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G Protein-Coupled Receptor 17 Inhibition as a Prospective Treatment for Multiple Sclerosis: A Research Protocol

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Abstract

Introduction: Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease. MS is the most prevalent neurological disability that often leads to severe cognitive or physical incapacitations in young adults. As MS is currently an incurable disease, more effective treatments need to be investigated. The disease is associated with axonal degenerations and the development of demyelinated plaques, due to episodic autoimmune destruction of oligodendrocytes. Usually, demyelination is followed by remyelination as the brain attempts to reconstruct the myelin sheaths. Oligodendrocyte precursor cells are essential for remyelination as these cells proliferate and differentiate into mature oligodendrocytes. An important regulator of oligodendrocyte development is G protein-coupled receptor 17 (GPR17), whose inhibition has been suggested in previous studies to the promote oligodendrocyte differentiation and remyelination. As such, a potential therapy for MS patients is through decreasing GPR17 expression via inhibition of one of its ligands, LTC4.

Methods: We aim to promote remyelination in MS patients using the LTC4 synthase inhibitor AZD9898 to indirectly partially inhibit GPR17 in proteolipid protein-induced experimental autoimmune encephalomyelitis (PLP-induced EAE) MS mice models. There will be 6 experimental groups and 8 control groups. All experimental groups will receive a three mg dose of AZD9898. After approximately zero, one, five and ten days, the mice will be sacrificed, and magnetic resonance imaging will be conducted. The myelin water fraction will then be determined to visualize *in vivo* myelination of the central nervous system through myelin water imaging. Western blotting will be used to verify AZD9898's indirect inhibition of GPR17. The resulting data will be analyzed with the Kolomogorov–Smirnov test, Pearson's correlation coefficient (linear) and two-tailed paired *t*-tests and ImageJ software.

Anticipated Results: It is expected that AZD9898 administration in PLP-induced EAE mice models will result in an increased myelin water fraction, indicating remyelination, alongside decreased GPR17 expression.

Discussion: These results will provide a potential treatment for MS by illustrating how AZD9898 is effective at indirectly inhibiting GPR17 in mice models, and thus promoting remyelination.

Conclusion: This study will provide insight on the treatment of demyelinating diseases by demonstrating how pharmacological inhibition of GPR17 ligand LTC4 can promote remyelination in MS patients.

Keywords: multiple sclerosis; leukotriene C4; myelin; GPR17; AZD9898; myelin water imaging

Introduction

Multiple sclerosis (MS) is an incurable chronic inflammatory neurodegenerative disease that affects the central nervous system (CNS). MS is the most prevalent neurological disability that often leads to neurological complications and severe cognitive or physical incapacitations in young adults [1]. It is typically associated with axonal degenerations, destruction of oligodendrocytes, and the development of large, demyelinated plaques.

A lipid rich insulating layer called myelin surrounds the axons of nerve cells and in the CNS is composed of oligodendrocytes. Oligodendrocytes are specialized cells which can modify their plasma membrane to spirally enclose nerve axons [2,3]. They are responsible for providing nutritional support to neurons and are associated with fast axonal transport [4]. Myelin sheaths are important for the rapid and efficient propagation of action potentials along nerve cells. Periodic interruptions in these myelin

sheaths are referred to as the nodes of Ranvier, where small axonal portions are left uncovered. These nodes are essential for rapid conduction of nerve impulses [5]. Furthermore, myelin protects and stabilizes neuronal axons which prevents axonal degeneration [6].

Demyelination in MS occurs due to episodic autoimmune destruction of oligodendrocytes. Typically, demyelination is followed by remyelination as the brain attempts to reconstruct the myelin sheaths [7,8]. Remyelination involves the activation and proliferation of oligodendrocyte precursor cells (OPCs), followed by their migration to demyelinated axons. As the OPCs interact with the axons, they begin to differentiate and remyelination occurs [4]. OPCs are essential for the remyelination process as these cells proliferate and differentiate into mature oligodendrocytes. However, despite abundant levels of OPCs, remyelination in MS is often incomplete due to a lack of OPC differentiation [6]. This eliminates OPC recruitment and migration as potential barriers of remyelination. Therapies that venture to promote remyelination have the potential to slow down or even reverse the effects of demyelination.

