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R-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone (WIN-2) ameliorates experimental autoimmune encephalomyelitis and induces encephalitogenic T cell apoptosis: Partial involvement of the CB_2 receptor

Antonio J. Sánchez [a], *Paz González-Pérez* [a], *Ismael Galve-Roperh* [b], *Antonio García-Merino* [a,*]

[a] *Neuroimmunology Unit, Hospital Universitario Puerta de Hierro, Universidad Autónoma de Madrid, San Martin de Porres 4, 28035 Madrid, Spain*

[b] *Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain*

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EAE, experimental autoimmune encephalomyelitis
MS, multiple sclerosis
TMEV, Theiler's murine encephalomyelitis virus
CNS, central nervous system
CREAE, chronic/relapsing EAE
CB, cannabinoid receptors

ABSTRACT

Many reports have shown that cannabinoids might be beneficial in the symptomatic treatment of multiple sclerosis (MS). We have investigated the therapeutic properties of the non-selective cannabinoid receptor agonist WIN-2 as a suppressive drug in the experimental autoimmune encephalomyelitis (EAE) model of MS. In the passive variety of EAE, induced in Lewis rats by adoptive transfer of myelin-reactive T cells, WIN-2 ameliorates the clinical signs and diminishes the cell infiltration of the spinal cord. Due to the involvement of cannabinoids in the regulation of cell death and survival, we investigated the effects of WIN-2 on the encephalitogenic T cell population. WIN-2 induced a profound increase of apoptosis in a dose- and time-dependent manner. The potential involvement of cannabinoid receptors (CB) was investigated by encephalitogenic T cell stimulation in the presence of the CB_1 (SR141716A) and CB_2 (SR144528) antagonists, pertussis toxin (PTX) and the inactive enantiomer WIN-3. WIN-2-induced apoptosis was partially blocked by SR144528 and PTX, whereas, WIN-3 only exerted a mild effect on cell viability. These results point to the partial involvement of CB_2 receptor together with other receptor-independent mechanism or by yet unknown cannabinoid receptors. Moreover, WIN-2 induced the extrinsic pathway of apoptosis, as shown by caspase-10 and -3 activation. These results suggest that cannabinoid-induced apoptosis of encephalitogenic T cells may cooperate in their anti-inflammatory action in EAE models. The partial involvement of CB_2 receptors in WIN-2 action may open new therapeutic doors in the management of MS by non-psychoactive selective cannabinoid agonists.

* *Corresponding author*. Tel.: +34 913445446; fax: +34 913162578.
E-mail address: jgarciam.hpth@salud.madrid.org (A. García-Merino).

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WIN-2, R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone
WIN-3, S-(−)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone
gp-MBP, guinea pig myelin basic protein
LNCs, lymph node cells
TRPV1, transient receptor potential vanilloid 1
SR1 (SR141716A), N-(piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
SR2 (SR144528), N-[(1S)-endo-1,3,3-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DAPI, 4,6-diamidino-2-phenylindole
PTX, pertussis toxin
JWH-015, (2-methyl-1-propyl-1H-indol-3-yl)-1-napthalenylmethanone
Δ^9-THC, delta(9)-tetrahydrocannabinol
Δ^8-THC, delta(8)-tetrahydrocannabinol
ACEA, arachidonoyl-2-chloroethylamide
JWH-133, 3-(1′-dimethylbutyl)-1-deoxy-Δ^8-THC
HU-211, (+)-(3S,4S)-7-hydroxy-delta(6)-tetrahydrocannabinol 1,1-dimethylheptyl
DMSO, dimethylsulfoxide
PBS, phosphate buffered saline
FCS, fetal calf serum
SDS, sodium dodecyl sulfate
DMF, dimethylformamide
PMSF, phenylmethylsulfonyl fluoride
DMEM, Dulbecco's modified Eagle medium

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a well-known model of multiple sclerosis (MS), the most common chronic neuroinflammatory, demyelinating disease of the central nervous system (CNS) in humans [1]. EAE can be induced by active immunization with myelin antigens (active EAE) or by adoptive transfer of myelin-reactive T cells into naïve recipients (passive EAE), both forms of the disease are mediated by $CD4^+$ Th1 myelin-specific cells.

