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# A CB2 Receptor Agonist Reduces the Production of Inflammatory Mediators and Improves Locomotor Activity in Experimental Autoimmune Encephalomyelitis

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## Abstract

***Background:*** Cannabinoids (CBs) have been found to regulate the immune system, affect innate and adaptive immune responses, and reduce inflammatory reactions. This study assessed the therapeutic effects of GW-405833 synthetic CB2 agonist on inflammatory factors as well as locomotor activity in experimental autoimmune encephalomyelitis (EAE).

***Methods:*** In this experimental study, 48 adult male C57BL/6 mice were randomly and equally assigned to eight groups. By injecting 250 mg of MOG35-55 peptide, EAE was induced. Every other day for 17 days after EAE onset, EAE-afflicted mice in groups 1–3 received an intraperitoneal injection of GW-405833 at a dose of 3, 10, and 30 mg/kg, respectively. Clinical status and locomotor activity, measured using the beam walking assay, were assessed every other day during the first 17 days after EAE onset. Mice were euthanized in day 17th of treatment and the serum levels of the IL-1ß, IL-12, CRP, and TNF-α proinflammatory cytokines as well as IL-4 and TGF-ß anti-inflammatory cytokines were measured by ELISA method.

***Results:*** Clinical manifestations of EAE in groups 2 and 3 were significantly milder than group 4 and locomotor activity in groups 1–3 was significantly better than group 4 in days 5–17 ($p< 0.05$). GW-405833 also significantly decreased the levels of IL-12, TNF-α, and CRP and significantly increased the levels of IL-4 and TGF-ß but had no significant effects on the level of IL-1ß. GW-405833 was not associated with significant side effects.

**Conclusions:** The CB2 receptor agonist GW-405833, improves clinical conditions and reduces inflammation in mice with EAE.

**Keywords:** Clinical evaluation, Experimental autoimmune encephalomyelitis, GW-405833, Locomotor activity, Multiple sclerosis, Proinflammatory cytokines.

## Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease in which inflammatory lesions damage the myelin sheath of the nerve fibers in the central nervous system (1). Its manifestations are fatigue, numbness, loss of coordination, impaired vision, dizziness, pain, cognitive impairments, depression, and bladder and bowel dysfunction (2). From 1990

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to 2016, the age-standardized prevalence of MS increased by 10.4%, resulting in more than 2.2 million people with MS in the world in 2016 (3).

Some studies reported that the symptoms and severity of MS were milder among those who used cannabis (4, 5). Cannabis contains cannabinoids (CBs) which play roles in pain transmission, pain perception, and neuroinflammation (6). The expression patterns of CB receptors are also indicative of their role in these processes. The expression of CB1 receptor is primarily limited to the central and peripheral nervous systems including primary afferent nociceptive neurons, while the expression of CB2 receptors primarily happens in the cells of the immune system (7). Some studies showed that CB2 receptors are also found in peripheral primary afferent nociceptive neurons. These receptors belong to a superfamily of G protein-coupled receptors (8, 9). CB1 and CB2 receptors share 48% identity at amino acid level. The signals of these receptors inhibit adenylate cyclase through activating pertussis toxin sensitive G proteins and thereby, activate mitogen-activated protein kinases (7).

