Design and Synthesis of the CB1 Selective Cannabinoid Antagonist AM281: A Potential Human SPECT Ligand

Submitted: March 17, 1999; Accepted June 14, 1999;.Published June 20, 1999.

Ruoxi Lan, Qian Lu and Pusheng Fan
Department of Pharmaceutical Sciences and Molecular and Cell Biology, U-92, University of Connecticut, Storrs, CT 06269
John Gatley and Nora D. Volkow
Medical Department, Brookhaven National Laboratory, Upton, NY 11973
Susanthi R. Fernando and Roger Pertwee
Department of Biomedical Sciences, University of Aberdeen, Aberdeen AB9 1AS, Scotland
Alexandros Makriyannis
Department of Pharmaceutical Sciences and Molecular and Cell Biology and Institute of Material Sciences, University of Connecticut, Storrs, CT 06269

ABSTRACT In the search for a radioligand capable of imaging cannabinoid CB1 receptors in the living human brain by SPECT (single photon emission computed tomography), *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM281) was synthesized. This compound is an analog of the potent, CB1 receptor selective antagonist SR141716A [*N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]. AM281 bound to brain and spleen membrane preparations (CB1 and CB2 receptors, respectively) with K_i values of 12 nM and 4200 nM, respectively. AM281 also inhibited the response of guinea-pig small intestine preparation to a cannabinoid receptor agonist. Thus, AM281 behaves as a CB1 receptor selective antagonist. Methods for the rapid, high-yield synthesis and purification of [^{123}I]AM281 were developed, and transaxially reconstructed brain SPECT images obtained after continuous infusion of [^{123}I]AM281 in baboons. Thus [^{123}I]AM281 may be suitable for imaging CB1 receptors in humans.

Key Words: AM281, cannabinoid CB1 receptor, cannabinoid receptor antagonist, CB1 selective ligand, SPECT image.

INTRODUCTION

Marijuana is one of the oldest and most widely used drugs in the world. Although the pharmacological and biochemical properties of cannabinoids, the major psychoactive components of marijuana, have been studied extensively (1) their molecular mechanisms of action are not yet well understood. Cannabinoid receptors have been the focus of much recent work, since the discovery of the receptor subtypes in brain (CB1) (2) and spleen (CB2) (3).

Radiolabeled ligands are required to characterize the binding sites and visualize the distributions of the cannabinoid receptors in humans and other species. We have previously reported the synthesis of [^{123}I]*N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide ([^{123}I]AM251) (Figure 1), a high-affinity and selective brain cannabinoid receptor antagonist, structurally related to the Sanofi compound SR141716A (4-8), and its use to study cannabinoid receptor binding sites in mouse and rat brain (9,10). The distribution of cannabinoid receptor binding sites for [^{123}I]AM251 in rat brain was very similar to the published distributions for the non-classical cannabinoid [^{3}H]CP-55,940 (11-13). However, we were unable to visualize cannabinoid receptor binding sites in baboon brain with [^{123}I]AM251 by SPECT (single photon emission computed tomography) imaging because of negligible brain uptake in this species (9). With the hypothesis that the inability to enter the brain may be associated with the high lipophilicity of AM251, we replaced the piperidine ring of AM251 with the more polar morpholino moiety. The k' values on reverse-phase high-performance liquid chromatography (HPLC) were 3.7 for AM281, 4.8 for SR141716A, and 5.7 for AM251, in agreement with the expectation that lipophilicity will increase upon replacing Cl with I, and decrease when replacing a piperidine group with a morpholine

Corresponding Author: Dr. Alexandros Makriyannis, Department of Pharmaceutical Sciences and Molecular and Cell Biology, U-92, University of Connecticut, Storrs, CT 06269; telephone: (860) 486-2133; facsimile: (860) 486-3089; e-mail: makriyan@uconnvm.uconn.edu

group. The resulting compound, AM 281 [*N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide], proved able to penetrate the baboon blood brain barrier and has been used in a preliminary study of cannabinoid CB1 receptors in the baboon brain (14).

In the present article, we report details of the synthesis of AM281 (Scheme 1) together with further biological evaluation data.

