTI by the Drug Enforcement Administration (DEA). For the in vivo tests, the vehicle for all compounds was 7.8% Polysorbate 80 N.F. (VWR, Marietta, GA), and 92.2% sterile saline USP (Butler Schein, Dublin, OH). All compounds were injected at a volume of 10 ml/kg.

Guanosine 5' diphosphate, bovine serum albumin, ammonium acetate, and formic acid were purchased from Sigma Chemical Company (St. Louis, MO). GTP γ S was purchased from Roche Diagnostics (Indianapolis, IN). [³⁵S]GTP γ S (1150-1300 Ci/mmol) and scintillation fluid (MicroScint 20) were obtained from Perkin Elmer Life Sciences (Boston, MA). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Fairlawn, NJ). Reference standards

and metabolites reference standards for all compounds were obtained from Cayman Chemical (Ann Arbor, MI).

Data analysis

Binding data analysis

Specific binding was calculated by subtracting nonspecific binding from total binding for each concentration of displacing ligand. 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 [³⁵S]GTPγS binding was defined as agonist-stimulated minus basal [³⁵S]GTPγS binding, and percent stimulation was defined as (net-stimulated/basal [³⁵S]GTPγS binding) x 100%. Nonlinear iterative regression analyses of agonist concentration-effect curves were performed with GraphPad Prism. Significance was defined as $p \leq 0.05$.

Separate factorial ANOVAs (compound X receptor) were used to determine differences in k_i, EC₅₀, and B_{max}. Significant differences were further analyzed with Tukey post hoc tests ($\alpha = 0.05$) as necessary.

Mouse Tetrad

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: $[(\text{test-control})/(10\text{-control})] \times 100$. Rectal temperature values were expressed as the difference between control temperature (before injection) and temperature following drug administration ($\Delta^{\circ}\text{C}$). 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 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 flick, -6 $^{\circ}\text{C}$ change in rectal temperature, and 100% ring immobility. Separate between-subjects ANOVAs were also used to analyze the four measures for each compound. Significant differences from control (vehicle) were further analyzed with Tukey post hoc tests ($\alpha = 0.05$) as necessary. Factorial ANOVAs (rimonabant dose X 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.

Drug Discrimination

For each session, percentage of responses on the drug-associated manipulandum and response rate (responses/s) were calculated. Full substitution was defined as $\geq 80\%$ responding on the drug-associated manipulandum (Vann et al., 2009). ED_{50} values were calculated on the linear part of the drug manipulandum selection dose-response curve for each drug using least squares linear regression analysis, followed by calculation of 95% confidence intervals (CI). Since mice that responded less than 10 times during a test session did not respond on either manipulandum a sufficient number of times to earn a reinforcer, their data were excluded from analysis of drug manipulandum selection, but their response rate data were included. Response-rate data were analyzed using repeated-measures ANOVA across dose. Significant ANOVAs were further analyzed with Tukey post hoc tests ($\alpha = 0.05$) to specify differences between means.

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, precursor ion and fragment ion searching techniques (Grabenaus 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 at the same retention time. Metabolites were provisionally identified by their molecular weight, retention time, and fragment ions. Metabolites were compared to reference standards as available.

Results

Cannabinoid Receptor Binding and Agonist-Stimulated [35 S]GTP γ S Binding

All three test compounds displaced [3 H]CP-55,940 at the CB₁ receptor binding site (Figure 2, panel A), but with varying affinities which ranged from inhibition constants (i) 0.59 nM for CP55,940 to 296 nM for FUBIMINA (Table 1). In contrast, only two of the three test compounds (AB-CHMINACA and AB-PINACA) stimulated GTP- γ -[35 S] turnover with reasonable potency (Figure 2, panel B). Rank order of potency was AB-CHMINACA > CP55,940 > AB-PINACA (Table 1). Further, AB-CHMINACA and AB-PINACA exhibited enhanced efficacy compared to CP55,940 (Table 1) [drug X receptor interaction for B_{max}: F(3,46)=10.95, p <0.05]. Although FUBIMINA also stimulated GTP- γ -[35 S] turnover with efficacy comparable to that obtained with the positive control CP55,940 (Figure 2, panel B), it did so only at very high concentrations (Table 1), suggesting it serves as a weakly potent agonist at the CB₁ receptor.

Similar to their effects at the CB₁ receptor, all three test compounds displaced [3 H]CP-55,940 at the CB₂ receptor binding site (Figure 2, panel C). Further, affinities of all compounds for the CB₂ receptor exceeded those obtained at the CB₁ receptor by 1.7- to 12.6-fold (Table 1) [main effect of receptor type for k_i : F(1,24)=60.37, p <0.05]. In contrast with the greater affinities of the test compounds for the CB₂ receptor, they showed reduced efficacy at this receptor compared to the CB₁ receptor (Figure 2, panel D) [main effect of receptor type for B_{max}: F(1,46)=169.91, p <0.05]. Whereas CP55,940 and FUBIMINA produced similar efficacies at the CB₂ receptor, efficacies for AB-PINACA and AB-CHMINACA were somewhat lower (Table 1). Potencies for stimulating GTP- γ -[35 S] turnover at the CB₂ receptor varied greatly across the compounds (Table 1). Whereas AB-PINACA was approximately 7-fold less potent than

CP55,940, AB-CHMINICA and FUBIMINA were 111- and 604-fold less potent than CP55,940 (Table 1) [main effect of compound for EC_{50} : $F(3,46)=10.58$, $p<0.05$].

Mouse tetrad effects

For all ANOVAs performed on tetrad test data, significant F values are presented in figure legends of the appropriate figures. Δ^9 -THC, AB-CHMINACA and AB-PINACA exhibited the complete profile of cannabinoid effects in the tetrad tests in mice, with each compound producing dose-dependent suppression of spontaneous activity, antinociception, hypothermia, and ring immobility (Figure 3, panels A-D, respectively). Across the four tests, AB-CHMINACA was 11- to 58-fold more potent than Δ^9 -THC whereas AB-PINACA was 2- to 14-fold more potent than Δ^9 -THC (Table 2). With the exception of the effect of Δ^9 -THC on spontaneous activity, one or more doses of Δ^9 -THC, AB-CHMINACA, and AB-PINACA significantly affected each measure. Although Δ^9 -THC showed a trend toward decreased spontaneous activity, the effect did not reach statistical significance. In addition to its effects on the tetrad measures, 30 mg/kg AB-PINACA also produced convulsions, flattened body posture (splayed limbs), and labored breathing in most mice within 1 min after i.p. injection. By the end of tetrad testing, mice had started to recover and were walking around their home cages.

In contrast, FUBIMINA did not affect spontaneous activity or rectal temperature and did not produce ring immobility at i.p. doses up to 100 mg/kg (Figure 3). Although i.p. doses of 30 and 100 mg/kg FUBIMINA produced statistically significant increases in antinociception, the magnitude of these increases was small and did not approach the maximal effects observed with Δ^9 -THC, AB-CHMINACA, and AB-PINACA. Despite measurable affinity for the CB_1 receptor, FUBIMINA did not exhibit cannabinoid effects in the tetrad tests following i.p. injection.

Consequently, an i.v. dose of 56 mg/kg FUBIMINA was tested (Figure 4, panels A-D). At this dose, FUBIMINA suppressed locomotor activity and produced antinociception, hypothermia and catalepsy (Figure 4, right sides of panels A-D, respectively), albeit the magnitudes of its antinociceptive and hypothermic effects were somewhat less than a comparable i.p. dose of Δ^9 -THC.

Figure 4 also shows the results of antagonism tests. Alone, i.p. doses of 56 mg/kg Δ^9 -THC, 30 mg/kg AB-PINACA, and 3 mg/kg AB-CHMINACA significantly suppressed locomotor activity (panel A) and produced antinociception (panel B), hypothermia (panel C), and catalepsy (panel D). In each instance, these effects were attenuated by co-administration of 3 mg/kg rimonabant (i.p.). Rimonabant (3 mg/kg, i.v.) also attenuated the cannabinoid effects of 56 mg/kg FUBIMINA (panels A-D).

