0022-3565/03/3061-363–370$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 306, No. 1
49924/1071944
Printed in U.S.A.

JPET 306:363–370, 2003

Synthesis and Characterization of NESS 0327: A Novel Putative Antagonist of the CB_1 Cannabinoid Receptor

STEFANIA RUIU, GÈRARD A. PINNA, GIORGIO MARCHESE, JEAN-MARIO MUSSINU, PIERLUIGI SABA, SIMONE TAMBARO, PAOLA CASTI, ROMINA VARGIU, and LUCA PANI

Neuroscienze S.c.a r.l., Cagliari, Italy (S.R., G.M., S.T., P.C.); Department of Pharmacology, Chemistry, and Toxicology, University of Sassari, Sassari, Italy (G.A.P., J.-M.M.); "B.B. Brodie" Department of Neuroscience, University of Cagliari, Cagliari, Italy (P.S.); Section of Human Physiology and Nutrition, Department of Applied Sciences to Biosystems, University of Cagliari, Cagliari, Italy (R.V.); and C.N.R. Institute of Neurogenetics and Neuropharmacology and Neuroscienze S.c.a r.l, Selargius, Italy (L.P.)

Received February 3, 2003; accepted March 26, 2003

ABSTRACT

The compound *N*-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo [6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide] (NESS 0327) was synthesized and evaluated for binding affinity toward cannabinoid CB_1 and CB_2 receptor. NESS 0327 exhibited a stronger selectivity for CB_1 receptor compared with *N*-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (SR 141716A), showing a much higher affinity for CB_1 receptor (K_i = 350 ± 5 fM and 1.8 ± 0.075 nM, respectively) and a higher affinity for the CB_2 receptor (K_i = 21 ± 0.5 nM and 514 ± 30 nM, respectively). Affinity ratios demonstrated that NESS 0327 was more than 60,000-fold selective for the CB_1 receptor, whereas SR 141716A only 285-fold. NESS 0327 alone did not produce concentration-dependent stimulation of guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding in rat cerebella membranes. Conversely, NESS 0327 antagonized [*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrolol [1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate] (WIN 55,212-2)-stimulated [^{35}S]GTPγS binding. In functional assay, NESS 0327 antagonized the inhibitory effects of WIN 55,212-2 on electrically evoked contractions in mouse isolated vas deferens preparations with pA_2 value of 12.46 ± 0.23. In vivo studies indicated that NESS 0327 antagonized the antinociceptive effect produced by WIN 55,212-2 (2 mg/kg s.c.) in both tail-flick (ID_{50} = 0.042 ± 0.01 mg/kg i.p.) and hot-plate test (ID_{50} = 0.018 ± 0.006 mg/kg i.p.). These results indicated that NESS 0327 is a novel cannabinoid antagonist with high selectivity for the cannabinoid CB_1 receptor.

Interest in the pharmacology of cannabinoids (CBs) has rapidly increased after the cloning of cannabinoid receptors and the discovery of their endogenous ligand: arachidonylethanolamide (anandamide) (Devane et al., 1988, 1992; Munro et al., 1993). Two types of cannabinoid receptors, CB_1 and CB_2, have been characterized, both of which have distinct anatomical distributions and ligand binding profiles. Cannabinoid CB_1 receptors are present in the central nervous system with the highest densities in the hippocampus, cerebellum, and striatum (Herkenham et al., 1990; Howlett, 1998), and to a lesser extent in several peripheral tissues. Cannabinoid CB_2 receptors seem to be predominantly located in peripheral tissues (Pertwee, 1997, 1999; Galiègue et al., 1995). Both receptors belong to the G protein-coupled family of receptors that negatively regulate adenylate cyclase and control the release of arachidonic acid (Howlett, 1995). Naturally occurring [Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^8-THC] and synthetic cannabinoid agonists (HU-210, CP 55,940, and WIN 55,212-2) produce a number of effects in mice (hypoactivity, catalepsy, hypothermia, and antinociception) that are collectively known as the tetrad of cannabinoid-induced behaviors (Abood and Martin, 1992; Compton et al., 1992, 1993). These behaviors are of a central origin and are thought to be mediated via the cannabinoid CB_1 receptor (Rinaldi-Carmona et al., 1994; Compton et al., 1996; Lichtman and Martin, 1997), whereas the CB_2 receptor may mediate some of the peripheral effects of Δ^9-THC, such as immunosuppression (Martin, 1986).

The cloning of CB_1 and CB_2 receptors and the subsequent development of selective tools have advanced the concept of

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.103.049924.

ABBREVIATIONS: CB, cannabinoid; THC, tetrahydrocannabinol; WIN 55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrolol[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate; NESS 0327, *N*-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo [6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide]; SR 141716A, *N*-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate; DMSO, dimethyl sulfoxide; EtOH, ethanol; mp, melting point; BSA, bovine serum albumin; ANOVA, analysis of variance; %MPE, percent maximal possible effect; Hu-210, *R*(−)-7-hydroxy-Δ-tetrahydrocannabinol-dimethylheptyl; CP 55,960, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-3-hydroxy-propyl)cyclohexan-1-ol; IR, infrared spectroscopy.

therapeutically targeting cannabinoid receptors. Besides their established clinical antiemetic action (Voth and Schwartz, 1997; Gralla, 1999), cannabinoid receptor agonists also possess appetite stimulant, anticonvulsant, antinociceptive, hypothermic, and antiglaucoma properties (Formukong et al., 1989; Mattes et al., 1994; Pertwee, 1999; Porcella et al., 2001).

Recently, several groups have become interested in the development of cannabinoid antagonists, hoping to develop new drugs to cure diseases connected with possible malfunctions of "cannabinoid/anandamide" system.

We report the synthesis of a putative cannabinoid ligand, code named NESS 0327, its differential binding to CB_1 and CB_2 cannabinoid receptors, its ability to stimulate [^{35}S]GTPγS binding in rat brain, its effect on mouse vas deferens, and its action on an in vivo assay known to be affected by cannabinoids.