Chemistry

The synthesis of *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide AM281 is outlined in Scheme 1. 4-Bromo-propiophenone reacted with diethyl oxalate in anhydrous diethyl ether under basic conditions to afford the lithium salt of ethyl 2,4-dioxo-3-methyl-4-(4-bromophenyl) butanoate 1, which reacted with 2,4-dichlorophenylhydrazine hydrochloride to provide 5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxylic acid ethyl ester 2. The procedure for the conversion of ester **2** to amide **3** via three steps in our earlier publication with AM251 (10) was now shortened to a single step in which ester 2 was allowed to react with 4-aminomorpholine under basic conditions. Subsequently, the bromo derivative **3** reacted with bis(tributyltin) in the presence of a catalytic amount of tetrakis(triphenylphosphine)palladium(0) to afford the tributyltin derivative 4. Iododestannylation of compound 4 by the method used previously (10) for preparation of AM251 involving sodium iodide and chloramine-T in the presence of either acetic acid or phosphoric acid gave the desired compound 5 in a low yield (15-21%), and the product was especially difficult to purify. The alternative method where the tributyltin compound 4 was allowed to react with iodine in a carbon tetrachloride solution (15) gave AM281 in almost quantitative yield.

Radiolabeling was conducted similarly to that of [^{123}I]AM251 (10). The addition of a trace of carrier iodide, and use of SEP-PAK methodology instead of HPLC as the purification procedure, increased the net yield of AM281 from about 50% to about 75%, while resulting in a product of sufficiently high specific activity (about 5 Ci/μmol) to visualize CB1 receptors *in vivo*.

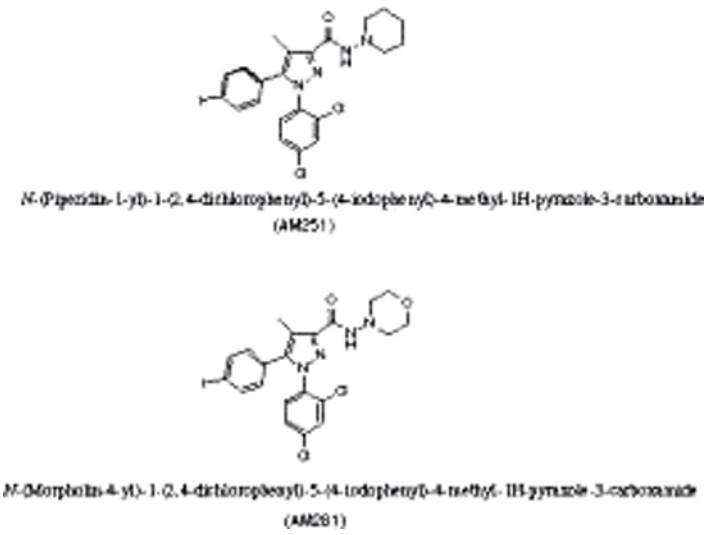
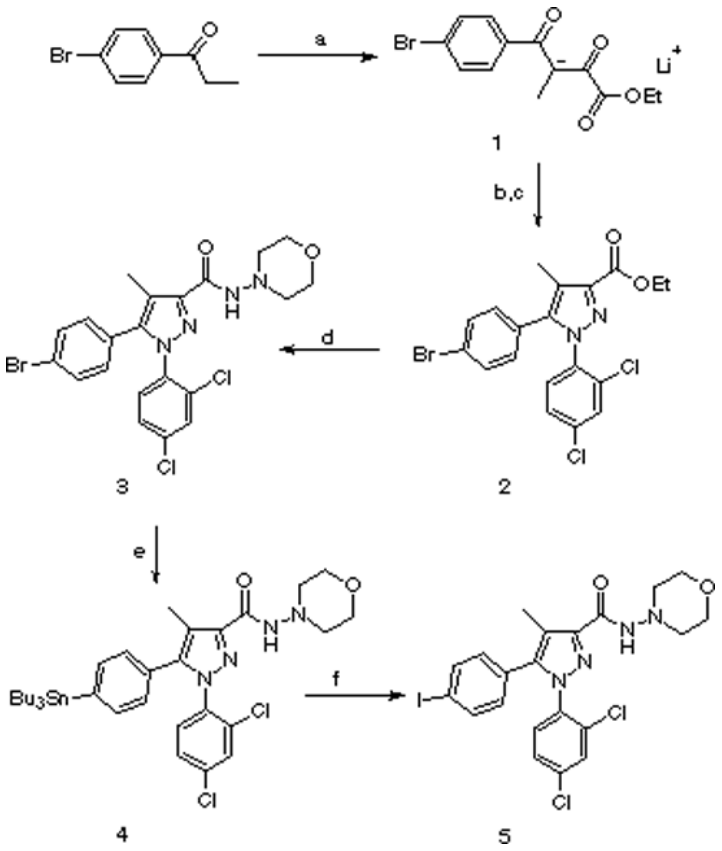