Drug Discrimination in Mice

Mice trained to lever press for food reward in a Δ^9 -THC discrimination procedure showed full dose-dependent substitution for the 5.6 mg/kg Δ^9 -THC training dose (Figure 5, top panel), with a potency similar to that obtained with mice trained in the nose poke procedure (Table 3). The aminoalkylindole WIN55,212-2, a bicyclic cannabinoid CP47,497, and the prototypic indole-derived synthetic cannabinoid JWH-018 also fully and dose-dependently substituted for Δ^9 -THC (Figure 5, top panel). Rank order of potency for substitution was JWH-018 > CP47,497 > WIN55,212-2 > Δ^9 -THC (Table 3). Whereas CP47,497 produced response rate decreases at higher doses [$F(4,28)=9.18$, $p<0.05$], Δ^9 -THC and JWH-018 did not (Figure 5, bottom panel), although the highest dose of JWH-018 tested (1 mg/kg) was relatively low

compared to those of the other compounds. WIN55,212-2 substantially decreased response rates; however, the small number of mice tested prevented attainment of significance.

Δ^9 -THC also produced full dose-dependent substitution for the 5.6 mg/kg Δ^9 -THC training dose in all mice responding in the nose poke procedure (Figure 6, panel A), regardless of whether they had received initial training in Δ^9 -THC discrimination with a different response topography (i.e., transition from lever presses to nose pokes; ED_{50} = 5.4 μ mol/kg; 95% CI: 4.8-5.7 μ mol/kg) or had been trained *de novo* with the nose poke response (ED_{50} = 5.7 μ mol/kg; 95% CI: 4.5-7.6 μ mol/kg). Further, Δ^9 -THC did not alter response rates (compared to vehicle) in any of the groups across the dose range tested (Figure 6, panel B). For the purpose of comparison with the test compounds, data from all mice trained to discriminate Δ^9 -THC using the nose poke response were combined, with a resulting ED_{50} = 5.7 μ mol/kg (95% CI: 4.8-6.7 μ mol/kg) [Table 3; Figure 6, panel C]. Response rates were significantly increased by 3 mg/kg Δ^9 -THC [$F(5,95) = 5.97$, $p < 0.05$], but significant decreases (compared to vehicle) were not observed at any dose (Figure 6, panel D). During all control tests with vehicle and 5.6 mg/kg Δ^9 -THC across the course of the study, mice responded predominantly on the vehicle- and Δ^9 -THC-associated apertures, respectively (Figure 6, left side of panels A and C).

As shown in Figure 6 (panel C), AB-CHMINACA and AB-PINACA produced full, dose-dependent substitution for Δ^9 -THC. Both compounds were more potent than Δ^9 -THC by 16- and 1.5-fold for AB-CHMINACA and AB-PINACA, respectively (Table 3). Of the two test compounds, the effects of AB-CHMINACA bore the most resemblance to those of Δ^9 -THC, in that the lowest dose producing full substitution (0.3 mg/kg) did not affect response rates (Figure 6, panel D). Although full substitution also occurred at 1 mg/kg AB-CHMINACA, this effect

was accompanied by an overall reduction in response rates [$F(3,21)=36.09$, $p<0.05$]. In fact, of the 7 mice tested at this dose, only 1 responded on either aperture during the entire session.

The profile of AB-PINACA was distinct from that produced by Δ^9 -THC and AB-CHMINACA, in that full substitution was observed *only* at a dose (3 mg/kg; Figure 6, panel C) that also severely reduced response rates [$F(4,32)=16.48$, $p<0.05$] (Figure 6, panel D). Only 2 of 9 mice tested with this dose responded on either aperture. Further, the response rate dose-effect function was steep, with no effect at 1.7 mg/kg and nearly complete suppression at a dose (3 mg/kg) only $\frac{1}{2}$ log higher (Figure 6, panel D).

Results of substitution tests with FUBIMINA revealed considerable variability in choice of aperture across the mice. While FUBIMINA did not fully substitute for Δ^9 -THC (i.e., $>80\%$ Δ^9 -THC-aperture responding), dose-dependent increases in responding on the Δ^9 -THC aperture (partial substitution) were observed (Figure 6, panel C; Table 3). FUBIMINA did not significantly affect response rates (Figure 6, panel D).

Metabolite Identification

In urine from mice administered AB-PINACA, only monohydroxylations and their corresponding glucuronide conjugates were observed. Three distinct monohydroxylated metabolites were observed, none of which matched the retention times of 4-hydroxy AB-PINACA or 5-hydroxy AB-PINACA reference standards. Fragment ions observed for one of the hydroxylated metabolites were m/z 231, m/z 302, and m/z 330, where m/z 231 is indicative of hydroxylation located on the 1-pentyl-1*H*-indazole moiety with an attached carbonyl. Other metabolites were identified in in vitro AB-PINACA and confirmed in human urine specimens (Wohlfarth et al., 2015), but not observed in the present study.

Similar to AB-PINACA, a single hydroxylated metabolite and its corresponding glucuronide conjugate were identified in the urine from mice dosed with AB-CHIMINACA. Characteristic fragments observed at both retention times (RT) were m/z 328, m/z 356, and m/z 257. The fragment ion at m/z 257 is indicative of hydroxylation on the 1-(cyclohexylmethyl)-1*H*-indazole moiety with an attached carbonyl. At the time of analysis, no reference standard was available for hydroxylated AB-CHIMINACA; however, Cayman Chemical AB-CHIMINACA metabolites M2, M3A, M4, M5A, and M6 were run as reference standards and were not observed in the in vivo sample.

A summary of the metabolites found in urine from mice dosed FUBIMINA is shown in Table 4, where I represents the phase I metabolites and II represents the phase II glucuronide conjugates. Unlike for AB-PINACA and AB-CHIMINACA, non-metabolized, intact parent compound was observed in the pooled in vivo urine sample. Major metabolites identified were multiple monohydroxylations and their corresponding glucuronide conjugates (Figure 7). Hydroxylation for FUBIMINA was confirmed to occur on the 1-(5-fluoropentyl)-1*H*-benzimidazole moiety by the presence of a fragment ion at m/z 249 (with the carbonyl attached). Several of the hydroxylated metabolites also had a fragment ion at m/z 161, indicating that hydroxylation was on the benzimidazole moiety. Hydroxylation was also observed on the naphthyl moiety as determined by the presence of fragment ions at m/z 171, m/z 143, and m/z 233.

Discussion

Psychoactive cannabinoid agonists produce a characteristic profile of in vitro and in vivo pharmacological effects, including binding to and activating CB₁ receptors, dose-dependent activity in a tetrad battery of tests in mice, and Δ^9 -THC-like discriminative stimulus effects (Wiley and Martin, 2009). In the present study, the in vitro positive control CP55,940 showed low nM K_i for both CB₁ and CB₂ receptors, similar to a number of indole and pyrrole-derived synthetic cannabinoids (Huffman and Padgett, 2005; Wiley et al., 2014a). Further, it stimulated [³⁵S]GTP γ S turnover with high potency and efficacy at both receptors, suggesting that CP55,940 would act as a potent CB₁ receptor agonist in vivo. Indeed, this prediction has proved true, as CP55,940 produces cannabimimetic effects in the tetrad battery in mice (Compton et al., 1992b) and substitutes and cross-substitutes for Δ^9 -THC in drug discrimination in rats (Gold et al., 1992; Wiley et al., 1995a). Similarly, the in vivo positive control Δ^9 -THC suppressed locomotor activity and showed hypothermic, antinociceptive, cataleptic and Δ^9 -THC-like discriminative stimulus effects in mice. Δ^9 -THC-like discriminative stimulus effects were also observed with JWH-018, WIN55,212-2, and CP47,497, as has been shown previously with the former two compounds (Compton et al., 1992a; Wiley et al., 2014b) and with the C-8 homolog of CP47,497 (Gatch and Forster, 2014). Previous research has shown that these three compounds bind to the CB₁ receptor with high affinity and produce cannabimimetic effects in the tetrad battery (Compton et al., 1992a; Compton et al., 1992b; Wiley et al., 1998). Together, these data show that representative compounds from the tetrahydrocannabinol, bicyclic and aminoalkylindole cannabinoid classes produce similar in vivo and in vitro pharmacological profiles in these assays.

