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Synthesis and characterization of NESS 0327: a novel putative antagonist of the CB_1 cannabinoid receptor

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NESS 0327: a novel putative CB1 cannabinoid receptor antagonist.

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Abbreviations: 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, 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]

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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 towards cannabinoid CB_1 and CB_2 receptor. NESS 0327 exhibited a stronger selectivity for CB_1 receptor when 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, while 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 values 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 CB1 receptor.

Interest in the pharmacology of cannabinoids 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 (CNS) 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 appear 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 behaviours (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 (Compton et al., 1996; Lichtman and Martin, 1997; Rinaldi Carmona et al., 1994), while 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 has advanced the concept of therapeutically targeting cannabinoid receptors. Besides their established clinical antiemetic action (Gralla 1999; Voth and Schwartz, 1997), 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).

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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.

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 dimethylsulfoxide (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 (3x25 ml). The combined extracts were washed with brine, water and then dried over anhydrous sodium sulphate, 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): 3200-2500 (OH), 1720 (C=O), 1590 (Ar); ^{1}H-NMR: 2.40-2.75 (m, 8H), 5.60-5.75 (m, 2H), 6.15-6.45 (m, 2H), 7.10-7.28 (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, m.p.56-58°C. Rf 0.84 (petroleum ether/ethyl acetate 1/1); IR(nujol): 3300 (OH), 1710 (C=O), 1600 (Ar); ^{1}H-NMR: 1.60-1.80 (m, 4H), 2.30-2.45 (m, 2H), 2.55-2.71 (m, 2H), 7.04 (d, 1H, J = 6.4 Hz), 7.12-7.35 (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.

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Thionyl chloride in excess was subsequently removed under reduced pressure and the residue was added for 3 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 (3x5 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, b.p. 94-97°C/0.05 mm Hg (lit[1] 128-131/0.35 mmHg); Rf 0.65 (petroleum ether/ethyl acetate 9/1); IR (film): 3350 (OH), 1680 (C=O), 1590 (Ar); ^{1}H-NMR: 1.72-1.98 (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-5H-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 then poured onto crushed ice and the whole acidified with 2 N hydrochloric acid and extracted with chloroform (3x15 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); b.p. 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), 2.13-2.40 (m, 2H), 2.67 (t, 2H, J = 6.4 Hz), 3.09-3.40 (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: 2.20-2.39 (m, 2H), 2.50-3.35 (m, 4H), 6.61 (d, 1H, J = 8.2 Hz), 7.03 (dd, 1H), 7.32 (d, 1H, J = 1.8 Hz), 7.39-7.49 (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).

***N*-Piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6 tetrahydrobenzo [6,7] cyclohepta [1,2-*c*] pyrazole-3-carboxamide] (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 (3x5 ml) and evaporated to yield the crude carboxylic chloride. A solution of the above carboxylic chloride in dichloromethane (6 ml) was added dropwise to a solution of 1-aminopiperidine (10) (0.19

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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 (3x15 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: 1.35-1.53 (m, 2H), 1.58-1.89 (m, 6H), 2.15-2.36 (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), 7.37-7.54 (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 20-25 g (Charles River, Calco, LC, Italy) were housed in the animal care quarters; temperatures were 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 E.C. regulations for care and use of experimental animals (EEC N°86/609).

Mice were killed by cervical dislocation, brains (minus cerebellum) and spleens were rapidly removed and placed on an ice-cold plate. After thawing, tissues were homogenated in 20 vol. (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 1,086 x g for 10 min at 4°C, and the resulting supernatants were centrifuged at 45,000 x g for 30 min at 4°C.

[^{3}H]-CP 55,940 binding was performed by a modification of the method previously described (Rinaldi-Carmona et al., 1994). Briefly, the membranes (30-80 µg of protein) were incubated with 0.5 nM of [^{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 of fatty acid-free bovine serum albumin (BSA). Non-specific binding was estimated in the presence of 1 µM of CP 55,940. All binding studies were performed in

disposable glass tubes pre-treated with Sigma-Cote (Sigma Chemical Co. Ltd., Poole, UK), in order to reduce non-specific binding. The reaction was terminated by rapid filtration through Whatman GF/C filters pre-soaked in 0.5% polyethyleneimine using a Brandell 96-sample harvester (Gaithersburg, MD, USA). 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, Packard, Meridien, USA) with 4 ml of scintillation fluid (Ultima Gold MV, Packard). 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 non-linear regression analysis of a Sigmoid Curve using Graph Pad Prism program (Graph Pad Software, Inc. San Diego, CA, USA). 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 25-40 g. Tissue was mounted in 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, Va, 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 (2Biological Instruments, Besozzo, Va, 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 pM, 10 pM 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 pre-drug 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 prior to 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 (M) vs. 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, Como, Italy), weighing 200-250 g, were used in all experiments. Rats were killed by decapitation, their brains rapidly removed and cerebella were dissected on ice. Cerebella tissue was suspended in 20 volumes of cold centrifugation buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and homogenated using a homogenizer system (Glas-Col, Terre Haute, IN, USA). The homogenate was centrifuged at 48,000 x g for 10 min at 4°C. The pellet was then resuspended in the same buffer, homogenized, and centrifuged as previously. The final P2 pellet was subsequently resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 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, Milan, Italy). 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 guanosine-5'-diphosphate (GDP), in a final volume of 1 ml. The reaction was terminated by rapid filtration using a Packard Unifilter-GF/B, washed 2 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, Packard, Meridien, USA) using 50 µl of scintillation fluid (Microscint ^{TM} 20, Packard, Meridien, USA).

