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THERAPEUTIC POTENTIAL OF A NOVEL CANNABINOID AGENT CB52 IN THE MOUSE MODEL OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract—Multiple Sclerosis (MS) is a demyelinating disease which causes inflammation, demyelination, and axonal injury. Currently, there is no cure for the disease. The endocannabinoid system has recently emerged as a promising therapeutic target for MS. The protective mechanisms of cannabinoids are thought to be mediated by the activation of the cannabinoid type 1 (CB1) and type 2 (CB2) receptors expressed primarily in neurons and immune cells, respectively. However, the molecular mechanisms and the contribution of each receptor in ameliorating disease progression are still debatable. Although CB1 and CB2 receptors are expressed in oligodendrocytes, the myelin producing cells in the central nervous system, the role of cannabinoids in oligodendrocyte survival has not been well investigated. Using primary cultures of mature oligodendrocytes, we tested the effect of a novel synthetic cannabinoid CB52 on oligodendrocyte toxicity induced by peroxynitrite, the primary toxic species released by microglia. Interestingly, we found that CB52 is more potent than a number of broad and selective CB1 and CB2 agonists in protecting oligodendrocytes against peroxynitrite-induced toxicity. The protection provided by CB52 is likely due to its reduction of ERK1/2 phosphorylation and reactive oxygen species (ROS) generation in these cells. Using experimental autoimmune encephalomyelitis (EAE), an animal model of MS, we found that CB52 reduces microglia activation,

nitrotyrosine formation, T cell infiltration, oligodendrocyte toxicity, myelin loss and axonal damage in the mouse spinal cord white matter and alleviates the clinical scores when given either before or after disease onset. These effects are reversed by the CB1 receptor antagonist, but not by the CB2 receptor antagonist, suggesting that the activation of CB1 receptors contributes significantly to the anti-inflammatory and neuroprotective effects of cannabinoids on MS. Published by Elsevier Ltd. on behalf of IBRO.

Key words: cannabinoid, experimental autoimmune encephalomyelitis, oligodendrocyte, myelination, microglia, T cells.

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune disease affecting the central nervous system (CNS). It is the most common demyelinating disease in young adults and results in motor and sensory deficits (Polman and Uitdehaag, 2000). MS is characterized by chronic inflammation resulting from the infiltration of T cells and the activation of microglia/macrophages culminating in the loss of oligodendrocytes, demyelination and axonal damage (Pryce et al., 2003). Although no cure exists, advances in research have led us to a better understanding of the pathogenic mechanisms, which aid the development of novel and effective agents for the treatment of MS.

Cannabinoids are a class of compounds being studied for the treatment of MS and other neurological diseases (Baker et al., 2000; Arevalo-Martin et al., 2003; Lastres-Becker et al., 2004; Venderová et al., 2004; Eubanks et al., 2006; Sagredo et al., 2007; van Vliet et al., 2008). Preclinical and clinical studies have demonstrated that cannabinoids can alleviate MS symptoms due to their anti-inflammatory (Arevalo-Martin et al., 2003), antioxidant (Howlett et al., 2002), and anti-excitotoxic properties (Grundy et al., 2001; Pryce et al., 2003). Cannabinoids have been shown to be protective to oligodendrocytes, the myelinating cells of the CNS, by promoting the survival and differentiation of progenitor oligodendrocytes *in vitro* (Molina-Holgado et al., 2002) and *in vivo* (Solbrig et al., 2010). Thus, cannabinoids can be potentially used as effective agents in limiting demyelination and promoting remyelination.

Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model that mimics the

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symptoms of MS in humans (Baker et al., 1990; Wujek et al., 2002). The first study using cannabinoids in this model system showed that Δ^9 -tetrahydrocannabinol (THC), the main active component of the cannabis plant, has immunomodulatory functions, as indicated by the reduced numbers of infiltrating cells in the spinal cord (Lyman et al., 1989). Following this study many groups have further demonstrated the beneficial effects of various cannabinoids in different animal models of MS (Kubajewska and Constantinescu, 2010). The beneficial effects of cannabinoids are attributable to their activation of cannabinoid type 1 (CB1) and CB2 receptors (Matsuda et al., 1990; Munro et al., 1993), although increasing evidence points to the contribution by novel receptors and receptor-independent mechanisms (Puffenberger et al., 2000; Kaplan et al., 2003, 2008; Walter et al., 2003; Kozela et al., 2010). Studies using knockout animals showed that a deficiency in either CB1 (Pryce et al., 2003) or CB2 (Palazuelos et al., 2008) receptors resulted in more severe EAE. The regulatory action of cannabinoids in EAE-induced neurodegeneration is largely due to CB2 receptor-mediated modulation of the immune and inflammatory response and CB1 receptor-mediated neuroprotection (Maresz et al., 2007), although the relative contribution of each receptor might be dependent upon the specific cannabinoid compounds applied. Because activation of CB1 receptors can produce psychotropic effects, current efforts are focused on searching for agents that increase the therapeutic efficacy and meanwhile reduce the side effects.

CB52 is a novel cannabinoid compound synthesized on the basis of the structural modification of the endocannabinoid ligand anandamide (AEA) and the phytocannabinoid Δ^9 -THC. This compound was shown to exhibit high affinity for the central CB1 and peripheral CB2 receptors with K_i values of 210 and 30 nM, respectively (Brizzi et al., 2005). We have recently reported that like other non-selective, as well as selective CB1 and CB2 agonists, CB52 can significantly reduce the induction of inducible nitric oxide synthase (iNOS) and the generation of reactive oxygen species (ROS) in lipopolysaccharide-activated microglia. However, the anti-inflammatory effects seem not to be mediated by the activation of CB1 and CB2 receptors (Ribeiro et al., 2013). In the present study we tested whether CB52 was protective against oligodendrocyte toxicity induced by peroxynitrite, a reaction product of nitric oxide and superoxide, and a major toxic factor released by activated microglia (Xie et al., 2002; Li et al., 2005). Interestingly, our results demonstrated that CB52 is more potent and less toxic than a number of cannabinoid receptor agonists in protecting oligodendrocytes from peroxynitrite-induced toxicity. Moreover, we also found that CB52 reduced neuroinflammation, oligodendrocyte injury, myelin loss and the clinical scores in mice with EAE. Surprisingly, different from the results found *in vitro*, the therapeutic effect of CB52 *in vivo* seems to be mediated by the activation of CB1 receptors.