In contrast, the three novel synthetic cannabinoids tested herein produced distinct profiles in the battery of in vitro and in vivo assays, with quantitative differences compared to typical

cannabinoid effects. The profiles of AB-CHMINACA and FUBIMINA most closely matched those obtained previously with other synthetic cannabinoids, differing only quantitatively in their respective affinities, potencies and efficacies. Similar to CP55,940 (present study) and other full dual CB₁/CB₂ agonists (Huffman and Padgett, 2005), AB-CHMINACA and FUBIMINA displaced [³⁵H]CP55,940 from both cannabinoid receptor types. Although both compounds showed higher CB₂ receptor affinity, FUBIMINA exhibited several-fold greater selectivity than AB-CHMINACA or CP55,940. Both compounds also activated CB₁ receptors with full efficacy comparable to (FUBIMINA) or greater than (AB-CHMINACA) that produced by CP55,940 (present study) or other full agonists such as WIN55,212-2 (Griffin et al., 1998), albeit FUBIMINA showed limited potency that was consistent with its lower binding affinity. Consistent with the magnitudes of their respective CB₁ receptor affinities, AB-CHMINACA produced the full profile of cannabinoid effects in the tetrad battery whereas FUBIMINA was inactive when administered i.p. and produced a cannabimimetic profile only at a 56 mg/kg i.v. dose. Tetrad effects of AB-CHMINACA and FUBIMINA were attenuated by rimonabant, suggesting CB₁ receptor mediation. In Δ^9 -THC discrimination, 0.3 mg/kg AB-CHMINACA, a dose that did not affect response rates, fully and potently substituted for Δ^9 -THC. This pattern of results resembles that obtained with the control compounds from different chemical classes of cannabinoids (see Figure 6). In contrast, FUBIMINA only partially substituted for Δ^9 -THC and did so with considerable intra-subject variability. Although tested up to the limits of solubility, FUBIMINA failed to decrease response rates. Together, these results suggest that AB-CHMINACA is a potent and efficacious psychoactive CB₁ receptor agonist that is likely to possess abuse liability in humans, a finding that has been implicitly supported through its recent placement in Schedule I (U.S. Drug Enforcement Administration, 2015). FUBIMINA also

appears to share cannabimimetic effects with Δ^9 -THC; however, its low potency may limit its abuse, as illicit manufacturers tend to focus on compounds with greater CB₁ receptor affinity and high potency. Nevertheless, FUBIMINA has been detected in products in Japan (Uchiyama et al., 2014) and with sufficient concentrations, cannabimimetic effects are likely. Metabolic transformations observed for these compounds were similar to those described for many similarly structured synthetic cannabinoids and primarily consisted of monohydroxylation and glucuronide conjugation, with FUBIMINA also undergoing defluorination, as is typical for synthetic cannabinoids with a 5-fluoropentyl group (e.g., AM-2201).

While in vitro results showed that AB-PINACA resembled AB-CHMINACA in its high affinities for CB₁ and CB₂ receptors and its high efficacy for stimulation of CB₁ receptors, differences between their profiles emerged in the in vivo experiments. Both compounds produced rimonabant-reversible effects in the complete tetrad battery; however, administration of 30 mg/kg (i.p.) AB-PINACA was accompanied by short-lived convulsive behavior, an effect that we do not typically observe with Δ^9 -THC or other cannabinoids at doses that produce tetrad effects. In the Δ^9 -THC discrimination procedure, AB-PINACA substituted fully and dose-dependently for Δ^9 -THC, but full substitution was achieved only at a dose that was accompanied by substantial decreases in response rate, with only a small percentage (22%) of mice responding at this dose. Previously, we have observed response rate decreases only with doses of other synthetic cannabinoids that were suprathreshold for full substitution (Figure 5; Wiley et al., 1995a; Wiley et al., 2013). Hence, AB-PINACA's lack of separation between doses that were Δ^9 -THC-like and those that substantially suppressed responding was unusual compared to the profile seen with other synthetic cannabinoids. These results suggest that AB-PINACA is a potent psychoactive CB₁ receptor agonist, but they also suggest that the doses that induce

intoxication may be very close to (or indistinguishable from) doses associated with behavioral toxicity.

In summary, synthetic cannabinoids that were originally developed as research tools or as candidate medications have been diverted to drugs of abuse in the form of products labeled with such terms as “herbal incense,” “fake weed,” “spice,” and “K2.” AB-CHMINACA, AB-PINACA, and FUBIMINA are among the chemicals that have been identified in recent confiscations. The results of the present study demonstrate that the pharmacological effects of AB-CHMINACA, AB-PINACA and FUBIMINA overlap with those of psychoactive cannabinoids from different chemical classes, including Δ^9 -THC, JWH-018, CP47,497, and WIN55,212-2. Each of these three compounds binds to and activates CB₁ and CB₂ cannabinoid receptors, produces a characteristic tetrad of cannabimimetic effects in mice, and produces dose-dependent increases in responding on the Δ^9 -THC-associated aperture in Δ^9 -THC discrimination. A primary difference among the compounds is their potency, with rank order of potency being correlated with their CB₁ receptor binding affinities: FUBIMINA < Δ^9 -THC < AB-PINACA < AB-CHMINACA. Notably, all three of these compounds are high efficacy agonists in the [³⁵S]GTPγS binding assay, as compared to the low partial agonism of Δ^9 -THC. Ironically, AB-PINACA and AB-CHMINACA are of potential interest to the scientific community as research tools due to their unique chemical structures and their high CB₁ receptor efficacies (i.e., > CP55,940). Further use of these chemicals is likely to include greater emphasis on the original purpose for which they were developed: research with a primary goal of increased understanding of cannabinoid receptors and other components of the endocannabinoid system that underlie the abuse of plant-derived and synthetic cannabinoids.

Acknowledgements

The authors would like to thank Dr. Jordan Trecki of the Drug Enforcement Agency for helpful discussion on the human aspects of the abuse of these novel synthetic cannabinoids.

Authorship Contributions

Participated in research design: Wiley, Thomas, Lefever.

Conducted experiments: Antonazzo, Cortes, Moore, Patel, Wallgren.

Performed data analysis: Wiley, Thomas, Grabenauer, Moore.

Wrote or contributed to the writing of the manuscript: Wiley, Thomas, Marusich, Lefever, Grabenauer, Moore.

References

- Bateman RH, Carruthers R, Hoyes JB, Jones C, Langridge JI, Millar A and Vissers JPC (2002)
A novel precursor ion discovery method on a hybrid quadrupole orthogonal acceleration
time-of-flight (Q-TOF) mass spectrometer for studying protein phosphorylation. *Journal
of the American Society for Mass Spectrometry* **13**:792-803.
- Compton DR, Gold LH, Ward SJ, Balster RL and Martin BR (1992a) Aminoalkylindole analogs:
cannabimimetic activity of a class of compounds structurally distinct from delta 9-
tetrahydrocannabinol. *J Pharmacol Exp Ther* **263**:1118-1126.
- Compton DR, Johnson MR, Melvin LS and Martin BR (1992b) Pharmacological profile of a
series of bicyclic cannabinoid analogs: Classification as cannabimimetic agents. *J
Pharmacol Exp Ther* **260**:201-209.
- Compton DR, Rice KC, De Costa BR, Razdan RK, Melvin LS, Johnson MR and Martin BR
(1993) Cannabinoid structure-activity relationships: Correlation of receptor binding and
in vivo activities. *J Pharmacol Exp Ther* **265**:218-226.
- Devane WA, Dysarz FA, Johnson MR, Melvin LS and Howlett AC (1988) Determination and
characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34**:605-613.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D,
Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain
constituent that binds to the cannabinoid receptor. *Science* **258**:1946-1949.
- Gaoni Y and Mechoulam R (1964) Isolation, structure, and partial synthesis of an active
constituent of hashish. *J Amer Chem Soc* **86**:1646-1647.
- Gatch MB and Forster MJ (2014) - Delta9-Tetrahydrocannabinol-like discriminative stimulus
effects of compounds. *Behav Pharmacol* **25**:750-757.