Figure 1. Pyrazole CB1 receptor radioligands.



Scheme 1. Synthesis of AM281[a]. [a]Reagents: (a) LiHMDS, ether, then EtO_2CCO_2Et; (b) 2,4-dichlorophenylhydrazine hydrochloride, EtOH; (c) AcOH; (d) 4-aminomorpholine, LiHMDS, THF; (e) Bu_6Sn_2, $Pd(PPh_3)_4$, Et_3N; (f) I_2, CCl_4.

BIOLOGICAL RESULTS AND DISCUSSION

Receptor Binding Studies of AM281 In Vitro

The affinity of AM281 for the cannabinoid receptor in rat forebrain membranes was evaluated using the competitive binding assay showed a K_i value of 12 nM (Figure 2). In contrast, AM281 had very low affinity for the CB2 cannabinoid receptor as measured in mouse spleen membrane preparation (K_i value of 4200 nM). These results indicated that AM281 is a selective cannabinoid receptor ligand with a 340-fold selectivity for CB1 compared to CB2 receptors.

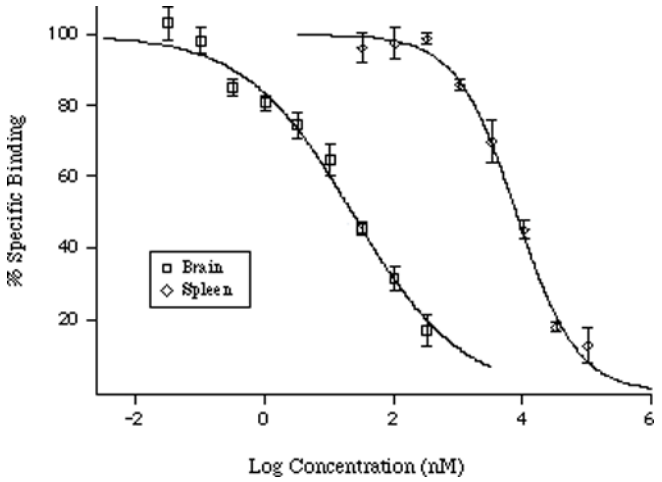


Figure 2. Equilibrium binding of AM281 to the cannabinoid receptors in rat forebrain and mouse spleen membranes.

Antagonism Studies

At a concentration of 1 μM, AM281 produced a significant dextral shift in the log concentration-response curve of the cannabinoid receptor agonist, WIN55,212-2 (Figure 3) (16). This shift was parallel and not accompanied by any change in the size of the maximal response to the agonist. The mean K_d value of AM281 calculated from these data was 110 nM, the 95% confidence limits being 42 nM and 710 nM. The data suggest that AM281 shares the ability of SR141716A to behave as a competitive cannabinoid receptor antagonist in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine (16). We have also shown that AM281 behaves as a cannabinoid receptor antagonist in the electrically stimulated rat hippocampal slice model (17).

