Synthesis and characterization of a peripherally restricted CB_1 cannabinoid antagonist, URB447, that reduces feeding and body weight gain in mice

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SUPPLEMENTARY DATA

Experimental

Chemistry

All chemicals were purchased from Sigma-Aldrich or Tocris in the highest quality commercially available. Rimonabant was provided by RBI as part of the Chemical Synthesis Program of the National Institutes of Health (NIH). Solvents were RP grade, unless otherwise indicated. Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 60, 0.040-0.063 mm, Merck). TLC analyses were performed on precoated silica gel on aluminium sheets (Kieselgel 60 F_{254}, Merck). Melting points were determined on a Büchi SMP-510, or a Kofler Reichert, capillary melting point apparatus. MS (EI) analyses (70 eV) were recorded with a Fisons Trio 1000 spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer and analyzed using the WIN-NMR software package; chemical shifts were measured by using the central peak of the solvent. IR spectra were obtained on a Shimadzu FT-8300, or a Nicolet Avatar, spectrometer. Elemental analyses were performed on a Carlo Erba analyzer. Fresh drug solutions were prepared immediately before use in a vehicle of 0.9% sterile saline/10% polyethylene glycol (PEG)-400/10% Tween-80.

General procedure for the synthesis of 1-(4-chlorobenzyl)pyrrole derivatives (2, 7, 8a). To a stirred, cooled (0 °C) solution (contained in a N_2 atmosphere) of the suitable pyrrole derivative **1**, **5a**, **6** (1 mmol) in dry DMF (2.5 mL), NaH (0.035 g of an 80% mineral oil dispersion; 1.15 mmol) was added with constant stirring that was continued for 0.5 h before addition of the 4-chlorobenzylchloride (0.185 g; 1.15 mmol). After stirring the mixture for 2 h at room temperature a further amount of NaH (0.035 g; 1.15 mmol) and 4-chlorobenzylchloride (0.185 g; 1.15 mmol) were added and the mixture was allowed to react for 4 h (**8a**: 1 h; **2**, **7**: 4 h). CH_2Cl_2 and H_2O were then slowly added, the organic layer was separated, washed with H_2O, dried (Na_2SO_4) and concentrated. Purification of the residue by column chromatography (cyclohexane/EtOAc 9:1: **8a**; 8:2: **7**; 7:3: **2**) and recrystallization yielded **2**, **7**, and **8a** as solids.

1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-carboxylic acid ethyl ester (2). White solid. Yield: 48% (0.169 g). Mp 112–114 °C (Et_2O/petroleum ether). MS (EI): *m/z* 353 (M^+), 125 (100). ^1H NMR ($CDCl_3$): δ 1.37 (t, 3H); 2.46 (s, 3H); 4.31 (q, 2H); 5.10 (s, 2H); 6.69 (s, 1H); 6.84 (m, 2H); 7.24 (m, 7H) ppm. IR (neat): 3063, 2976, 2929 cm^{-1}.

{1-(4-chlorobenzyl)-4-[(4-chlorobenzylidene)amino]-2-methyl-5-phenyl-1*H*-pyrrol-3-yl}(phenyl) methanone (7). Yellow amorphous solid. Yield: 78% (0.408 g). MS (EI): *m/z* 523 (M^+), 77 (100). ^1H NMR ($CDCl_3$): δ 2.34 (s, 3H); 5.15 (s, 2H); 6.94 (d, 2H); 7.18–7.36 (m, 14H); 7.83 (m, 3H) ppm.

[1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-yl](phenyl)methanone (8a). White crystals. Yield: 70% (0.270 g). Mp 109–110 °C (Et_2O/petroleum ether). MS (EI): *m/z* 385 (M^+), 125 (100). ^1H NMR ($CDCl_3$): δ 2.52 (s, 3H); 5.16 (s, 2H); 6.51 (s, 1H); 6.90 (d, 2H); 7.23–7.51 (m, 10H); 7.88 (m, 2H) ppm. IR (nujol): 1635 cm^{-1}. Anal. calcd for $C_{25}H_{20}ClNO \cdot 0.25H_2O$ (390.39): C, 76.92; H, 5.29; N, 3.59. Found: C, 77.13; H, 5.10; N, 3.48.

Synthesis of 1-(4-chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-carboxylic acid. To a solution of **2** (0.118 mg; 0.33 mmol) in MeOH (2.7 mL) and H_2O (0.66 mL), was added $LiOH \cdot H_2O$. After

refluxing the mixture for 14 h, the solvent was evaporated. The residue was cooled, neutralized with 2N HCl and extracted with EtOAc. The organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated. Purification of the residue by crystallization gave the desired compound as a white solid. Yield 90% (0.097 g). Mp 185–186 °C (EtOAc/petroleum ether). MS (EI): *m/z* 326 (M^+), 125 (100). ^{1}H NMR ($CDCl_3$): δ 2.48 (s, 3H); 5.11 (s, 2H); 6.74 (s, 1H); 6.85 (d, 2H); 7.24–7.35 (m, 7H) ppm. IR (nujol): 1662 cm^{-1}.

