Synthesis and Characterization of NESS 0327: A Novel Putative Antagonist of the CB₁ Cannabinoid Receptor

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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 CB₁ and CB₂ receptor. NESS 0327 exhibited a stronger selectivity for CB₁ receptor compared with N-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR 141716A), showing a much higher affinity for CB₁ receptor (Kᵢ = 350 ± 5 nM and 1.8 ± 0.075 nM, respectively) and a higher affinity for the CB₂ receptor (Kᵢ = 21 ± 0.5 nM and 514 ± 30 nM, respectively). Affinity ratios demonstrated that NESS 0327 was more than 6,000-fold selective for the CB₁ receptor, whereas SR 141716A only 285-fold. NESS 0327 alone did not produce concentration-dependent stimulation of guanosine 5’-O-(3-[35S]thio)-triphosphate ([35S]GTP·S) binding in rat cerebella membranes. Conversely, NESS 0327 antagonized [3H]-2,3-dihydro-5-methyl-3-[morpholinyl]methylpyrrolo[1,2,3-de]-1,4-benzoxazin-yl-(1-naphthalenyl)methanone mesylate (WIN 55,212-2)-stimulated [35S]GTP·S 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 pA₂ value of 12.46 ± 0.23. In vivo studies indicated that NESS 0327 antagonized the antinoceptive effect produced by WIN 55,212-2 (2 mg/kg s.c.) in both tail-flick (ID₅₀ = 0.042 ± 0.01 mg/kg i.p.) and hot-plate test (ID₅₀ = 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 CB₁ receptor.

Interest in the pharmacology of cannabinoids (CBs) has rapidly increased after the cloning of cannabinoid receptors and the discovery of their endogenous ligand: arachidonylthanolamide (anandamide) (Devane et al., 1988, 1992; Munro et al., 1993). Two types of cannabinoid receptors, CB₁ and CB₂, have been characterized, both of which have distinct anatomical distributions and ligand binding profiles. Cannabinoid CB₁ 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 CB₂ 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 (Δ⁶-tetrahydrocannabinol (Δ⁶-THC) and Δ⁹-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 CB₁ receptor (Rinaldi-Carmona et al., 1994; Compton et al., 1996; Lichtman and Martin, 1997), whereas the CB₂ receptor may mediate some of the peripheral effects of Δ⁹-THC, such as immunosuppression (Martin, 1986). The cloning of CB₁ and CB₂ receptors and the subsequent development of selective tools have advanced the concept of

ABBREVIATIONS: CB, cannabinoid; THC, tetrahydrocannabinol; WIN 55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[morpholinyl]methylpyrrolo[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-1H-pyrazole-3-carboxamide; [35S]GTP·S, guanosine 5’-O-(3-[35S]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-Δ⁹-tetrahydrocannabinol-dimethylheptyl; CP 55,960, (1R,3R,4R)-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 (Voith 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 malfunction of "cannabinoid/anandamide" system.

We report the synthesis of a putative cannabinoid ligand, code named NESS 0327, its differential binding to CB₁ and CB₂ cannabinoid receptors, its ability to stimulate [35S]GTPγS binding in rat brain, its effect on mouse vas deferens, and its action in 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 brownish compound. The crude compound was purified by flash chromatography on silica gel eluting with petroleum ether/dichloromethane (9:1) to afford the desired diastereomeric mixture of 3 (42% yield); Rf 0.51 (dichloromethane/acetone, 9:1); IR (film): 3200 (OH), 1730 (C=O), 1605 (Ar); 1H NMR: 1.43 (t, 3H, J = 7 Hz), 2.13 to 2.40 (m, 2H), 2.72 (t, 2H, J = 7 Hz), 2.73 (t, 2H, J = 6.2 Hz), 7.23 (d, 1H, J = 1.8 Hz), 7.34 (dd, 1H, J = 8.2 Hz), 7.53 (s, 1H, exch. with D2O). Anal. C15H15ClO4 (C, H, Cl).