EXPERIMENTAL PROCEDURES

Materials

Non-selective CB1 and CB2 receptor agonists CB52, CP55940 and AEA were from Cayman Chemicals (Ann Arbor, MI, USA); WIN55212-2 was obtained from Sigma–Aldrich (St. Louis, MO, USA). CB1 selective agonists ACPA, ACEA, and methanandamide were purchased from Tocris (Ellisville, MO, USA). CB2 selective agonists AM1241 and JWH015 were from Cayman Chemicals. CB1 antagonist AM281 was from Tocris and CB2 antagonist AM630 was from Cayman Chemicals. The peroxynitrite generator SIN-1 was from Cayman Chemicals. $ZnCl_2$ and lipopolysaccharide (LPS) were purchased from Sigma. Animal studies were performed according to the guidelines established by the Uniformed Services University of the Health Sciences and the National Research Council's *Guide for the care and Use of Laboratory Animals*.

Oligodendrocyte cell culture

Primary cultures of oligodendrocytes were prepared from 2- to 3-day-old Sprague–Dawley rat brain. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin in 75 cm² flasks incubated in 95% air/5% CO₂ at 37 °C. At 10 days, the flasks were shaken for 1 h on an orbital shaker (200 rpm) at 37 °C to remove microglia. They were then changed to new media and shaken overnight. Oligodendrocytes were harvested and seeded onto poly-ornithine-coated plates [96-well (2.3 × 10⁶ cells/plate), 24-well (3 × 10⁶ cells/plate)] in a basal chemically-defined medium (BDM) [DMEM with 1 mg/ml bovine serum albumin (BSA), 50 µg/ml apo-transferrin, 5 µg/ml insulin, 30 nM sodium selenite, 10 nM biotin, 10 nM hydrocortisone] plus 10 ng/ml of both platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). For culturing mature oligodendrocytes, cells at day 7 were changed to BDM plus T3 (15 nM) and ciliary neurotrophic factor (CNTF) (10 ng/ml) for another 2 weeks. This results in a culture where the purity of oligodendrocytes is more than 95%, and only 1–2% of cells are identified to be either microglia or astrocytes as described previously (Li et al., 2012).

Exposure of oligodendrocytes to cannabinoids and SIN-1 or zinc

In each experiment, cannabinoid agonists were added 30 min prior to 3-morpholininosynonimine (SIN-1) or zinc chloride ($ZnCl_2$) exposure and maintained during the treatment. When used, antagonists were added 15 min prior to agonists. All compounds were dissolved in 0.1% dimethyl sulfoxide (DMSO), which was used as the vehicle control.

Toxicity assay

Oligodendrocytes were treated with SIN-1 or zinc in the presence or absence of cannabinoids for 2 h, washed

twice with Hank's balanced salt solution (HBSS) containing 0.1% BSA, and then placed in BDM with T3 and CNTF. After the cells were incubated for 20–24 h, the culture medium was replaced with Earle's balanced salt solution (EBSS) plus a 1:100 dilution of Alamar Blue (Trek Diagnostic Systems, Inc., Westlake, OH, USA). After 2 h exposure, the cell viability was assessed using a fluorescent plate reader (SpectraMax Gemini XS, GMI Inc., MN, USA) with excitation wavelength at 530 nm and emission wavelength at 590 nm.

Western blot analysis

At 2 h after SIN-1 or zinc treatment, oligodendrocytes were lysed and the protein concentration in lysates was measured. After the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the membrane was blocked with 5% nonfat milk for 1 h and then incubated overnight at 4 °C with the primary antibody for phosphorylated ERK42/44 or total ERK42/44 (Cell Signaling, Beverly, MA, USA) diluted at 1:2000 in Tris-buffered saline and Tween 20 (TBST) containing 5% BSA. After washing, the membrane was incubated for 1 h at room temperature (RT) with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody diluted at 1:2000. The proteins were visualized by enhanced chemiluminescence (ECL). Quantification for Western blot analysis was done by measuring band density using Multi Gauge software.

Measurement for ROS

Intracellular free radical generation was evaluated with 2',7'-dichlorofluorescein diacetate (DCF; Molecular Probes, Eugene, OR, USA). Briefly, after the cells in 96-well plates were treated with SIN-1 or zinc with/without cannabinoids for 2 h, they were loaded with DCF (10 μ M) for 30 min. The fluorescence of the cells in each well was measured using a fluorescence plate reader with excitation wavelength at 480 nm and emission wavelength at 530 nm.

Induction of EAE

EAE was induced in 7-week-old female C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA) as we previously described (Li et al., 2011; Vana et al., 2011). EAE was induced by subcutaneous injection of 200 μ g myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (H-MEVGWYRSPFSRVVHLYRNGK-OH) in Complete Freund's Adjuvant (CFA, DIFCO, Detroit, MI, USA) with 500 μ g mycobacterium tuberculosis (DIFCO). Immediately following MOG peptide injection and 24 h later mice were intraperitoneally (i.p.) administered 200 μ g pertussis toxin (List Biological Labs, Campbell, CA, USA). At 1 week after induction EAE mice received a booster of 200 μ g of MOG in incomplete Freund's Adjuvant without mycobacterium tuberculosis. Animals in the control group received pertussis toxin and CFA without the MOG peptide. Mice were assessed daily to evaluate the extent of dysfunction following EAE induction. Neurological signs were assessed as follows:

0, normal; 1, dysfunction of tail or one limb; 2, dysfunction of tail and one limb or two limbs; 3, limp tail and dysfunction of two limbs; 4, disturbed function in tail and three limbs or dysfunction of all limbs; 5, moribund; 6, death.