- Gold LH, Balster RL, Barrett RL, Britt DT and Martin BR (1992) A comparison of the discriminative stimulus properties of delta 9-tetrahydrocannabinol and CP 55,940 in rats and rhesus monkeys. *J Pharmacol Exp Ther* **262**:479-486.
- Grabenauer M, Krol WL, Wiley JL and Thomas BF (2012) Analysis of synthetic cannabinoids using high-resolution mass spectrometry and mass defect filtering: implications for nontargeted screening of designer drugs. *Anal Chem* **84**:5574-5581.
- Griffin G, Atkinson PJ, Showalter VM, Martin BR and Abood ME (1998) Evaluation of Cannabinoid Receptor Agonists and Antagonists Using the Guanosine-5'-O-(3-[³⁵S]thio)-triphosphate Binding Assay in Rat Cerebellar Membranes¹. *JPharmacolExpTher* **285**:553-560.
- Hanus L, Abu-Lafi S, Fride E, Breuer A, Vogel Z, Shalev DE, Kustanovich I and Mechoulam R (2001) 2-Arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci U S A* **98**:3662-3665.
- Huffman JW and Padgett LW (2005) Recent developments in the medicinal chemistry of cannabimimetic indoles, pyrroles and indenenes. *Curr Med Chem* **12**:1395-1411.
- Manera C, Tuccinardi T and Martinelli A (2008) Indoles and related compounds as cannabinoid ligands. *Mini Rev Med Chem* **8**:370-387.
- Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R and Ward SJ (1991) Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. *Pharmacol Biochem Behav* **40**:471-478.
- National Research Council (2011) *Guide for the care and use of laboratory animals*. National Academies Press, Washington, D.C.

- Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Néliat G, Caput D, Ferrara P, Soubrié P, Brelière JC and Le Fur G (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350**:240-244.
- Showalter VM, Compton DR, Martin BR and Abood ME (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *J Pharmacol Exp Ther* **278**:989-999.
- U.S. Drug Enforcement Administration OoDC (2015) - Schedules of controlled substances: temporary placement of three synthetic. *Fed Regist* **80**:5042-5047.
- Uchiyama N, Shimokawa Y, Matsuda S, Kawamura M, Kikura-Hanajiri R and Goda Y (2014) Two new synthetic cannabinoids, AM-2201 benzimidazole analog (FUBIMINA) and (4-methylpiperazin-1-yl)(1-pentyl-1H-indol-3-yl)methanone (MEPIRAPIM), and three phenethylamine derivatives, 25H-NBOMe 3,4,5-trimethoxybenzyl analog, 25B-NBOMe, and 2C-N-NBOMe, identified in illegal products. *Forensic Toxicol* **32**:105-115.
- Vann RE, Warner JA, Bushell K, Huffman JW, Martin BR and Wiley JL (2009) Discriminative stimulus properties of Delta9-tetrahydrocannabinol (THC) in C57Bl/6J mice. *Eur J Pharmacol* **615**:102-107.
- Vardakou I, Pistos C and Spiliopoulou C (2010) Spice drugs as a new trend: mode of action, identification and legislation. *Toxicol Lett* **197**:157-162.
- Wiley JL, Barrett RL, Lowe J, Balster RL and Martin BR (1995a) Discriminative stimulus effects of CP 55,940 and structurally dissimilar cannabinoids in rats. *Neuropharmacology* **34**:669-676.

- Wiley JL, Compton DR, Dai D, Lainton JA, Phillips M, Huffman JW and Martin BR (1998) Structure-activity relationships of indole- and pyrrole-derived cannabinoids. *J Pharmacol Exp Ther* **285**:995-1004.
- Wiley JL, Lowe JA, Balster RL and Martin BR (1995b) Antagonism of the discriminative stimulus effects of delta 9-tetrahydrocannabinol in rats and rhesus monkeys. *J Pharmacol Exp Ther* **275**:1-6.
- Wiley JL and Martin BR (2009) Preclinical pharmacological and brain bioassay systems for CB1 cannabinoid receptors, in *The cannabinoid receptors* (Reggio PH ed) pp 329-360, Humana Press, New York.
- Wiley JL, Marusich JA and Huffman JW (2014a) Moving around the molecule: Relationship between chemical structure and in vivo activity of synthetic cannabinoids. *Life Sci* **97**:55-63.
- Wiley JL, Marusich JA, Lefever TW and Cortes RA (2014b) Cross-substitution of delta-9-tetrahydrocannabinol and JWH-018 in drug discrimination in rats. *Pharmacol Biochem Behav* **124**:123-128.
- Wiley JL, Marusich JA, Lefever TW, Grabenauer M, Moore KN and Thomas BF (2013) Cannabinoids in disguise: Delta-9-Tetrahydrocannabinol-like effects of tetramethylcyclopropyl ketone indoles. *Neuropharmacology* **75**:145-154.
- Wohlfarth A, Castaneto MS, Zhu M, Pang S, Scheidweiler KB, Kronstrand R and Huestis MA (2015) Pentyndole/pentyndazole synthetic cannabinoids and their 5-fluoro analogs produce different primary metabolites: Metabolite profiling for AB-PINACA and 5F-AB-PINACA. *AAPS J* **17**:660-677.

Zhang Y, Gilliam A, Maitra R, Damaj MI, Tajuba JM, Seltzman HH and Thomas BF (2010)

Synthesis and biological evaluation of bivalent ligands for the cannabinoid 1 receptor. *J*

Med Chem **53**:7048-7060.

Footnotes

The research described in this manuscript was supported by the Department of Justice, Drug Enforcement Agency [Contract DJD-14-HQ-P-0713]; the National Institutes of Health National Institute on Drug Abuse [Grant R01DA-003672]; and the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice [Contract 2012-R2-CX-K001]. None of the funding agencies had any other role in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the Department of Justice, the Drug Enforcement Agency, or the National Institutes of Health.

Reprint requests should be addressed to Jenny L. Wiley (jwiley@rti.org) or Brian F. Thomas (bft@rti.org), 3040 Cornwallis Road, Research Triangle Park, NC 27709-2194.

Figure Legends

Figure 1: Chemical structures of JWH-018, AM-2201, FUBIMINA, AB-CHMINACA, and AB-PINACA.

Figure 2: Effects of CP55,940 (filled squares), AB-PINACA (unfilled squares), AB-CHMINACA (filled circles), and FUBIMINA (unfilled circles) on [^3H]CP55,940 displacement (panel A) and [^{35}S]GTP γ S turnover (panel B) in hCB $_1$ receptors expressed in HEK-293 cells. Effects of CP55,940 (filled squares), AB-PINACA (unfilled squares), AB-CHMINACA (filled circles), and FUBIMINA (unfilled circles) on [^3H]CP55,940 displacement (panel C) and [^{35}S]GTP γ S turnover (panel D) in hCB $_2$ receptors expressed in HEK-293 cells. Each concentration-effect curve represents the mean (\pm SEM) of 3-6 repetitions.

Figure 3: Effects of Δ^9 -THC (filled squares), AB-PINACA (unfilled squares), AB-CHMINACA (filled circles), and FUBIMINA (unfilled circles) on locomotor activity (panel A), antinociception (panel B), rectal temperature (panel C) and catalepsy (panel D). Values represent the mean (\pm SEM) of 6 male ICR mice, with the exception that n=5 for ring immobility for the 100 mg/kg dose of FUBIMINA due to data excluded for one mouse that fell off the ring 10 times. Asterisks (*) indicate significant differences ($p < 0.05$) compared to respective vehicle (at left side of each panel). Significant F values are as follows: For panel A, AB-PINACA [$F(6,35)=6.45$, $p < 0.05$] and AB-CHMINACA [$F(5,30)=9.18$, $p < 0.05$]. For panel B, Δ^9 -THC [$F(3,20)=21.69$, $p < 0.05$], AB-PINACA [$F(6,35)=11.97$, $p < 0.05$], AB-CHMINACA

[F(5,30)=23.43, $p<0.05$], and FUBIMINA [F(3,20)=7.27, $p<0.05$]. For panel C, Δ^9 -THC [F(3,20)=63.92, $p<0.05$], AB-PINACA [F(6,35)=56.87, $p<0.05$], and AB-CHMINACA [F(5,30)=23.09, $p<0.05$]. For panel D, Δ^9 -THC [F(3,20)=27.30, $p<0.05$], AB-PINACA [F(6,35)=11.75, $p<0.05$], and AB-CHMINACA [F(5,30)=17.48, $p<0.05$].