Materials and Methods

(*Z*,*E*)-5-(3-Chlorophenyl)-pent-4-enoic Acid (3). A solution of (3-carboxypropyl) triphenylphosphonium bromide (2) (14 g, 32.61 mM) in anhydrous dimethyl sulfoxide (DMSO) (40 ml), with 2.6 M of the sodium salt of DMSO in anhydrous DMSO (24 ml, 62.25 mM), below 10°C, was added to a solution of 3-chlorobenzaldehyde (3.06 g, 21.74 mM) in anhydrous tetrahydrofuran (8 ml). The resulting solution was heated at 50°C for 18 h; subsequently, it was allowed to return to room temperature and poured into water. The mixture was acidified with 6 N hydrochloric acid and extracted with ethyl acetate (3 × 25 ml). The combined extracts were washed with brine, water, and then dried over anhydrous sodium sulfate, to provide a browning compound after evaporation. The crude compound was purified by flash column chromatography on silica gel eluting with dichloromethane/acetone (9:1) to afford the desired diastereomeric mixture 3 (42% yield); Rf 0.51 (dichloromethane/acetone, 9:1); IR (nujol): 3200 to 2500 (OH), 1720 (C=O), 1590 (Ar); ^{1}H NMR: 2.40 to 2.75 (m, 8H), 5.60 to 5.75 (m, 2H), 6.15 to 6.45 (m, 2H), 7.10 to 7.28 (m, 6H), 7.32 (s, 2H), 9.50 (br s, 2H, exch. with D_2O). Anal. $C_{11}H_{11}ClO_2$ (C, H, Cl).

5-(3-Chlorophenyl)-pentanoic Acid (4). A suspension of the diastereomeric mixture of pentenoic acid derivate 3 (1 g, 4.75 mM) was subjected to catalytic hydrogenation over PtO_2 [Adams' catalyst, 0.1 g, 10% (w/w)] in ethanol (EtOH) (50 ml) for 2.5 h at room temperature and 45 psi of hydrogen pressure. The mixture was filtered through a paper filter and the filtrate concentrated under reduced pressure to yield the desired acid 4 (100% yield) as a yellow solid, mp 56–58°C. Rf 0.84 (petroleum ether/ethyl acetate, 1:1); IR (nujol): 3300 (OH), 1710 (C=O), 1600 (Ar); ^{1}H NMR: 1.60 to 1.80 (m, 4H), 2.30 to 2.45 (m, 2H), 2.55 to 2.71 (m, 2H), 7.04 (d, 1H, J = 6.4 Hz), 7.12 to 7.35 (m, 3H), 9.65 (br s, 1H, exch. with D_2O). Anal. $C_{11}H_{13}ClO_2$ (C, H, Cl).

2-Chloro-6,7,8,9-tetrahydro-benzocyclohepten-5-one (5). A suspension of pentanoic acid 4 (0.5 g, 2.36 mM) and thionyl chloride (0.63 ml, 8.5 mM) was heated for 30 min at 50°C. Thionyl chloride in excess was subsequently removed under reduced pressure and the residue was added for three times to dichloromethane (3 ml), which was evaporated under reduced pressure. A solution of the crude acyl chloride in dichloromethane (3 ml) was added drop wise to a magnetically stirred suspension of $AlCl_3$ (0.32 g, 2.36 mM) in dichloromethane (3 ml). The resulting mixture was stirred at room temperature overnight then poured into ice and the whole extracted with dichloromethane (3 × 5 ml). The combined extracts were washed with (5%) aqueous sodium bicarbonate solution, water, and after drying over anhydrous sodium sulfate, filtered and evaporated to provide a brownish compound. The crude compound was purified by flash chromatography on silica gel eluting with petroleum ether/ethyl acetate (9:1) to afford the attempt compound 5 (77% yield) as a yellow orange oil, boiling point 94–97°C/0.05 mm Hg (lit[1] 128–131/0.35 mm Hg); Rf 0.65 (petroleum ether/ethyl acetate, 9:1); IR (film): 3350 (OH), 1680 (C=O), 1590 (Ar); ^{1}H NMR: 1.72 to 1.98 (m, 4H), 2.73 (t, 2H, J = 6.2 Hz), 2.91 (t, 2H, J = 6.0 Hz), 7.21 (s, 1H), 7.28 (d, 1H, J = 8.8 Hz), 7.68 (d, 1H, J = 8.6 Hz). Anal. $C_{11}H_{11}ClO$ (C, H, Cl).

(2-Chloro-5-oxo-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-6-yl)-oxo-acetic Acid Ethyl Ester (6). A mixture of EtONa (7.5 mM) in absolute EtOH (3.5 ml) and diethyl oxalate (0.51 ml, 3.75 mM) was stirred for 30 min at room temperature, and a solution of compound 5 (0.73 g, 3.75 mM) in absolute ethanol (27 ml) was added over 30 min. The resulting mixture was reacted at room temperature for 9 h and then poured onto crushed ice and the whole acidified with 2 N hydrochloric acid and extracted with chloroform (3 × 15 ml). The combined extracts were washed with water, dried over anhydrous sodium sulfate, filtered, and evaporated to afford the β-dichetoester 6 as an orange oil, which was used in the next step without further purification (84% yield); boiling point 95–98°C/0.05 mm Hg; Rf 0.78 (petroleum ether/ethyl acetate, 9:1); IR (film): 3440 (OH), 1730 (C=O), 1680 (C=O), 1600 (Ar); ^{1}H NMR: 1.41 (t, 3H, J = 7 Hz), 2.08 (quint, 2H), 2.32 (t, 2H, J = 7.2Hz), 2.72 (t, 2H, J = 7 Hz), 3.88 (q, 2H, J = 7 Hz), 7.23 (d, 1H, J = 1.8 Hz), 7.34 (dd, 1H), 7.58 (d, 1H, J = 8.2 Hz), 15.37 (br s, 1H, exch. with D_2O). Anal. $C_{15}H_{15}ClO_4$ (C, H, Cl).

8-Chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxylic Acid Ethyl Ester (8). 2,4-Dichlorophenylhydrazine hydrochloride (7) (0.72 g, 3.38 mM) was added to a magnetically stirred solution of ester 6 (0.9 g, 3.05 mM) in EtOH (21 ml) and the resulting mixture heated under reflux for 3 h; subsequently the solvent was removed under reduced pressure to yield the crude ester. Purification by flash chromatography on silica gel eluting with petroleum ether/ethyl acetate, 8.5:1.5, gave the attempt compound 8 as a yellow solid (58% yield); mp 160–161°C (crumbled with petroleum ether); Rf 0.47 (petroleum ether/ethyl acetate, 9:1); IR (nujol): 1725 (C=O), 1605 (Ar); ^{1}H NMR: 1.43 (t, 3H, J = 7 Hz), 2.13 to 2.40 (m, 2H), 2.67 (t, 2H, J = 6.4 Hz), 3.09 to 3.40 (m, 2H), 4.46 (q, 2H, J = 7 Hz), 6.60 (d, 1H, J = 8.2 Hz), 7.02 (dd, 1H), 7.31 (d, 1H, J = 2.2 Hz), 7.36–7.49 (m, 2H), 7.54 (d, 1H, J = 9.2 Hz). Anal. $C_{21}H_{17}Cl_3N_2O_2$ (C, H, Cl, N).