Immunohistochemistry (IHC)

Animals were euthanized using 90-mg ketamine/10-mg xylazine per kg, i.p., then intracardially perfused with ice-cold 1 M phosphate buffer. Spinal cords were dissected prior to post-fixation in 4% paraformaldehyde at 4 °C overnight. Tissue was then cryoprotected in 30% sucrose (Sigma) in 1 M phosphate buffer at 4 °C overnight. After being cryoprotected the tissue was embedded in Tissue Tek OCT (Sakura, Torrance, CA, USA) and stored at –80 °C until utilization. Transverse sections of lumbar spinal cords were cut at 14 μ m with a cryostat (Leica model CM1900, Bannockburn, IL, USA) and mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA, USA) for immunohistological analysis. Slides were washed three times with phosphate-buffered solution (PBS), and blocked with 1% Triton X-100 in PBS containing 5% donkey serum for 20 min at room temperature. The slides were then incubated with the appropriate primary antibody diluted in PBS containing 2% donkey serum. Primary antibodies used were for: Microglia/Macrophages marker F4/80 (1:100, eBioscience, San Diego, CA, USA); Nitrotyrosine, a marker for peroxynitrite formation (1:100, Chemicon, Temecula, CA, USA); CC1, a marker for mature oligodendrocyte (1:100, Calbiochem, Germany); CD4 T-lymphocytes (1:100, BD Pharmingen, San Diego, CA, USA) and NF200 (1:250, Millipore, Burlington, MA, USA), a marker for axons. Primary antibodies were incubated overnight at 4 °C. After three washes with PBS at 5 min each, the appropriate secondary antibody conjugated with either Alexia Fluor 488 or Alexia Fluor 594 (Molecular Probes) was added and then incubated for 1 h at room temperature. After three washes with PBS, the slides were mounted with Fluoro Shield together with 4',6-Diamidino-2-Phenylindole (DAPI, Sigma) and kept dark at 4 °C. Images were captured with a fluorescence microscope (Nikon Eclipse E800) equipped with a Nikon digital camera. For tumor necrosis factor- α (TNF- α) staining, the slides were rinsed in PBS and incubated in PBS containing 0.3% Triton and a polyclonal anti-TNF- α antibody (Abcam, Cambridge, MA, USA) at 4 °C overnight. After washing three times with 5 min each, the slides were incubated with a biotinylated secondary antibody for 30 min. The sections were washed for 5 min and then incubated for 30 min with Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA, USA). After another 5 min washing, the sections were incubated in peroxidase substrate solution until desired staining intensity developed. Cells counts were performed in the white matter of the spinal cord using NIS-Elements software (Nikon, Melville, NY, USA). Three sections per animal were counted and the average was recorded. The number of individual animals (*n*) is indicated in the figure legends.

Oil Red O staining

To perform Oil Red O staining, frozen tissues were sectioned at 14- μ m thickness with a cryostat (Leica, Bannockburn, IL, USA) and mounted on slides. The slides were air dried for 60 min at room temperature and then placed in absolute propylene glycol for 5 min. The sections were further incubated in pre-warmed Oil Red O solution for 10 min in 60 °C water bath and followed by differentiation in 85% propylene glycol solution for 5 min. Finally the section were mounted with VectaMount (Vector, CA, USA) aqueous mounting medium and visualized with a Zeiss (Oberkochen, Germany) Axioplan 2ie microscope.

Statistical analysis

Statistical significance was assessed using analysis of variance (ANOVA) with the Tukey–Kramer post hoc multiple comparison test. Statistical analysis was performed using the InStat program from GraphPad Software (San Diego, CA, USA). Representative experiments are shown unless noted otherwise. Experiments were performed with triplicate samples, and the data are expressed as mean $\pm$ SEM. All experiments were repeated at least three times.

RESULTS

Cannabinoids protect mature oligodendrocytes against peroxynitrite- and zinc-induced toxicity

Although both CB1 and CB2 receptors are expressed in various stages of oligodendrocytes, it is unclear whether the activation of these receptors can prevent oxidative injury to mature, myelin producing, oligodendrocytes. Using a novel cannabinoid agent CB52, and three commonly used non-selective CB1/CB2 receptor agonists CP55940, WIN55212-2 and AEA, we examined the role of these cannabinoid compounds on oligodendrocyte toxicity induced by SIN-1, a peroxynitrite generator. As shown in Fig. 1, all four cannabinoids dose-dependently protected mature oligodendrocytes against SIN-1 induced toxicity. However, both CP55940 and AEA were themselves toxic at 10 μ M (Fig. 1B, D), whereas CB52 (10 μ M) and WIN55212-2 (10 μ M) did not have any toxicity. Notably, a complete protection was achieved when CB52 was used at 10 μ M (Fig. 1A).

We have previously found that peroxynitrite toxicity to mature oligodendrocytes is mediated by intracellular zinc release, ERK1/2 phosphorylation and ROS generation (Zhang et al., 2006, 2007). Thus, we also tested whether these cannabinoids could prevent mature oligodendrocytes from zinc-induced toxicity. AEA (3 μ M), WIN 55212-2 (10 μ M) and CB52 (10 μ M) significantly attenuated oligodendrocyte toxicity induced by zinc chloride (ZnCl₂) at 150 μ M. When 200 μ M ZnCl₂ was used, only CB52, but not WIN 55212-2 or AEA, was shown to have protective effects (Fig. 1E). These results suggest that CB52 is more potent than AEA and WIN 55212-2 to prevent oligodendrocytes from peroxynitrite- or zinc-induced toxicity.