During the course of EAE, lymphocytes and macrophages enter the CNS and elicit variable degrees of demyelination and inflammation [2]. The ongoing inflammation is manifested by clinical signs, such as paresis and paralysis of the limbs. During spontaneous recovery, apoptosis is a leading mechanism for the clearing of CNS infiltrating cells [3]. Based on anecdotal reports from MS patients indicating relief of pain and spasticity after self-medication with cannabis [4], a number of clinical studies were carried out to investigate the potential therapeutic action of cannabinoids [5]. In addition, different animal models, including acute EAE chronic/relapsing EAE (CREAE) and Theiler's murine encephalomyelitis virus (TMEV), have been employed to investigate the anti-inflammatory and neuroprotective properties of cannabinoids [6]. Plant-derived cannabinoids, synthetic agonists (such as WIN-2), as well as endogenous cannabinoids, have been shown to influence the regulation of cell death and survival [7]. In particular, they induce T cell lymphocyte growth arrest [8] and apoptosis [9] and thus may contribute to neuroprotection [10]. Cannabinoid regulation of cell-fate decision, involves cells of different origins, including neural (e.g. neurons, oligodendrocytes and astrocytes) and peripheral cells (e.g. lymphocytes, macrophages, B-cells and dentritic cells). Two subtypes of receptors mediate most cannabinoid actions, namely the CB_1 receptor [11], located in the CNS and in peripheral tissues, and the CB_2 receptor [12], found in the periphery, mainly in cells of the immune system. Both receptors belong to the G-protein-coupled receptor family, and have been shown to be involved in immunomodulation at different levels [13]. In addition, endocannabinoids may also act via transient activated vanilloid receptors 1 (TRPV1) [14]. Cannabinoids regulate T cell cytokine production [15], rolling and adhesion of venous leukocytes [16], and antigen processing and presentation in macrophages [17]. Induction of apoptosis might contribute to down-regulation of T cell activity and, thereby, would terminate the inflammatory process that precedes the clinical signs of EAE. In order to study the mechanisms of action of the therapeutic properties of cannabinoids in EAE, we investigated the effects of WIN-2 in the passive variety of this disease. This experimental model allows for a direct examination of the effects that these compounds may exert on its aetiological agents, the encephalitogenic T cell. In particular, the survival of these cells and the signalling pathways implicated were studied. Our data show that WIN-2 induces apoptosis of encephalitogenic T cells, at least in part through the CB_2 receptors. Thus, the partial involvement of CB_2 receptors in cannabinoid action may open new therapeutic doors in the management of MS by non-psychoactive selective cannabinoid agonists.

2. Materials and methods

2.1. Chemicals

WIN-2 (R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone) and WIN-3 (S-(−)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The CB_1 receptor antagonist SR141716A (SR1) and the CB_2 receptor antagonist SR144528 (SR2) were kindly provided by Sanofi-Aventis (Montpellier, France). These compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted with phosphate buffered saline (PBS). Pertussis toxin (PTX) and other chemicals were purchased from Sigma–Aldrich.

2.2. Induction, clinical evaluation and treatment protocols of passively transferred EAE

Blasting encephalitogenic T cells (20×10^6) cultured as described below, were resuspended in 0.5 ml Dulbecco's modified Eagle medium (DMEM) (BioWittaker, Walkersville, MD, USA) and transferred intravenously, through the tail vein, into naïve Lewis rats on day 0. The following scale of clinical signs was employed: 0 = no signs; 1 = partial loss of tail tonicity; 2 = loss of tail tonicity; 3 = unsteady gait and mild paraparesis; 4 = hind-limb paralysis; 5 = death was employed. Grades 3 and 4 were often accompanied by urinary and fecal incontinence.

Based on preliminary experiments aimed to determine active cannabinoid concentration, EAE rats ($n = 5$) were treated daily with a single i.p. dose of WIN-2 according to two different regimes: treatment I, 2 mg/kg starting on day 0 post-transfer (PT) until day 8 PT and treatment II, 4 mg/kg (days 0–2 PT); 4.5 mg/kg (day 3 PT); 5 mg/kg (day 4 PT); 5.5 mg/kg (day 5 PT);

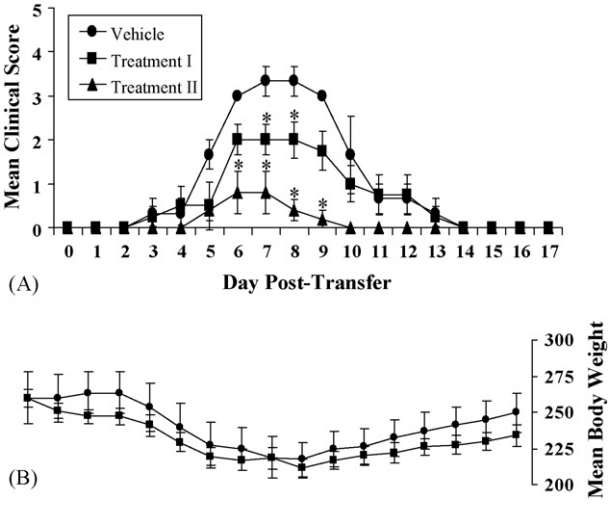

Fig. 1 – WIN-2 suppresses clinical signs of EAE induced by adoptive transfer of encephalitogenic T cells into naïve Lewis rats. (A) Five animals per group were treated with a daily single intraperitoneal dose of WIN-2. Treatment I (■): 2 mg/kg, starting on day 0 PT and continuing until day 8 PT. Treatment II (▲): 4 mg/kg (days 0–2 PT); 4.5 mg/kg (day 3 PT); 5 mg/kg (day 4 PT); 5.5 mg/kg (day 5 PT); 6 mg/kg (day 6 PT); 6.5 mg/kg (day 7 PT); 7 mg/kg (day 8 PT). Vehicle-immunized (●) EAE rats were sham-treated with saline/Tween 80. Compared with the vehicle-treated group, treatments I and II reduced significantly the mean clinical score ($^*p < 0.05$; Mann–Whitney non-parametric ranking test) on days 7–8 PT and 6–9 PT, respectively. (B) None of the treatments had any effect on the curve of body weight. Values indicate mean clinical score ± S.E.M. on each day of clinical disease.