8-Chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxylic Acid (9). A solution of potassium hydroxide (0.17 g, 2.94 mM) in methanol (5 ml) was added to a magnetically stirred solution of ester 8 (0.64 g, 1.47 mM) in methanol (7 ml), the mixture was refluxed for 9 h and the cooling reaction mixture poured onto crushed ice and acidified with 1 M hydrochloric acid. The precipitate was filtered, washed with water, and dried under vacuum to yield the corresponding acid as a white solid (97% yield); mp 270°C (EtOH); Rf 0.51 (chloroform/methanol, 9:1); IR (nujol): 3410 (OH), 1690 (C=O); ^{1}H NMR: 2.20 to 2.39 (m, 2H), 2.50 to 3.35 (m, 4H), 6.61 (d, 1H, J = 8.2 Hz), 7.03 (dd, 1H), 7.32 (d, 1H, J = 1.8 Hz), 7.39 to 7.49 (m, 2H), 7.53 (d, 1H, J = 8.2 Hz), 13.25 (br s, 1H, exch. with D_2O). Anal. $C_{19}H_{13}Cl_3N_2O_2$ (C, H, Cl, N).

NESS 0327. A solution of the acid 9 (0.50 g, 1.23 mM) and thionyl chloride (0.24 ml, 3.69 mM) in toluene (10 ml) was refluxed for 3 h. Solvent was evaporated under reduced pressure and the residue redissolved in toluene (3 × 5 ml) and evaporated to yield the crude carboxylic chloride. A solution of the above-mentioned carboxylic chloride in dichloromethane (6 ml) was added dropwise to a solution of 1-aminopiperidine (10) (0.19 ml, 1.65 mM) and triethylamine (0.23 ml, 1.65 mM) in dichloromethane (6.2 ml). After stirring at room temperature for 1 h, the reaction mixture was added with brine and extracted with dichloromethane (3 × 15 ml). The combined extracts were washed with brine, dried over anhydrous sodium sulfate, filtered, and evaporated to give a yellowish compound. The crude compound was purified by flash chromatography on silica gel eluting with petroleum ether/ethyl acetate, 1:1, to afford the desired carboxamide NESS 0327 as a white solid (93% yield); mp 205–206°C (acetone), (lit[2] 202°C); Rf 0.68 (petroleum ether/ethyl acetate, 1:1); IR (nujol): 3200 (NH), 1650 (C=O), 1600 (Ar); ^{1}H NMR: 1.35 to 1.53 (m, 2H), 1.58 to 1.89 (m, 6H), 2.15 to 2.36 (m, 2H), 2.66 (t, 2H, J = 6.4

Hz), 2.87 (t, 4H, J = 5.0 Hz), 6.56 (d, 1H, J = 8.2 Hz), 7.01 (dd, 1H), 7.31 (d, 1H, J = 1.8 Hz), 7.37 to 7.54 (m, 3H), 7.66 (br s, 1H, exch. with D_2O). Anal. $C_{24}H_{23}Cl_3N_4O$ (C, H, Cl, N).

Radioligand Binding Methods. Male CD1 mice weighing 20 to 25 g (Charles River, Calco, Italy) were housed in the animal care quarters; temperature was maintained at 22 ± 2°C on a 12-h light/dark cycle and food and water were available ad libitum. All experimental protocols were authorized by the Ethical Committee at the University of Cagliari and performed in strict accordance with the EC regulations for care and use of experimental animals (EEC no. 86/609).

Mice were killed by cervical dislocation, and brains (minus cerebellum) and spleens were rapidly removed and placed on an ice-cold plate. After thawing, tissues were homogenated in 20 volumes (w/v) of ice-cold TME buffer (50 mM Tris-HCl, 1 mM EDTA, and 3.0 mM $MgCl_2$, pH 7.4). The homogenates were centrifuged at 1,086g for 10 min at 4°C, and the resulting supernatants were centrifuged at 45,000g for 30 min at 4°C.

[^{3}H]CP 55,940 binding was performed by a modification of the method described previously (Rinaldi-Carmona et al., 1994). Briefly, the membranes (30–80 μg of protein) were incubated with 0.5 nM [^{3}H]CP 55,940 for 1 h at 30°C in a final volume of 0.5 ml of TME buffer containing 5 mg/ml fatty acid-free bovine serum albumin (BSA). Nonspecific binding was estimated in the presence of 1 μM CP 55,940. All binding studies were performed in disposable glass tubes pretreated with Sigma-Cote (Sigma Chemical, Poole, Dorset, UK), to reduce nonspecific binding. The reaction was terminated by rapid filtration through GF/C filters (Whatman, Maidstone, UK) presoaked in 0.5% polyethyleneimine using a 96-sample harvester (Brandel, Inc., Gaithersburg, MD). Filters were washed five times with 4-ml aliquots of ice-cold Tris HCl buffer (pH 7.4) containing 1 mg/ml BSA The filter bound radioactivity was measured in a liquid scintillation counter (Tricarb 2900; PerkinElmer Life Sciences, Boston, MA) with 4 ml of scintillation fluid (Ultima Gold MV; PerkinElmer Life Sciences). Protein determination was performed by means of Bradford (1976) protein assay using BSA as a standard, according to the protocol of the supplier (Bio-Rad, Milan, Italy). Drugs were dissolved in DMSO. To avoid possible undesired effects on radioligand binding, DMSO concentration in the different assays never exceeded 0.1% (v/v). All experiments were performed in triplicate, and results were confirmed in at least four independent experiments. Data from radioligand inhibition experiments were analyzed by nonlinear regression analysis of a Sigmoid Curve using GraphPad Prism program (Graph Pad Software, Inc., San Diego, CA). IC_{50} values were derived from the calculated curves and converted to K_i values as described previously (Cheng and Prusoff, 1973).

Mouse Vas Deferens Experiments. Vasa deferentia were obtained from albino CD1 mice weighing 25 to 40 g. Tissue was mounted in a 10-ml organ bath at an initial tension of 0.5 g using the method described by Pertwee et al. (1993). The bath contained Krebs-Henseleit solution (118.2 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 11.0 mM glucose, and 2.54 mM $CaCl_2$), which was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. Isometric contractions were evoked by stimulation with 0.5-s trains of three pulses of 110% maximal voltage (train frequency, 0.1 Hz; pulse duration, 0.5 ms) through platinum electrodes attached to the upper end of each bath and a stainless steel electrode attached to the lower end. Stimuli were generated by Grass S88K stimulator then amplified (multiplexing pulse booster 316S; Ugo Basile, Comerio, Italy) and divided to yield separate outputs to four organ baths. Contractions were monitored by computer using a data recording and analysis system (PowerLab 400) linked via preamplifiers (QuadBridge) to an F10 transducer (Biological Instruments, Besozzo, Italy).

