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Article

# Therapeutic modulation of neuroinflammatory and apoptotic pathways by PEPITEM in an EAE model of multiple sclerosis

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**Abstract: Objective:** To examine the therapeutic effects of PEPITEM on neuroinflammatory and apoptotic pathways in an Experimental Autoimmune Encephalomyelitis (EAE) model of Multiple Sclerosis (MS), focusing on the modulation of key biomarkers: SIRT1, NRF2, NF-κB p65, Bax, and Bcl2. **Methods:** We utilized a controlled experimental design involving five groups of female C57BL/6 mice, aged 9–12 months to assess the effects of PEPITEM administered therapeutically and prophylactically. Groups included a normal healthy mice group (G1), an EAE-induced group receiving scrambled peptide therapeutically (post-induction) (G2), an EAE-induced group treated with PEPITEM therapeutically (G3), and an EAE-induced group given scrambled peptide (G4) prophylactically or, an EAE-induced group treated with PEPITEM prophylactically (G5). Following induction and PEPITEM treatment, weight and EAE scores were compared among the designated groups. Additionally, spinal cord tissues were harvested for protein lysate preparation and Western blot analysis quantified the expression levels of the selected biomarkers. **Results:** Analysis of the weight and EAE scores reveals that G3 and G5 exhibit trends toward recovery, potentially indicating the effectiveness of the treatment. Moreover, PEPITEM treatment significantly upregulated the expression of SIRT1 and NRF2, suggesting an enhanced neuroprotective and antioxidant response. Conversely, NF-κB p65 and Bax levels were notably decreased, indicating a suppression of inflammatory and apoptotic pathways. Additionally, Bcl2 expression was significantly increased, highlighting a shift toward cell survival mechanisms. **Conclusion:** Our findings demonstrate that PEPITEM exerts a multifaceted therapeutic effect in the EAE model of MS by mitigating the symptoms of EAE as evidenced by modulating crucial biomarkers involved in neuroprotection, inflammation, and apoptosis. The significant alterations in the expression of the biomarkers highlight the potential of PEPITEM as a promising therapeutic agent for MS, offering insights into its mechanism of action and paving the way for future clinical investigations.

**Keywords:** multiple sclerosis; PEPITEM; neuroinflammation; apoptosis; EAE model; biomarkers; SIRT1; NRF2; NF-κB; Bax; Bcl2

## 1. Introduction

Multiple sclerosis (MS) is a chronic, often disabling disease that attacks the central nervous system (CNS), comprising the brain, spinal cord, and optic nerves. It is characterized by the immune-mediated destruction of the myelin sheath, the protective covering of nerve fibers, which leads to impaired transmission of nerve signals and a multitude of neurological symptoms [1]. Despite advances in understanding the disease, MS remains incurable, and its etiology is not fully understood, presenting a significant challenge for the development of effective

therapies [2]. The search for reliable biomarkers in MS research is of paramount importance, as they hold the potential for early diagnosis, prognostic predictions, and monitoring therapeutic responses [3]. Biomarkers that reflect the underlying pathology could develop the management of MS by allowing for personalized therapeutic strategies and real-time assessment of disease dynamics [4,5]. Animal models, particularly experimental autoimmune encephalomyelitis (EAE), have been instrumental in MS research, providing a platform for dissecting the complex immunopathogenic mechanisms and testing novel therapeutic approaches [6]. EAE replicates several key aspects of MS, including CNS inflammation, demyelination, and neurodegeneration, making it an essential model for studying these complex interactions within the CNS environment [7]. The insights gained from EAE models have been pivotal in shaping our understanding of MS and have driven the discovery of several therapeutic agents currently in use [8].