Figure 4: The left side of each panel shows the effects of i.p. injections of vehicle, Δ^9 -THC (56 mg/kg), AB-PINACA (30 mg/kg), and AB-CHMINACA (3 mg/kg) tested in combination with vehicle (unfilled bars) or 3 mg/kg rimonabant (filled bars). The right side of each panel shows the effects of i.v. injections of vehicle and FUBIMINA (56 mg/kg) tested in combination with vehicle (unfilled bars) or 3 mg/kg rimonabant (filled bars). Dependent measures are spontaneous activity (panel A), antinociception (panel B), rectal temperature (panel C), and catalepsy (panel D). Values represent the mean (\pm SEM) of 6 mice per group. Dollar signs (\$) indicate significant main effects ($p < 0.05$) of compound dose, as compared to vehicle. Asterisks (*) and number symbols (#) indicate significant interactions (with post-hoc confirmation of difference) between compound ($p < 0.05$), relative to the vehicle/vehicle condition or the compound plus vehicle condition, respectively. Significant F values are as follows: For panel A, Δ^9 -THC, AB-PINACA, and AB-CHMINACA [main effect: F(3,40)=7.81, $p<0.05$]; FUBIMINA [main effect: F(1,20)=31.80, $p<0.05$]. For panel B, Δ^9 -THC, AB-PINACA, and AB-CHMINACA [interaction: F(3,40)=15.28, $p<0.05$]; FUBIMINA [interaction: F(1,20)=14.44, $p<0.05$]. For panel C, Δ^9 -THC, AB-PINACA, and AB-CHMINACA [interaction: F(3,40)=5.66, $p<0.05$]; FUBIMINA [interaction: F(1,20)=23.30, $p<0.05$]. For panel D, Δ^9 -THC, AB-PINACA, and AB-CHMINACA [interaction: F(3,40)=28.55, $p<0.05$]; FUBIMINA [interaction: F(1,20)=19.74, $p<0.05$].

Figure 5: Effects of Δ^9 -THC (filled squares), WIN55,212-2 (unfilled squares), JWH-018 (filled circles), and CP47,497 (unfilled circles) on percentage of responses that occurred on the Δ^9 -THC-associated lever (top panel) and response rate (bottom panel). Each point represents the mean (\pm SEM) of data for male C57/Bl6J mice: all doses of Δ^9 -THC (n=6-7); all doses of JWH-018 (n=11); at doses of 0.1, 0.3 and 1 mg/kg of CP47,497 (n=8) and WIN55,212-2 (n=3); at 3 mg/kg CP47,497 (n=5 for % Δ^9 -THC-lever responding and n=6 for response rate); at 5.6 mg/kg CP47,497 (n=1 for % Δ^9 -THC-lever responding and n=2 for response rate); at 3 mg/kg WIN212-2 (n=2 for % Δ^9 -THC-lever responding and n=3 for response rate); at 5.6 mg/kg WIN55,212-2 (n=1) for response rate. Asterisks (*) indicate significant differences ($p < 0.05$) compared to vehicle.

Figure 6: *Left panels:* Effects of Δ^9 -THC on percentage of responses that occurred on the Δ^9 -THC-associated aperture (panel A) and response rate (panel B) in mice trained to discriminate Δ^9 -THC from vehicle in a nose poke procedure following transition from a lever press procedure (filled squares) or *de novo* (unfilled squares). Each point represents the mean (\pm SEM) of data for 7 male C57/Bl6J mice for the transitional group and n=11-13 for the *de novo* group. *Right panels:* Effects of Δ^9 -THC (filled squares), AB-PINACA (unfilled squares), AB-CHMINACA (filled circles), and FUBIMINA (unfilled circles) on percentage of responses that occurred on the Δ^9 -THC-associated aperture (panel C) and response rate (panel D). Each point represents the mean (\pm SEM) of data for 18-20 male C57/Bl6J mice for Δ^9 -THC and n=6-9 for the synthetic compounds, except for % Δ^9 -THC-associated aperture responding at 3 mg/kg AB-PINACA

(n=2) and 1 mg/kg AB-CHMINACA (n=1). For all panels, asterisks (*) indicate significant differences compared to respective vehicle.

Figure 7: Structure of FUBIMINA showing characteristic fragment masses for monohydroxylated metabolites. Hydroxylation was confirmed on the 1-(5-fluoropentyl)-1*H*-benzimidazole moiety by the presence of a fragment ion at m/z 249. Hydroxylation was also confirmed on the benzimidazole moiety by the presence of a fragment ion at m/z 161 and on the naphthyl moiety by the presence of fragment ions at m/z 171, m/z 143, and m/z 233.

Table 1. Binding affinity and potency and efficacy for stimulation of GTP- γ -[³⁵S] turnover at hCB₁ and hCB₂ receptors

Compound	CB ₁ K _i ^a	GTP- γ -[³⁵ S] Turnover ^b		CB ₂ K _i ^a	GTP- γ -[³⁵ S] Turnover ^b	
		CB ₁ EC ₅₀ ^c	CB ₁ E _{max} ^d		CB ₂ EC ₅₀ ^c	CB ₂ E _{max} ^d
CP55,940	0.59	23.3	124	0.30	2.1	63
	(0.06)	(4.7)	(9)	(0.04)	(0.8)	(4)
	<i>7</i>	<i>12</i>	<i>12</i>	<i>6</i>	<i>10</i>	<i>10</i>
AB-CHMINACA	0.78	7.4	205	0.45	232.4	35
	(0.11)	(1.5)	(14)	(0.03)	(231.2)	(2)
	<i>3</i>	<i>6</i>	<i>6</i>	<i>3</i>	<i>4</i>	<i>4</i>
AB-PINACA	2.87	71	192	0.88	14.9	41
	(0.69)	(20.9)	(25)	(0.00)	(8.4)	(1)
	<i>3</i>	<i>6</i>	<i>6</i>	<i>3</i>	<i>4</i>	<i>4</i>
FUBIMINA	296.1	2466.3	122	23.45	1269.3	56
	(33.5)	(1037.2)	(7)	(3.21)	(557.1)	(9)
	<i>4</i>	<i>6</i>	<i>6</i>	<i>3</i>	<i>6</i>	<i>6</i>

^a Values represent K_is (± SEM) in nM for [³H]CP55,940 displacement at specified (hCB₁ or hCB₂) receptor.

^b For each measure in all columns, n is shown in italics below the SEM.

^c Values represent EC₅₀s (± SEM) in nM for [³⁵S]GTP γ S binding at specified (hCB₁ or hCB₂) receptor.

^d Values represent % maximal increase (± SEM) for [³⁵S]GTP γ S binding over basal at specified (hCB₁ or hCB₂) receptor.

Table 2. Potencies in the tetrad tests.^a

Compound (MW)	Tetrad Tests ED ₅₀ s (μmol/kg)			
	SA	MPE	RT	RI
Δ ⁹ -THC (314)	104 (51 - 216)	34 (20 - 58)	30 (23 - 39)	30 (17 - 53)
AB-CHMINACA (356)	1.8 (0.7 – 4.5)	2.0 (1.3 – 3.0)	1.1 (0.7 – 1.6)	2.7 (1.9 – 3.9)
AB-PINACA (330)	7.6 (3.3 – 17.4)	13.7 (5.5 – 34.2)	5.3 (3.5 – 8.0)	13.9 (6.8 – 28.4)
FUBIMINA (360)	Not active (> 278)	Not active (> 278)	Not active (> 278)	Not active (> 278)

^a Values represent ED₅₀s (± 95% confidence limits) in μmol/kg. All compounds were administered i.p. SA = % inhibition of spontaneous activity, MPE = percentage of maximum possible effect in tail flick test, RT = change in rectal temperature in °C, RI = ring immobility, and MW = molecular weight.