Each tissue was subject to several periods of stimulation. The first of these began after the tissue had equilibrated in the buffering medium but before drug administration, and continued for 10 min. The stimulator was then switched off for 15 min, after which the tissues were subjected to further periods of stimulation each lasting 5 min and separated by a stimulation-free period. The drugs were added once the contractile responses to electrical stimulation were reproducible. Preparations were exposed to cumulative increasing concentrations of WIN 55,212-2 to obtain concentration-response curves either in the absence (control) or in the presence of NESS 0327 (1, 10, or 100 pM) added at a fixed concentration 20 min before the first concentration of WIN 55,212-2. It was not possible to reverse the inhibitory effect of cannabinoid on the twitch response by washing them out of the organ bath. Consequently, only one concentration-response curve was constructed per tissue. DMSO was added instead of the drug. The control dose of DMSO was the same as the dose added in combination with the highest dose of drug used. DMSO alone did not inhibit the twitch response (n = 6) at the maximum concentration used in the bath (4 μl/ml).

Drug additions were performed in volumes of 10 μl. The effects of the antagonists or agonists were calculated as percentage of decrease in the predrug twitch force. Inhibition of the electrically evoked twitch response is expressed in percentage terms and has been calculated by comparing the amplitude of the twitch response after each addition of an agonist with the amplitude immediately before the first addition of the agonist. The pA_2 values for competitive antagonists were calculated by Schild regression analysis (Arunlakshana and Schild, 1959). Data were plotted as log antagonist concentrations (molar) versus log (concentration-ratios, −1). It is assumed that when the slope value of the regression line in the Schild plot does not differ statistically from unity, the pA_2 value represents the dissociation constant of the antagonist (pK_B). In each estimate, eight isolated tissue preparations were used. Statistical significance was determined by use of Student's test and $P < 0.05$ was considered significant.

[^{35}S]GTPγS Binding Assay. Male Sprague-Dawley rats (Charles River), weighing 200 to 250 g, were used in all experiments. Rats were killed by decapitation, their brains rapidly removed, and cerebella dissected on ice. Cerebella tissue was suspended in 20 volumes of cold centrifugation buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4) and homogenated using a homogenizer system (Glas-Col, Terre Haute, IN). The homogenate was centrifuged at 48,000g for 10 min at 4°C. The pellet was then resuspended in the same buffer, homogenized, and centrifuged as described previously. The final P2 pellet was subsequently resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4), homogenized, and diluted to a concentration of ~2 μg/μl with assay buffer. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard according to the protocol of the supplier (Bio-Rad). Membrane aliquots were then stored at −80°C until use.

[^{35}S]GTPγS binding was measured as described by Selley et al. (1996). Briefly, rat cerebella membranes (15 μg of protein) were incubated with drugs for 60 min at 30°C in assay buffer containing 0.1% fatty acid-free bovine serum albumin in the presence of 0.05 nM [^{35}S]GTPγS and 30 μM GDP, in a final volume of 1 ml. The reaction was terminated by rapid filtration using a Unifilter-GF/B (PerkinElmer Life Sciences), washed two times with 1 ml of ice-cold 50 mM Tris-HCl, pH 7.4, buffer, and dried 1 h at 30°C. The radioactivity on the filters was counted in a liquid microplate scintillation counter (TopCount NXT' PerkinElmer Life Sciences) using 50 μl of scintillation fluid (Microscint 20; PerkinElmer Life Sciences).

Stock solution of WIN 55,212-2 and NESS 0327 were prepared in DMSO and then diluted in assay buffer. The final concentration of DMSO was <0.01%, which had no effect either on basal or stimulated [^{35}S]GTPγ binding. WIN 55,212-2 concentration effect curves were determined by incubating membranes with various concentrations of WIN 55,212-2 (10–10,000 nM) in the presence of 0.05 nM [^{35}S]GTPγS and 30 μM GDP.

Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. Basal binding was assayed in the absence of agonist and in the presence of GDP. The stimulation by agonist was defined

as a percentage increase above basal levels (i.e., {[dpm(agonist) − dpm (no agonist)]/dpm (no agonist)} × 100).

Data are reported as mean ± S.E.M. of three to six experiments, performed in triplicate. Nonlinear regression analysis of concentration-response data was performed using Prism 2.0 software (GraphPad Prism program) to calculate E_{max} and EC_{50} values.

The resulting ED_{50} values were used to determine K_e values for antagonism of the agonist-stimulated response by antagonist, using the relationship K_e = [Ant]/(Dr − 1), where [Ant] is the concentration of antagonist, and DR is the ratio of ED_{50} values in the presence and absence of antagonist (Sim et al., 1995). Statistical analyses were carried out using one-way ANOVA followed by Newman-Keuls post hoc test.

Determination of Mouse Antinociception. Male CD1 mice, weighing 20 to 25 g (Charles River), were used to assess antinociception by means of the tail-flick and hot-plate test. A tail-flick meter (Ugo Basile) equipped with an irradiant heat source that focused 2.5 cm of the distal tip of the tail was used. A 15-s cut-off time for heat exposure was used to avoid cutaneous damage and the intensity of the thermal source was adjusted to produce a 3- to 5-s latency in vehicle-treated rats.

The effect of the compounds on the reaction time of mice placed on the hot-plate (Ugo Basile) (55 ± 0.8°C) was assessed determining the time at which animals first displayed a nociceptive response (licking the front paws, fanning the hind paws, or jumping). To avoid skin damage, after 40 s (cut-off) the animal was removed from the hot-plate. In both tests, each animal was tested before drug administration to determine control latency and the animals were used only in the determination of one time point. Data were transformed to the %MPE by the following equation (Harris and Pierson, 1964): %MPE = [(test latency − control latency)/(cut-off − basal latency)] × 100; where the latencies were expressed in seconds and the cut-off varied depending on the test (tail-flick = 15 s; hot-plate = 40 s). To establish the dose-dependent curves, at least four drug doses were used on 10 mice per each dose and each animal group was used only in the determination of one time point. Mice were tested 30 min after WIN 55,212-2 (2 mg/kg s.c.) or vehicle and up to 120 min. NESS 0327 (0.01–1 mg/kg i.p.) or vehicle were given 20 min before WIN 55,212-2 administration. WIN 55,212-2 was dissolved (5 ml/kg) in an emulsion of ethanol/cremophor/saline (1:1:18); NESS 0327 was dissolved in two drops of Tween 80 diluted in distilled water to a volume of 5 ml/kg. Three independent experiments were carried out for ID_{50} ± S.E.M. determination. Statistical analyses were carried out using two-way ANOVA followed by Newman-Keuls post hoc test.