8-Chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzoxepin-6-one (5). A suspension of pentanoic acid 4 (0.5 g, 2.36 mM) and thionyl chloride in dichloromethane (6 ml) was added dropwise to a solution of the crude acyl chloride 3 (0.9 g, 3.05 mM) in dichloromethane (6.2 ml). After stirring at room temperature for 1 h, the reaction mixture was added with brine and then evaporated under reduced pressure. A solution of the crude acyl chloride 3 (0.5 g, 2.36 mM) 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 ml). After stirring at room temperature for 1 h, the reaction mixture was added with brine and 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 128–131/0.35 mm Hg); Rf 0.65 (petroleum ether/ethyl acetate, 9:1); IR (film): 3350 (OH), 1680 (C=O), 1590 (Ar); 1H NMR: 1.72 to 1.98 (m, 4H), 2.73 (t, 2H, J = 6.2 Hz), 2.91 (2H, J = 6.0 Hz), 3.12 (1H), 7.28 (d, 1H, J = 8.8 Hz), 7.68 (d, 1H, J = 8.6 Hz). Anal. C17H16ClO2 (C, H, Cl).

(2-Chloro-5-oxo-6,7,8-trihydroxy-5H-benzo[6,7]cyclohepten-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-dicetone 6 as an orange oil, which was used in the next step without further purification (84% yield); boiling point 161°C (crumbled with petroleum ether); Rf 0.47 (petroleum ether/ethyl acetate, 9:1); IR (nujol): 1725 (C=O), 1600 (Ar); 1H 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. C17H16ClO4 (C, H, Cl, N).

C15H15ClO4 (C, H, Cl).

8-Chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzoxepin-6-one (7). A suspension of potassium hydroxide (0.17 g, 2.94 mM) in methanol (5 ml) 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 solution 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 (84% yield); boiling point 161°C.

C17H16ClO4 (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-aminooxycarbonyl-1H-1,2,4-triazole (10) (0.19 ml, 1.65 mM) and triethylamine (0.23 ml, 1.65 mM) in dichloromethane (6 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 (97% yield); mp 270°C (EtOH); Rf 0.51 (chloroform/methanol, 9:1); IR (nujol): 3410 (OH), 1690 (C=O); 1H 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 D2O). Anal. C17H16ClO4 (C, H, Cl, N).

NESS 0327, its differential binding to CB₁ and CB₂ cannabinoid receptors, its ability to stimulate [35S]GTPγS binding in rat brain, its effect on mouse vas deferens, and its action in an in vivo assay known to be affected by cannabinoids.
Nicotine use and the risk of sudden death: A systematic review and meta-analysis of prospective studies

Nicotine is a highly addictive drug that is widely used for smoking, and its effects on the cardiovascular system are well-known. However, the role of nicotine in the risk of sudden death is not well understood. In this study, we conducted a systematic review and meta-analysis of prospective studies to determine the relationship between nicotine use and the risk of sudden death.

We included 14 prospective studies that were published between 1978 and 2017. The studies were reported in 12 languages, and the majority were published in English. The studies included a total of 1,517,807 participants, of whom 7,617 died suddenly during follow-up.

The results of our meta-analysis showed that nicotine use was associated with an increased risk of sudden death. The pooled relative risk was 1.64 (95% CI: 1.34-1.99) for current smokers compared to non-smokers. The risk was even higher for ex-smokers, with a pooled relative risk of 2.20 (95% CI: 1.55-3.11).

We also found that the risk of sudden death was higher in younger smokers and those with a history of myocardial infarction. However, the risk was lower in those with a history of hypertension or diabetes.

In conclusion, our study provides evidence that nicotine use is associated with an increased risk of sudden death. These findings highlight the need for further research to better understand the mechanisms underlying this relationship and to develop effective interventions to reduce the risk of sudden death in smokers.
as a percentage increase above basal levels (i.e., [(dpm(agonist) - dpm (no agonist))/dpm (no agonist)] × 100).

Data are reported as means ± 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 \( E_{\text{max}} \) and EC\(_{50} \) values.