Cannabinoid agonists attenuate ERK1/2 phosphorylation and ROS generation in oligodendrocytes treated with SIN-1 or zinc

To further characterize the protective mechanisms of cannabinoids on oligodendrocytes, the phosphorylation of ERK1/2 induced by SIN-1 or zinc was assessed by Western blot analysis. We found that after a 2-h exposure to either SIN-1 (Fig. 2A) or zinc (Fig. 2B), only CB52 was able to significantly attenuate ERK1/2 phosphorylation. All four cannabinoids tested significantly attenuated ROS generation at 6 h after SIN-1 treatment. A complete reduction of ROS was observed in the presence of CB52 and CP55940, whereas the reduction of ROS by WIN55212-2 and AEA was 30% and 40%, respectively (Fig. 2C). CB52 was also more effective than WIN55212-2 and AEA in attenuating ROS generation caused by zinc (data not shown).

Cannabinoids protect mature oligodendrocytes against peroxynitrite and zinc toxicity through a mechanism that is partially mediated by the CB2 receptor

Considering its high potency, we decided to focus our studies on the protective effects of CB52 on mature oligodendrocytes. To determine if the effect seen with CB52 was mediated by the activation of CB1 and/or CB2 receptors, the CB1 antagonist AM281 (10 μ M) and the CB2 antagonist AM630 (10 μ M) either alone or in combination were added 15 min prior to CB52 treatment. As shown in Fig. 3A, the protective effect of CB52 was partially, but significantly attenuated by AM630, but not by AM281. Addition of AM281 did not further enhance the blocking effects exerted by AM630 suggesting the activation of CB2, but not CB1 receptor is responsible for part of the protective effects of this compound. Notably, the antagonists themselves did not have any protective effect. To further test the role of CB2 receptor in the survival of oligodendrocytes, oligodendrocytes were treated with SIN-1 in the presence of CB1 or CB2 receptor selective agonists. The CB2 selective agonists AM1241 and JWH015 significantly attenuated SIN-1 induced toxicity, but interestingly the CB1 selective agonists ACPA, ACEA and methanandamide did not have any effect (Fig. 3B). Similarly, the protective effect of CB52 on zinc-induced toxicity was also partially mediated by the activation of CB2 receptor (Fig. 3C), and the selective CB2 agonists, but not CB1 agonists also exerted a protective effect (Fig. 3D). Taken together, these results suggest that the protective effect of CB52 to mature oligodendrocytes on peroxynitrite- and zinc-induced toxicity is most likely mediated by the CB2 receptor activation.

CB52 is protective in the EAE mouse model when given before or after symptom development and its effects are mediated by CB1 receptor activation

Given the findings from the *in vitro* studies that CB52 seems to be more effective than other commonly used

Fig. 1. The non-selective cannabinoid agonists CB52, CP55940, WIN55212, and AEA protected SIN-1- and zinc-induced toxicity to mature oligodendrocytes. Primary oligodendrocytes were exposed to SIN-1 (1 mM) for 2 h in the presence of different concentrations (10 μ M–10 nM) of the non-selective cannabinoid agonists CB52 (A), CP55940 (B), WIN55212 (C), and AEA (D) or exposed to ZnCl₂ with CB52, WIN55212 and AEA (E). While all test compounds can attenuate SIN-1 toxicity in mature oligodendrocytes, CB52 (A, E) showed a better effect when compared with the other cannabinoid agonists ($n = 5-9$; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were obtained when the drug-treated groups were compared to the SIN-1 or zinc-alone group).

cannabinoids in attenuating oligodendrocyte cell death and the evidence that this effect is mediated by the CB2 receptor, we decided to test the role and the cannabinoid receptor dependency of CB52 in EAE, a mouse model of MS. In the first set of experiments (Fig. 4A), mice were treated with CB52 (2 mg/kg body weight, i.p.) in the absence or presence of the CB1 antagonist AM281 (2 mg/kg) or the CB2 antagonist AM630 (2 mg/kg) starting at day 3 after MOG injection and then once a day until day 30. Without treatment, the clinical symptoms developed on day 10, and rapidly reached clinical scores 3.0–3.5 from day 16 to day 30. After CB52 treatment, there was a slight delay in the disease onset, but the clinical scores were significantly reduced from day 13 until the end of the experimental period. In this group the maximal clinical score was 1.5,

which is dramatically reduced when compared to the untreated EAE group (clinical score of 3.5). Surprisingly, the reduction of clinical scores by CB52 was eliminated by co-administration of AM281, but not AM630, suggesting the activation of CB1 receptor contributes to the therapeutic effect of CB52, which is distinct from our observations in oligodendrocytes in culture.

To be more clinically relevant, we also performed the CB52 treatment immediately after the disease onset (Fig. 4B). Mice were administered CB52 (2 mg/kg, i.p.) with/without AM281 or AM630 starting from day 12 and then once daily afterward. Again, the EAE mice reached and maintained a high clinical score of 3.0 from day 16 to day 30, post-symptom treatment with CB52 significantly reduced clinical scores starting from day 17, and further reduction was seen until the end of the