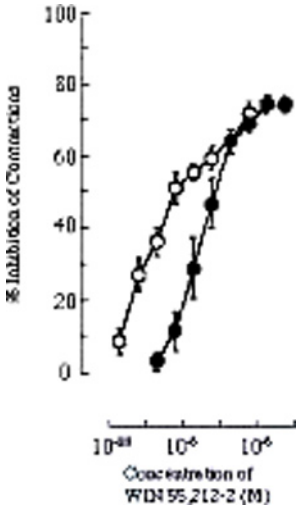


Figure 3. Mean concentration-response curves for WIN55,212-2 constructed in the presence of 1000 nM AM281 (filled symbols) or Tween 80 vehicle (open symbols). Each symbol represents the mean value ± SE of inhibition of electrically evoked contractions of strips of myenteric plexus-longitudinal muscle expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of WIN55,212-2 to the organ bath (n = 6 different preparations). The E_{max} of WIN55,212-2, with its 95% confidence limits shown in brackets, was 68.84% (59.14 and 78.53%) in the presence of Tween 80 and 74.87% (72.03 and 77.71%) in the presence of AM281. The dextral shift in the log concentration-response curve of WIN55,212-2 did not deviate significantly from parallelism ([2 + 2] dose parallel line assay; $P > 0.05$).

Brain Imaging

The transaxial SPECT images confirmed uptake of radioactivity in cerebellum and in cortical areas (Figure 4). The spatial resolution of current SPECT scanners is insufficient to permit visualization of the subcortical areas of highest CB1 receptor density, such as the substantia nigra and globus pallidus in the monkey brain. However, heterogeneous distribution of [^{123}I]AM281 was clearly seen in rat brain autoradiograms obtained using a phosphor imaging plate (Figure 5).

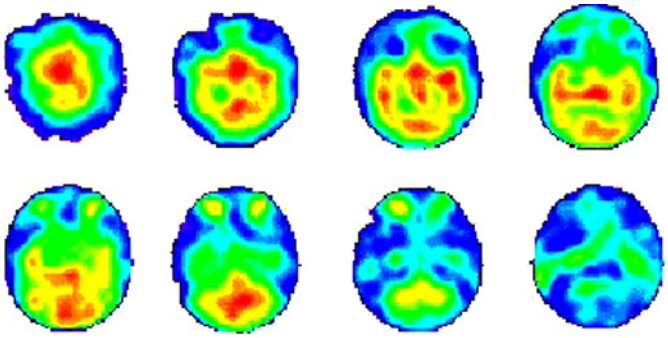


Figure 4. Visualization of cannabinoid CB1 receptors. A "rainbow" color scale (black>red>yellow>green>blue>white) was used. Transaxial SPECT images of the baboon brain. Slice width was 8.6 mm. Slices are ordered from top to bottom of the brain (left-to-right and top-to-bottom of the panel). The cerebellum is visualized towards the lower edge of the middle two slices of the bottom row.

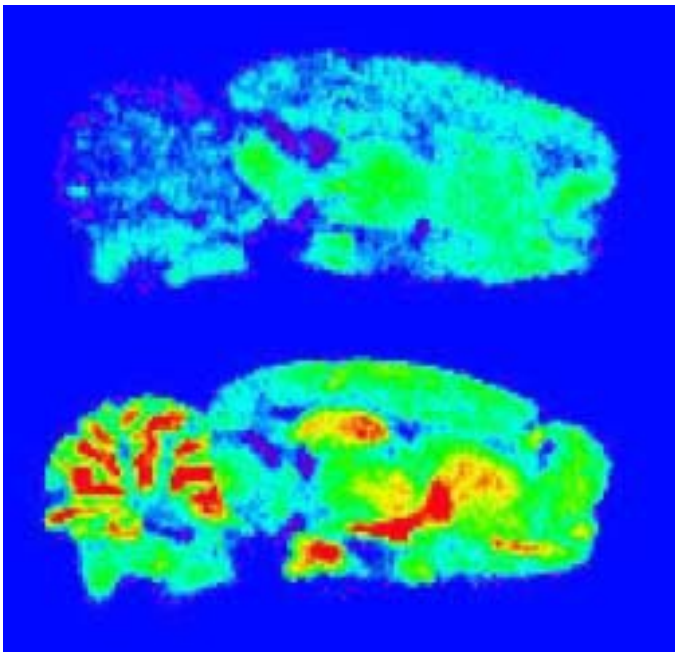


Figure 5. Phosphor imaging autoradiograms of a sagittal rat brain section after incubation in [^{123}I]AM281, washing and drying. *Upper section:* Specific binding was blocked with 10 µM SR141716A. *Bottom section:* Cerebellum is the "striped" region at the left, where CB1 receptors are strongly localized in the molecular layer. The intense area in the middle of the image towards the right is the globus pallidus.