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 of [^{35}S]GTPγS and 30 μM GDP.

Non specific 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)}x 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, San Diego, CA, USA) 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 20-25 g (Charles River, Calco, LC, Italy) were used to assess antinociception by means of the tail flick and hot plate test. A tail flick meter (Ugo Basile Instruments, Italy) 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 3-5 s latency in vehicle treated rats.

The effect of the compounds on the reaction time of mice placed on the hot plate (Ugo Basile Instruments, Italy) (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 prior to 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)] X 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 minutes after WIN 55,212-2 (2 mg/kg s.c.) or vehicle and up to 120 minutes. 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 ways 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 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 TMS as the internal standard and coupling constants in Hz. 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 Hz. IR spectra were recorded as thin films or nujol mulls on NaCl plates with a Perkin-Elmer 781 IR spectrophotometer and are expressed in ν (cm^{-1}). Melting points were

determined on a Kőfler 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/mmole) were purchased from New England Nuclear (Boston, MA, USA). CP 55,940 and WIN 55,212-2 were obtained from Tocris Cookson Ltd (Bristol, UK). GDP and GTPγS were obtained from Sigma/RBI (St. Louis, MO, USA). SR 141716A and SR 144528 were kindly provided by Sanofi-Synthélabo (Bagneux, France).

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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, that 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 nM, 514 ± 30 nM 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).

In order 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, 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, while, 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.

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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. 4A and fig. 4B, 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 ways 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 ways 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 ($P>0.05$ vs. vehicle treated rats) and of 0.05 mg/kg in the hot plate test ($P>0.05$ vs. 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. 30 min 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.

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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 an high selectivity for CB_1 vs. 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 since the relative binding affinity of NESS 0327 for the CB1 receptor is about 5,000 times more than that of SR 141716A, the *in vivo* experiment whereas the relative difference in activity is only ten 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 (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 analogues 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 CB1 receptor, NESS 0327 may prove to be more advantageous when compared to 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 described 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 conceptualisation of the rather complex mode of cannabinoid receptor/ligand interaction, since 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). Since recent data using SR 141716A seem to suggest a ligand-independent activity for cannabinoid receptor signalling (Mato et al., 2002), NESS 0327 could be employed as a more selective antagonist for the CB_1 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 signalling (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 labelling of the latter, thus enabling the study of CB1 cellular and tissutal 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 area of possible interest for a novel CB_1 selective antagonist.

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Footnotes

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Legends

Fig.1. Schematic synthesis of NESS 0327

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.

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 "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.

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 were administered i.p. 20 minutes 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 ways ANOVA followed by Newman Keuls post-hoc test (* $P<0.05$ and ** $P<0.01$).

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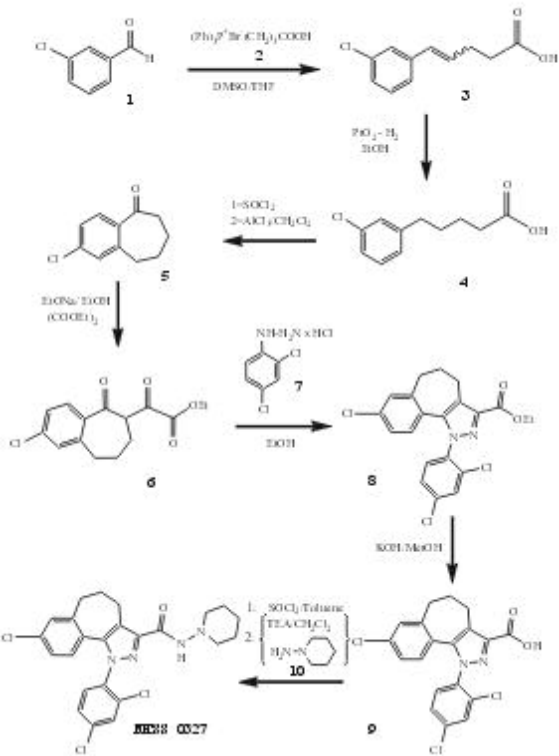
Table 1