A G protein-coupled receptor 17 (GPR17) is an important regulator of oligodendrocyte development and remyelination. During the course of OPCs maturation, GPR17 expression is almost undetectable in early OPCs, but levels gradually increase in mature OPCs. Levels of GPR17 expression plateaus in pre-oligodendrocytes before it begins to decrease during the terminal differentiation of oligodendrocytes [4]. In particular, MS animal models and MS patients show high GPR17 expression in active white matter plaques [9]. Therefore, GPR17 overexpression may be responsible for impairing remyelination in MS [10].

Studies have reported that GPR17 can be activated by cysteinyl leukotrienes (ex: leukotriene C4), uridine diphosphate, and purines including adenosine triphosphate [4,11,12,13]. Therefore, a potential therapy for MS patients is through indirectly decreasing GPR17 expression via partially inhibiting / decreasing the synthesis of one of its ligands, LTC4, thus having downstream effects on GPR17. This study ventures to explore the feasibility of using AZD9898, a small molecule inhibitor of leukotriene C4 synthase (LTC4S), to indirectly partially inhibiting GPR17 by inhibiting one it's ligands, and subsequently promote remyelination in proteolipid protein (PLP)-induced experimental autoimmune encephalomyelitis (EAE) MS mice models.

Methods

Animal Models

We will be using 15-week-old male and female PLP-induced EAE mice models. PLP-induced EAE mice models have developed a chronic demyelinating disease with a similar relapse-remitting pattern to human MS, allowing it to serve as an effective model for MS treatment studies [14]. 15-week-old mice will be used to allow ample time for the advanced symptoms of the chronic demyelinating disease to occur, under which a larger area of demyelination can be evaluated. These mice will be bred under specific pathogen-free conditions of creative biolabs [14]. The experimental groups and subsequent investigation steps are outlined in Figure 1. Seven mice will be used in each group, with fourteen groups in total (seven consisting of male mice and seven consisting of female mice).

Administration of LTC4S Inhibitor

The LTC4S inhibitor, AZD9898, will be obtained from Adooq Bioscience. We will orally administer a single three mg dose of AZD9898 through gavage to the experimental mice, excluding control group mice. The dosage concentration was determined using the half-maximal inhibitory concentration value of 0.28 nM as reference [15] and in a previous safety study, 100 mg/kg of AZD9898 was used to determine dosage [16]. In general, 15-week-old mice weigh approximately 30 grams, therefore, a three milligram dose was determined to be ideal. The administration will be done at one time (zero time).

Magnetic Resonance Imaging (MRI)

After approximately one, five and ten days, the experimental mice will be sacrificed by intraperitoneal injections of 150 mg/kg sodium pentobarbital [17]. Two control mice groups (female and male) will be sacrificed by the same method as the experimental mice at day zero. MRI tests will then be conducted to evaluate the brains and spinal cords of the mice. It is essential to conduct MRIs to then conduct myelin water imaging.

Myelin Water Imaging

In order to visualize *in vivo* myelination of the CNS in the PLP-induced EAE mice, myelin water imaging will be conducted via MRI [18]. When analyzing MRI data, it is shown that intracellular water has a T2 time greater than 60 ms in contrast to myelin water, which has a T2 time between 10 and 20 ms. A plot of signal amplitude vs T2 time will be used to display the T2 decay curve's exponential components [18]. This will allow us to determine the myelin water fraction (MWF), which is the ratio of the area in the T2 distribution that is due to myelin water compared to the overall of the T2 distribution. The MWF may be represented as a figure or image of myelin water graphically [18]. The picture of the myelin water will be obtained from the use of 3D multi echo gradient and spin echo (GRASE) data, using a multi-voxel spatial regularization (MVSR) approach [19].