6 mg/kg (day 6 PT); 6.5 mg/kg (day 7 PT); 7 mg/kg (day 8 PT). Control EAE rats were sham-treated with saline/Tween 80 ($n = 5$). Rats were examined daily 24 h after the last drug administration in order to avoid potential analgesic or psychoactive effects that might interfere with the assessment of neurological signs. Animal procedures were performed according to the European Union guidelines (86/609/EU) for the use of laboratory animals.

2.3. Histopathological examination

At the peak of clinical signs, 7 days PT, rats were anesthetized and perfused with sterile saline buffer via cardiac puncture before removal of their spinal cords. Part of the spinal cord, at the lumbar level, was fixed in 10% formaldehyde and embedded in paraffin. Sections (5 μm thick) were stained with haematoxylin–eosin, and examined by light microscopy to study the presence of mononuclear cell infiltrates.

2.4. Myelin basic protein (MBP)-encephalitogenic T cell culture

Lewis rats (Charles River, France) were inoculated as described elsewhere [18]. Briefly, an inoculum containing 50 μg of guinea pig myelin basic protein (gp-MBP) (Sigma–Aldrich) and 500 μg of *Mycobacterium tuberculosis* (strain *H37Ra*, Difco, Detroit, MI, USA) in incomplete Freund's adjuvant (Difco) was injected subcutaneously into the hind footpads.

Ten days after immunization, rat popliteal and inguinal lymph nodes from gp-MBP immunized rats were removed, passed through a 200-μm stainless steel mesh sieve and the lymph node cells (LNCs) suspension was washed and resuspended in Click's medium (RPMI 1640 with 2 mM glutamine, 1 mM sodium pyruvate, essential and non-essential amino acids, 10 mg/ml streptomycin and 100 U/ml penicillin and 0.1 M 2-mercaptoethanol (Gifco Lab., USA) and supplemented with 10% heat-inactivated fetal calf serum (FCS) (Cambrex Bio Science Walkersville, Walkersville, MD, USA). To establish a rat MBP-specific T cell line, LNCs (2×10^6 cells/ml) were cultured with gp-MBP (20 μg/ml) in Click's medium supplemented with 10% heat-inactivated FCS for 3 days at 37 °C and 5% CO_2. The cells were expanded in the presence of recombinant rat IL-2 (1.6 ng/ml) (R&D systems, Abingdon, UK) for 10 days and stimulated with mitomycin C-treated spleen cells from Lewis rats at 0.5×10^6 cells/ml, in the presence of 15 μg/ml gp-MBP. The culture was maintained through additional rounds of stimulation and expansion. On repeated experiments, these cells exhibited a high encephalitogenic capacity when injected intravenously to naïve Lewis rats (data not shown).

2.5. Cell viability MTT assay

Cells were cultured in 96-well plates in the presence of the indicated concentrations of WIN-2 or WIN-3 in DMSO or vehicle alone. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) stock solution was added to each well to

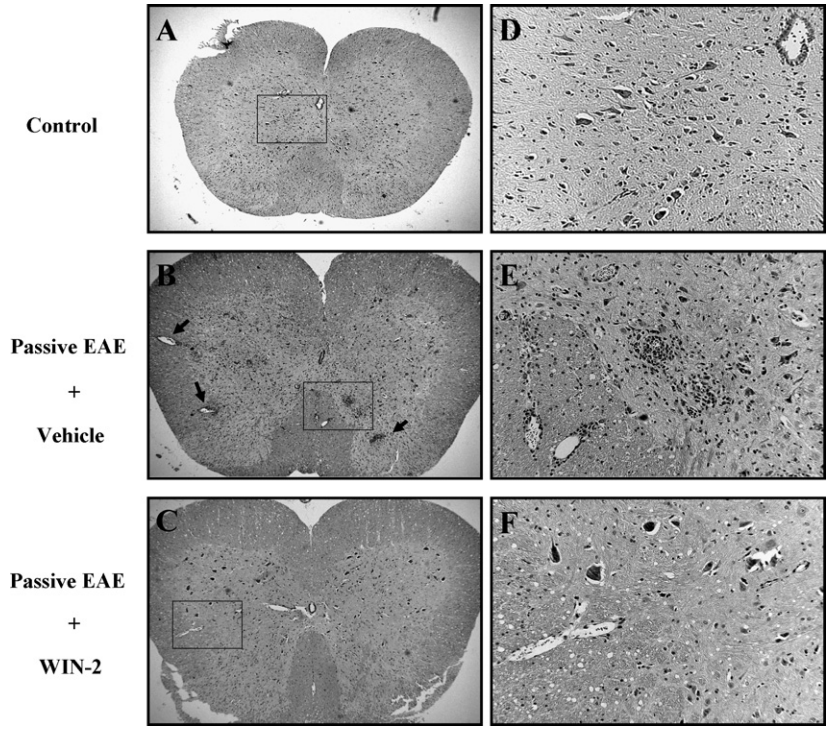

Fig. 2 – Reduction of inflammatory cellular infiltration by WIN-2 in the spinal cord of passive EAE. Lumbar spinal cords were removed at the peak of clinical score 7 days post-transfer; tissue sections were stained with haematoxylin–eosin. Compared with the control group (A), rats with passively (B) induced EAE demonstrated inflammatory infiltrates; this effect was attenuated in sections from passive EAE + WIN-2-treated animals (treatment II as in Fig. 2) (C). Insets from (A)–(C) (2.5×) are shown at higher magnification (10×) in (D)–(F) images, respectively. Vehicle-treated rats (B) showed multifocal intense inflammatory cellular infiltration areas, indicated by arrowheads. Representative images of at least five animals per group.

