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Potent immunomodulatory activity of a highly selective cannabinoid CB2 agonist on immune cells from healthy subjects and patients with multiple sclerosis

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ABSTRACT

COR167, a novel CB2-selective high affinity agonist, was found to significantly inhibit, in a dose-dependent manner, the proliferation of both peripheral blood mononuclear cells and myelin basic protein-reactive T cell lines from normal healthy subjects and patients with relapsing-remitting multiple sclerosis (MS). In MS, a significantly higher inhibition was observed in patients on treatment with disease modifying drugs compared to those naive to treatment. The inhibitory activity of COR167 was exerted through a mixed mechanism involving atypical and incomplete shift of Th1 phenotype towards Th2 phenotype associated with slight reduction of IL-4 and IL-5 as well as strongly reduced levels of Th17-related cytokines. COR167 was also able to reduce in vitro migration of stimulated immunocompetent cells through human brain endothelium associated with a significant reduction of levels of several chemokines. These findings demonstrate that COR167 exerts potent immunomodulatory effects and confirm the cannabinoid CB2 receptor as a novel pharmacological target to counteract neuroinflammation.

1. Introduction

Over the past few years, a number of clinical trials have provided evidence that cannabinoids extracted from cannabis such as Delta9-Tetrahydrocannabinol (Delta9-THC), nabilone, and/or Delta9-THC hemisuccinate may ameliorate symptoms in multiple sclerosis (MS) including perceived spasticity, spasms and pain (reviewed by Moreno Torres et al., 2014) despite lack of effects of orally administered THC on spasticity (Zajicek et al., 2003; Killestein et al., 2002; Centonze et al., 2009). The relative binding specificity for cannabinoid CB1 and CB2 receptors remains a strong limiting factor for the broad clinical use of cannabinoids and seriously hampers their use for the treatment of neurological disorders. Ideally, cannabinoids for chronic use in MS should not stimulate CB1 receptors in order to avoid unwanted psychoactive effects, a serious limiting factor of Delta9-THC based treatments. On the contrary, drugs targeting CB2 receptors would be free of strong psychotropic actions and might favor modulation of autoreactive cells in the periphery and neuroprotection against acute and chronic inflammatory damage. For clinical testing, a highly selective agonist for CB2, devoid of CB1 affinity, would be required (Pertwee, 2009). The relevance of CB2 receptors in inflammatory neurological disorders has been the subject of several reviews (Ashton and Glass, 2007; Benito et al., 2008). All the cannabinoids used in MS including Delta9-THC act as agonists for both CB1 and CB2 cannabinoid receptors mainly located on neurons and immunocompetent cells, respectively (Galiegue et al., 1995), attenuating the respective role played by each receptor type. Nevertheless, there is still a lack of CB2 full agonists able to bind selectively and with high affinity to this receptor which could be used in an MS treatment not merely as a symptomatic therapy but also finalized to a disease-modifying outcome. Recently, COR167, a new synthetic cannabinoid agonist possessing high affinity and selectivity for the CB2 receptor (Pasquini et al., 2008) was found to display significant in vivo analgesic activity in a model of formalin-induced pain in mice (Cascio et al., 2010). Notably, COR167 showed the potency required for a drug candidate, eliciting its effects at a concentration as low as 10 nM, that is two orders of magnitude lower than that of other CB2 ligands used in similar investigations (Pacher and Hasko, 2008). In this study, we evaluated the immunomodulatory and anti-inflammatory effects of COR167 on immunocompetent cells including peripheral blood mononuclear cells (PBMC) and myelin basic protein (MBP)-reactive T cell lines isolated from normal healthy subjects and relapsing-remitting MS patients with different disease activity and in relationship to any disease modifying drug (DMD).

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2. Materials and methods

2.1. CB2 agonist synthesis

Compound (COR167) was synthesized at the Department of Biotechnology, Chemistry and Pharmacy of the University of Siena according to established procedures (Pasquini et al., 2008).

2.2. Patients and control subjects

Twenty-five patients with definite relapsing-remitting MS (McDonald et al., 2001) were recruited for the study. Fourteen of 25 subjects displayed active disease defined as occurrence of new clinical neurological signs or symptoms not associated with fever and new gadolinium enhancing T1-weighted lesions at brain magnetic resonance imaging (MRI). In patients with active disease, blood samples were collected prior to any treatment with intravenous corticosteroids. Eight of 25 patients were on treatment with disease modifying drugs (DMD) including interferon-beta (7 subjects) and glatiramer acetate (1 subject). None of the remaining 17 patients was on any treatment at the time of blood collection including intravenous corticosteroids within 30 days of the sample collection nor did they receive any DMD in the past time with the exception of one patient voluntarily stopping interferon-beta 1a at 6 months prior to the blood drawing. Twenty-one sex- and age-matched normal healthy subjects (NHS), blood donors at the Blood Transfusion Center of the Medical School of University of Siena, were recruited as healthy control subjects. The demographic and clinical characteristics of the patients and control subjects are summarized in Table 1.

All patients and healthy subjects gave informed consent. This study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of the Medical School of the University of Siena.