Table 3. Potencies for substitution in Δ^9 -THC discrimination.

THC DD (Lever)			THC DD (Nose poke)		
Compound	CB ₁ K _i (nM)	ED ₅₀ (μ mol/kg)	Compound	CB ₁ K _i (nM)	ED ₅₀ (μ mol/kg)
Δ^9 -THC (n = 7) (314)	41 ^a (2)	4.5 (3.5 – 5.7)	Δ^9 -THC (n = 20) (314)	41 ^a (2)	5.7 (4.8 – 6.7)
JWH-018 (n = 11) (341)	9.5 ^a (4.5)	0.35 (0.32 – 0.50)	AB-CHMINACA (n = 8) (356)	0.78 (0.11)	0.34 (0.22 – 0.50)
CP47,947 (n = 8) (318)	9.5 ^b (0.35)	0.85 (0.47 – 1.45)	AB-PINACA (n = 9) (330)	2.87 (0.69)	3.78 (2.42 – 5.87)
WIN 55,212-2 (n = 3) (522)	1.9 ^a (0.1)	0.80 (0.46 – 1.38)	FUBIMINA (n = 6) (360)	296.1 (33.5)	131 (72 – 233)

* ED₅₀s (\pm 95% confidence limits) are expressed in μ mol/kg. Molecular weights are provided in parentheses. All compounds were administered i.p.

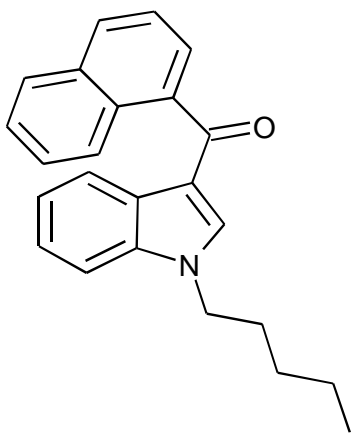
^a (Showalter et al., 1996)

^b (Compton et al., 1993)

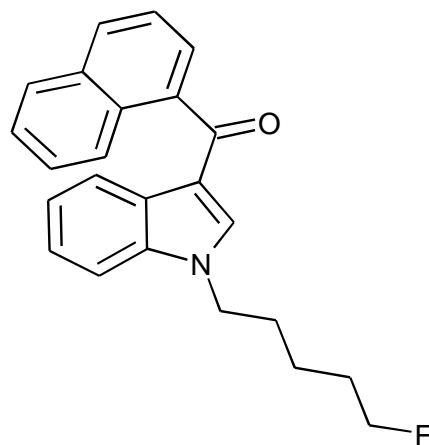
Table 4. Metabolic transformations observed in pooled
urine from mice dosed with FUBIMINA

Metabolite	<u>FUBIMINA</u>	
	I	II
Parent	X	
Monohydroxylation	X	X
Dihydrodiol formation	X	X
Saturation	X	
Hydration	X	X
Defluorination + hydroxylation	X	X
Defluorination+ carboxylation	X	
Defluorination+ carboxylation + hydroxylation	X	