One of the derivatives of cannabis is tetrahydrocannabinol (THC). Evidence shows that THC, endogenous ligands, and some small synthetic molecules can affect CB receptors (10). *In vivo* and *in vitro* pharmacologic evidence also shows that most these compounds exert their effects through affecting CB1 receptor or both CB1 and CB2 receptors. CB1 receptor agonists have many different therapeutic effects (11). However, they are associated with adverse psychoactive side effects due to the abundant expression of CB1 receptors in the central nervous system. For example, they can cause dry mouth, eye redness, dizziness, anxiety, depression, and drowsiness (12). Therefore, selective CB2 receptor agonists are more preferred for neuroinflammation management (13). A study showed that a non-selective cannabinoid agonist called HU-210 reversed carrageenan-induced edema through CB2 receptor, while the selective CB2 receptor antagonist SR144528 blocked this effect. Moreover, some pain models approved the effectiveness of compounds with greater selectivity for CB2 receptors than CB1 receptor. A study also found that HU-308, a CB2-selective derivative of THC, reduced the inflammation associated with ear swelling caused by arachidonic acid and reduced late phase formalin-induced pain behavior in mice, while the effects of this compound was blocked by SR-144528 (14). It has also been shown that the local or systemic administration of the selective CB2 receptor agonist AM-1241 had anti-inflammatory and analgesic effects against inflammatory and neuropathic pain; meanwhile, the effects of AM-1241 were reversed with selective CB2 receptor antagonists but were not reversed with selective CB1 receptor antagonists (15-17).

GW-405833 is an alternative selective CB2 receptor agonist which can both reduce edema and prevent the oversensitivity associated with intraplantar injection of carrageenan, while these effects were blocked by the CB2 receptor antagonist SR-144528. Evidence shows that the effects of GW-405833 are mediated by CB2 receptors (7). GW-405833 also blocks the activity of myeloperoxidase, reduces the recruitment of leukocytes and neutrophils, and reduces malondialdehyde concentration during carrageenan-induced acute inflammation. It also reverses the serum levels of tumor necrotizing factor alpha (TNF-α) and interleukin (IL) 1 beta (IL-1ß) to their normal levels. These studies confirmed the suppression of inflammation by GW-405833 (18).

Because of its potential anti-inflammatory effects, GW-405833 may be effective in reducing oxidative stress in different inflammatory conditions such as MS. It has been suggested that specific CB2 receptor agonists can be used to prevent the production of both stress oxidative and proinflammatory cytokines in inflammatory conditions (18). Nonetheless, there are no empirical data about the effects of the CB2 receptor agonist GW-405833 on MS-associated symptoms. Therefore, the present study was conducted to assess the therapeutic effects of GW-405833 on proinflammatory and anti-inflammatory factors



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as well as locomotor activity in experimental autoimmune encephalomyelitis (EAE) as a model of MS. EAE is an autoimmune inflammatory demyelinating disease in the central nervous system of rodents.

## Materials and Methods

### *Design*

This experimental study was conducted in the Clinical Biochemistry and the Physiology Departments of the Faculty of Medicine of Kashan University of Medical Sciences, Kashan, Iran.

### *Ethical approval*

The study has been approved by the Ethics Committee of Kashan University of Medical Sciences, Iran and also all experiments were reviewed and approved following the current European Union Directive (2010/63/EU) guideline on the protection of animals used for scientific purposes.

### *Experimental animals*

Forty-eight adult male C57BL/6 mice aged 6–8 weeks and weighed 16–22 grams were purchased from the Pasteur Institute of Iran, Tehran, Iran. They were housed and maintained in pathogen-free animal laboratory conditions with a humidity of 50%–70%, a temperature of 25±0.5 °C, and 12/12 light/dark cycles (07:00–19:00).

### *Induction of EAE*

EAE was induced using myelin oligodendrocyte glycoprotein peptide MOG35-55 (NH2–MEVGWYRSPFSRVVHLYRNGK–OH; ALEXIS). For injection, this peptide was emulsified with complete Freund's adjuvant (CFA; Sigma-Aldrich) containing *Mycobacterium tuberculosis* bacteria (strain H37Ra). Each mouse was immunized in day 1 through a subcutaneous injection in the flank with the emulsion contained MOG 250 micrograms prepared in 150 micro-liters of phosphate-buffered saline (PBS) and 150 microliters of CFA and then, 400 nanograms of pertussis toxin diluted in 100 micro-liters of sterile PBS was injected intraperitoneally (PTX; Sigma-Aldrich). In day 3, the booster injection of pertussis toxin (PTX) 400 nanograms was given (19, 20).