a final concentration of 0.5 mg/ml MTT. After 4 h of incubation at 37 °C, water-insoluble dark blue formazan crystals formed from MTT cleavage in actively metabolizing cells were dissolved in lysis buffer containing 20% sodium dodecyl sulfate (SDS) and 50% dimethylformamide (DMF). Optical densities were measured at a wavelength of 570 nm, using a scanning multiwell spectrophotometer. All measurements were carried out in triplicate.

2.6. Flow cytometric analysis of apoptosis

After cannabinoid stimulation, cultured cells were collected and washed with PBS. Cells undergoing apoptosis were identified by staining with Annexin-V FITC (BD-Pharmingen, San Diego, CA, USA) and propidium iodide in 0.01 M HEPES, pH 7.4/0.14 M NaCl/2.5 mM $CaCl_2$ for 15 min at room temperature. Samples were analysed on a FACScan™ flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentage of apoptosis was measured using a gate on forward and side scatter in the lymphocyte area. Results were analysed using Lysis II and Paint-a-gate softwares (Becton Dickinson).

2.7. DAPI

In addition, apoptosis was measured with the fluorescent dye, 4,6-diamidino-2-phenylindole (DAPI). Briefly, after cannabinoid incubation, cells were washed and attached to poly-L-lysine covered slides. The slides were incubated 15 min at room temperature and washed twice with PBS. DAPI (1 μg/ml; 100 μl) was applied to each slide, and the slides were incubated for 10 min under dark at room temperature. The slides were washed twice with PBS, covered with a cover slip, and analysed under a fluorescence microscope (Nikon Eclipse E800). The apoptotic cells were identified by bright blue nuclei, characteristic of either condensed or fragmented chromatin, while normal cells were characterized by faint blue nuclei.

2.8. Western blot analysis

Cells were treated and collected in lysis buffer (25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 20 mM β-glycerolphosphate) in the presence of protease and phosphatase inhibitors (0.2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM Na_3VO_4). Fifty micrograms samples were loaded onto 10% SDS-PAGE, transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK), blocked by incubation with 5% non-fat milk in TBS-T buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) for 1 h, and blotted against the different proteins using specific antibodies: anti-caspase-10, anti-caspase-3, anti-Bax (Cell Signaling Technology, Beverly, MA, USA), anti-caspase-12 (ProSci Inc., Poway, CA, USA), anti-Bcl-2 (BD PharMingen) and anti-α-tubulin (Sigma–Aldrich). After washings with TBS, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, UK). The blots were developed using enhanced chemoluminescence reagents (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions and exposure to Kodak X-Omatlm® images were scanned and quantified by densitometry analysis software (PCBas software). When necessary, the blot was stripped by incubating the membrane for 30 min at 55 °C in 62.5 mM Tris/HCl (pH 6.7) containing 100 mM 2-mercaptoethanol and 2% (w/v) SDS, then washed five times with TBS-T buffer and treated as described above.

2.9. Statistical analysis

All data are expressed as mean ± S.E.M. Clinical study results were represented as the mean group clinical score and the statistical difference calculated by the Mann–Whitney non-parametric ranking test. Analysis of apoptosis was performed using one-way ANOVA followed by Dunnet's multiple comparison test.

3. Results

3.1. WIN-2 suppresses the clinical signs of passively transferred EAE

Passively transferred EAE had a disease onset from days 3 to 4 PT, and reached a maximum score on days 6–8 PT (grades 3 or