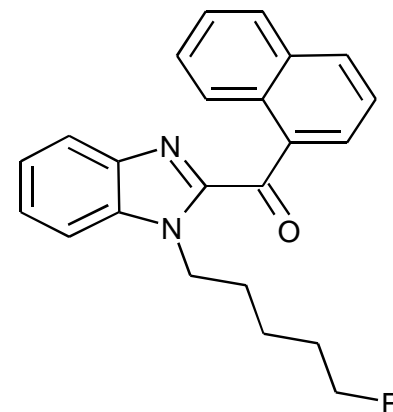
Figure 1 – JPET 225326



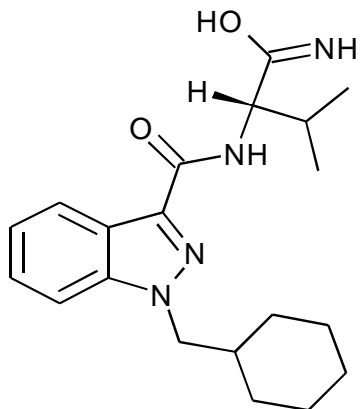
JWH-018



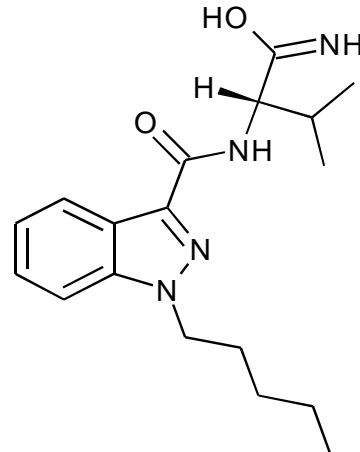
AM-2201



FUBIMINA



AB-CHMINACA



AB-PINACA

Figure 2 – JPET 225326

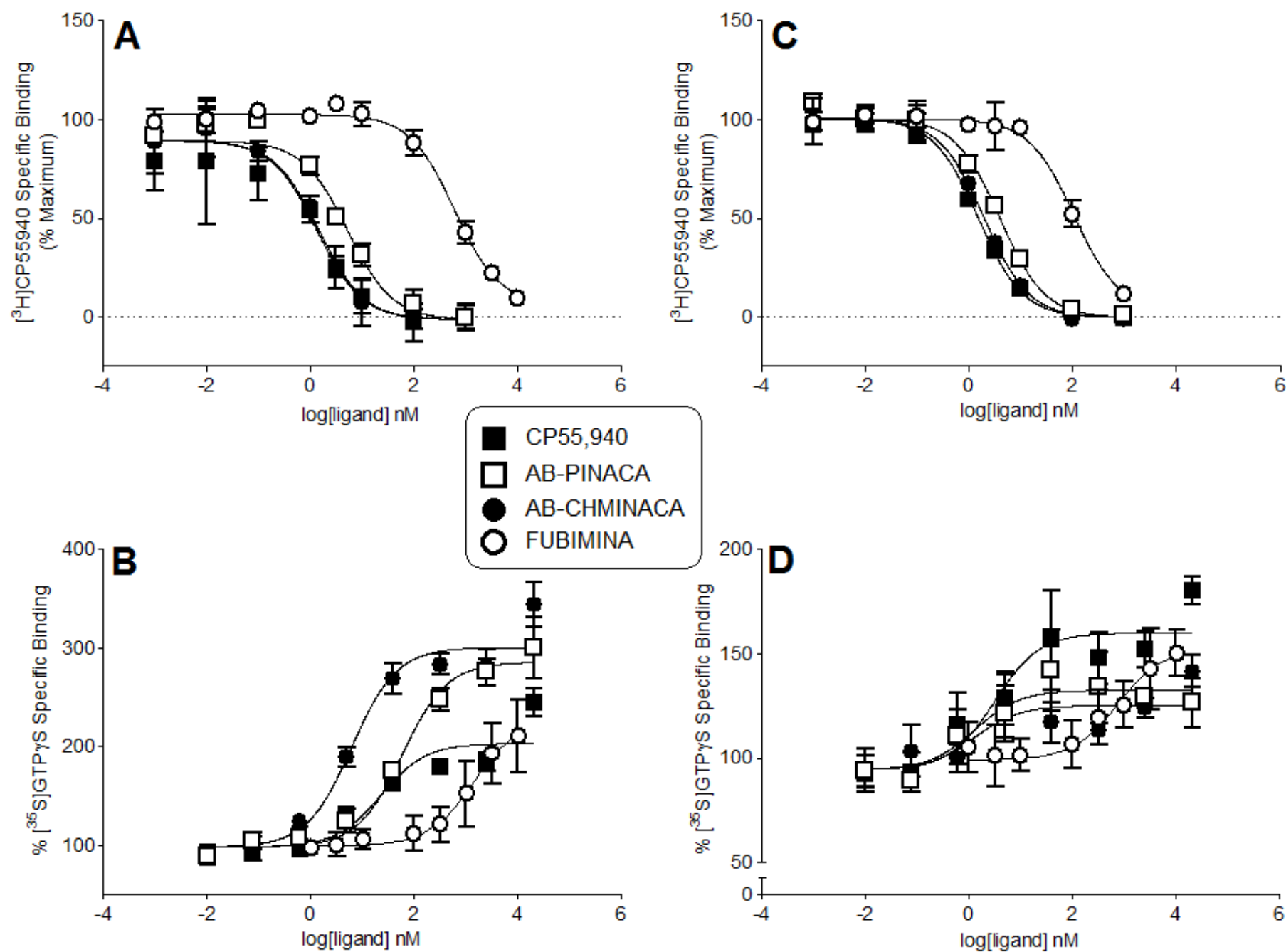