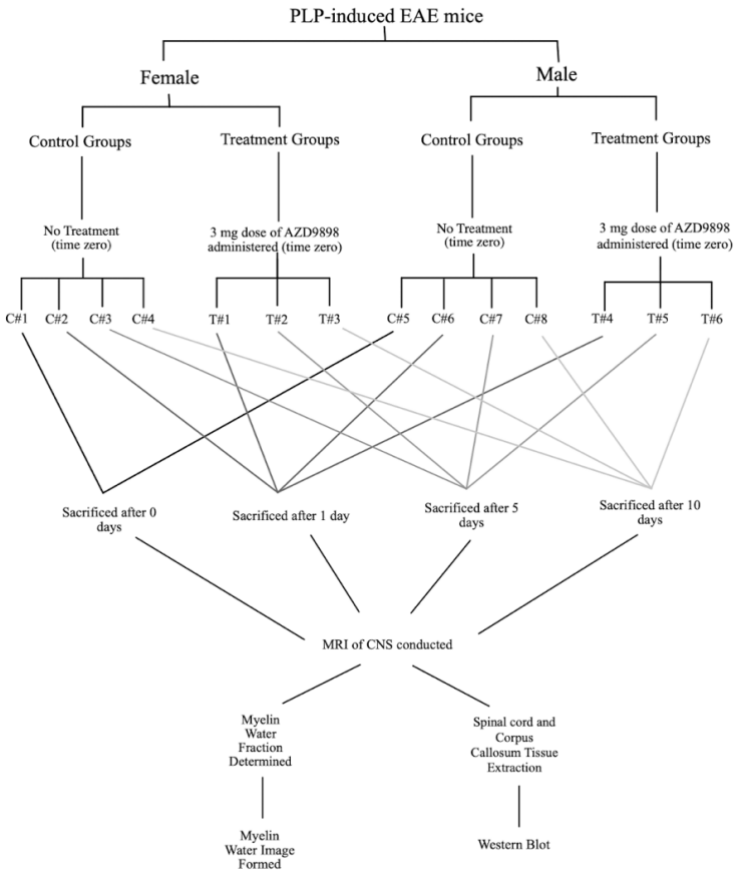

Figure 1. An experimental overview of the proposed study. N = 7 mice for each treatment and control group. A total of 98 mice will be used. PLP = Proteolipid protein; EAE = Experimental autoimmune encephalomyelitis; C = Control Group, T = Treatment Group. Figure made using Goodnotes 5.

Tissue Extraction and Preparation

In order to conduct western blotting, 50 mg tissue samples of the spinal cord and corpus callosum will be isolated from the mice in both the treatment and control groups. The extraction will be conducted under sterile conditions. The tissue samples will then be frozen. Thermo Fisher's Western Blot Sample Preparation Protocol [20] will be used as reference to prepare the tissue samples for western blotting. Approximately 1,000 µL of ice-cold radioimmunoprecipitation assay lysis buffer will be added to each tissue sample, which will then homogenize on ice. Each sample will then be centrifuged for five minutes at 10,000 x g. The supernatant will then be transferred to a new microcentrifuge tube and the pellet will be discarded. The supernatant's protein concentration will then be measured by Thermo Fisher's Bicinchonic Acid Protein Assay Kit [20].

Western Blot

Western blotting will be used verify GPR17 expression is being indirectly decreased by AZD9898's inhibition of LTC4 synthesis by evaluating GPR17's key downstream effectors protein kinase A (PKA) and exchange protein activated by cyclic adenosine monophosphate (EPAC), which are upregulated during GPR17 expression [10]. The western blot will also be used to evaluate LTC4 levels. Using the protocol of Chen et al. [9] as reference, on a 4–12% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) equal amounts of the protein from the supernatant will be separated and then transferred over to a Hybond Polyvinylidene difluoride membrane. A NuPage 4–12% Bis-Tris gel transfer system will then be used to conduct the western blot, in which the primary antibodies used will be mouse anti- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), mouse anti-LTC4, rabbit anti-PKA, and rabbit anti-EPAC. GAPDH will be used as a loading control. Horseradish peroxidase conjugated secondary antibodies will also be used. The top and bottom of the membrane will then be covered with an enhanced chemiluminescence mixture, which will allow the protein blots to be visualized in a dark room [9]. The number of proteins in the sample will be indicated by the thickness of the protein blots. This process will be conducted for all the control and experimental samples separately.