PEPITEM is an endogenous immunomodulator that has shown promising anti-inflammatory effects in EAE models. Recent data suggest that PEPITEM not only attenuates the inflammatory response of MS but also modulates a range of molecular pathways relevant to the disease process [9,10]. In an interesting study, Pezhman et al. [11] investigate the immunomodulatory effects of PEPITEM on systemic inflammation induced by obesity in mice, highlighting its potential as a therapeutic agent. Administered both prophylactically and therapeutically, PEPITEM reduced the size of pancreatic beta cells and limited the migration of T-cells and macrophages in visceral adipose tissue, demonstrating its potential as a novel therapy to reduce obesity-associated low-grade systemic inflammation and its metabolic consequences [11]. Another study examines the potential of PEPITEM as a therapeutic intervention in rheumatoid arthritis (RA), the authors identifies a defect in the adiponectin-PEPITEM pathway in RA patients, characterized by impaired suppression of T-cell migration due to reduced adiponectin receptor expression on B-cells. Administration of synthetic PEPITEM both prophylactically and therapeutically in a murine model of collagen induced arthritis (CIA) significantly ameliorated disease symptoms, including reducing leukocyte infiltration and bone erosion, suggesting the re-establishment of this pathway could be a promising treatment for RA [12]. The most recent study by Lewis et al. [13] reveals the anabolic osteogenic capabilities of PEPITEM in promoting bone growth and curbing bone loss. The study demonstrates for the first time PEPITEM direct action on osteoblasts, enhancing their maturation and boosting trabecular bone growth through NCAM-1 signaling. Additionally, PEPITEM induces an inhibitory loop by stimulating osteoblasts to release osteoprotegerin, thereby reducing osteoclast activity and overall bone resorption, highlighting its potential as a novel therapeutic avenue for bone repair and the management of bone loss disease [13].

These findings have opened new opportunities for the exploration of PEPITEM as a potential therapeutic candidate, with implications that extend beyond symptomatic relief to modifying the disease course itself. The analysis of protein expression patterns in disease models is fundamental to understanding the molecular foundations of pathogenesis and the identification of potential therapeutic targets [14]. Physiologically, certain marker proteins stand out for their key roles in regulating key biological processes. Sirtuin 1 (SIRT1) is well-known for its involvement in aging,

metabolism, and stress resistance, acting through the deacetylation of various substrates to influence cellular longevity and metabolic balance [15,16]. Similarly, Nuclear factor erythroid 2-related factor 2 (NRF2) serves as a guardian against oxidative stress, regulating the expression of antioxidant defenses and thus protecting cellular integrity [17]. The NF-κB p65 subunit, part of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) complex, plays a critical role in inflammatory responses and immune regulation, highlighting its importance in conditions characterized by chronic inflammation and autoimmunity [18]. Conversely, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) are key regulators of apoptosis, with Bcl-2 acting to inhibit cell death and Bax promoting it [19–21]. These markers not only offer insights into the mechanisms of cell survival and death but also underscore the delicate balance that governs cellular fate under stress. Together, SIRT1, NRF2, NF-κB p65, Bax, and Bcl-2 encapsulate a complex network of signaling pathways integral to understanding and manipulating cellular responses in health and disease. In the context of EAE, an established model for MS, we chose key proteins such as SIRT1, NRF2, NF-κB p65, Bax, and Bcl2 to study the immunomodulation function of PEPITEM on these markers to provide a significant insight into the disease's molecular characteristics and potential therapeutic interventions.

This study aims to study the modulation of key proteins SIRT1, NRF2, NF-κB p65, Bax, and Bcl2 in the EAE model following PEPITEM treatment therapeutically and prophylactically. By examining the influence of PEPITEM on these biomarkers, we aim to provide a deeper understanding of its therapeutic mechanisms and further establish the relevance of these proteins as biomarkers in MS research. The outcomes of this study have the potential to enhance the predictive power of these biomarkers, facilitate early intervention, and ultimately contribute to the development of precision medicine approaches for MS management.

## 2. Materials and methods

### 2.1. Ethics approval

The Institutional Animal Care and Use Committee (IACUC) at King Abdullah International Medical Research Center (KAIMRC) (RC-19/084/R) approved the experimental design, and the Research Ethics Committee in King Saud University (KSU) (KSU-SE-19-10) approved it.

### 2.2. Animal

This study utilized five groups of female C57BL/6 mice, aged 9–13 weeks, to establish an EAE model, which closely mimics human multiple sclerosis. The mice were obtained from Experimental Surgery Center in King Saud University's (KSU), Riyadh, Saudi Arabia. The mice were housed in groups within standard cages equipped with adequate bedding and nesting supplies. The ambient conditions were regulated to keep the temperature at 22 ± 2 ℃ and the humidity at approximately 50 ± 10%. A steady 12-hour cycle of light and darkness was maintained, with lights on from 7:00 a.m. to 7:00 p.m. Continuous access to water, a water gel placed at the

bottom of the cage, and food was provided. A veterinarian conducted daily assessments of their weight and general health.

## 2.3. Experimental design

The groups were divided as follows: G1 served as the healthy control; G2 represented the EAE-induced group receiving a scrambled peptide from day 10 ±1 post-induction; G3 was the EAE-induced group receiving PEPITEM treatment from day 10 ±1; G4 included EAE-induced mice receiving a prophylactic scrambled peptide from day 0; and G5 included of EAE-induced mice receiving prophylactic PEPITEM from day 0.