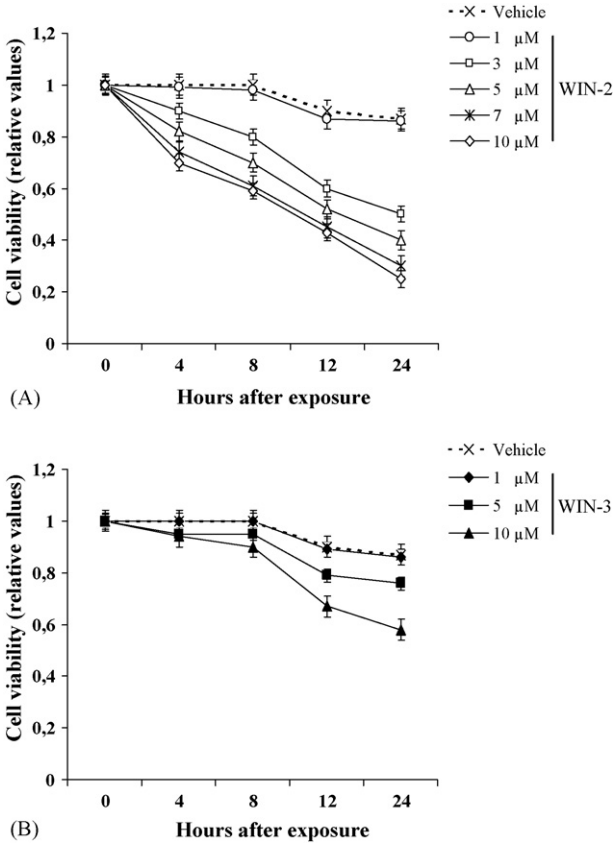

Fig. 3 – WIN-2 decreases encephalitogenic T cell viability. The effect of different concentrations of WIN-2 (A) and WIN-3 (B) on cell viability analysed by MTT assay at the indicated times are shown. Data are expressed as relative values to vehicle-treated cells and correspond to the mean ± S.E.M. of three independent experiments.

4) in nearly 100% of the animals. WIN-2 treatment I (2 mg/kg, starting on day 0 PT and continuing until day 8 PT) resulted in a lesser clinical disease on days 7 and 8 PT; the higher and increasing dose employed in treatment II (4 mg/kg (days 0–2 PT); 4.5 mg/kg (day 3 PT); 5 mg/kg (day 4 PT); 5.5 mg/kg (day 5 PT); 6 mg/kg (day 6 PT); 6.5 mg/kg (day 7 PT); 7 mg/kg (day 8 PT)), increased the length of the therapeutic action from days 6 to 9 PT and reduced the clinical score in EAE as shown in Fig. 1. No differences in the weight curve were observed between treated and untreated animals.

3.2. *WIN-2 reduces cell infiltration in the CNS*

Using haematoxylin–eosin staining, a massive inflammatory infiltrate within the spinal cord of vehicle-treated rats was evident. However the number of inflammatory infiltrates on day 7 PT, at the peak of the clinical signs, were reduced in the WIN-2 treated group (Fig. 2). Of importance, there was a very clear correlation between clinical score and the degree of inflammatory cell infiltration rats with grade 0, showed no infiltrates, whereas, animals with a mild clinical score, grades 1 or 2 (not shown) showed partial infiltration of the CNS that reached the maximal degree only in animals with grades 3 and 4.

3.3. *WIN-2 induces apoptosis of MBP-specific T cells*

Cellular viability was determined by using the MTT assay by incubating gp-MBP-specific T cells with increasing concentrations of WIN-2. As shown in Fig. 3A, the cell viability was reduced in a time-dependent manner when cultured in the presence of WIN-2. In contrast, WIN-3 (Fig. 3B), the inactive enantiomer of WIN-2, induced cytotoxicity only at doses much higher, suggesting the involvement of cannabinoid receptors. To assess if the observed reduction in viability was due to apoptosis, cells were incubated with WIN-2, and the percentage of them staining with Annexin-V FITC and propidium iodide was measured by flow cytometry. As shown in Fig. 4A, induction of apoptosis was observed after 8 h by the use of WIN-2 starting at the 5 μM concentration. By the use of the