EXPERIMENTAL SECTION

Chemistry

Melting points were determined using an Electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Brucker DMX-500 MHz spectrometer. Chemical shifts are reported in ppm (parts per million) relative to tetramethylsilane as the internal standard, and signals are quoted as s (singlet), d (doublet), t (triplet) or m (multiplet). Mass spectra (electron ionization, 70 eV) were determined on a KRATOS MS-50RFA instrument. Elemental analyses were performed at Analytical Services Center of Baron Consulting Company. Compounds indicated by the molecular formula followed by the symbols for the elements (C, H, N) were found to be within 0.4% of their theoretical values. The HPLC system was an Alltech Econosil cyanopropyl column (10-µ particles; 4.6 ×250 mm column length) eluted with acetonitrile/water (34:66, v/v) containing 25 mM ammonium acetate. Flash column chromatography was carried out by using Whatman active silica gel (230-400 mesh), and eluents were distilled before use. Solvents for reactions were dried or purified if necessary. Reactions were carried out under nitrogen atmosphere unless otherwise noted.

***N*-(Morpholin-4-yl)-5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (3).** To a magnetically stirred solution of ester 2 (3.00 g, 6.60 mmol) and 4-aminomorpholine (772 µL, 7.90 mmol) in dry tetrahydrofuran (25 mL) was added 1.0 M solution of lithium bis(trimethylsilyl)amide in hexane (10.0 mL, 10.0 mmol). The resulting mixture was stirred at room temperature for 2 hours, and then quenched with saturated aqueous ammonium chloride and extracted with chloroform (3 ×50 mL). The combined extracts were washed with brine, dried over anhydrous sodium sulfate, filtered, and evaporated. Purification by flash column chromatography on silica gel with chloroform and ethyl acetate (1:1) afforded amide **3** as a white solid (3.30 g, 98% yield): mp 255-257 °C (dec.); ^{1}HNMR ($CDCl_3$) δ7.69 (s, 1H, NH), 7.45 (d, J = 8.4 Hz, 2H, ArH), 7.42 (d, J = 1.9 Hz, 1H, ArH), 7.31-7.27 (m, 2H, ArH), 6.98 (d, J = 8.4 Hz, 2H, ArH), 3.84 (t, J = 4.4 Hz, 4H, OCH_2), 2.94 (t, J = 4.2 Hz, 4H, NCH_2), 2.35 (s, 3H, CH_3); MS *m/e* 508 (M^+), 407 (M^+ - $C_4H_9N_2O$), 379 (M^+ - $C_5H_9N_2O_2$). Anal. ($C_{21}H_{19}BrCl_2N_4O_2$) C, H, N.

***N*-(Morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-tributyltinphenyl)-4-methyl-1H-pyrazole-3-carboxamide (4).** To a magnetically stirred suspension of **3** (2.40 g, 4.70 mmol) in fresh distilled triethylamine (150 mL) was added bis(tributyltin) (3.16 mL, 6.25 mmol) followed by tetrakis(triphenylphosphine)palladium (0) (272 mg, 0.23 mmol) at room temperature under an argon atmosphere and the reaction mixture was heated to reflux for 30 hours. After cooling to room temperature, the precipitate was removed by filtration. The filtrate was then concentrated under reduced pressure and purified by flash column chromatography on silica gel with petroleum ether and acetone (4:1) to give the tributyltin derivative 4 (470 mg, 21% yield based on the recovery of starting material): mp 64-68 ^{o}C; ^{1}HNMR ($CDCl_3$) δ 7.71 (s, 1H, NH), 7.43 (s, 1H, ArH), 7.39 (d, J = 7.3 Hz, 2H, ArH), 7.26-7.25 (m, 2H, ArH), 7.04 (d, J = 7.2 Hz, 2H, ArH), 3.86 (t, J = 4.4 Hz, 4H, OCH_2), 2.96 (t, J = 4.3 Hz, 4H, NCH_2), 2.39 (s, 3H, CH_3), 1.53-1.48 (m, 6H, CH_2), 1.32-1.27 (m, 6H, CH_2), 1.04 (t, J = 8.3 Hz, 6H, CH_2), 0.86 (t, J = 7.3 Hz, 9H, CH_3); MS *m/e* 718 (M^+). Anal. ($C_{33}H_{46}Cl_2N_4O_2Sn$) C, H, N.