**Table 1.** Experimental design.

| Group ID | Group condition |
|---|---|
| G1 | Healthy mice |
| G2 | EAE-induced group receiving scrambled peptide (Therapeutically) |
| G3 | EAE-induced mice treated with PEPITEM therapeutically (Therapeutically) |
| G4 | EAE mice given scrambled peptide prophylactically (prophylactically) |
| G5 | EAE-induced mice treated with PEPITEM prophylactically (prophylactically) |

## 2.4. EAE induction and PEPITEM administration

EAE was induced in the mice using a standard protocol from Hooke Laboratories involving the injection of myelin oligodendrocyte glycoprotein (MOG) peptide emulsified in complete Freund's adjuvant, followed by pertussis toxin administration (Cat EK-2110, Hooke Laboratories, LLC, Lawrence, MA, USA). Briefly, to induce the disease, EAE kits supplied by Hooke Laboratories were used. These kits were freshly prepared, and kept refrigerated at 2-8 degrees Celsius. Following the manufacturer's protocol, immunization of the mice was achieved by injecting an emulsion of MOG35-55 peptide in complete Freund's adjuvant (CFA). Additionally, all mice were administered two doses of pertussis toxin (PTX) in PBS on consecutive days, day 0 and day 1. Specifically, the antigen emulsion was administered subcutaneously at two sites along the dorsal midline, upper and lower back, using a mouse restraint cage, with each site receiving 0.1 mL for a total of 0.2 mL per mouse. Two hours post-emulsion injection, PTX toxin was prepared ~~a~~fresh, diluted according to the manufacturer's recommendations, and given intraperitoneally (IP) in 0.1 mL doses on both days. G1 mice received injections of normal saline.

Since the study is both therapeutic and prophylactic, the daily injections started on day 0 for the G4 and G5 groups, whereas; the daily injections started on day 10 ±1 post-induction for G2 and G3, when the first sign of inflammation appeared until the end of the experiment on day 21. Treatment with PEPITEM or scrambled peptide was administered according to the group designations. PEPITEM was administered IP at a dose optimized from our published established protocols (concentration of 100 mg/mL; 200 µL total volume per injection) [9,10]

## 2.5. Animal observation

Mice were observed daily to assess variations in weight, EAE score, behavior, occurrences, and mortality rates. The scoring system for EAE, adapted from the manual of the Hooke Laboratories induction kit, was applied. Scores were recorded daily by both the animal care staff and the research team. A score of 0 was indicative of no evident symptoms, 0.5 suggested minor tail weakness, 1 was for total loss of tail muscle tone, 1.5 indicated a combination of tail limpness and an unsteady gait, 2 was for limp tail with some hind leg mobility restriction, 2.5 showed tail limpness with noticeable weakness in the hind legs, 3 was for complete paralysis of both the tail and hind legs, 3.5 denoted the inability of the mouse to correct its posture when placed on its side, combined with tail and hind leg paralysis, 4 was for full paralysis of the tail and hind legs along with some front limb involvement, and a score of 5 was given for severe total paralysis or death, at which point euthanasia was advised.

## 2.6. Harvesting of spinal cord tissue and lysate preparation

At the experimental endpoint, mice were euthanized through an anesthesia overdose, and spinal cord tissues were carefully extracted. The tissues were then homogenized in a lysis buffer containing protease and phosphatase inhibitors to prepare the lysates (The Protease/Phosphatase Inhibitor Cocktail (100X) (Cell Signaling technology, Danvers, MA, USA, Cat No. 5872). The homogenates were centrifuged, and the supernatant containing soluble proteins was collected for analysis.