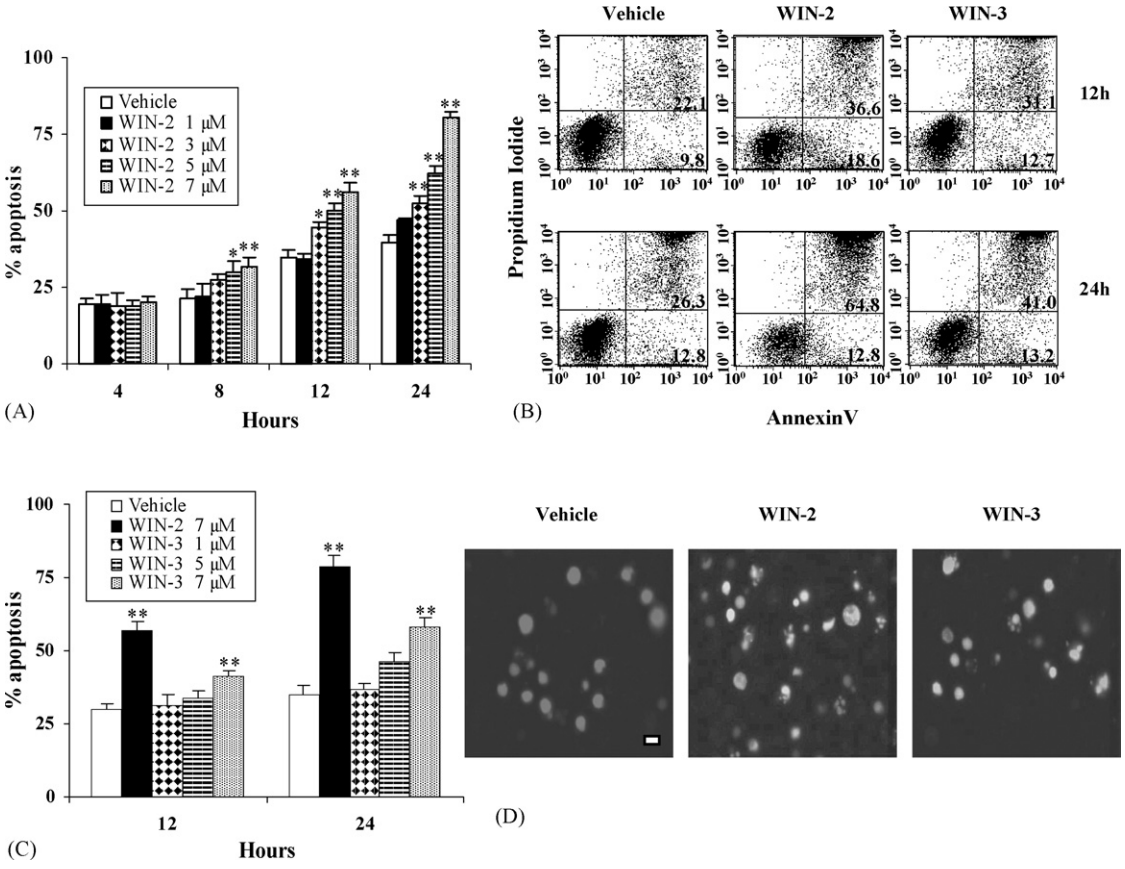

Fig. 4 – WIN-2-induced apoptosis in an encephalitogenic T cell line. (A) Apoptosis was determined and quantified by the percentage of Annexin-V/propidium iodide positive cells showing an increase in a time (4, 8, 12 and 24 h) and dose-dependent (1, 3, 5 and 7 μM) manner, when compared with vehicle. (B) Representative dot plots of Annexin V/propidium iodide labelled cells are shown for the comparison of WIN-2 and WIN-3 action. The percentage of cells found in each quadrant is indicated. (C) Quantification of the apoptotic effects of different concentrations of WIN-3 and 7 μM WIN-2 at 12 and 24 h. (D) DAPI staining of DNA condensation status in cells treated with 7 μM WIN-2 and WIN-3 or vehicle after 12 h. Scale bar 10 μm. Asterisks denote statistically significant difference ($^{*}p < 0.01$ and $^{**}p < 0.001$). One-way ANOVA followed by Dunnet's multiple comparison test) as compared with the vehicle-treated group. Data represent the mean ± S.E.M. of three experiments.

same concentrations of the inactive enantiomer WIN-3 we could only observe a minor effect on apoptosis thus, indicating the stereo-selectivity of the process. A comparison of the effect of WIN-3 and WIN-2 is depicted in Fig. 4B and C. The morphological analysis of apoptotic cells was performed after staining with DAPI, as marker for nuclear morphology changes during apoptosis. Condensed and fragmented nuclei were found in cells treated with WIN-2 (Fig. 4D), but these changes were minoritary in cells treated with WIN-3.

3.4. *WIN-2-induced encephalitogenic T-cell apoptosis occurs with the partial involvement of CB_2 receptor*

In order to test if the cannabinoid-induced apoptosis of encephalitogenic T cells was dependent on CB receptors, we employed SR1 and SR2, selective antagonists for the CB_1 and CB_2 receptors, respectively. Cells were preincubated in the presence or absence of SR1 or SR2 at the same concentration than WIN-2 (7 μM) or vehicle treatment during the following hour. SR2 partially antagonized the induction of apoptosis by WIN-2, whereas, in contrast SR1 failed to reverse it, thus pointing to the involvement of CB_2 but not CB_1 receptors (Fig. 5A). SR1 and SR2 treatment alone had no effect on the induction of apoptosis (data not shown). To asses the involvement of a heterotrimeric Gi protein coupling we investigated the effect of PTX. Pretreatment of MBP-specific T cells with PTX, partially inhibited the WIN-2-induced apoptosis (Fig. 5B), indicating that the effect of WIN-2 was, at least in part, mediated through a Gi-coupled receptor. PTX alone had no effect on the induction of apoptosis (data not shown). The fact that neither the SR2 antagonist nor PTX were able to completely suppress cannabinoid-induced apoptosis indicates the contribution of a receptor independent component or the interaction with still uncharacterized receptors.