Synthesis of 3-(*N*-substituted)carbamoyl-1-(4-chlorobenzyl)-2-methyl-5-phenylpyrroles (3a–c). To a stirred, cooled (0 °C) solution of the above carboxylic acid (0.108 g; 0.33 mmol) in dry DMF (1.2 mL) under N_2 atmosphere, $(COCl)_2$ (0.169 g; 0.12 mL; 1.33 mmol) was added. After stirring the mixture for 6 h at room temperature, the solvent and the excess of oxalyl chloride were evaporated. Dry CH_2Cl_2 was added and distilled away twice in order to remove the last traces of oxalyl chloride. The residue was dissolved in dry CH_2Cl_2 (2.5 mL) and added dropwise to a solution of the appropriate amine (0.54 mmol; 0.45 mmol in the case of **3c**) and TEA (0.055 mg; 0.076 mL; 0.54 mmol) in dry CH_2Cl_2 (3.3 mL). The mixture was stirred 14 h at room temperature, then was washed with H_2O, 2N NaOH, 2N HCl and H_2O, dried (Na_2SO_4) and concentrated. Purification of the residue by column chromatography (cyclohexane/EtOAc 7:3: **3c**; 1:1: **3a**) and recrystallization yielded **3a** and **3c** as solids. In the case of **3b** the residue was purified directly by crystallization.

1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-carboxylic acid piperidin-1-ylamide (3a). White crystals. Yield: 74% (0.099 g). Mp 189–192 °C (CH_2Cl_2/petroleum ether). MS (EI): *m/z* 407 (M^+), 308 (100). ^{1}H NMR ($CDCl_3$): δ 1.44–1.78 (m, 6H); 2.47 (s, 3H); 2.86 (m, 4H); 5.08 (s, 2H); 6.32 (br d, 1H); 6.52 (s, 1H); 6.84 (d, 2H); 7.20–7.35 (m, 7H) ppm. IR (nujol): 3244, 1638 cm^{-1}. Anal. calcd for $C_{24}H_{26}ClN_3O$ (407.94): C, 70.66; H, 6.42; N, 10.30. Found: C, 70.35; H, 6.40; N, 10.22.

1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-carboxylic acid bicyclo[2.2.1]-hept-2-ylamide (3b). White solid. Yield: 78% (0.108 g). Mp 214–215 °C (EtOAc). MS (EI): *m/z* 418 (M^+), 125 (100). ^{1}H NMR ($CDCl_3$): δ 1.15-1.53 (m, 7H); 1.83-1.94 (m, 1H); 2.31 (m, 2H); 2.48 (s, 3H); 3.89

(m, 1H); 5.08 (s, 2H); 5.64 (br d, 1H); 6.30 (s, 1H); 6.84 (d, 2H); 7.21–7.29 (m, 7H) ppm. IR (nujol): 3325, 1622 cm^{-1}. Anal. calcd for $C_{26}H_{27}ClN_2O$ (418.96): C, 74.54; H, 6.50; N, 6.69. Found: C, 74.23; H, 6.37; N, 6.53.

1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-carboxylic acid adamantan-2-ylamide (3c). White solid. Yield: 59% (0.089 g). Mp 190–192 °C (EtOAc/petroleum ether). MS (EI): *m/z* 458 (M^+), 125 (100). ^1H NMR ($CDCl_3$): δ 1.72–2.05 (m, 14H); 2.49 (s, 3H); 4.24 (br d, 1H); 5.09 (s, 2H); 6.18 (br d, 1H); 6.37 (s, 1H); 6.85 (d, 2H); 7.22–7.34 (m, 7H) ppm. IR (nujol): 3334, 1616 cm^{-1}. Anal. calcd for $C_{29}H_{31}ClN_2O$ (459.02): C, 74.01; H, 7.01; N, 5.57. Found: C, 74.37; H, 6.92; N, 5.67.