### *Clinical evaluation of EAE mice*

After the first injection, mice were daily assessed. The clinical manifestations of EAE appeared 22±2 days after MOG injection. Clinical manifestations were scored as follows: zero: "No clinical manifestation"; 0.5: "Partial tail weakness or slight loss of muscle tone"; 1.0: "Tail weakness"; 1.5: "Slightly clumsy gait"; 2.0: "Hind limb paresis"; 2.5: "marked hind limb paresis and partial dragging of the hind limbs"; 3: "Hind limb paralysis"; 3.5: "Hind limb paralysis and forelimb paresis"; 4.0: "Complete paralysis (tetraplegia)"; and 5.0: "Moribund/dead" (21). Mice with scores 1 or greater were included in the study and assessed respecting clinical scores and locomotor activity every other day for seventeen consecutive days (i.e. in days 1, 3, 5, 7, 9, 11, 13, 15, and 17). In each assessment day, each mouse was assessed three times and the total mean score was documented. Mice were sacrificed seventeen days after the onset of EAE signs and blood samples were collected.

### *Treatment of animals*

In order to assess the effects of GW-405833 on the course of EAE, 48 mice were randomly and equally assigned to eight groups. Every other day during the first seventeen days after the appearance of EAE manifestations, mice in groups 1–3 (the intervention groups) received GW-405833 at a dose of respectively 3, 10, and 30 milligrams per kilogram (mg/kg) of body weight with 100-microliter PBS. In group 4 (the control group), mice solely received PBS 100 microliters every other day for seventeen days. Mice in group 5 were healthy, did not receive any injection, and were assessed for seventeen days. Mice in groups 6–8 were healthy and received GW-405833 at a dose of respectively 3, 10, and 30 mg/kg with 100-microliter PBS. Groups 6–8 were



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considered for assessing the side effects of GW-405833.

### *Beam walking assay*

The beam walking assay was used to assess motor coordination and balance of all mice in all study groups every other day during the first seventeen days after the appearance of EAE manifestations. In this test, each mouse is placed on an elevated narrow beam to pass it to a safe platform at its end. Balance beams in this study were square and made out of medium-density fiberboard (MDF) in four widths, namely 9, 12, 15, and 18 millimeters with a length of 100 centimeters. Beams were mounted on an elevation of forty centimeters from the ground with a pad under them to cushion any fall.

Ability to pass the beams was assessed using the following scoring system: zero: "Complete inability to walk on the beam"; 0.5: "Ability to pass half of the beam despite problems in hind limb and forelimb"; 1.0: "Ability to pass the whole beam despite problems in hind limb and forelimb"; 1.5: "Partial ability to pass the beam without fall despite problems in hind limb"; and 2.0: "Normal weight support and accurate foot placement". Each mouse passed each beam three times and the mean score of the three assessments was documented. The total possible score of the four beams was 0–8 (22, 23).

### *Measuring the concentration of cytokines using enzyme-linked immunosorbent assay (ELISA)*

After seventeen-day treatment, animals were sacrificed and the serum levels of the IL-1ß, IL-12, C-reactive protein (CRP), and TNF-α proinflammatory cytokines as well as IL-4 and transforming growth factor beta (TGF-ß) anti-inflammatory cytokines were measured using sandwich ELISA kits (Axxora Co.) according to the manufacturer's instruction.

### *Statistical analysis*

The SPSS software was used for data analysis. Between-group mean comparisons were made using the one-way analysis of variance (ANOVA) with the post hoc Tukey's test. The variations of disease severity across the measurement time points were also tested through the repeated measures ANOVA. The level of significance was set at less than 0.05.