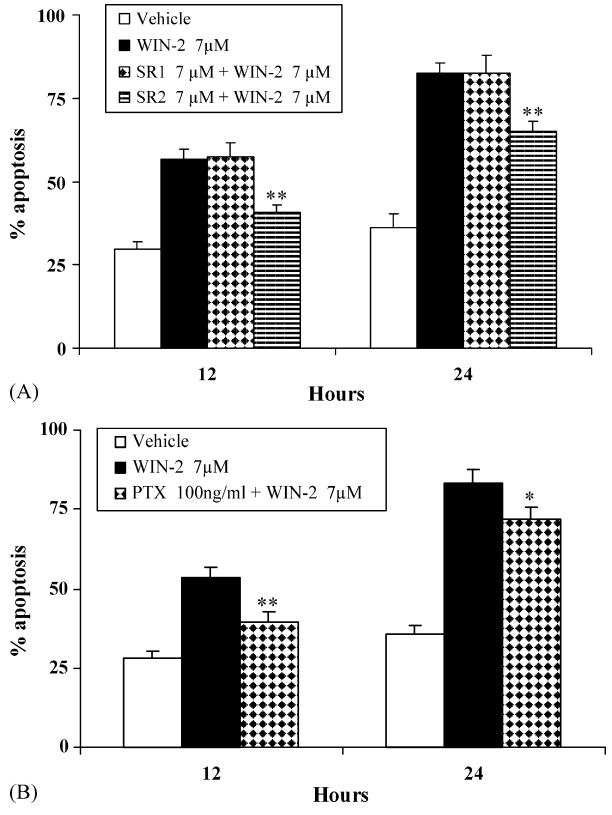

Fig. 5 – Involvement of cannabinoid receptors on WIN-2-induced apoptosis. (A) Cells were cultured with 7 μM antagonist (SR1 and SR2, respectively) for 1 h before treatment with 7 μM WIN-2 or vehicle. (B) PTX 100 ng/ml pre-treatment for 3 h decreased the effect on apoptosis induced by 7 μM WIN-2. Apoptosis was quantified by the percentage of Annexin V/propidium iodide staining. Asterisks denote statistically significant difference ($^{*}p < 0.01$ and $^{**}p < 0.001$). One-way ANOVA followed by Dunnet's multiple comparison test) as compared with the WIN-2-treated group. Data represent the mean ± S.E.M. of three experiments.

3.5. *WIN-2 induces activation of caspase-3 and -10*

To address the mechanism of WIN-2-induced cell death, we analysed by Western blot the activation stage of caspase-3 and -10. As shown in Fig. 6, the 17-kDa protein, corresponding to the active products (cleaved caspase-3) of inactive pro-caspase-3 and the processing of the full length form, 64-kDa, of caspase-10 were generated in WIN-2 treated cells, whereas, caspase-12 activation was not observed. Vehicle (DMSO) incubations up to the longer time studied did not show activation of caspase-3 and -10 (data not shown). In addition, changes in cytosolic-mitochondrial levels of the pro- and anti-apoptotic proteins, Bax and Bcl-2, respectively, did not seem to be involved in the apoptotic pathway induced by WIN-2. The apoptotic effect of WIN-2 associated with the activation of caspase-10 and -3 was not observed with the inactive enantiomer WIN-3 (data not shown).

4. Discussion

Cannabis derivatives are among the most commonly used non-conventional therapies for MS. A number of anecdotal reports of symptomatic relief [4] have led to the completion of controlled clinical trials aimed to evaluate their efficiency in different symptoms of the disease [5]. Besides the therapeutic potential of cannabinoids as symptomatic medication, the possibility exists that these drugs might also exert some beneficial effects on the evolution of the disease itself. In fact, in non-related neurological disorders, such as Parkinson's [19], Alzheimer's [20] and Huntington diseases [21], cannabinoids might play a neuroprotective role in addition to symptomatic improvement [10]. In MS, there exists an increasing interest on the possible role of these compounds as agents able to modulate the immune response against the CNS, and also to cooperate in neural protection in the chronic stages of the disease [10].

Previous experiences with several cannabinoids have shown their ability to improve different experimental models of autoimmune demyelination: Δ^9-THC in EAE [22,23], Δ^8-THC in EAE [24], WIN-2, ACEA and JWH-015 in TMEV [25,15], JWH-133, WIN-2 and Δ^9-THC in CREAE [26] and WIN-2 in actively induced EAE [16]. This work shows, for the first time, how WIN-2, a synthetic cannabinoid, efficiently