Figure 3 – JPET 225326

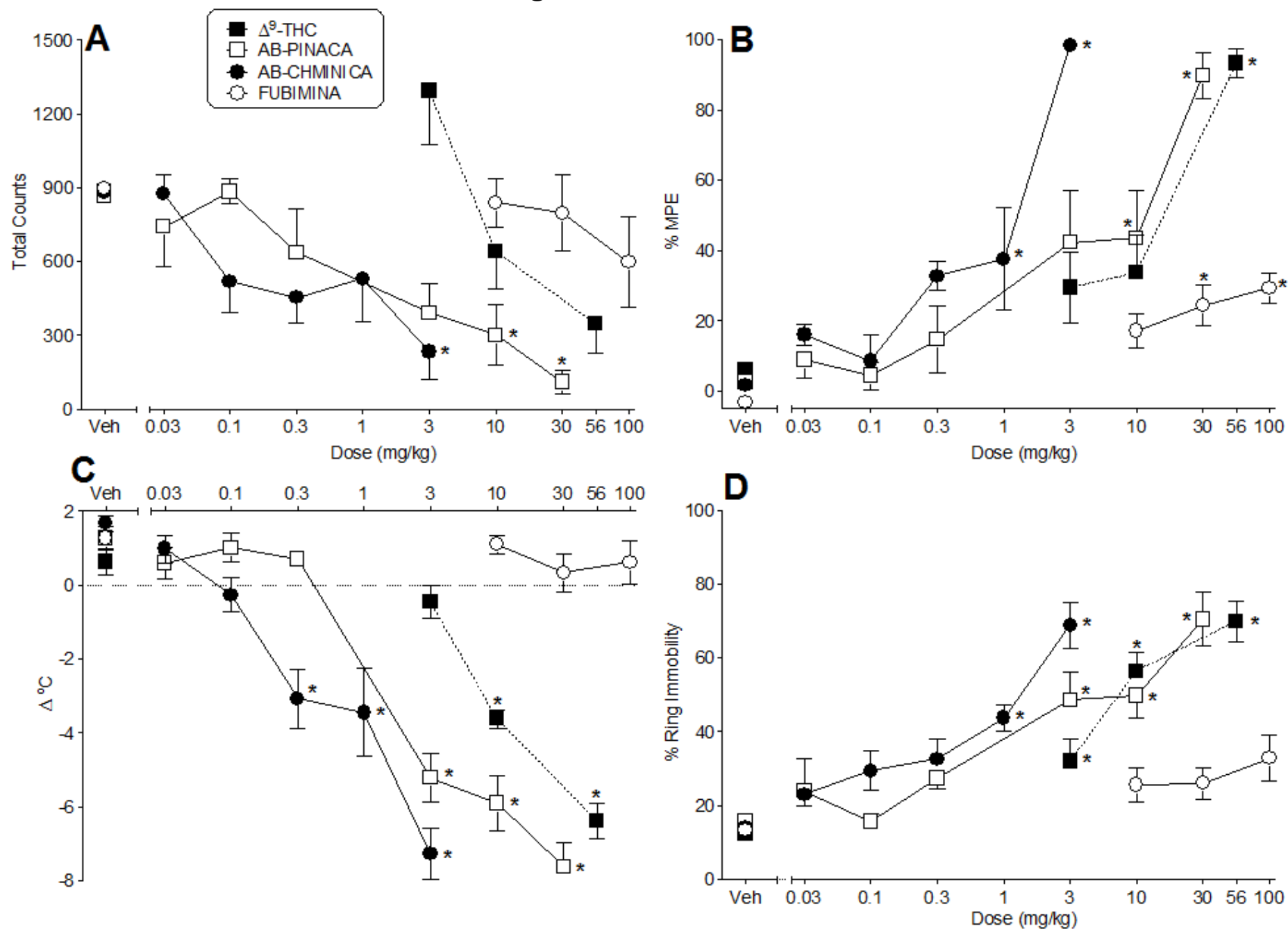


Figure 4 – JPET 225326

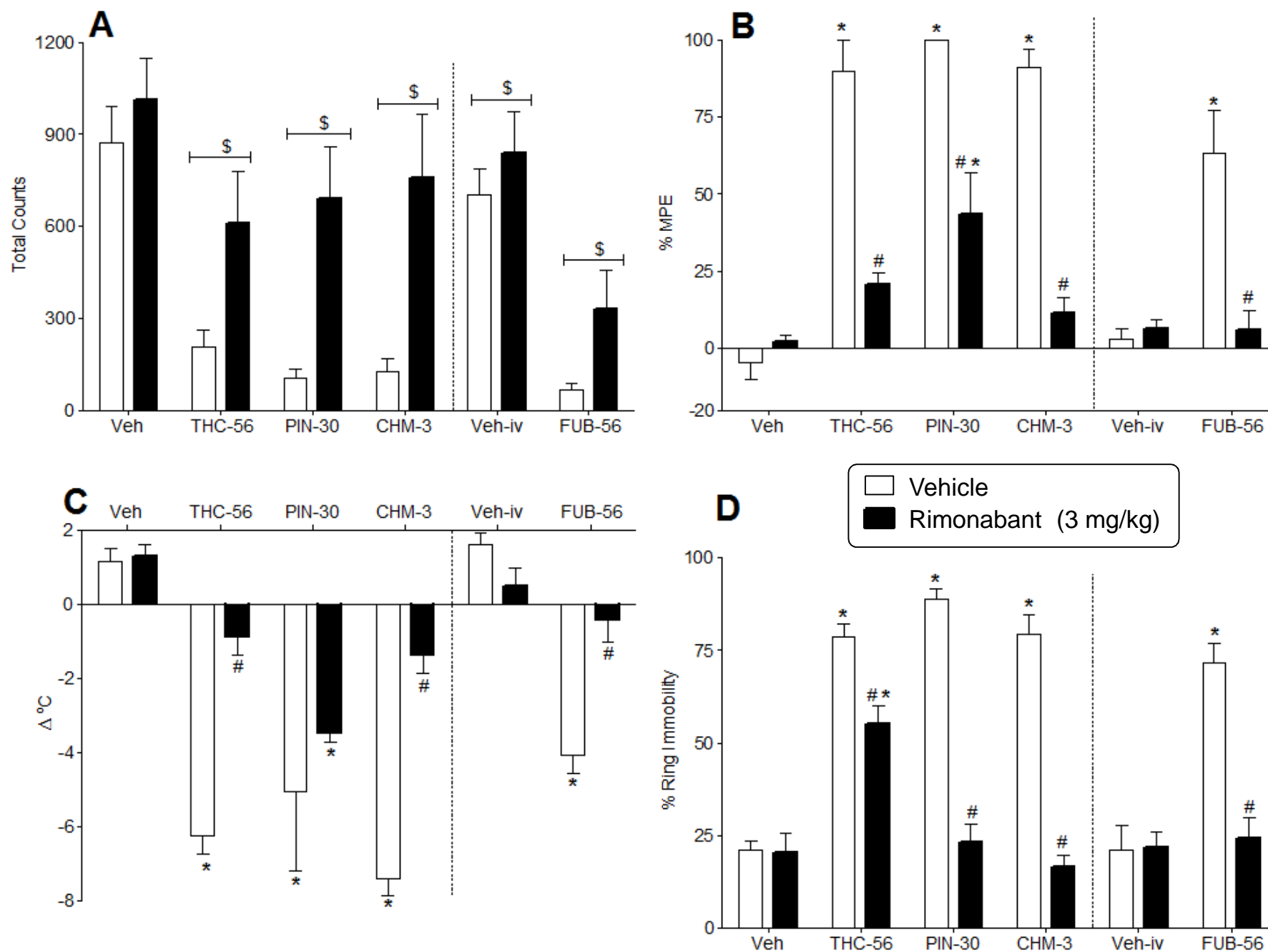


Figure 5 – JPET 225326

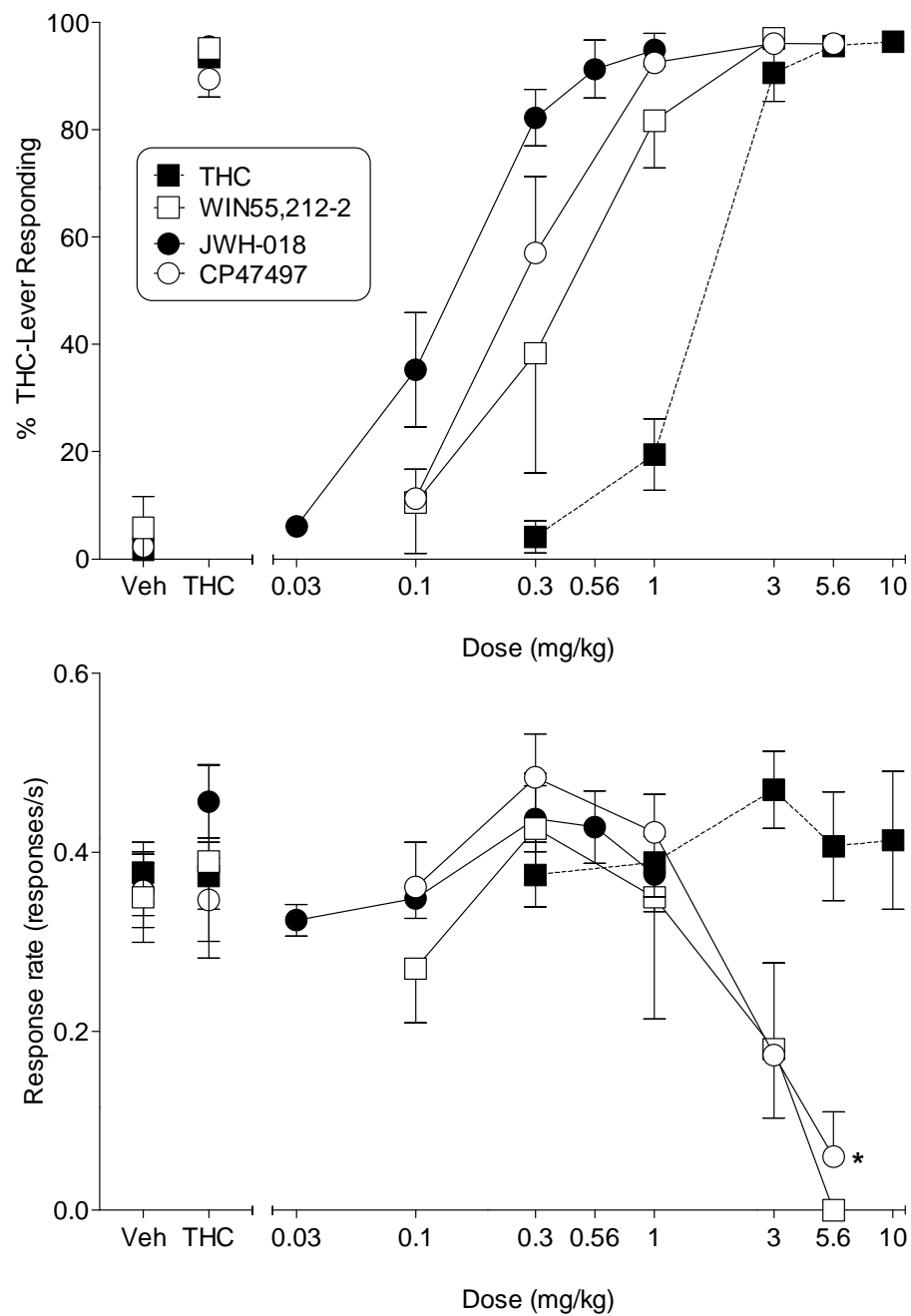


Figure 6 – JPET 225326

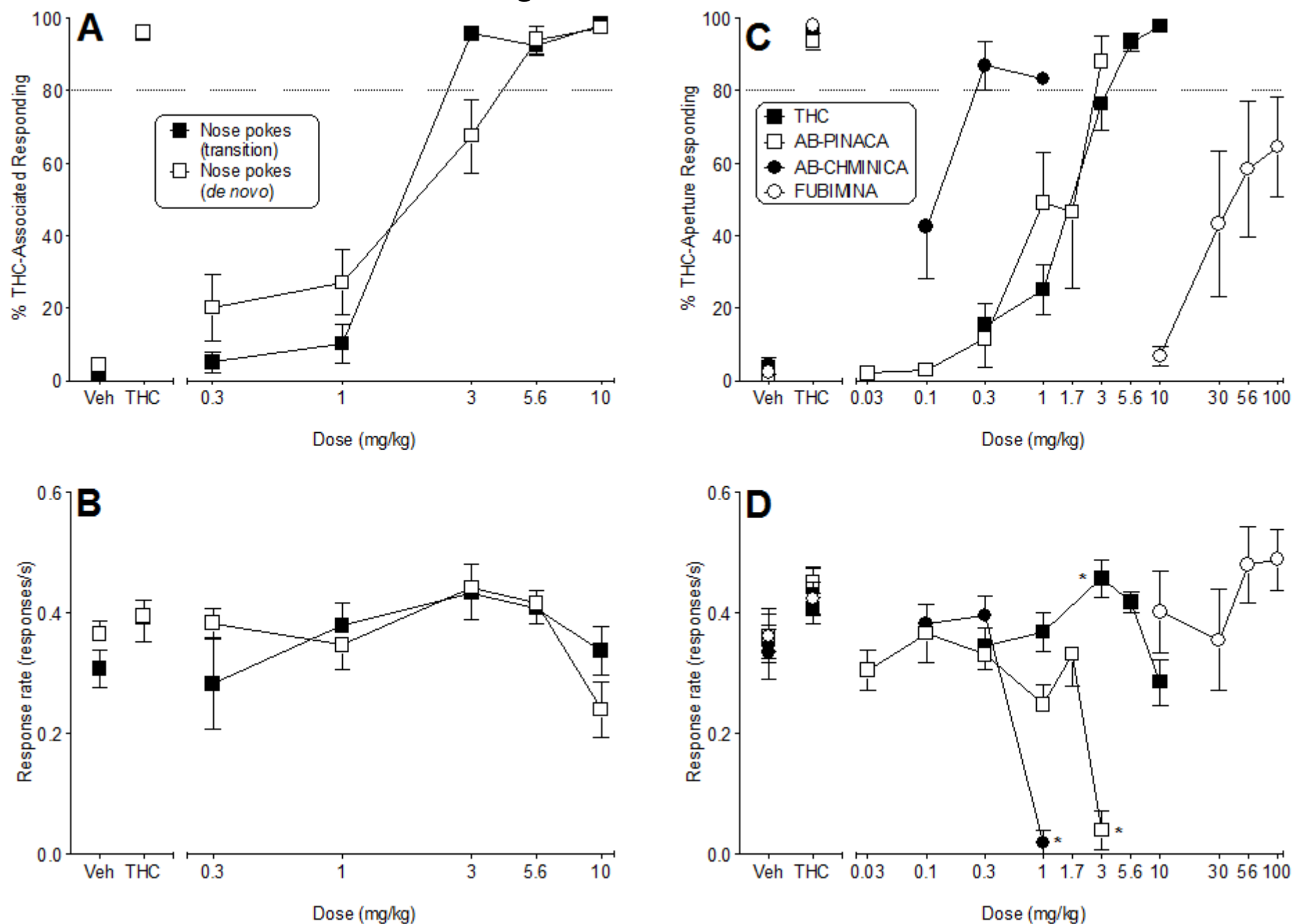


Figure 7 – JPET 225326