**Table 1.** Within- and between-group comparisons respecting the means of clinical and beam walking scores.

| Outcome | Time / Group | Day 1 | Day 3 | Day 5 | Day 7 | Day 9 | Day 11 | Day 13 | Day 15 | Day 17 | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Clinical score | 1: EAE+3mg | 1.52±0.2 | 1.58±0.2 | 1.65±0.17 | 1.58±0.08 | 1.25±0.28 | 1.41±0.15 | 1.36±0.24 | 1.5±0.05 | 1.45±0.07 | 0.02†<br>0.014‡ |
| | 2: EAE+10mg | 1.66±0.16 | 1.66±0.16 | 1.5±0.05 | 1.33±0.10 | 1.16±0.24 | 1±0.15 | 0.8±0.25 | 0.7±0.15 | 0.8±0.11 | 0.07†<br>0.007‡ |
| | 3: EAE+30mg | 1.41±0.15 | 1.33±0.21 | 1±0.12 | 1.08±0.15 | 1±0.12 | 0.8±0.18 | 0.83±0.16 | 0.91±0.2 | 0.83±0.08 | 0.03†<br>0.00‡ |
| | 4: EAE+PBS | 1.51±0.10 | 1.64±0.17 | 1.75±0.14 | 1.7±0.10 | 1.78±0.10 | 1.47±0.13 | 1.45±0.09 | 1.65±0.23 | 1.65±0.23 | 0.02† |
| | **P value** | 0.91#<br>0.080^<br>0.20* | 0.58#<br>0.83^<br>**0.018*** | 0.29#<br>**0.002**^<br>**< 0.001*** | **0.04**#<br>**< 0.001**^<br>**< 0.001*** | **< 0.001**#<br>**< 0.001**^<br>**< 0.001*** | 0.47#<br>**< 0.001**^<br>**< 0.001*** | 0.41#<br>**< 0.001**^<br>**< 0.001*** | 0.14#<br>**< 0.001**^<br>**< 0.001*** | 0.06#<br>**< 0.001**^<br>**< 0.001*** | — |
| Beam walking | 1: EAE+3mg | 1.58±0.09 | 1.60±012 | 1.77±0.12 | 1.54±0.11 | 1.69±0.1 | 1.74±0.14 | 1.59±0.17 | 1.67±0.13 | 1.60±0.13 | 0.00†<br>0.007‡ |
| | 2: EAE+10mg | 1.57±0.08 | 1.65±0.1 | 1.76±0.13 | 1.69±0.07 | 1.73±0.12 | 1.64±0.15 | 1.55±0.16 | 1.59±0.1 | 1.58±0.15 | 0.00†<br>0.00‡ |
| | 3: EAE+30mg | 1.54±0.05 | 1.63±0.05 | 1.66±0.1 | 1.67±0.09 | 1.67±0.09 | 1.73±0.11 | 1.82±0.14 | 1.82±0.14 | 1.75±0.10 | 0.00†<br>0.00‡ |
| | 4: EAE+PBS | 1.5±0.04 | 1.5±0.05 | 1.56±0.14 | 1.54±0.08 | 1.54±0.1 | 1.49±0.07 | 1.35±0.09 | 1.36±0.13 | 1.36±0.08 | 0.00† |
| | **P value** | 0.0747#<br>0.0842^<br>0.1570* | 0.89#<br>**0.01**^<br>**< 0.001*** | **0.01**#<br>**0.02**^<br>0.18* | 1.00<br>**0.01**^<br>**0.02*** | **0.01**#<br>**0.02**^<br>**0.03*** | **0.01**#<br>0.507^<br>**< 0.001*** | **0.01**#<br>**0.02**^<br>**< 0.001*** | **0.01**#<br>**< 0.001**^<br>**< 0.001*** | **< 0.001**#<br>**0.01**^<br>**< 0.001*** | — |

#: Comparison between groups 1 and 4; ^: Comparison between groups 2 and 4; *: Comparison between groups 3 and 4.
†: The results of within-group comparisons; ‡: The results of between-group comparisons.



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## Results

### *GW-405833 reduces the clinical manifestations of EAE*

The severity of clinical signs in groups 2 and 3 was significantly less than group 4 ($p< 0.05$; Table 1). In group 3, the severity of clinical manifestations reduced by 29%–41% in days 5–17 of treatment.

### *GW-405833 improves the scores of the beam walking assay among mice with EAE*

The mean score of the beam walking assay in groups 1–3 was significantly greater than group 4 ($p< 0.05$; Table 1).