## 2.7. Protein quantification and western blot analysis

Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Cat. 23225, Thermo Fisher Scientific (Waltham, Massachusetts, United States). The experimental procedure began by subjecting 1 µg of mouse spinal cord homogenate to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. The proteins were then transferred onto a PVDF membrane (GE Healthcare, Germany, Catalog No. 10600021). The membrane underwent blocking with 5% skimmed milk for 60 minutes, followed by overnight incubation at 4°C with primary antibodies, including anti-beta Actin (1:2000 dilution, Cat. Sc-8432, Santa Cruz Biotechnology, TX, USA), SIRT1 Antibody (1:1000 dilution, Cat. Sc-74465, Santa Cruz Biotechnology, TX, USA), NRF2 (1:1000 dilution, Cat. Sc-365949, Santa Cruz Biotechnology, TX, USA), NF-κB p65 (1:2000 dilution, Cat. Sc-8008, Santa Cruz Biotechnology, TX, USA), Bax (1:1000 dilution, Cat. Sc-20067, Santa Cruz Biotechnology, TX, USA), and Bcl2 (1:1000 dilution, Cat. Sc-7382, Santa Cruz Biotechnology, TX, USA) diluted in blocking solution. After three 5-minute washes, the membrane was incubated with goat anti-mouse secondary antibodies at a 1:10,000 dilution. Following four additional 5-minute washes, the membrane was exposed to an ECL western blotting substrate for 2 minutes. The protein bands were visualized using a ChemiDoc Touch imaging system from Bio-Rad, Hercules, California, USA. Semi-quantitative data analysis was conducted using Image Lab software. For optimization purposes, we run two controls with each gel, which explains the six bands on the gel.

## 2.8. Statistical analysis

The results were expressed as mean values ± standard error of the mean (SEM), analyzed with GraphPad Prism 8 software (GraphPad Prism Inc., La Jolla, CA, USA). The analysis is done using students-test. A p-value of less than 0.05 was considered statistically significant.

# 3. Results

## 3.1. PEPITEM exhibits neuroprotective and anti-inflammatory efficacy in experimental autoimmune encephalomyelitis model of multiple sclerosis

### 3.1.1. Body weight

G1 serves as a baseline for healthy weight gain. Control groups G2 and G4, treated with scrambled peptide therapeutically or prophylactically. G3 and G5 treated with PEPITEM therapeutically or prophylactically. G1 shows a consistent upward trend in weight over the 19 days, indicating healthy growth. Regarding the therapeutic effect of PEPITEM, G3 shows a decline in weight but then a more significant and sustained recovery after PEPITEM treatment is administered. G2 (EAE + Scrambled Peptide Post-Induction), there appears to be a sharp decline in weight initially, followed by a period of fluctuation and then a slight recovery towards the end. The initial decline may indicate the impact of EAE on the mice, and the scrambled peptide seems not to have a stabilizing effect. This suggests that PEPITEM has a therapeutic effect when given post-induction. In case of Prophylactic PEPITEM administration, G5 shows a modest decline followed by stabilization and recovery of weight, which suggests that prophylactic PEPITEM may help in mitigating the effects of EAE on weight loss compared with it, designated control G4 (**Figure 1**).

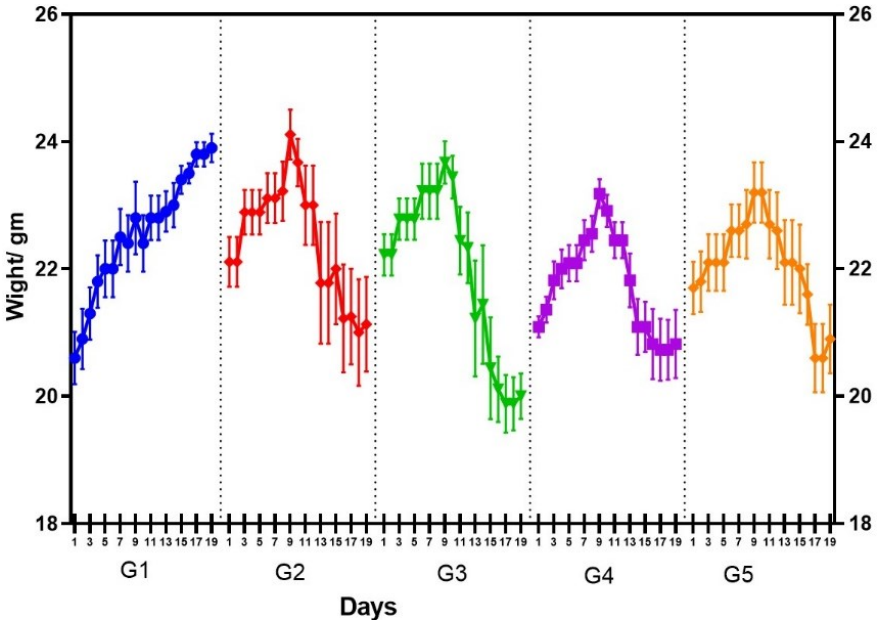


**Figure 1.** Time series analysis of mouse weight across five experimental groups.

The graph describes the daily weight measurements of mice across five distinct experimental groups over a 19-day trial. G1 is represented by blue color, G2 by red color, G3 by green color, G4 by purple color s, and G5 by orange color. Each data point conveys the average weight of the group for the respective day, and the accompanying vertical lines denote the standard deviation, indicating the spread of weights within each group.