Synthesis of 4-bromo-1-(4-chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole-3-carboxylic acid adamantan-2-ylamide (4). To a solution of **3c** (0.069 g; 0.15 mmol) in dioxane (0.6 mL) and CH_3COOH (0.3 mL), *N*-bromosuccinimmide (0.027 g; 0.15 mmol) was added portionwise and the reactants were allowed to react at room temperature for 2 h. The mixture was then poured onto a cooled (0 °C) 2N NaOH solution, and extracted with CH_2Cl_2. The combined organic layers were washed with 2N HCl and brine, dried (Na_2SO_4), and concentrated. Purification of the residue by column chromatography (CH_2Cl_2) and recrystallization gave **4** as a white solid. Yield: 45% (0.036 g). Mp 155–157 °C (Et_2O/petroleum ether). MS (EI): *m/z* 538 (M^+), 125 (100). ^1H NMR ($CDCl_3$): δ 0.83–2.04 (m, 15H); 2.51 (s, 3H); 4.30 (br d, 1H); 4.96 (s, 2H); 6.78 (d, 2H); 7.12–7.35 (m, 7H) ppm. IR (nujol): 3428, 1647 cm^{-1}. Anal. calcd for $C_{29}H_{30}BrClN_2O$ (537.92): C, 63.69; H, 5.71; N, 5.12. Found: C, 63.66; H, 5.54; N, 4.97.

Synthesis of {4-[(4-chlorobenzylidene)amino]-2-methyl-5-phenyl-1*H*-pyrrol-3-yl}(phenyl) methanone (6). To a stirred suspension of **2** (0.574 g; 2 mmol) in toluene (9 mL), 4-chlorobenzaldehyde (0.281 g; 2 mmol) and a catalytic amount of *p*-toluensulphonic acid were added. The mixture was refluxed in a Dean-Stark apparatus for 2 h and then concentrated. Purification of the residue by column chromatography (cyclohexane/EtOAc 6:4) gave **6** as a yellow solid. Yield 65% (0.528 g). MS (EI): *m/z* 406 (M^+), 301 (100).

[^{35}S]GTPγS and receptor binding assays

CB_1 and CB_2 binding assays were performed on rat cerebellar membranes or in CHO-K1 cells overexpressing CB_2 receptors (Receptor Biology, Beltsville, Maryland), respectively. Binding studies were performed at 25 ºC with 20 μg of receptor protein in a 20 mM HEPES/1mM MgCl (pH = 7.4) buffer along with [^{3}H]WIN-55212-2 (Perkin-Elmer, 40-60 Ci/mmol, 10 nM) at a concentration of 10 nM and test compounds at various concentrations. Final assay volume was 0.5 mL and the incubation time was 20 min. Receptor-bound [^{3}H]WIN-55212-2 was separated from free [^{3}H]WIN-55212-2 by filtration. The filter paper was washed twice with 0.2 mL of cold assay buffer and radioactivity measured by liquid scintillation counting.

GTPγS binding reactions were conducted on rat cerebellar membranes (20 μg) in the presence of various concentrations of test compounds for 90 min at 30 °C with 0.05 nM of [^{35}S]GTPγS (New England Nuclear, Boston, Massachusetts, specific activity of 1200 Ci/mmol) in 20 mM Tris pH 7.4, 5 mM $MgCl_2$, 0.1 M NaCl, 0.5 M EGTA, 30 μM GDP, 3 mU-mL^{-1} adenosine deaminase, 0.02% BSA and 50 μM phenylmethylsulphonyl fluoride at a total reaction volume of 50 μL. Each assay was conducted in the presence or absence of 10 μM cold WIN-55212-2. Reactions were stopped by centrifugation at 4 °C and the resulting supernatants aspirated, followed by washing of the pellet with 1 mL cold buffer. Membrane bound [^{35}S]GTPγS was measured in the pellets by liquid scintillation counting.

Membranes were prepared from freshly dissected rat cerebella. Tissues were homogenized in 20 mM Tris (pH 7.5) containing 0.32 M sucrose, followed by centrifugation at 1000 g for 10 min at 4 °C. The resulting pellet was discarded and the supernatant centrifuged at 27,000 g for 30 min at 4 °C. The supernatant was aspirated and the pellet resuspended in 50 mM Tris (pH 7.5). A Bradford assay was used to measure protein concentration.

cAMP quantification

HEK cells stably expressing mouse CB_2 were rinsed once with HEPES buffer (15 min), pre-incubated in HEPES buffer plus IBMX (10 mM, 15 min) and incubated in HEPES plus IBMX with or without isoproterenol (10 μM, 15 min). These steps were performed at 37 °C in a shaking water bath. Cannabinoids were added in both preincubation and incubation. Cells were then lyzed with water containing HEPES (20 mM), followed by sonication on ice. cAMP levels in homogenates were quantified using a cAMP [^{125}I] radioimmunoassay kit (PerkinElmer NEK033) according to manufacture's instructions.