### *GW-405833 reduces the serum levels of proinflammatory cytokines*

The serum levels of TNF-α and CRP in groups 1–3 were significantly less than group 4 ($p< 0.05$; Tables 1 and 2). Moreover, the serum level of IL-12 in groups 2 and 3 was significantly less than group 4 ($p< 0.05$), while the serum level of IL-12 in group 1 did not significantly differ from group 4 ($p> 0.05$; Table 2). The serum level of IL-1ß in groups 1–3 did not significantly differ from group 4 ($p> 0.05$; Table 2). The serum levels of proinflammatory cytokines among healthy mice are also shown in Table 2.

**Table 2.** Between-group comparisons respecting the mean scores of proinflammatory and anti-inflammatory cytokines.

| Outcome / Group | TNF-α | IL-12 | CRP | IL-1 | TGF-ß | IL-4 |
|---|---|---|---|---|---|---|
| 1: EAE+3mg | 203.4±15.3 | 178.83±23.3 | 225.8±11.8 | 880.5±48.3 | 125.5±5.0 | 409.3±10.8 |
| 2: EAE+10mg | 209.2±8.7 | 173.6±16.4 | 187±14.6 | 866.4±38.1 | 137.8±6.2 | 382±18.9 |
| 3: EAE+30mg | 170.2±9.2 | 155.4±14.4 | 160.2±5.1 | 845.4±67.1 | 149.7±8.5 | 436.4±16.8 |
| 4: EAE+PBS | 234.4±14.6 | 194.2±9.8 | 276.5±11.8 | 882±19.7 | 95.2±8.6 | 351.6±11.3 |
| 5. Healthy | 163.3±12.8 | 134.5±7.9 | 142.4±9.6 | 516.8±6.2 | 91.1±7.8 | 350.66.1±15.3 |
| p value | **0.01**#<br>**< 0.001**^<br>**< 0.001*** | 0.1672#<br>**0.02**^<br>**< 0.001*** | **< 0.001**#<br>**< 0.001**^<br>**< 0.001*** | 0.94#<br>0.39^<br>0.22* | **< 0.001**#<br>**< 0.001**^<br>**< 0.001*** | **< 0.001**#<br>**0.01**^<br>**< 0.001*** |

#: Comparison between groups 1 and 4; ^: Comparison between groups 2 and 4; *: Comparison between groups 3 and 4.

### *GW-405833 increases the serum levels of anti-inflammatory cytokines*

The levels of both IL-4 and TGF-ß in groups 1–3 were significantly greater than group 4 (P 0.05; Table 2). The levels of IL-4 and TGF-ß in group 3 were also significantly greater than all other groups ($p< 0.05$; Table 2). The serum levels of anti-inflammatory cytokines among healthy mice are also shown in Table 2.

### *Side effects of GW-405833*

Groups 6–8 were considered for assessing the side effects of GW-405833. Mice in these groups were healthy but received GW-405833 at a dose of 3, 10, and 30 mg/kg, respectively. No significant side effects of GW-405833 were observed in these groups.

## Discussion

This study assessed the therapeutic effects of GW- 405833 on proinflammatory and anti-inflammatory cytokines and locomotor activity among mice with EAE. Findings showed the positive effects of the 3-, 10-, and 30-mg/kg doses of GW-405833 on locomotor activity and cytokine profile in EAE.

Findings showed that GW-405833 at doses 10 and 30 mg/kg significantly reduced the mean scores of clinical manifestations of EAE. Moreover, GW-405833 at 3, 10, and 30 mg/kg doses significantly improved the mean score of beam walking assay. In line with our findings, a former study showed that the administration of cannabis extracts significantly improved neurologic status in the relapse phase of EAE (24). Another study found that Dexanabinol, a synthetic cannabinoid, significantly reduced inflammatory responses in the brain and the spinal cord in animals and thereby, reported that Dexanabinol may be an alternative treatment for managing the acute



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exacerbations of MS (25). Beam walking is an appropriate method for assessing motor coordination and balance and is considered as a sensitive test for assessing the ability to keep balance among animals with EAE. The successful performance of this assay necessitates the complex function of neural networks in the brain including inter-hemispheric communication.