### 3.1.2. EAE scoring

The EAE score for G1 remains at 0 throughout the study. Regarding the therapeutic effect of PEPITEM, G2 exhibits a consistent increase in EAE score over time, suggesting a progression of disease symptoms. When compared with G3, after an initial increase, there is a leveling off in the EAE score, suggesting that post-induction treatment with PEPITEM may help stabilize the disease and prevent further progression of symptoms. In case of Prophylactic PEPITEM administration, G5 shows a delayed and more gradual increase in EAE score compared to G4, which could indicate that PEPITEM has a prophylactic effect, potentially slowing down the onset and progression of EAE. For G4 shows, a delayed onset of symptoms compared to G2, but eventually, the EAE score increases similarly (**Figure 2**).

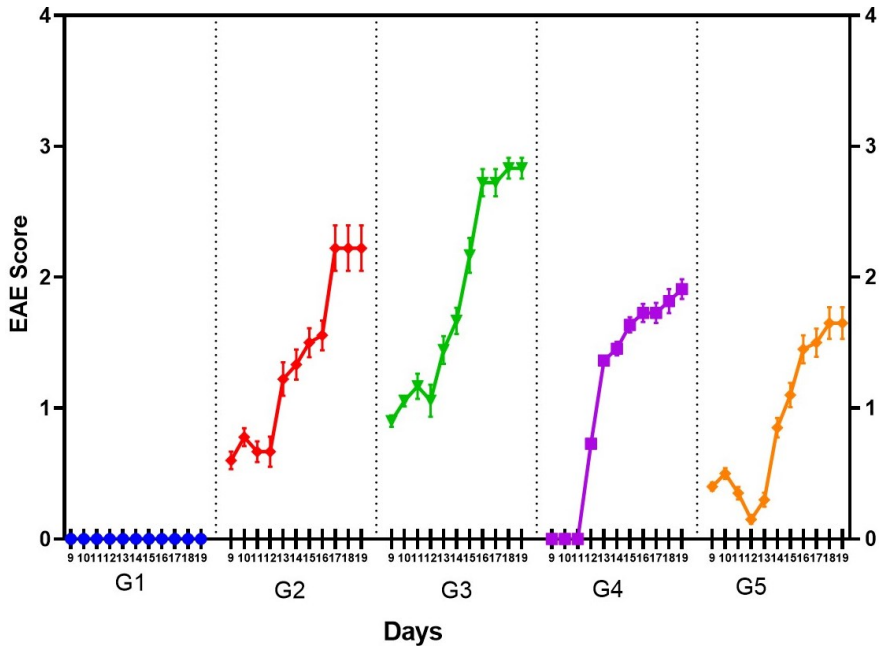


**Figure 2.** Daily progression of mouse EAE scores among five experimental groups.

The graph shows the progression of the Experimental Autoimmune Encephalomyelitis (EAE) score for five groups of mice across a 19-day period, with each group represented by a distinct color. G1 is illustrated with blue color, G2 with red color, G3 with green color, G4 with purple color, and G5 with orange color. The EAE score, an index reflecting the severity of the disease, is plotted on the vertical axis, while the horizontal axis represents the days of the experiment. Each point on the graph reflects the mean EAE score of each group for that day. Scores span from zero (indicating the absence of symptoms) to five (representing severe, complete paralysis or death).