***N*-(Morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (5).** To a magnetically stirred solution of organotin compound 4 (290 mg, 0.40 mmol) in carbon tetrachloride (15 mL) was added dropwise a 0.02 M solution of iodine in carbon tetrachloride (25 mL, 0.50 mmol) at room temperature. After TLC showed that the reaction was completed, carbon tetrachloride was removed under reduced pressure. The residue was purified by flash column chromatography on silica gel with petroleum ether and ethyl acetate (1:1) to afford the desired product 5 as a white solid (215 mg, 97% yield): mp 265-268 ^{o}C (dec.); ^{1}HNMR ($CDCl_3$) δ 7.68 (s, 1H, NH), 7.66 (d, J = 8.2 Hz, 2H, ArH), 7.44-7.43 (m, 1H, ArH), 7.32-7.27 (m, 2H, ArH), 6.85 (d, J = 8.2 Hz, 2H, ArH), 3.86 (t, J = 4.2 Hz, 4H, OCH_2), 2.95 (t, J = 4.0 Hz, 4H, NCH_2), 2.36 (s, 3H, CH_3); MS *m/e* 556 (M^+), 471 (MH^+ - C_4H_8NO). Anal. ($C_{21}H_{19}Cl_2IN_4O_2$) C, H, N.

Receptor Binding Assay

For CB1 receptor binding studies, rat forebrain membranes were prepared following earlier procedures (18,19). For CB2 receptor binding studies, membranes were prepared from frozen mouse spleen according to Dodd (20). Silanized centrifuge tubes were used throughout to minimize receptor loss due to the adherent properties of the CB2 containing macrophages (18). The binding of the novel probes to the cannabinoid receptors was assessed as previously described (18,21). Briefly, approximately 50 µg of rat forebrain or mouse spleen membranes were incubated in silanized 96-well microtiter plates with TME containing 0.1% essentially fatty acid free bovine serum albumin (BSA), 0.8 nM [^{3}H]CP-55,940, and various concentrations of AM281 in a final volume of 200 µL. The assays were incubated for 1 hour at 30^{o}C and then immediately filtered on Unifilter GF/B filterplates using a Packard Filtermate 196 harvester, followed by four washes with ice cold wash buffer containing 0.5% BSA. Radioactivity was detected by adding MicroScint 20 scintillation cocktail directly to the dried filterplates, which were counted using a Packard Instruments TopCount Microplate Scintillation Counter. Nonspecific binding was assessed using 100 nM CP-55,940. Data collected from three independent experiments performed with duplicate determinations were normalized between 100 and 0% specific binding for [^{3}H]CP-55,940. The normalized data were then analyzed using a 4-parameter nonlinear logistic equation to yield IC_{50} values. The IC_{50} values from three independent experiments were combined and converted to K_i values using the assumptions of Cheng and Prusoff (22).

Pharmacological Studies In Vitro

The *in vitro* pharmacology of AM281 was investigated using the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine assay. The measured response was WIN55,212-2-induced inhibition of electrically evoked contractions (18,19). Both drugs were mixed with two parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline). The size of maximal responses to WIN55,212-2 (E_{max} values) and their 95% confidence limits were calculated by

non-linear regression analysis using GraphPAD InPlot (GraphPAD Software, San Diego). The K_d value of AM281 was calculated using the equation $(x\text{-}1) = B/K_d$, where x (the "dose ratio") is the concentration of WIN55,212-2 that produces a particular degree of inhibition in the presence of the antagonist at a concentration, B, divided by the concentration of WIN55,212-2 that produces an identical degree of inhibition in the presence of Tween 80 (18,19,23,24). The dose ratio and its 95% confidence limits have been determined by symmetrical (2 + 2) dose parallel line assay (25), using responses to pairs of WIN55,212-2 concentrations located on the steepest part of each log concentration-response curve.