2.3. Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated by standard density gradient centrifugation procedures on Ficoll-Hypaque from NHS as well as from MS patients. After isolation, PBMC were stimulated with phytohemagglutinin-L (PHA-L) (5 μg/ml) for 72 h. In all assays, unstimulated cultures were prepared. MBP-reactive T cell lines were prepared from 4 MS patients and 4 healthy blood donors according to our previously established method (De Santi et al., 2009).

2.4. Cytotoxicity assay

To test whether test compound and the respective vehicle (0.1% DMSO in 0.9% NaCl) might cause cytotoxic effects on PBMC, preliminary cytotoxic assay was performed at different compound concentrations ranging between 10^{-5} M and 10^{-9} M. Cell viability was assessed with a previously described MTT assay (Tada et al., 1986).

Table 1
Clinical and demographic characteristics of the cohorts.

	MS (n = 25)	NHS (n = 21)
Gender (F/M)	19/6	16/5
Age (years)	36.4 ± 2	35.4 ± 1.2
Relapsing subjects	14/25	–
Treatment^{+} subjects	8/25	–

The results are expressed a mean ± SEM. F = female subjects; M = male subjects.
MS = multiple sclerosis subjects; NHS = normal healthy subjects.
Treatment^{+} = subjects on treatment with disease modifying drugs (DMD).

2.5. PBMC and T cell line proliferation assay

Proliferation of PBMC cultures (1×10^5 cells/well) and MBP-reactive T cell lines (1×10^5 cells/well) was assessed by a previously established MTT assay suitable for measuring cell proliferation (Hussain et al., 1993) in the presence or absence of COR167 in 0.1% DMSO-0.9% NaCl added to the cultures 30 min prior to stimulation with PHA-L or specific pulse with human MBP (25 μg/ml), respectively (Sigma, St. Louis, USA). To test whether the eventual ability of COR167 to inhibit T cell line proliferation is due to its binding to CB2 receptor, all assays were also performed in the presence of the commercially available CB2 antagonist AM630 (10 μg/ml) (Tocris Bioscience, Europe) added 15 min prior to addition of COR167. To rule out the eventual role of CB1 receptor, possibly expressed on immunocompetent cells, all assays were performed in the presence of the commercially available CB1 antagonist AM251 (10 μg/ml) (Tocris Bioscience) added 15 min prior to addition of COR167. In all assays, a wide concentration curve of COR167 ranging10^{-9} M to10^{-5} M was used. Other concentrations outside this range were not considered because very far from the Ki value of 6.3 nM reported for COR167 to displace [^{3}H]CP-55,940 from CB2 receptors (Pasquini et al., 2008). The results are expressed as inhibition of proliferation rate calculated with the following formula:

100−(Absorbance COR167^{+}−PHA
−stimulated PBMC or COR167^{+}−MBP
−stimulated MBP−reactive T cell lines
−absorbance unstimulated PBMC or unstimulated MBP
−reactive T cells)/(Absorbance PHA−stimulated PBMC or MBP
−stimulated MBP−reactive T cell lines
−absorbance unstimulated PBMC or unstimulated MBP
−reactive T cells) × 100.

2.6. Comparative analysis of inhibition of T cell proliferation assay

Proliferation of PHA-stimulated PBMC cultures was assessed respectively by previously established MTT assay (Hussain et al., 1993) in the presence or absence of COR167 or JWH133 (Tocris Bioscience) used as reference CB2 agonist, in 0.1% DMSO in 0.9% NaCl added to cultures 30 min prior to stimulation with PHA-L.

2.7. Immunoblotting detection of CB2 expression

To test whether COR167 could change CB2 receptor expression, western blot analysis was performed. Briefly, PBMC, previously stimulated with PHA-L and MBP-reactive T cell lines (stimulated with at least two consecutive MBP pulses), in the presence or absence of COR167 10^{-5} M and the CB2 antagonist AM630 (10 μg/ml), after washing in cold phosphate buffered saline (PBS) pH 7.4 were lysed on ice for 30 min in buffer containing 50 mM Tris-HCl pH 6.8, 2 mM EDTA, 1% aprotinin and 2 mM PMSF. Lysates were centrifugated at 12,000 rpm for 10 min to remove small cell debris, proteins measured and 40 μg of proteins was then separated by 10% Tricine-SDS-polyacrylamide gel electrophoresis (Schagger and von Jagow, 1987). The proteins were then electro-transferred to a nitrocellulose sheet by a Bio-Rad apparatus and, after overnight blocking at 4 °C with 50 mM Tris-buffered saline (TBS) pH 7.5 containing 3% bovine serum albumin (BSA), the membrane was exposed for 2 h at room temperature (RT) with mouse monoclonal antibody anti-human β-tubulin (2 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After four washes with TBS-0.05% Tween 20, the nitrocellulose sheet was then incubated with rabbit polyclonal antibody anti-human cannabinoid CB2 receptor (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) (1:250 in TBS-0.5% BSA) overnight at 4 °C. The membrane was then washed four times with TBS-0.05%