### 3.1.3. Neuroinflammatory and apoptotic markers

To evaluate the impact of PEPITEM on the expression levels of key biomarkers within an EAE mouse model, we focused on the following markers: SIRT1, NRF2, NF-κB p65, Bax, and Bcl-2 proteins**. Figures 3–5** shows the levels of biomarkers protein in the spinal cord sections relative to β-actin for five different experimental groups of mice. The levels of SIRT1 expression between G2 and G3 are very close, indicating a minimal difference that is not statistically significant. This suggests that the effect of therapeutic PEPITEM treatment on SIRT1 protein expression, when compared directly with the scrambled peptide treatment in an EAE model, does not significantly alter the levels of SIRT1 in the spinal cord. Nonetheless, G5 shows a higher level of SIRT1 expression compared to G4. This suggests that prophylactic PEPITEM administration may lead to increased SIRT1 protein levels in the spinal cord (**Figure 3**). In case of Nrf2, the level of Nrf2 expression in G3 is higher than in G2. Similarly, G5 shows a higher level of Nrf2 expression compared to G4. This suggests that the therapeutic and prophylactic treatment with PEPITEM may be beneficial in increasing Nrf2 protein levels in the spinal cord (**Figure 4**). In the same figure, the level of NF-κB p65 expression in G3 is lower than in G2. This suggests that PEPITEM treatment is associated with a decrease in NF-κB p65 protein levels in the spinal cord. The same pattern is seen when comparing level of NF-κB p65 expression in G4 and G5. Hence, therapeutic and prophylactic treatment with PEPITEM is associated with a significant reduction in NF-κB p65 levels in the spinal cord. The levels of protein of Bax and Bcl2 in the spinal cord sections for five different experimental groups. G3 has a slightly lower expression level of Bax compared to G2, but the difference is not marked significant. However, the level of Bax expression in G5 is markedly lower than in G4, which suggests that prophylactic PEPITEM administration may lead to decreased in Bax protein levels in the spinal cord. In case of Bcl2, the figure shows a significant increase in Bcl2 expression in G3 compared to G2. Similarly, there is a marked increase in Bcl2 expression in G5 compared to G4. Therefore, therapeutic and prophylactic administration of PEPITEM is associated with a significant increase in Bcl2 levels in the spinal cord (**Figure 5**).

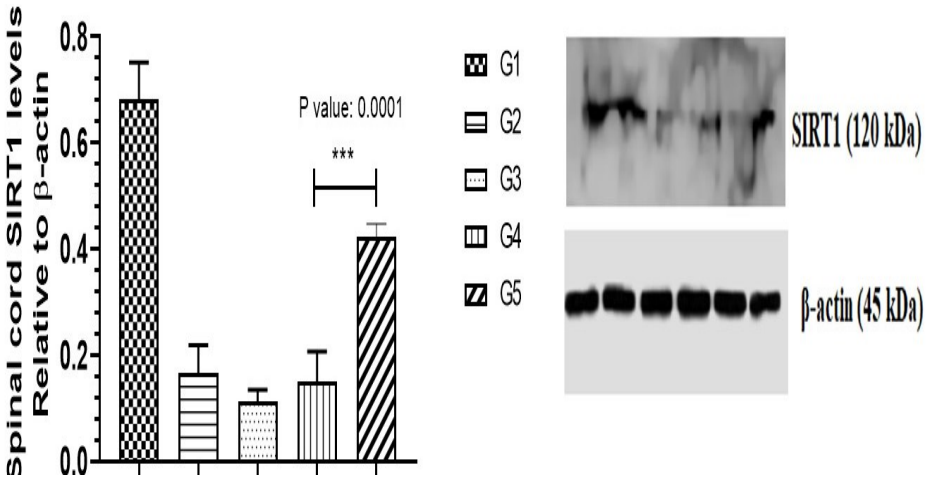


**Figure 3.** Differential expression of Protein levels of SIRT1 in the spinal cord of all groups of EAE mice as detected by western blotting and relatively expressed to β-actin.

Bar graphs representing SIRT1 protein expression in the spinal cord lysate of EAE mice. EAE mice received a daily IP injection of scramble peptide (G2) and (G4) or PEPITEM (G3) and (G5) until day 21 post EAE induction. The protein expression was evaluated by western blot. Mean ± SEM are depicted (n = 3 per group in triplicate). For optimization purposes, we run two controls with each gel, which explains the six bands on the gel. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

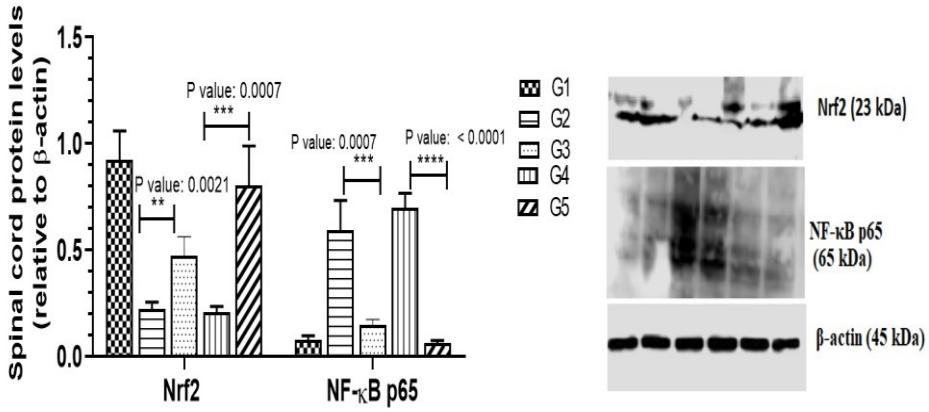


**Figure 4.** Differential expression of Protein levels of NRF2 and NF-κB p65 in the spinal cord of all groups of EAE mice as detected by western blotting and relatively expressed to β-actin.