Materials. Unless otherwise stated, all materials were obtained from commercial suppliers and used without purification. Anhydrous solvents such as ethanol, tetrahydrofuran, and DMSO were obtained from Sigma-Aldrich (St. Louis, MO) in sure-seal bottles. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere. Flash column chromatography was carried out using Merck Silica gel 60 (230–400 mesh ASTM). Thin-layer chromatography was performed with Polygram SIL N-HR-/HV_{254} precoated plastic sheet (0.2 mm). ^{1}H NMR spectra were determined in $CDCl_3$ with super conducting FT-NMR using a XL-200 Varian apparatus at 200 MHz. Chemical shifts are reported in δ (ppm) relative to tetramethylsilane as the internal standard and coupling constants in Hertz. Significant ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double doublet; br s, broad singlet), number of protons, coupling constants (*J*) in Hertz. IR spectra were recorded as thin films or nujol mulls on NaCl plates with a PerkinElmer 781 IR spectrophotometer and are expressed in ν (per centimeter). Melting points were determined on a Köfler melting point apparatus and are uncorrected. Compounds are indicated by the molecular formula followed by the symbols for the elements (C, H, N) and were found to be within ± 0.4% of their theoretical values. [^{3}H]CP 55,940 (180 Ci/mmol) and [^{35}S]GTPγS (1200–1350 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). CP 55,940 and WIN 55,212-2 were obtained from Tocris Cookson, Inc. (Bristol, UK). GDP and GTPγS were obtained from Sigma-Aldrich. SR 141716A and SR 144528 were kindly provided by Sanofi-Synthélabo (Bagneux, France).

Results

Chemistry. Target compound NESS 0327 was prepared as shown in Fig. 1. Acid 9, prepared via the ester 8 by saponification, was activated with thionyl chloride and, without isolation of the intermediate acyl chloride, reacted with a stoichiometric amount of *N*-amino-piperidine, in presence of triethylamine. Ester 8 was prepared starting from the aldehyde 1, which submitted to a Wittig condensation with the phosphonium bromide 2 yielded the pentenoic acid derivate 3. Reduction of the double bond of 3 to give 4 with H_2 over PtO_2 in EtOH (Adams' catalyst), followed by transformation into the corresponding acyl chloride with thionyl chloride, and cyclization, with $AlCl_3$ in dichloromethane, afforded the benzocycloheptanone 5. This benzocyclanone reacted with diethyl oxalate by means of NaOEt in EtOH to provide the α,γ-diketoester 6, which was allowed to react with 2,4-dichlorophenylhydrazine hydrochloride 7 to yield the educt 1*H*-pyrazole-3-carboxylic acid ethyl ester 8. (Fig. 1).

Biology. The affinity of NESS 0327 for the cannabinoid CB_1 receptor in mouse forebrain membranes was evaluated using competitive binding assay. As shown in Fig. 2A, the specific binding of [^{3}H]CP 55,940 to its high-affinity receptor in mouse brain synaptosomal membranes was totally displaced by NESS 0327 in a concentration dependent manner with K_i values of 350 ± 5 fM (n = 4). Both SR 141716A and SR 144528 compete for CB_1 receptor with K_i values of 1.8 ± 0.075 nM and 70 ± 7 nM (n = 4), respectively, in close agreement with published values (Rinaldi-Carmona et al., 1994, 1998). The affinities of NESS 0327, SR 141716A, and SR 144528 for CB_2 receptor were determined in mouse spleen (Fig. 2B). The concentration-response gave K_i values of 21 ± 0.5, 514 ± 30, and 0.28 ± 0.04 nM (n = 4) for NESS 0327, SR 141716A, and SR 144528, respectively. These results show that NESS 0327 is over 60,000-fold selective for the CB_1 receptor versus CB_2 receptor. NESS 0327 was screened for cannabinoid agonist activity using mouse vas deferens model. Cannabinoid agonists inhibit the electrically induced contractions of the mouse vas deferens via activation of inhibitory CB_1 receptors present on the sympathetic nerve terminals (Pertwee, 1997). As shown in Fig. 3, WIN 55,212-2 induced a concentration-dependent inhibition of the twitch contractions in the mouse isolated vas deferens preparations, with pD_2 values of 8.45 ± 0.05. NESS 0327, which alone had no effect up to 1 μM, produced a concentration-dependent rightward and almost parallel shift of the concentration response-curve for WIN 55,212-2, showing that it behaved as a competitive antagonist versus the synthetic cannabinoid agonist with pA_2 value of 12.46 ± 0.23 and with a slope in the Schild plot not significantly different from unity (1.03 ± 0.05, $P > 0.05$).

Efficacy of the compound at the CB_1 receptor was measured using ligand stimulation of [^{35}S]GTPγ binding to cerebellar membranes. [^{35}S]GTPγ binding was stimulated in a concentration-dependent and saturable manner by WIN 55,212-2 with ED_{50} and E_{max} values of 0.16 ± 0.01 μM and 286 ± 24% (stimulation above basal binding), respectively (Table 1).