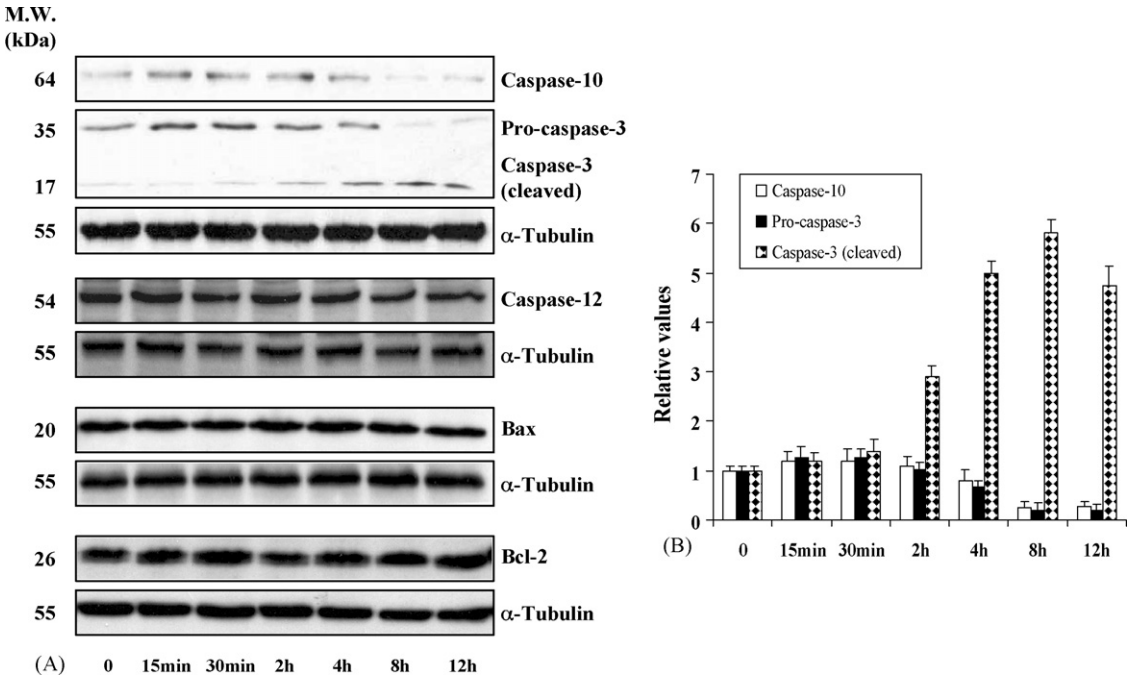

Fig. 6 – Caspase activation by WIN-2. Western blot analysis from cells treated with 7 μM WIN-2 at the indicated times. (A) The blots show decreased levels of full-length caspase-10 (64 kDa) and procaspase-3 (35 kDa) and the corresponding increase in the cleaved forms of caspase-3 (17 kDa). Caspase-12 (54 kDa), Bax (20 kDa) and Bcl-2 (26 kDa) did not seem to be associated with the apoptosis induced by WIN-2. Loading control was performed with anti-α-tubulin (55 kDa) antibody. The relative molecular weights (M.W.) of proteins are given on the left. Representative images of three independent experiments are shown. (B) The relative levels of caspase-10 and full pro-caspase-3/cleaved caspase-3 were quantified by densitometric analysis and normalized with α-tubulin. Data in values relative to control represent the mean ± S.E.M. of three experiments.

suppresses the passive variety of EAE, diminishing both clinical signs and cell infiltration of the spinal cord. These observations were correlated with the induction of apoptosis of encephalitogenic cells mediated by this drug, and suggest this effect to be one of the possible underlying mechanisms of the therapeutic efficacy. Our results are in line with previous reports of cannabinoid-induced apoptosis of activated T cells [9,27].

EAE amelioration was achieved in adoptive transfer EAE, with the two therapeutic regimes of cannabinoid administration employed. Some of the immunomodulatory actions described for cannabinoids, such as prevention or inhibition of antigen processing and presentation by macrophages [17], production of inflammatory mediators [28], and migration of encephalitogenic cells into the CNS [16], could be relevant for the down-regulation of the effector's phase of EAE. Microscopically, the improvement of clinical signs in treated rats with the passive form of EAE correlated with diminished cell infiltration of the spinal cord, as reported in EAE [29,23] and in TVME [25].

Taking into account the proapoptotic effect of cannabinoids described in several cell types [30], induction of lymphocyte apoptosis may participate, in addition to immunomodulatory actions, in the mechanism of the therapeutic improvement induced by WIN-2. Induction of encephalitogenic T cell apoptosis showed, by the use of an inactive enantiomer and the use of selective receptor antagonists [31,32], the partial involvement of cannabinoid receptors and in particular CB_2. Furthermore, the remaining fraction of cell death, after WIN-3, or antagonist co-incubations, suggests the existence of additional CB_2 cannabinoid receptor-independent mechanisms of citotoxicity that may involve additional cannabinoid targets or receptor independent mechanisms. In this sense it has recently been proposed an alternative mechanism for cannabinoid immunosupression relying in nucleoside transporter modulation [33]. Taken together, these results indicate that lymphocyte apoptosis induced by WIN-2 was the result of a combination of mechanisms dependent on CB_2 receptors, but also independent of them [34]. Besides the agonism of CB_2 receptors, the mechanism of the action of WIN-2 on programmed death of encephalitogenic cells may involve passage across membrane lipid rafts [35] or the mediation of still uncharacterized receptors [14].

Experiments aimed at identifying the mechanism of WIN-2-induced cell death revealed the involvement of caspase-10 and -3, proteins implicated in the extrinsic pathway of apoptosis; these findings are consistent with a previous work with T cells [36]. In contrast, Bax and Bcl-2, two proteins regulated in the intrinsic pathway of apoptosis, were unmodified after incubation with WIN-2. Caspase-12, a protein activated by stress of the endoplasmic reticulum after calcium influx, was also unresponsive to the cannabinoid challenge. It is important to note that an increase of intracellular calcium in T lymphocytes has been reported

with some cannabinoid ligands, but not with WIN-2 or JWH-133, a selective CB2 agonist [37]. In other cell types, WIN-2 has been shown to increase intracellular calcium trough CB1 receptors only [38]. These observations along with the scarce expression of CB1 receptors in T lymphocytes, suggest that the absence of caspase-12 activation in our experiments might be due to an absent or non-significant increase of calcium in encephalitogenic T cells after culture with WIN-2.All these data indicate that the death receptor-mediated pathway is probably the predominant one in these cells, even though WIN-2-mediated T cell apoptosis may occur via additional pathways.

In summary, the present work shows the suppressive effect of WIN-2 on passive EAE, both on clinical and histological grounds. The observed induction of apoptosis in encephalitogenic cells, suggests that this mechanism may play a significant therapeutic role, participating in the elimination of the cells responsible for CNS inflammation. The partial involvement of the CB_2 receptors in cannabinoid action in EAE, together with the selective expression of these receptors in the immune system, opens the way to explore the ability of specific CB_2 agonists devoid of psychoactive effects as anti-inflammatory agents for MS management.

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