Preparation of Radioiodinated AM281

Procedures were essentially as described for [^{123}I]AM251 (10), except for the following. No carrier added [^{123}I]NaI was purchased in 20 mCi lots from Nordion (Kanata, Ont.) as the dried product in serum capped vials. To the shipping vial were added in turn with shaking between additions: sufficient water (about 100 µL) to dissolve the dried residue; 4 µL of a 1 mM aqueous solution of NaI; 50 µL of 0.5 M H_3PO_4; 100 µL of a 1 mg/mL solution in DMSO of the tributyltin analog of AM281, sufficient ethanol (about 300 µL) to make a clear solution, and 20 µL of a 10 mg/mL aqueous solution of chloramine-T. The mixture was shaken, left to stand at room temperature for 3 min, and then mixed with 5 mL of water and passed through a SEP-PAK containing 300 mg octadecylsilane (Alltech Associates, Deerfield, IL). The SEP-PAK was washed in turn with 5 mL water and 3 mL 35% methanol. The radiolabeled product was eluted in 5 mL 70% methanol. The solvent was removed with a stream of N_2 with warming at 50^{o}C, and the residue was re-dissolved in 0.1 mL absolute ethanol.

Brain Imaging

A Picker 3000 camera, equipped with low energy ultra high resolution collimators, was employed for SPECT studies. A 128 ×128 acquisition matrix was used, and the energy peak was set at 158 keV with a 15% window. Image reconstruction was performed using the predefined brain SPECT protocol supplied with the camera. Transverse sections were reconstructed using a Butterworth filter. A 3-D post filter (low pass with order of 4.0 and cutoff 0.26) was then applied. Attenuation correction was performed using an ellipse fitting with a factor of 0.11. Transaxial images were obtained with a slice width of 8.6 mm. Using sterile techniques, the ethanolic solution of [^{123}I]AM281 was rapidly mixed with 2 mL of blood plasma prepared immediately beforehand from the experimental subject, and then diluted to 20 mL with 0.9% NaCl. One third of the saline solution was injected into the anesthetized baboon over about 1 min, and the remainder was infused over a 2-hour period while the animal was scanned. The distribution of [^{123}I]AM281 in slide-mounted rat brain sections was visualized using a phosphor imaging plate (Molecular Dynamics, Sunnyvale, CA) essentially as previously described (14).

ACKNOWLEDGEMENTS

This work was supported by Grants DA-3801, DA-152, DA-7215, and DA-9158 from the National Institute on Drug Abuse to Dr. A. Makriyannis, by the U. S. Department of Energy and its Office of Health and Environmental Research to S. J. Gatley and N. D. Volkow, and by Wellcome Trust Grant 039538 and NIDA Grant DA-9158 to Dr. R. Pertwee. We thank Dr. L. S. Melvin and Pfizer Inc. for providing us with CP-55,940 and Packard Instruments Inc. for providing the Filtermate 196 and Top-Count.

REFERENCES

1. Martin BR. Cellular effects of cannabinoids. Pharmacol Rev. 1986;38:45-74.

2. Devane WA, Dysarz FA 3d, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol. 1988; 34:605-613.

3. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature (London) 1993;365:61-65.

4. Barth F, Casellas P, Congy C, Martinez S, Rinaldi M, Anne-Archard G. Pyrazole derivatives, method of preparing them and pharmaceutical compositions in which they are present. US Patent 5,624,941 Apr. 29, 1997.

5. 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, Breliere JC, Le Fur G. SR141716A, A potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 1994;350:240-244.

6. Rinaldi-Carmona M, Barth F, Héaulme M, Alonso R, Shire D,

Congy C, Soubrié P, Breliere JC, Le Fur G. Biochemical and pharmacological characterization of SR141716A, the first potent and selective brain cannabinoid receptor antagonist. Life Sci. 1995;56:1941-1947.

7. Shire D, Calandra B, Delpech M, Dumont X, Kaghad M, Le Fur G, Caput D, Ferrara P. Structural features of the central cannabinoid CB1 receptor involved in the binding of the specific CB1 antagonist SR 141716A. J Biol Chem. 1996;271:6941-6946.