The resulting \( E_{\text{50}} \) values were used to determine \( K_e \) values for antagonism of the agonist-stimulated response by antagonist, using the relationship \( K_e = \frac{[\text{Ant}]}{[\text{DR} - 1]} \), where \([\text{Ant}]\) is the concentration of antagonist, and \([\text{DR}]\) is the ratio of \( E_{\text{50}} \) 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 infrared 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 Pirenne, 1964): %MPE = [(test latency – control latency)/control 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 \( E_{\text{50}} \) 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-HRP/H\(_\text{V}_{60} \) precoated plastic sheet (0.2 mm). 1H 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 \( \delta \) (ppm) relative to tetramethylsilane as the internal standard and coupling constants in Hertz. Significant 1H 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 \( \nu \) (per centimeter). Melting points were determined on a Kofler 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]GTP\(_\gamma\)S (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 GTP\(_\gamma\)S 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 derivative 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 benzylocycloheptanone 5. This benzocyclanone reacted with diethyl oxalate by means of NaOEt in EtOH to provide the \( \gamma, \gamma\)-diketoester 6, which was allowed to react with 2,4-dichlorophenylhydrazine hydrochloride 7 to yield the educt 1H-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 \( K_e \) values of 350 ± 5 FM (\( n = 4 \)). Both SR 141716A and SR 144528 compete for CB\(_1\) receptor with \( K_e \) 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\(_1\) receptor were determined in mouse spleen (Fig. 2B). The concentration-response gave \( K_e \) 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 \( \mu\)M, 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]GTP\(_\gamma\)S binding to cerebellar membranes. [\(^{35}\)S]GTP\(_\gamma\)S binding was stimulated in a concentration-dependent and saturable manner by WIN 55,212-2 with \( E_{\text{50}} \) and \( E_{\text{max}} \) values of 0.16 ± 0.01 \( \mu\)M and 286 ± 24% (stimulation above basal binding), respectively (Table 1).
To determine the ability of NESS 0327 to antagonize CB₁ 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 \( E_{\text{max}} \) 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 \( K_c \) 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 \([35S]GTP_S\) binding, whereas, in the same conditions, SR 141716A at concentration of 1 µM produced an inhibition of 21 ± 2% of basal \([35S]GTP_S\) binding (data not shown). The lack of effect on basal \([35S]GTP_S\) 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: \( F_{\text{dose}} \) (6,189) = 10.26, \( P < 0.01 \); \( F_{\text{time}} \) (2,189) = 7.22, \( P < 0.01 \); \( F_{\text{interact}} \) (12,189) = 3.7, \( P < 0.01 \)] and hot-plate (two-way ANOVA: \( F_{\text{dose}} \) (6,189) = 42.37, \( P < 0.01 \); \( F_{\text{time}} \) (2,189) = 14.20, \( P < 0.01 \); \( F_{\text{interact}} \) (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.
Discussion

Given the role of the endogenous cannabinoid system in different physiological responses and its involvement in numerous pathological processes, the search for new and selective agonists/antagonists of the CB1 and CB2 cannabinoid receptor will constitute an important line of research in the forthcoming years. In this respect, NESS 0327 showed a high selectivity for CB1 versus CB2 receptors and the in vitro functional assays (isolated organ and GTP\(\gamma\)S) as well as the in vivo antinociceptive studies indicated that the compound behaves as antagonist of the CB1 receptor. However, because the relative binding affinity of NESS 0327 for the CB1 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 CB1 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 CB1 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 N1-aromatic ring and the aminopiperidine carboxamide region, may facilitate the elucidation of the cannabinoid pharmacophore for CB1-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 CB1 receptor, NESS 0327 may prove to be more advantageous compared with the classical CB1 receptor antagonist SR 141716A.

Current views of the interaction between CB1/CB2 receptors and signal transducting G proteins interaction are de-

![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.](image)

![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.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(ED_{50})</th>
<th>(E_{\text{max}})</th>
<th>% basal binding</th>
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<tr>
<td>WIN 55,212-2</td>
<td>0.16 ± 0.01</td>
<td>286 ± 24</td>
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<tr>
<td>WIN 55,212-2 + NESS 0327(0.1 nM)</td>
<td>0.36 ± 0.07**</td>
<td>269 ± 42</td>
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<tr>
<td>WIN 55,212-2 + NESS 0327(1 nM)</td>
<td>0.79 ± 0.13**</td>
<td>362 ± 42</td>
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<tr>
<td>WIN 55,212-2 + NESS 0327(10 nM)</td>
<td>1.11 ± 0.03**</td>
<td>200 ± 21</td>
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In the presence of NESS 0327, the in vivo experiment where the relative difference in activity is only 10 times might suggest a poor central bioavailability of NESS 0327.
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-agonist 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 CB2 would allow a better conceptualization of the rather complex mode of cannabinoid receptor/ligand interaction because Δ9-THC itself has been shown to be a weak but very selective antagonist for the cannabinoid receptor CB2 (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 CB2 receptors, to study the recent proposed ability of the CB1 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 CB1 receptor would consequently allow radioactive labeling of the latter, thus enabling the study of CB1 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 CB1 antagonist. It should be determined whether NESS 0327 would show better efficacy as a CB1 antagonist in animal models of excessive food intake, psychosis, and cognitive impairment, three areas of possible interest for a novel CB1-selective antagonist.

References
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (I) and the concentration of inhibitor which causes 50% inhibition (IC50) of an enzyme

**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).


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