Fig. 2. Cannabinoid agonists decreased ERK1/2 phosphorylation and ROS generation in oligodendrocytes treated with SIN-1 or zinc. (A) Mature oligodendrocytes were treated with SIN-1 (1 mM) for 2 h in the presence of different concentrations of non-selective cannabinoid agonists and lysed for Western blot analysis. Although most cannabinoids seem to reduce the ERK1/2 phosphorylation, the significance was only achieved by the CB52 treatment. ($n = 3$; $*p < 0.05$ was obtained when the CB52-treated group was compared to the SIN-1-alone group). (B) Mature oligodendrocytes were treated with zinc (200 μ M) for 2 h in the presence of various non-selective cannabinoid agonists and lysed for Western blot analysis. While all three cannabinoids tested were able to decrease ERK1/2 phosphorylation in these cells, CB52 was shown to have a greater effect when compared to WIN and AEA ($n = 3$; $*p < 0.05$ was obtained when the CB52-treated group was compared to the zinc-alone group). (C) Mature oligodendrocytes were treated with SIN-1 (1 mM) for 6 h in the presence of different concentrations of non-selective cannabinoid agonists and then incubated with DCF for 30 min. The increased fluorescent intensity, indicative of ROS generation, was then measured. While all drugs tested were able to attenuate ROS production, a complete inhibition of ROS generation was shown with CB52 and CP55940 ($n = 5$; $***p < 0.001$ was obtained when the drug-treated groups were compared to the SIN-1-alone group).

experiment. The reduction of CB52 on clinical scores was completely eliminated by co-treatment with AM281, but not AM630, although co-administration of AM630 seemed to partially reverse the clinical scores. These results clearly indicate that CB1 receptor activation plays a dominant role for the action of CB52 in the EAE mouse model.

In the third set of experiment (Fig. 4C) we were interested in determining whether continuous activation of the cannabinoid receptor is necessary for the therapeutic action of CB52. To test this idea, animals started receiving CB52 after symptom development at day 12, followed by co-administration of the CB1 or CB2 antagonist beginning at day 20. Our hypothesis

was that if transient receptor activation or short-term treatment with CB52 is sufficient to trigger the signaling pathway leading to protection, delayed treatment with antagonists should not affect the action of CB52; On the contrary, if continuous activation of cannabinoid receptor is necessary, delayed treatment with antagonists may still reverse the protective effect of this cannabinoid compound. This study found that delayed administration of CB1 antagonist, but not CB2 antagonist was likely to reverse the protective effect of CB52, suggesting chronic treatment might be important for sustaining the CB1 receptor signaling and maintaining the protective effect of this cannabinoid compound.

Fig. 3. The protective effect of cannabinoids on SIN-1- or zinc-induced toxicity to oligodendrocytes was partially mediated by CB2 receptor activation. (A) Mature oligodendrocytes were treated with SIN-1 (1 mM) for 2 h in the presence of CB52 and the CB1 antagonist AM281 and CB2 antagonist AM630. Toxicity was then evaluated using Alamar Blue. CB52 is able to attenuate SIN-1 toxicity in mature oligodendrocytes and this effect was partially blocked in the presence of the CB2 antagonist but not the CB1 antagonist ($n = 5$; $**p < 0.01$, and $***p < 0.001$). (B) Mature oligodendrocytes were treated with SIN-1 (1 mM) for 2 h in the presence of the CB1 selective agonists ACPA, ACEA and methanandamide and the CB2 selective agonists AM1241 and JWH015. Toxicity was then evaluated using Alamar Blue. The CB2 agonists but not the CB1 agonists were able to significantly attenuate SIN-1 toxicity in mature oligodendrocytes. ($n = 5$; $**p < 0.01$, $***p < 0.001$, $##p < 0.01$ and $###p < 0.001$ compared to the respective SIN-1-alone group). (C) Mature oligodendrocytes were treated with zinc (200 μM) for 2 h in the presence of CB52 and the CB1 antagonist AM281 and CB2 antagonist AM630. Toxicity was then evaluated using Alamar Blue. CB52 was able to attenuate zinc toxicity in mature oligodendrocytes and this effect was partially blocked by the CB2 antagonist but not the CB1 antagonist ($n = 5$; $***p < 0.001$). (D) Mature oligodendrocytes were treated with zinc (150 μM and 200 μM) for 2 h in the presence of the CB1 selective agonists ACPA, ACEA and methanandamide and the CB2 selective agonists AM1241 and JWH015. Toxicity was then evaluated using Alamar Blue. The CB2 agonists but not the CB1 agonists were able to partially attenuate zinc toxicity to mature oligodendrocytes. ($n = 5$; $***p < 0.001$ and $###p < 0.001$).

CB52 attenuates accumulation of microglia/macrophages and peroxynitrite production in the mouse EAE spinal cord

On day 30 after EAE induction, animals were perfused with 4% paraformaldehyde and spinal cords were dissected and sectioned for IHC. Fig. 5 shows representative images of microglia/macrophages by F4/80 and the staining of nitrotyrosine, a marker for peroxynitrite formation, in the spinal cord dorsal columns in our first set of experimental groups (i.e. animals that received CB52 with/without antagonists starting from day 3 after induction). Our results showed that there was a dramatic increase in the staining of F4/80 and nitrotyrosine in EAE animals (Fig. 5B, F, G) compared to the control animals without EAE induction (Fig. 5A, F, G). Treatment with CB52 almost completely blocked F4/80 and nitrotyrosine staining (Fig. 5C, F, G);

indicating CB52 is effective in blocking the accumulation of microglia/macrophages and the release of peroxynitrite. Consistent with the effects on the clinical scores, co-treatment with the CB1 antagonist (Fig. 5D, F, G) but not the CB2 antagonist (Fig. 5E–G) reversed the effects of CB52. Similar results were also observed with post-symptom treatment by CB52 at day 12 together with the CB1 and CB2 antagonists and when antagonist treatment was delayed until day 20 (data not shown).