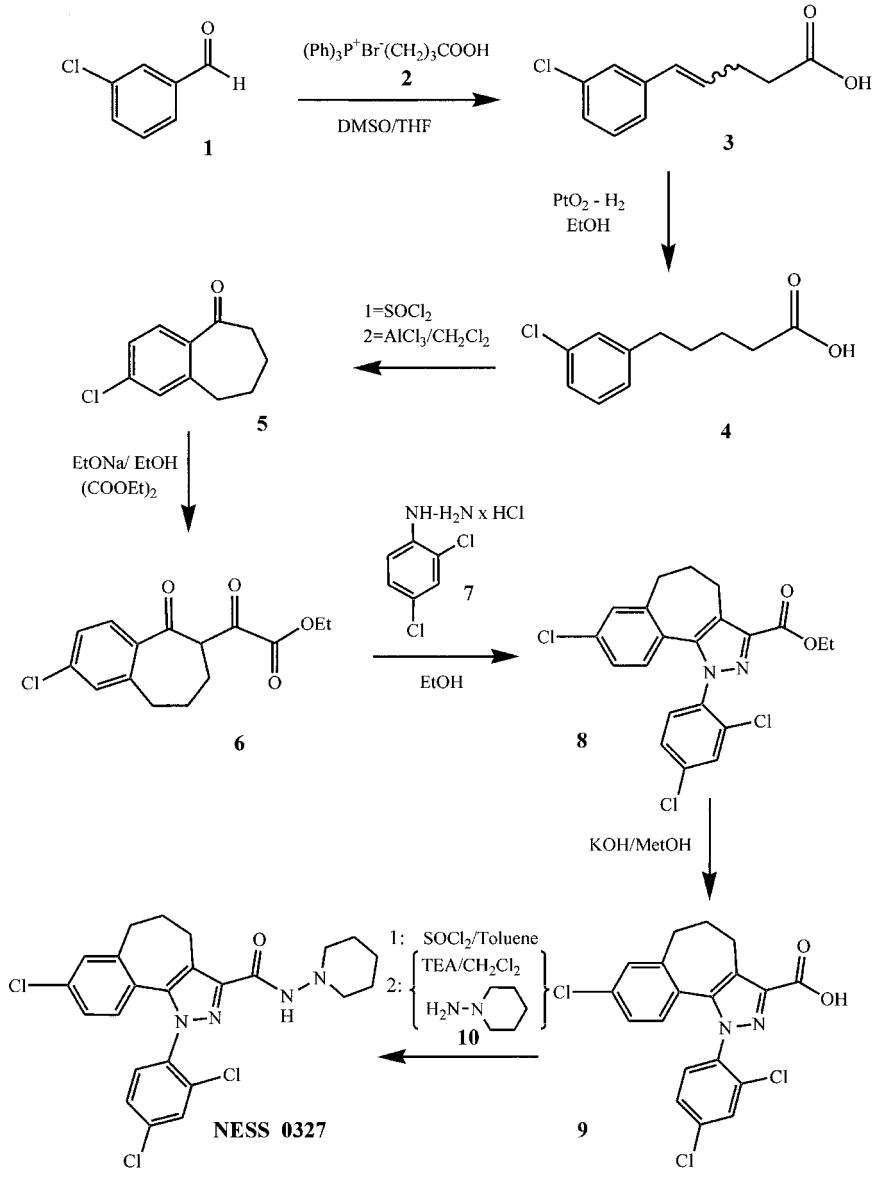

Fig. 1. Schematic synthesis of NESS 0327.

To determine the ability of NESS 0327 to antagonize CB_1 agonist-stimulated activation of G protein the effect of three concentrations of NESS 0327 (0.1, 1, and 10 nM) on the log concentration-response curve of WIN 55,212-2 was investigated. NESS 0327 produced concentration-dependent rightward shift of the WIN 55,212-2 concentration response-curve [one-way ANOVA: $F(3,14) = 43.35$, $P < 0.01$) without affecting the E_{max} of the agonist (Table 1). NESS 0327 at concentrations of 0.1, 1, and 10 nM shifted the dose-response curve for WIN 55,212-2 to the right with calculated K_e values of 80.3 ± 20, 283 ± 11, and 2016 ± 226 pM, respectively. NESS 0327, at concentrations from 0.1 through 1 μM, had no effect on [^{35}S]GTPγS binding, whereas, in the same conditions, SR 141716A at concentration of 1 μM produced an inhibition of 21 ± 2% of basal [^{35}S]GTPγS binding (data not shown). The lack of effect on basal [^{35}S]GTPγS binding suggests that NESS 0327 had no appreciable negative intrinsic activity in brain under the conditions used in this study.

The in vivo antagonism of NESS 0327 for the cannabinoid receptor was investigated in an animal model classically used to study cannabinoid drug effects. As shown in Fig. 4, A and B, NESS 0327 dose dependently reduced the analgesia induced by the cannabinoid agonist WIN 55,212-2 (2 mg/kg s.c.) on both tail-flick [two-way ANOVA: F_{dose} (6,189) = 10.26, $P < 0.01$; F_{time} (2,189) = 7.22, $P < 0.01$; $F_{interact}$ (12,189) = 3.7, $P < 0.01$] and hot-plate (two-way ANOVA: F_{dose} (6,189) = 42.37, $P < 0.01$; F_{time} (2,189) = 14.20, $P < 0.01$; $F_{interact}$ (12,189) = 5.4, $P < 0.01$]; a complete antagonism was reached at the dose of 0.1 mg/kg in the tail-flick test ($P > 0.05$ versus vehicle-treated rats) and of 0.05 mg/kg in the hot-plate test ($P > 0.05$ versus vehicle-treated rats). The ability of NESS 0327 to inhibit the antinociceptive effect induced by WIN 55,212-2 was maintained during the observation period. Thirty minutes after WIN 55,212-2 injection, NESS 0327 showed a ID_{50} = 0.042 ± 0.01 mg/kg i.p. in the tail-flick and ID_{50} = 0.018 ± 0.006 mg/kg i.p. in the hot-plate test. Furthermore, NESS 0327 did not show any antinociception activity per se (data not shown), suggesting that it is devoid of inverse agonist activity and it should be regarded as a pure antagonist.

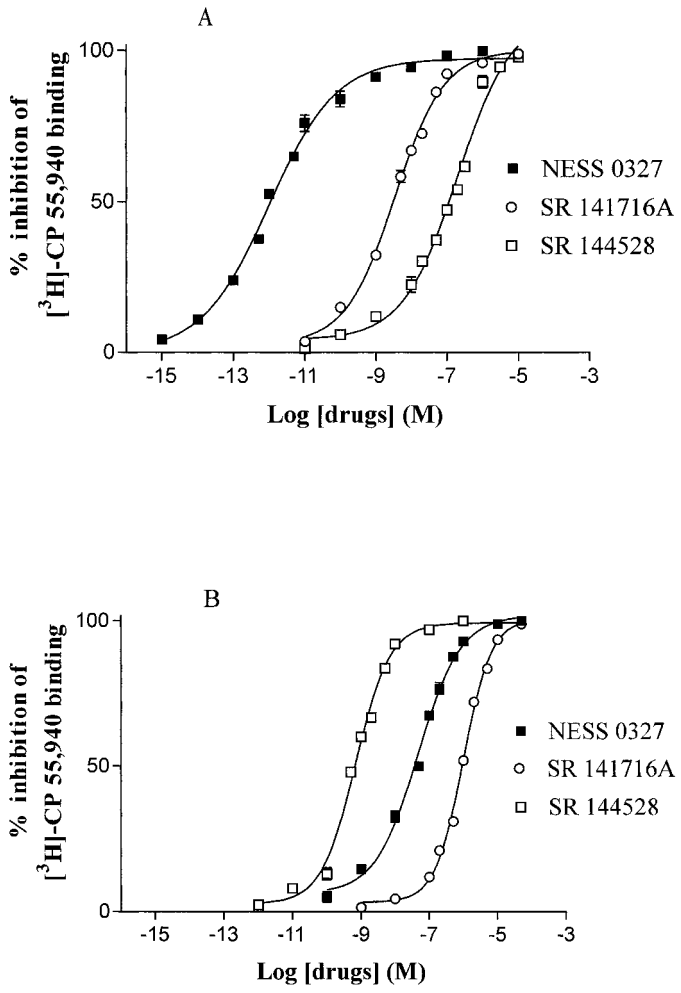

Fig. 2. Competitive inhibition of [^{3}H]CP 55,940 binding in mouse brain (A) and mouse spleen (B) by NESS 0327, SR 141716A, and SR 144528. Binding assays were carried out at 30°C using 0.5 nM [^{3}H]CP 55,940 and increasing concentrations of drugs. Data are mean ± S.E.M. of at least four different experiments, each performed in triplicate.