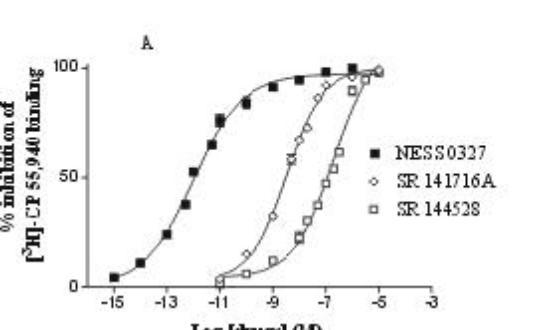
ED_{50} and E_{max} values of WIN 55,212-2 in stimulating [^{35}S]GTPγS binding in the presence or absence of NESS 0327

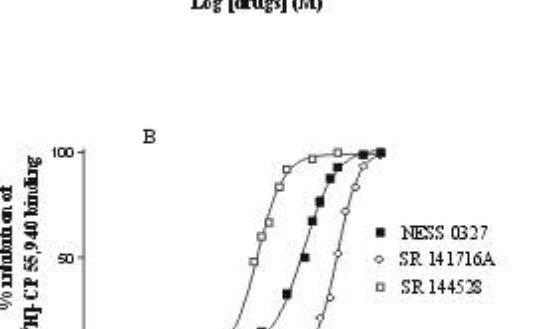
Compounds	ED_{50} (μM)	E_{max} (% Basal Binding)
WIN 55,212-2	0.16 ± 0.01	286 ± 24
WIN 55,212-2 + NESS 0327 (0.1 nM)	$0.36 \pm 0.07^{*}$	269 ± 42
WIN 55,212-2 + NESS 0327 (1 nM)	$0.79 \pm 0.13^{**}$	362 ± 42
WIN 55,212-2+ NESS 0327 (10 nM)	$1.11 \pm 0.03^{**}$	200 ± 21

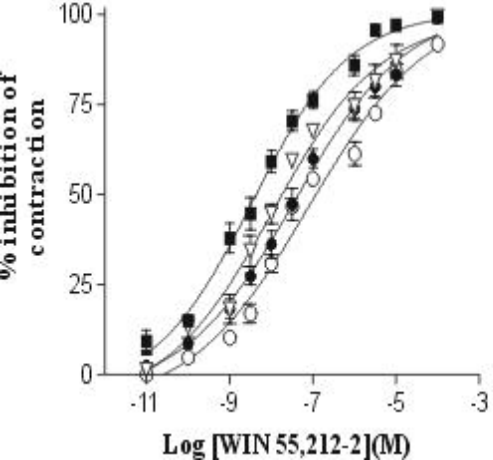
[^{35}S]GTPγS binding was performed in rat cerebella membranes with 30 μM of GDP and 8-10 concentrations of WIN 55,212-2 in the absence and presence of 0.1, 1, and 10nM of NESS 0327. Data are expressed as percent of basal [^{35}S]GTPγS binding. The values represent mean ± S.E.M. of 4-6 separate experiments, each performed in triplicate. ED_{50} and E_{max} values were calculated from non-linear regression curve fitting using Graphpad prism program.

The statistical significance of differences between the groups was assessed by one-way analysis of variance (ANOVA) following by the Neuman-Keuls test (*$P<0.05$ and **$P<0.001$ versus WIN 55,212-2)


1
2
DMSO/TBT
3
PtO2 - H2
EtOH
4
1-SOCl2
2-AlCl3/CH2Cl2
5
EtONa/ EtOH
(COOEt)2
6
7
EtOH
8
KOH/MeOH
9
10
1. SOCl2/Toluene
TEA/CH2Cl2
2.
MRS 0327


A
% inhibition of [3H]-CP 55,940 binding
100
50
0
-15
-13
-11
-9
-7
-5
-3
Log [drugs] (M)
NESS 0327
SR 141716A
SR 144528


B
% inhibition of [3H]-CP 55,940 binding
100
50
0
-15
-13
-11
-9
-7
-5
-3
Log [drugs] (M)
NESS 0327
SR 141716A
SR 144528


% inhibition of contraction
100
75
50
25
0
-11
-9
-7
-5
-3
Log [WIN 55,212-2](M)

A
B
Vehicle
WIN 55,212-2 (2 mg/kg, ip)
WIN 55,212-2 (2 mg/kg, ip) + NESS 0327 (0.01 mg/kg)
WIN 55,212-2 (2 mg/kg, ip) + NESS 0327 (0.05 mg/kg)
WIN 55,212-2 (2 mg/kg, ip) + NESS 0327 (0.1 mg/kg)
WIN 55,212-2 (2 mg/kg, ip) + NESS 0327 (0.5 mg/kg)
WIN 55,212-2 (2 mg/kg, ip) + NESS 0327 (1.0 mg/kg)
time (min)
time (min)