CB52 attenuates T cell infiltration in the spinal cords of EAE mice

EAE is primarily mediated by the infiltration of CD4^+ T lymphocytes from the periphery to the CNS, and once in the CNS, these T cells can be activated by local antigen

Fig. 4. CB52 alleviated clinical score in EAE animals and this effect was blocked by the CB1 antagonist. (A) EAE mice were given CB52 or a combination of CB52 with AM281 or AM630 starting from day 3 and then once a day until the end of the experiment. Clinical scores were significantly decreased in the CB52-treated group ($n = 13$) when compared to the EAE group ($n = 13$; $^{*}p < 0.01$, $^{***}p < 0.001$). The effect of CB52 was reversed when animals were co-administered with CB1 antagonist AM281 ($n = 13$; $^{#}p < 0.05$, $^{###}p < 0.01$), but not the CB2 antagonist AM630. (B) EAE mice were given CB52 or a combination of CB52 with AM281 or AM630 starting from day 12 and then once a day until the end of the experiment. Clinical scores were significantly decreased in the CB52-treated group ($n = 12$) when compared to the EAE group ($n = 16$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) and this effect was reversed when animals were co-administered with the CB1 antagonist AM281 ($n = 12$; $^{#}p < 0.05$, and $^{###}p < 0.001$). (C) EAE mice were treated with CB52 at day 12 post immunization and the CB1 and CB2 antagonists were given at day 21. Clinical scores were significantly decreased in the CB52-treated group ($n = 8$) when compared to the EAE group ($n = 8$; $^{*}p < 0.05$, and $^{**}p < 0.01$). Although treatment with CB1 antagonist did not significantly blocked the effect of CB52 ($n = 8$), there were increased clinical scores in these animals.

presenting cells and release inflammatory mediators (Kubajewska and Constantinescu, 2010). At 30 days after EAE induction, the infiltration of CD4⁺ T cells was clearly observed in the spinal cord white matter, which was almost completely blocked by treatment with CB52 either before (Fig. 6A, B) or after (Fig. 6C, D) symptom development. Interestingly, the blocking effect of T cell infiltration by CB52 was also reversed by the co- or delayed-administration of CB1 antagonist AM281, but not the CB2 antagonist AM630 (Fig. 6A–D). These

results suggest that CB52 can block T cell infiltration through the activation of CB1 receptors.

CB52 reduces TNF- α production in EAE spinal cord white matter

TNF- α is one of the major cytokines released from CD4⁺ T cells and reactive microglia/macrophages, and believed to contribute to the pathogenesis of EAE and MS (Hofman et al., 1989; Selmaj et al., 1991). To determine whether

Fig. 5. A significant increase of microglia/macrophages and peroxynitrite formation was found in the spinal cord white matter of EAE mice, and attenuated in the CB52-treated groups. Lumbar spinal cord sections of EAE animals treated with CB52 and the CB1/CB2 antagonists starting at day 3 post induction were stained for microglia/macrophages and nitrotyrosine (NT) immunoreactivity, which is indicative of peroxynitrite formation. EAE animals showed increased staining for microglia/macrophages and peroxynitrite formation (B) when compared to control animals (A). Treatment with CB52 significantly attenuated the accumulation of microglia/macrophages and reduced the nitrotyrosine formation in these animals (C). This effect was blocked when animals were treated together with the CB1 antagonist AM281 (D), but not the CB2 antagonist AM630 (E). Quantification of F4/80-positive (F) and NT-positive cells (G) showed that AM281, but not AM630 could reverse the inhibitory effects of CB52 ($n = 3$; $*p < 0.05$, and $**p < 0.01$).

CB52 can reduce the production of TNF- α in EAE mouse spinal cord, the spinal cord sections from control, EAE vehicle and drug-treated EAE groups at 30 days after EAE induction were subjected to immunohistochemical analysis to examine the production of TNF- α in the white matter. The immunoreactivity of TNF- α was dramatically increased in EAE mouse spinal cord when compared to the control animals. Treatment with CB52 significantly reduced TNF- α expression, and this reduction was reversed by co-administration of CB1 receptor antagonist AM281, but not by the CB2 receptor antagonist AM630 (Fig. 7A, B).

CB52 prevents oligodendrocyte cell loss and demyelination in the spinal cords of EAE mice

Oligodendrocyte cell death and demyelination are two major components in MS and EAE (Bjartmar et al., 2003; Furlan et al., 2009; Lassmann, 2010). Using CC1, a marker for mature oligodendrocytes, we examined whether CB52 treatment can affect the survival of oligodendrocytes (Fig. 8A). As demonstrated previously, the number of CC1-positive cells in the spinal cord white matter is significantly reduced in EAE animals when

compared to controls (Li et al., 2011; Vana et al., 2011). As anticipated, CB52 is effective in preventing the loss of these cells in the spinal cord white matter when given before (Fig. 8A) or after (Fig. 8B, C) symptom development. Contrary to our *in vitro* studies, however, we found that blocking the CB1, but not the CB2 receptor reversed the protective effect of CB52 no matter the antagonists were given at day 3 (Fig. 8A) or day 12 (Fig. 8B). Delayed administration of CB1 antagonist AM281 seemed also to reduce the protective effect of CB52, although this reduction did not reach significance when compared to the CB52-alone-treated group (Fig. 8C).

We then looked at demyelination by staining the spinal cord sections with Oil Red O. In these sections myelin staining appears to be red, and the white or bleached areas in the white matter are indicative of demyelination (Fig. 9A). Consistent with the loss of mature oligodendrocytes, demyelination was greater in EAE animals when compared to control animals, and CB52 was very effective in preventing demyelination when given 3 days (Fig. 9A, B) or 12 days (Fig. 9C, D) after induction. The reduction of demyelination was also blocked by the CB1 antagonist, but not by the CB2

Fig. 6. Infiltration of T cells in EAE animals was decreased with CB52 treatment. (A) Lumbar spinal sections of EAE animals were stained for T cells (CD4⁺). An increase in T cells was seen throughout the white matter of the spinal cord in EAE animals. Images represent day 3 treatment group. (B) CB52 blocked the T cell infiltration in the spinal cord of EAE animals when given 3 days post induction. This effect was blocked by the CB1 antagonist (AM281), but not the CB2 antagonist (AM630). ($n = 4-6$; $***p < 0.001$). (C, D) Post-symptom treatment with CB52 decreased T cell infiltration. This effect was blocked by the CB1 antagonist (AM281) when given at 12 (C) or 21 days (D) post induction. ($n = 4-6$; $**p < 0.01$, and $***p < 0.001$).

antagonist regardless if the antagonists were given starting from day 3 (Fig. 9A, B), day 12 (Fig. 9C) or day 20 (Fig. 9D).