Animals

All procedures met NIH guidelines for the care and use of laboratory animals. Male Swiss mice, weighing 20-25 g were obtained from Charles River (Wilmington, Massachusetts). Genetically obese mice, weighing 40-45 g (B6.V-Lep^{ob}/J) were obtained from Jackson Labs (Bar Harbor, Maine). All animals were maintained on a 12-h/12-h light/dark cycle with free access to water and chow (RMH 2500, Prolab, Framingham, Massachusetts). For blood and tissue collection animals were anaesthetized with halothane and blood was collected by cardiac puncture. Briefly, blood was collected by inserting a syringe, fitted with a 25.5 gauge needle into the right ventricle of anaesthetized mice. 500 μL of blood was withdrawn from the heart and placed into a glass tube containing EDTA (7.2 mg). Plasma was isolated by centrifugation. Tissues were snap-frozen in liquid N_2 and stored at -80 ºC.

Feeding analyses

Food intake was recorded with an automated system (Scipro, New York, NY), consisting of 24 individually housed cages containing feeding sensors and light beams for motor activity. Chow was accessible to mice through a wire grating at the bottom of a basket suspended from a calibrated sensor. Each time food was removed, the computer recorded the quantity removed. Weight variations in the baskets were monitored every second. Animals were habituated to the feeding/motor cages for 4 days

prior to the experiment. The animals were injected with vehicle or drugs i.p. min before the start of the 12 h dark cycle, the time at which monitoring was initiated. Food intake was normalized to body weight in kg. Recorded data were analyzed as food ingested per kg body weight across the test period.

Motor activity

Motor activity was determined as the number of light beam interruptions projecting across each cage that the animals interrupted. Motor data was collected automatically by the same cages used for the feeding analyses simultaneously with the feeding measurements (Scipro, New York, NY).

Formalin pain assay

Formalin (5% formaldehyde in a vehicle of 0.9% sterile saline/5% PEG-400/5% Tween-80, 10 μL) was injected into the plantar surface of mice using a 27-gauge needle fitted to a microsyringe. Vehicle or (*R*)-methanandamide was dissolved in the formalin solution. Following injections, animals were immediately transferred to a transparent observation chamber where pain behavior (time spent licking and biting the injected paw) was continuously monitored for 45 min (phase-I: 0-15 min; phase-II: 15-45 min) by an observer blind to the treatments. Antagonists were administered intraperitoneally 30 minutes before formalin.

Hypothermia

Body temperature was determined using a digital thermometer coupled to a mouse rectal probe (Stoelting, Wood Dale, Illinois). Results are expressed as change in body temperature from baseline.

Catalepsy

Catalepsy was determined by an observer blind to the treatment as the total time spent cataleptic during a 3-min observation period using a ring apparatus previously described.[1]

LC/MS analyses

Analytes were extracted from blood (0.5 mL total volume) and tissue homogenates (100-200 mg) by the addition and subsequent mixing in CH_3OH:$CHCl_3$:H_2O (1:2:1 mL). Following, centrifugation the organic layer was aspirated off, transferred to new vials, and evaporated under a stream of N_2. Analytes were resuspended in 100 μL of CH_3OH:$CHCl_3$ (1:1) for LC/MS analyses. URB447 was quantified using an Agilent model #1100-high performance liquid chromatography (HPLC) system coupled to an Agilent model #1946A-mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface. URB447 was separated on an Agilent Zorbax SB-CN (2.1 x 150 mm ID, 5 μm) column. A solvent gradient of H_2O:MeOH (30:70%) (containing 0.25% CH_3COOH and 5 mM CH_3COONH_4) was used at a flow rate of 0.4 mL/min. The solvent gradient was as follows: 0.0-5.0 min, 30% H_2O; 5.0-5.1 min, 20% H_2O; 5.10-6.00 min, 0% H_2O. MS detection was performed in the positive mode and the capillary voltage was set at 3 kV. N_2 was used as the drying gas at a flow rate of 13 L/min at 350 °C. Nebulizer pressure was set to 60 PSI. Rimonabant (500 pmol/ sample) was used as an internal standard to quantify the recovery of URB447 during the extraction process by adding rimonabant to the CH_3OH at the start of the lipid extraction. Calibration curves were established for both URB447 and rimonabant. The molecular ion used to quantify URB447 was m/z = 401 [$M+H^+$] and for rimonabant, m/z = 463 [$M+H^+$]. These ions were monitored in selected ion-monitoring (SIM) mode.

Statistical analyses

Graphpad Prism was used to calculate statistical significance by determining P values using either Student's t-test, ANOVA followed by a Tukey's post-hoc test, or 2-WAY repeated measures ANOVA followed by a Bonferroni post-hoc test where appropriate.

References and notes

1. Pertwee, R. G. *Br. J. Pharmacol.* **1972**, *46*, 753.