The significant positive effects of GW-405833 on locomotor activity are mediated through CB2 receptors (26). Evidence shows that the expression of CB2 receptors in the spinal cord increases after peripheral nerve injuries in mice (27). Therefore, administration of CB2 receptor agonists can reduce pain and inflammation (18). A study showed that intraperitoneal administration of selective CB2 receptor agonists reduced pain and inflammation in animals with peripheral neuropathic pain, while these effects were not observed in animals with CB2 knock out (7). The present study showed the same results in an EAE model of MS. We found that GW-405833 significantly reduced inflammation among mice with EAE in a dose-dependent manner. A study showed that MOG35-55 increased CB2 mRNA and protein in the spinal cord of animals with EAE and another study showed that CB2 reactivity was significantly up-regulated in the site of spinal injuries in patients with MS. The positive effects of GW-405833 on inflammation in the present study are consistent with anti-inflammatory and analgesic effects of cannabis observed among cannabis abusers with MS.

Our findings also showed that GW-405833 at a dose of 30 mg/kg significantly reduced the serum levels of IL-12 and TNF-α proinflammatory cytokines. Cytokines play significant roles in the pathogenesis and alleviation of MS and EAE. In the acute phase of EAE, lymphotoxin and IL-12 are the first cytokines and IL-6 and TNF-α are the second cytokines which appear in the central nervous system and the level of their expression is in accordance with clinical manifestations and infiltration of inflammatory cells (28). IL-12 is the main enhancer of T helper type 1 (Th1) cells and increases the production of cytokines such as TNF-α. Studies showed that the production of IL-12 increases immediately before the onset of EAE signs. Moreover, IL-12 increases EAE severity, while its reduction suppresses EAE (29, 30). The regulation of Th1 cell differentiation through the blocking of IL-12 or IL-12 receptors may have significant role in regulating the mechanism of EAE (31). Therefore, as shown in the present study, GW-405833 may reduce EAE severity through inhibiting TNF-α.

Study findings also showed that all three doses of GW405833 (i.e. 3, 10, and 30 mg/kg) significantly reduced the serum level of IL-4. IL-4 is an anti-inflammatory immune factor. Both IL-4 and IL-10 are correlated with protection against EAE and their levels increase in the central nervous system and lymphatic cells during recovery. Moreover, treatment with IL-4 is correlated with disease amelioration and remission. Much evidence shows that THC injection can affect the function of CB1 and CB2 receptors, reduce the production of IL-12 and the activity of IL-12 receptors, and thereby, suppress Th1 cell activity (32). GW-405833 is similar to THC and hence might have reduced EAE manifestations in the present study through the same mechanism. CB1 receptors may affect Th1 cells through the hypothalamus-pituitary-adrenal axis (33). On the other hand, expressed CB2 receptors in immune cells may regulate peripheral cytokines and Th1 cell maturation (34). Most animal experiments showed that CB-mediated immunosuppression is dose-dependent and is mostly attributable to the activation of CB2 receptors (35, 36). CB receptors are specifically expressed in peripheral cells such as lymphocytes, macrophages, and mast cells. Therefore, as macrophages play significant roles in the presentation of antigens and production of positive and negative regulatory proteins, GW-405833 at a dose of 30 mg/kg can suppress the immune system through inhibiting the activity of macrophages.

The CB2 receptor agonist GW-405833, particularly at a dose of 30 mg/kg, improves



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clinical conditions, regulates cytokine profile, and reduces inflammation in mice with EAE without exerting any significant side effect. Therefore, it can be considered as a potential treatment for MS. Studies into the effects of the simultaneous use of GW-405833 and CB2 receptor antagonists can provide better understanding about the mechanism of action of GW-405833.

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