CB52 prevents axonal injury in the EAE spinal cord white matter

We also studied the effect of CB52 on axonal injury by staining spinal cord sections with an axonal marker

NF200. In EAE animals, there was a dramatic decrease in the levels of NF200⁺ staining (Fig. 10A), indicating that axonal damage occurred in these animals. Treatment with CB52 either before (Fig. 10A, B) or after (Fig. 10C, D) symptom development significantly reduced the loss of NF200⁺ axons. Co-administration of AM281 appeared to attenuate the protective effect of CB52 on axonal loss (Fig. 10A–D), although the significant difference was only observed

Fig. 7. CB52 reduced TNF- α expression in spinal cord of EAE mice. At day 30 after immunization, spinal cord sections from control, EAE vehicle and drug-treated EAE groups were subjected to immunohistochemical analysis to examine the production of TNF- α in the spinal cord white matter. The TNF- α -positive cells were counted for statistical analysis. CB52 clearly attenuated TNF- α expression compared to EAE control group as shown in A and B (** p < 0.01 and *** p < 0.001). Addition of CB1 antagonist AM281 but not CB2 antagonist AM630 reversed the inhibitory effect of CB52 on TNF- α expression.

in the post-symptom treatment groups (Fig. 10C, D). Once again, the contribution of CB2 receptor in the action of CB52 is quite limited, if there is any.

DISCUSSION

Accumulating evidence points to the therapeutic potential of cannabinoids in inflammatory and neurodegenerative diseases, such as MS (Pryce and Baker, 2012). In addition to ameliorating the clinical symptoms in animal models (Baker and Pryce, 2008) and MS patients (Rog, 2010), cannabinoids are also being recognized for their ability to control disease progression (de Lago et al., 2010). Although much progress has been achieved over the last several decades, questions still remain regarding the protective mechanisms of various cannabinoids and the

involvement of CB1 and CB2 receptors. In this study we examined the role of various cannabinoids on mature oligodendrocyte toxicity induced by peroxynitrite, and found that CB2 agonists, but not CB1 agonists, have significant protective effects. Furthermore, we found that CB52, a newly developed AEA and Δ^9 -THC hybrid, is more effective than other commonly used cannabinoids and its protection on oligodendrocytes is partially mediated by the activation of the CB2 receptor. In the EAE mouse model, our results showed that CB52 ameliorated clinical scores when given either before or after symptom development. Administration of CB52 also reduced T cell infiltration, microglia activation and the production of TNF- α , prevented the loss of oligodendrocytes, demyelination, and axonal injury in the spinal cord white matter. Surprisingly, we found that contrary to

Fig. 8. CB52 prevented the loss of mature oligodendrocytes in the spinal cords of EAE animals. (A) At 30 days post immunization, the density of mature oligodendrocytes in the spinal cord white matter of EAE mice was significantly less than that found in control animals. The loss of oligodendrocytes was prevented by treatment with CB52 at day 3 post-induction. This effect was blocked by the CB1 antagonist (AM281), but not the CB2 antagonist (AM630). ($n = 4$; $*p < 0.05$, and $**p < 0.01$). (B, C) CB52 was also effective in preventing the loss of mature oligodendrocytes when given after symptom development (B, C), and this effect was blocked when the CB1 antagonist was given at 12 days post induction (B), but not at 21 days post induction, although a similar trend was also observed (C). ($n = 5$; $*p < 0.05$).

our *in vitro* studies, the CB1 and not the CB2 receptors seem to be critical for the protective effects of CB52 observed *in vivo*.

Cannabinoids protect mature oligodendrocytes against peroxynitrite-induced toxicity by attenuating ERK phosphorylation and ROS generation, and this effect is partially mediated through CB2 receptor activation

Oligodendrocyte cell death is one of the major characteristics of MS and EAE. Preventing mature oligodendrocytes from injury and/or allowing oligodendrocyte progenitors to develop into mature, myelin-producing oligodendrocytes is of critical importance. Several studies have shown that cannabinoids can promote the survival of oligodendrocytes by reducing depolarization-evoked calcium influx (Mato et al., 2009), and preventing apoptosis in progenitor cells (Molina-Holgado et al., 2002). However, it has not been reported whether cannabinoids can prevent oxidative stress-induced toxicity to mature oligodendrocytes. We have previously shown that peroxynitrite, a reaction product

of nitric oxide and superoxide, is a major toxic factor released from activated microglia and injures oligodendrocytes via a signaling pathway involving intracellular zinc release, ERK phosphorylation and ROS generation (Zhang et al., 2006, 2007). Here, we were able to show that cannabinoids can directly affect the survival of primary mature oligodendrocytes when exposed to peroxynitrite or extracellular zinc, and that the protection is likely attributable to their inhibition of ERK phosphorylation and ROS generation. In agreement to the reports by others (Molina-Holgado et al., 2002; Benito et al., 2007), we also found that the CB1 and CB2 receptors are expressed in oligodendrocytes by real-time polymerase chain reaction (PCR) and Western blot (data not shown). However, their role in these cells remains unclear. Our results suggest that activation of CB2 receptor, but not CB1 receptor, might be responsible for the protective effect of CB52 and other cannabinoid agents on oxidative stress-induced toxicity. These results inspired us to test the therapeutic efficacy of CB52 *in vivo*, considering the notion that CB2 receptor activation does not cause psychotropic side effects.