Bar graphs representing NRF2 and NF-κB p65 protein expression in the spinal cord lysate of EAE mice. EAE mice received a daily IP injection of scramble peptide (G2) and (G4) or PEPITEM (G3) and (G5) until day 21 post EAE induction. The protein expression was evaluated by western blot. Mean ± SEM are depicted (n = 3 per group in triplicate). For optimization purposes, we run two controls with each gel, which explains the six bands on the gel. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

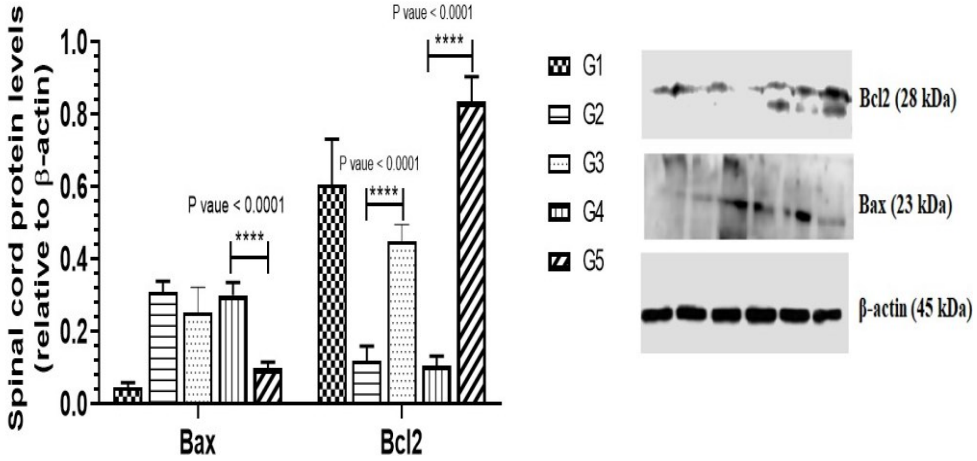


**Figure 5.** Differential expression of Protein levels of Bax, and Bcl2 in the spinal cord of all groups of EAE mice as detected by western blotting and relatively expressed to β-actin.

Bar graphs representing Bax, and Bcl2 protein expression in the spinal cord lysate of EAE mice on day 21. EAE mice received a daily IP injection of scramble peptide (G2) and (G4) or PEPITEM (G3) and (G5) until day 21 post EAE induction. The protein expression was evaluated by western blot. Mean ± SEM are depicted (n = 3 per group in triplicate). For optimization purposes, we run two controls with each gel, which explains the six bands on the gel. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

## 4. Discussion

The aim of this study is to assess the therapeutic and prophylactic efficacy of PEPITEM in an EAE mouse model. Our study demonstrates the therapeutic and prophylactic potential of PEPITEM in an EAE mouse model of Multiple Sclerosis. This claim is supported by the modulation of critical biomarkers, including increased SIRT1 and NRF2 expression and decreased NF-κB p65 levels, highlighting the activation of neuroprotective and antioxidant pathways while suppressing pro-inflammatory signals. The key findings indicate that PEPITEM effectively stabilizes body weight and reduces EAE scores since the weight loss correlates with the worsening of EAE scores, implying a direct relationship between disease severity and physical health. The stabilization and partial recovery alongside a plateau in EAE scores may indicate the effectiveness of PEPITEM in mitigating the progression of EAE symptoms when administered therapeutically. The prophylactic treatment is the most compelling data, with a moderate decline in body weight and a delayed, gradual increase in EAE score, which suggest that PEPITEM exerts a protective effect against EAE development when administered before disease induction [9]. Regarding the marker proteins, our study shows that the prophylactic administration of PEPITEM shows an increase in SIRT1 expressions which shows how timing influence neuroprotective mechanisms. The comparison between therapeutic and prophylactic administration of PEPITEM reveals differential effects on SIRT1 protein levels in spinal cord. Therapeutic administration of PEPITEM, initiated after the onset of EAE, appears not to modulate SIRT1 expression significantly. This observation suggests that once the neuroinflammatory cascade is activated and progresses, the capacity of PEPITEM to elicit changes in SIRT1 levels, and presumably its neuroprotective effects, may be limited. This could be attributed to the severe nature of neuroinflammatory processes and neurodegeneration at this stage, potentially reducing the efficacy of interventions aimed at modulating specific neuroprotective pathways [22–24]. Conversely, the prophylactic administration of PEPITEM, initiated prior to disease induction, results in a notable increase in SIRT1 expression. This finding aligns with recent literature that has associated SIRT1 in neuroprotection and the amelioration of neuroinflammatory conditions such Parkinson's disease [25]. This outcome highlights the potential of early intervention strategies to activate or enhance neuroprotective mechanisms, possibly by preemptively modulating the cellular stress response before the full onset of neuroinflammatory damage [16]. The increase in SIRT1 expression suggests that PEPITEM may exert a preconditioning effect on neural tissues, augmenting cellular defenses against the subsequent inflammatory assault characteristic of EAE. The observed upregulation of NRF2 in response to