8. Rinaldi-Carmona M, Pialot F, Congy C, Redon E, Barth F, Bachy A, Breliere JC, Soubrié P, Le Fur G. Characterization and distribution of binding sites for [3H]-SR 141716A, a selective brain (CB1) cannabinoid receptor antagonist, in rodent brain. Life Sci. 1996;58:1239-1247.

9. Gatley SJ, Gifford AN, Volkow ND, Lan R, Makriyannis A. ^{123}I-Labeled AM251: a radioiodinated ligand which binds *in vivo* to mouse brain cannabinoid CB_1 receptors. Eur J Pharmacol. 1996;307:331-338.

10. Lan R, Gatley SJ, Makriyannis A. Preparation of iodine-123 labeled AM251: a potential SPECT radioligand for the brain cannabinoid CB1 receptor. J Label Compds Radiopharma 1996;38:875-881.

11. Gatley SJ, Lan R, Pyatt B, Gifford AN, Volkow ND, Makriyannis A. Binding of the non-classical cannabinoid CP 55,940, and the diarylpyrazole AM251 to rodent brain cannabinoid receptors. Life Sci. 1997;61:PL191-197.

12. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, DeCosta BR, Rice KC. Cannabinoid receptor localization in brain. Proc Natl Acad Sci. 1990;87:1932-1936.

13. Herkenham M, Lynn AB, Johnson MR, Melvin LS, DeCosta BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative *in vitro* autoradiographic study. J Neurosci. 1991;11:563-583.

14. Gatley SJ, Lan R, Volkow ND, Pappas N, King P, Wong CT, Gifford AN, Pyatt B, Dewey SL, Makriyannis A. Imaging the brain marijuana receptor: Development of a radioligand that binds to cannabinoid CB1 receptors in vivo. J Neurochem. 1998; 70:417-23.

15. Hanson RN, El-Wakil H. Electrophilic destannylation: stereospecific introduction of electrophiles at the 21 position of (17α)-19-Norpregna-1,3,5(10),20-tetraene-3-17β-diols. J Org Chem. 1987;52:3687-3688.

16. Pertwee, RG, Fernando, SR, Nash, JE, Coutts, AA. Further evidence for the presence of cannabinoid CB1 receptors in guinea-pig small intestine. Br J Pharmacol. 1996;118:2199-2205.

17. Gifford AN, Tang Y, Gatley SJ, Volkow ND, Lan R, Makriyannis A. Effect of the cannabinoid receptor SPECT agent, AM 281, on hippocampal acetylcholine release from rat brain slices. Neurosci Lett. 1997;238:84-86.

18. Drake DJ, Jensen RS, Busch-Peterson J, Kawakami JK, Fernandez-Garcia MC, Fan P, Makriyannis A, Tius MA. Classical/nonclassical hydrid cannabinoids: southern aliphatic chain-functionalized C-6β methyl, ethyl, and propyl analogues. J Med Chem. 1998;41:3596-3608.

19. Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG, Makriyannis A. (R)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. J Med Chem. 1994;37:1889-1893.

20. Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, Delaunoy JP. A rapid method for preparing synaptosomes: comparison, with alternative procedures. Brain Res. 1981;226:107-118.

21. Charalambous A, Guo Y, Houston DB, Howlett AC, Compton DR, Martin BR, Makriyannis A. 5$^{'}$-Azido-Δ^8-THC: a novel photoaffinity label for the cannabinoid receptor. J Med Chem. 1992;35:3076-3079.

22. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent (IC_{50}) of an enzymatic reaction. Biochem Pharmacol. 1973;22:3099-3108.

23. Coutts AA, Pertwee RG. Inhibition by cannabinoid receptor agonists of acetylcholine release from the guinea-pig Myenteric plexus. Br J Pharmacol. 1997;21:1557-1566.

24. Tallarida RJ, Cowan A, Adler MW. pA2 and receptor differentiation: a statistical analysis of competitive antagonism. Life Sci. 1979;25:637-654.

25. Colquhoun D. Lectures on Biostatistics. Oxford: Oxford University Press; 1971.