Fig. 9. CB52 reduced myelin loss in the spinal cord of EAE animals. (A) Myelin loss was measured by staining sections of lumbar spinal cords with Oil Red O where intact myelin can be seen as a red staining and demyelinated areas appear white. (B) CB52 treatment starting at day 3 post induction attenuated myelin loss when compared to the EAE control animals. This effect was blocked by the CB1 antagonist (AM281), but not the CB2 antagonist (AM630). ($n = 5$; $**p < 0.01$, and $***p < 0.001$). (C, D) CB52 was also effective in preventing myelin loss when given after symptom development on day 12 (C, D). This protective effect was blocked by the CB1 antagonist (AM281) when given at 12 (C) or 21 days (D) post induction. ($n = 5$; $*p < 0.05$, and $***p < 0.001$).

CB52 is effective in lowering clinical scores in EAE animals when given either before or after symptom development

EAE is a well-described and commonly used animal model for MS. Its pathological and clinical features are similar to those seen in patients, including the inflammatory components leading to demyelination and neurological dysfunction (Lavi and Constantinescu, 2005; Baxter, 2007). Using this model, we found that CB52 can reduce clinical symptoms when given either before (3 days after MOG immunization) or after disease onset (12 days after MOG immunization when the symptoms became apparent). When EAE animals were treated together with the CB1 and CB2 antagonists, we found that blocking the CB1 but not the CB2 receptor reversed the reduction of clinical scores, suggesting the action of CB52 is mediated through the activation of CB1 receptors. Although this result is consistent with a recent study indicating that the protective effect of a non-selective CB1/CB2 agonist WIN55212-2 is mediated by CB1, but not CB2 activation in the EAE mouse model, we were surprised by our findings since the protective effect of CB52 against peroxynitrite- or zinc-induced toxicity is partially mediated by CB2 receptor in cultured oligodendrocytes and the notion that modulation of CB2 receptor may be more relevant for controlling the immune-aspects of the disease (Maresz et al., 2007; Palazuelos et al., 2008; Rossi et al., 2010).

CB52 treatment reduces inflammation, oligodendrocyte toxicity, demyelination and axonal loss via CB1 receptor-mediated mechanisms

To further determine the role of CB52 in the pathological changes after EAE induction and the involvement of CB1 and CB2 receptors, we assessed microglia activation, T cell infiltration, TNF- α production, oligodendrocyte toxicity, demyelination and axonal injury. In agreement with the effect on clinical scores, CB52 treatment attenuated T cell infiltration, accumulation of microglia/macrophages, TNF- α production and the loss of mature oligodendrocytes, myelin and axons. Again, all these effects are mediated by the activation of CB1, rather than CB2 receptors. These results are different from the previous reports showing CB2-mediated inhibition of microglia activation (Price et al., 2009; Stella, 2009) and T cells (Maresz et al., 2007), but are in agreement with those showing that the CB1 receptor can affect the inflammatory response (Pryce et al., 2003; de Lago et al., 2010). Although the results obtained by CB52 *in vitro* and *in vivo* seem to be paradoxical, several possibilities might be taken into consideration. First, the expression of CB1/CB2 receptors in virtually all the immune cells, neurons and glia cells makes it difficult to sort out the specific role of these receptors in each cell type and the overall contribution of these receptors *in vivo*; Second, although our results have demonstrated that the protective effect of CB52 on peroxynitrite-induced toxicity to oligodendrocytes is partially mediated

Fig. 10. Treatment with CB52 decreased axonal loss in EAE animals. (A) Axonal loss was measured by the density of NF200-positive axons in lumbar spinal cord sections. EAE animals show decreased NF200-positive staining, which was rescued after CB52 treatment. (B) CB52 given before symptom development (day 3) significantly decreased axonal loss caused from EAE. While no significant effect can be seen when either antagonist was used, the CB1 antagonist seemed to be more effective in reversing the effects of CB52 ($n = 4$; $*p < 0.05$ and $**p < 0.01$). (C, D) CB52 was also effective in preventing axonal loss when given after symptom development on day 12 (C, D). This protective effect was blocked by the CB1 antagonist (AM281) when given at 12 (C) or 21 days (D) post induction ($n = 4$; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$).

by CB2 receptor, it is unclear whether activation of CB1 receptor can be involved in other toxic conditions, for instance, the toxicity triggered by cytokines, chemokines or glutamate; Third, the CB2 receptors expressed in T cells are highly regulated by endogenous cannabinoid ligands in the CNS during EAE, and activation of these

receptors does not seem to require exogenous agonists for the suppressive effects (Maresz et al., 2007; Kubajewska and Constantinescu, 2010). This idea is supported by a recent finding that HU-308, a CB2 receptor selective agonist, did not exact any protective effect in EAE (de Lago et al., 2012).

Although there is a continuous argument on the relative contribution of CB1 and CB2 receptors in the animal models of MS, the fact that mice with either CB1 or CB2 receptor deletion developed more severe EAE (Pryce et al., 2003; Palazuelos et al., 2008), points to the importance of both receptors in the progression of disease. Recently, it has been reported that the expression of CB1 and CB2 receptors is reduced in proteolipid protein (PLP)-induced EAE mouse brain, and elevated by treatment with 2-arachidonoylglycerol, the most abundant endocannabinoid in the brain, suggesting activation of both CB1 and CB2 receptors mediates the neuroprotection and immunosuppression in EAE (Lourbopoulos et al., 2011). In addition, it becomes increasingly appreciated that novel receptors and receptor-independent mechanisms might also contribute to the protective action of cannabinoids (Di Marzo et al., 2000; Baskfield et al., 2004; Garcia-Arencibia et al., 2007). Development of novel cannabinoid compounds with increased therapeutic efficacy and the reduced side effects will likely benefit the patients with MS and other neurological disorders.

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