therapeutic or prophylactic administration of PEPITEM is particularly noteworthy as it could signify an induced endogenous antioxidant defense mechanism, potentially attenuating oxidative stress associated with EAE. This observation is consistent with the literature which associates NRF2 elevation with reduced oxidative damage and improved outcomes in an MS patients [17,26]. Furthermore, we showed that PEPITEM-induced downregulation of NF-κB p65, suggest a suppression of pro-inflammatory signaling pathways. This is consistent with findings from Liu et al. [27], where NF-κB inhibition was correlated with decreased production of inflammatory cytokines and amelioration of EAE severity, reinforcing the therapeutic potential of targeting this pathway [27,28]. The observed pattern of Bax protein expression in response to PEPITEM administration resembles the effects seen with SIRT1, providing further insight into the timing-dependent efficacy of PEPITEM in modulating pathways associated with cell death and survival. Just as with SIRT1, therapeutic administration of PEPITEM post-disease induction does not significantly alter Bax protein expression levels, whereas prophylactic administration before disease onset leads to a noticeable modulation in the expression of this pro-apoptotic marker. The lack of significant change in Bax levels following therapeutic administration suggests that, similar to the neuroprotective pathways indicated by SIRT1 expression, the window for influencing apoptosis-related processes may be limited once the neuroinflammatory and neurodegenerative mechanisms of EAE are fully activated. This could imply that the cellular and molecular environment established by ongoing disease processes may be less amenable to interventions targeting apoptosis modulation, such as those mediated by Bax. Conversely, the decrease in Bax protein expression levels observed with prophylactic PEPITEM administration highlights the potential of preventative interventions to influence the cellular landscape in favor of survival, potentially by reducing the initiation of apoptosis in neural tissues. This prophylactic effect suggests that PEPITEM might prime the neural tissue against the damaging effects of inflammation and degeneration typical of EAE, possibly through mechanisms that involve the suppression of pro-apoptotic signals [29].

## 5. Conclusion

Collectively, our findings demonstrate that PEPITEM exerts a multifaceted therapeutic effect in the EAE model of MS by mitigating the symptoms of EAE as evidenced by modulating crucial biomarkers involved in neuroprotection, inflammation, and apoptosis. The significant alterations in the expression of the biomarkers highlight the potential of PEPITEM as a promising therapeutic agent for MS, offering insights into its mechanism of action and paving the way for future clinical investigations.

Future studies employing pathway-specific inhibitors or activators, are required to identify the precise intracellular pathways influenced by PEPITEM. Moreover, quantification of the phosphorylated form of proteins will be very insightful. Additionally, using qPCR to measure the gene expression of the biomarkers will validate the Western Blot data. Dose-response studies and pharmacokinetic analyses would be essential for clinical translation, determining optimal dosing and safety

profiles. Furthermore, the potential for PEPITEM to complement existing MS treatments offers a promising path for integrated therapy.

The current study, while providing valuable insights into PEPITEM's therapeutic impact on neuroinflammatory and apoptotic pathways in EAE, is not without limitations. Analyzing the spinal cord via Western blot presents significant challenges due to the surrounding fatty tissues, which complicate the extraction of high-quality protein yields. Consequently, the bands observed in Western blots tend to be less defined and thinner compared to those derived from other organs. While other studies have opted for qPCR when working with spinal cord tissue, we chose the more difficult approach of Western blot analysis to obtain more detailed data. Moreover, our reliance on a single animal model, although well established, may not encapsulate the full heterogeneity of MS pathogenesis observed in humans.

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**Conflict of interest:** The author declares no conflict of interest.

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