Discussion

Given the role of the endogenous cannabinoid system in different physiological responses and its involvement in numerous pathological processes, the search of new and selective agonists/antagonists of the CB_1 and CB_2 cannabinoid receptor will constitute an important line of research in the forthcoming years. In this respect, NESS 0327 showed a high selectivity for CB_1 versus CB_2 receptors and the in vitro functional assays (isolated organ and GTPγS) as well the in vivo antinociceptive studies indicated that the compound behaves as antagonist of the CB_1 receptor. However, because the relative binding affinity of NESS 0327 for the CB_1 receptor is about 5000 times more than that of SR 141716A, the in vivo experiment where the relative difference in activity is only 10 times might suggest a poor central bioavailability of NESS 0327.

NESS 0327 was selected as a lead compound from a series of potential cannabinoid receptor antagonists (data not shown) because it displayed the highest affinity for the CB_1 subtype of the cannabinoid receptor. Structure relationship inferential reasoning would suggest that a proper low-energy constrained conformation of the NESS 0327 semirigid tricyclic unit may relate to its potent and selective affinity for the CB_1 receptor with respect to the parent compound SR 141716A. On the basis of the remarkable result further synthesis of analogs derived from manipulation in the tricyclic 1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole backbone and variation of substitution on either N_1-aromatic ring and the aminopiperidine carboxamide region, may facilitate the elucidation of the cannabinoid pharmacophore for CB_1-selective antagonist.

Development of cannabinoid receptor selective antagonists will provide the tools necessary for a better understanding of the cannabinoid receptor functions both in the central nervous system and in the peripheral immune system. In this respect, considering the higher selectivity for the CB_1 receptor, NESS 0327 may prove to be more advantageous compared with the classical CB_1 receptor antagonist SR 141716A.

Current views of the interaction between CB_1/CB_2 receptors and signal transducting G proteins interaction are de-

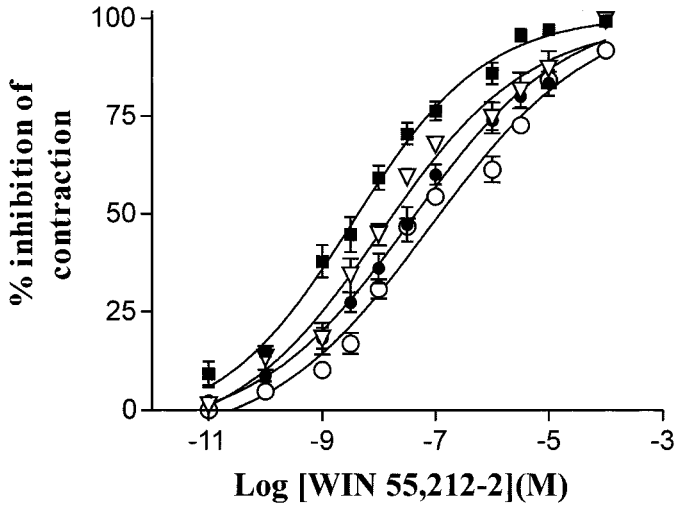

Fig. 3. Cumulative concentration-response curves for WIN 55,212-2 on the amplitude of twitch contractions elicited by electrical field stimulation of the mouse vas deferens obtained in the presence of its vehicle, DMSO (■) (control), and in the presence of NESS 0327 at 1 pM (▽), 10 pM (●), or 100 pM (○). Assays were performed as described under *Materials and Methods*. Each symbol represents the mean value ± S.E.M of inhibition of electrically evoked contractions of vasa deferentia expressed as a percentage of the amplitude of the twitch response measured before the first addition of WIN 55,212-2 to the organ bath (n = 6–8 different preparations). NESS 0327 was added 20 min before the first addition of WIN 55,212-2.

TABLE 1

ED_{50} and E_{max} values of WIN 55,212-2 in stimulating [^{35}S]GTPγS binding in the presence or absence of NESS 0327

[^{35}S]GTPγS binding was performed in rat cerebella membranes with 30 μM of GDP and 8 to 10 concentrations of WIN 55,212-2 in the absence and presence of 0.1, 1, and 10 nM NESS 0327. Data are expressed as percentage of basal [^{35}S]GTPγS binding. The values represent mean ± S.E.M. of four to six separate experiments, each performed in triplicate. ED_{50} and E_{max} values were calculated from nonlinear regression curve fitting using GraphPad Prism program. The statistical significance of differences between the groups was assessed by one-way ANOVA followed by the Newman-Keuls test (* $P < 0.05$ and ** $P < 0.001$ versus WIN 55,212-2).

Compounds	ED_{50}	E_{max}
	μM	*% basal binding*
WIN 55,212-2	0.16 ± 0.01	286 ± 24
WIN 55,212-2 + NESS 0327 (0.1 nM)	0.36 ± 0.07*	269 ± 42
WIN 55,212-2 + NESS 0327 (1 nM)	0.79 ± 0.13**	362 ± 42
WIN 55,212-2 + NESS 0327 (10 nM)	1.11 ± 0.03**	200 ± 21

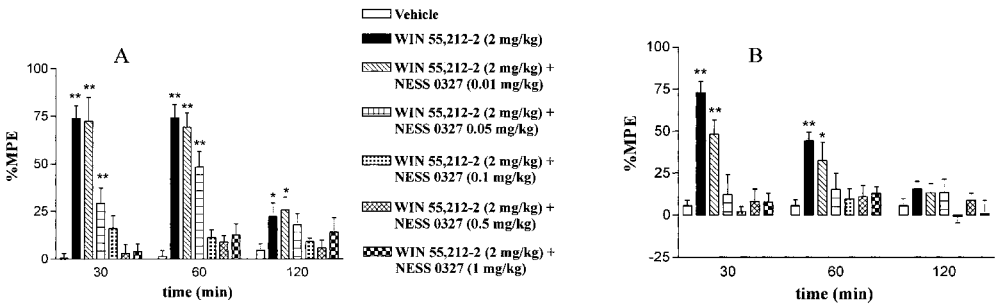

Fig. 4. Inhibition by NESS 0327 of WIN 55,212-2 induced antinociception in the tail-flick (A) and in the hot-plate test (B). Mice were tested after 30, 60, and 120 min after administration of WIN 55,212-2 (2 mg/kg s.c.) or vehicle. NESS 0327 (0.01–1 mg/kg) or vehicle was administered i.p. 20 min before WIN 55,212-2 injection. Each column represents the mean ± S.E.M of the %MPE obtained from ten animals. Statistical analysis was carried out using two-way ANOVA followed by Newman-Keuls post hoc test (*, $P < 0.05$ and **, $P < 0.01$).

scribed in the general framework of allosteric modulation, in which the receptor isomerizes between an active or inactive form (Samama et al., 1993; Nakamura-Palacios et al., 1999). Therefore, more detailed studies will be needed to address whether NESS 0327 may affect the distribution between the active or inactive states of the cannabinoid receptor (as for a neutral-competitive antagonist) or, on the contrary, may enhance the accumulation of the receptor in the inactive state (as for an inverse agonist). SR 141716A, for instance, has been shown to stimulate cAMP production, providing evidence for an inverse agonist effect (Mato et al., 2002). It has been further demonstrated that SR 141716A has a peculiar inverse-antagonist activity that is consistent with the stabilization of an inactive receptor/Gi protein complex. Accordingly, SR 141716A could cause a depletion of Gi and thus render the protein unavailable for the inhibitory action of other ligands (Bouaboula et al., 1997). The availability of new and selective ligands, such as NESS 0327, for the cannabinoid receptor CB_1 would allow a better conceptualization of the rather complex mode of cannabinoid receptor/ligand interaction because Δ^9-THC itself has been shown to be a weak but very selective antagonist for the cannabinoid receptor CB_2 (Bayewitch et al., 1996; Barth and Rinaldi-Carmona, 1999). Because recent data using SR 141716A seem to suggest a ligand-independent activity for cannabinoid receptor signaling (Mato et al., 2002), NESS 0327 could be used as a more selective antagonist for the CB_1 receptors, to study the recent proposed ability of the CB_1 receptor to sequester G proteins from a common pool and prevent other G protein-coupled receptors from signaling (Vasquez and Lewis, 1999).

The use of antagonists in studies investigating the biology of cannabinoid receptors may help to distinguish between receptor-dependent and receptor-independent effects elicited by cannabinoid agonists. A large arsenal of cannabinoid receptor antagonists will be instrumental in characterizing both the well known and eventually, newly discovered, cannabinoid receptor subtypes. The availability of a compound such as NESS 0327 displaying femtomolar affinity for the CB_1 receptor would consequently allow radioactive labeling of the latter, thus enabling the study of CB_1 cellular and tissue distribution in further detail. Stringent screening techniques might also be of use in the characterization of new cannabinoid receptors.

Additional in vivo experiments should provide further evidence for the clinical potential of this powerful CB_1 antagonist. It should be determined whether NESS 0327 would show better efficacy as a CB_1 antagonist in animal models of excessive food intake, psychosis, and cognitive impairment, three areas of possible interest for a novel CB_1-selective antagonist.

References

Abood ME and Martin BR (1992) Neurobiology of marijuana abuse. *Trends Pharmacol Sci* **13:**201–206.

Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. *Br J Pharmacol* **14:**48–58.

Barth F and Rinaldi-Carmona M (1999) The development of cannabinoid antagonists. *Curr Med Chem* **6:**745–755.

Bayewitch M, Rhee MH, Avidor-Reiss T, Breuer A, Mechoulam R, and Vogel Z (1996) (−)-Δ^9-Tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem* **271:**9902–9905.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, et al. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–22339.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

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

Compton DR, Aceto MD, Lowe J, and Martin BR (1996) In vivo characterization of a specific cannabinoid receptor antagonist (SR 141716A): inhibition of Δ^9-tetrahydrocannabinol-induced responses and apparent agonist activity. *J Pharmacol Exp Ther* **277:**586–594.

Compton DR, Jhonson MR, Melvin LS, and Martin BR (1992) 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 (Wash DC)* **258:**1946–1949.

Formukong EA, Evans AT, and Evans FJ (1989) The medicinal uses of cannabis and its constituents. *Psytother Res* **3:**219–231.

Galiègue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, and Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* **232:**54–61.

Gralla RJ (1999) Cannabinoids and the control of chemotherapy-induced nausea and vomiting, in *Marijuana and Medicine* (Nahas GG, Sutin KM, Harvey DJ, and Agurell S eds) pp 599–610, Humana Press, Totowa, NJ.

Harris LS and Pierson AK (1964) Some narcotic antagonists in the benzomorphan series. *J Pharmacol Exp Ther* **143:**141–148.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, and Rice KC (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* **87:**1932–1936.

Howlett AC (1995) Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* **35:**607–634.

Howlett AC (1998) The CB1 cannabinoid receptor in the brain. *Neurobiol Dis* **5:**405–416.

Lichtman AH and Martin BR (1997) The selective cannabinoid antagonist SR 141716A blocks cannabinoid-induced antininoception in rats. *Pharmacol Biochem Behav* **57:**7–12.

Martin BR (1986) Cellular effects of cannabinoids. *Pharmacol Rev* **38:**45–74.

Mato S, Pazos A, and Valdizan EM (2002) Cannabinoid receptor antagonism and inverse agonism in response to SR141716A on cAMP production in human and rat brain. *Eur J Pharmacol* **443:**43–46.

Mattes RD, Engelman K, Shaw LM, and Elsohly MA (1994) Cannabinoids and appetite stimulation. *Pharmacol Biochem Behav* **49:**187–195.

Munro S, Thomas KL, and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365:**61–65.

Nakamura-Palacios EM, Moerschbaecher JM, and Barker LA (1999) The pharmacology of SR 141716A: a review. *CNS Drug Rev* **5:**43–58.

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74:**129–180.

Pertwee RG (1999) Cannabis and cannabinoids: pharmacology and rationale for clinical use. *Fortsch Komplementarmed* **3:**12–15.
Pertwee RG, Stevenson LA, and Griffin G (1993) Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide. *Br J Pharmacol* **110:**1483–1490.
Porcella A, Maxia C, Gessa GL, and Pani L (2001) The synthetic cannabinoid WIN 55,212-2 decreases the intraocular pressure in human glaucoma resistant to conventional therapies. *Eur J Neurosci* **13:**409–412.
Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Neliat G, Caput D, et al. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350:**240–244.
Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, et al. (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284:**644–650.
Samama P, Cotecchia S, Costa T, and Lefkowitz RJ (1993) A mutation-induced activated state of the β2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268:**4625–4636.
Selley DE, Stark S, Sim LS, and Childers SR (1996) Cannabinoid receptor stimulation of guanosine-5′-*O*-(3-[^{35}S]thio) triphosphate binding in rat brain membranes. *Life Sci* **59:**659–668.
Sim LJ, Selley DE, and Childers SR (1995) In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S]thio]triphospate binding. *Proc Natl Acad Sci USA* **92:**7242–7246.
Vasquez C and Lewis DL (1999) The CB1 cannabinoid receptor can sequester G-proteins, making them unavailable to couple to other receptors. *J Neurosci* **19:**9271–9280.
Voth EA and Schwartz RH (1997) Medicinal applications of δ-9-tetrahydrocannabinol and marijuana. *Ann Intern Med* **126:**791–798.

Address correspondence to: Dr. Luca Pani, Institute of Neurogenetic and Neuropharmacology, Via Boccaccio 8, 09047 Selargius, Italy. E-mail: l.pani@inn.cnr.it