Statistical Analysis

The data we will obtain from the calculated MWF will be presented as a mean ± standard deviation. The normal distribution of this data will be tested with the Kolomogorov–Smirnov test. The MWF region-based associations will be tested using Pearson's correlation coefficient (linear). To test for the difference between mean MWFs of the two brain hemispheres, paired two-tailed *t*-tests will be conducted. These tests were determined using MacKay & Laule as reference [18]. The western blot results will be normalized against the loading control, GAPDH protein and levels and analyzed using ImageJ software.

Anticipated Results

PLP-induced EAE mice MS mice models will be used to inhibit the synthesis of GPR17 ligand LTC4. Fourteen experimental mice groups, seven mice per group, will receive a three mg dose of AZD9898 and the control group will receive no dose of AZD9898. The groups will be sacrificed after either one, five, or ten days to conduct MRI tests for examination of the brain and spinal cord. It is expected that an increase in myelin production will be observed in the mice if AZD9898 is sufficient at inhibiting LTC4 and thus indirectly decreasing GPR17 expression. Myelin water fraction imaging will detect the increase in myelin. It is anticipated that all groups receiving AZD9898 will show increased myelin water fraction with a short T2 component in contrast to the control groups. The increase in myelin water fraction and a short T2 component is an indication of remyelination. In the control group, it is expected that there will be no remyelination observed due to LTC4 being present to activate GPR17. As the dose concentration of AZD9898 is increased, we expect to observe an increase in myelin production. The mice groups sacrificed ten days following AZD9898 treatment are expected to display greater levels of remyelination than the groups sacrificed after one or five days. Furthermore, the western blots of the experimental groups are expected to show decreased levels of LTC4, PKA and EPAC in contrast to the control groups. The results will verify a decrease in LTC4 synthesis by AZD9898 and the indirect decrease in GPR17 expression by the decreased levels of its key downstream effectors, PKA and EPAC.

Discussion

These results will demonstrate how the effects of the LTC4S antagonist AZD9898 on GPR17 promote remyelination in MS patients. The mice treated with AZD9898 are expected to show a greater myelin water fraction than the control group mice. Myelin water imaging will serve as an indication of remyelination, since MS plaques contain less myelin water fraction [21]. These findings will provide insight on the role of GPR17 in the pathology of MS and determine if inhibiting one of GPR17's ligands, LTC4's, synthesis via AZD9898 is effective at increasing remyelination, alongside decreasing GPR17's expression, allowing it to be used as a treatment.

The application of studies using mouse models to research on human diseases can be limiting [22], so the applicability of AZD9898 from PLP-induced EAE models to human trials must be evaluated. However, PLP-induced EAE models have demonstrated to be effective demyelinating disease models since they activate the T-cells required for epitope spreading [23].

In previous studies it has been suggested that the myelin water fraction can be underestimated due to the movement of myelin water [24], which could be a possible limitation of this study. Although this is an important consideration, conducting an MRI along with myelin water imaging has been validated with histopathology to be a useful tool in the diagnosis of MS in human patients [25]. Another limitation of this study is AZD9898 only inhibits the synthesis of one of GPR17's ligands, and hence, the receptor will not be completely inhibited. Further studies may be conducted to evaluate the effect of complete inhibition of GPR17 on remyelination. It is also possible that multiple administrations of AZD9898 are required to observe greater remyelination, which could be explored in future studies. Future studies may also be performed to evaluate the effects of dosage and time course on the administration of AZD9898 on MS mice models.

Conclusions

This study will contribute to the research on demyelinating diseases and provide a potential treatment for MS, a currently incurable disease. It is expected that these findings will demonstrate how AZD9898 is effective at promoting remyelination in MS patients through the inhibition of the synthesis of LTC4, a GPR17 ligand. Future studies can be conducted to investigate the optimal AZD9898 dosage required to promote remyelination, and to evaluate how many days the effect may last.

List of Abbreviations Used

CNS: central nervous system
EAE: experimental autoimmune encephalomyelitis
EPAC: exchange protein activated by cyclic adenosine monophosphate
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GPR17: G protein-coupled receptor 17
GRASE: gradient and spin echo
LTC4S: leukotriene C4 synthase
MRI: magnetic resonance imaging
MS: multiple sclerosis
MVSR: multi-voxel spatial regularization
MWF: myelin water fraction
OPC: oligodendrocyte precursor cell
PKA: Protein Kinase A
PLP: proteolipid protein
SDS-PAGE: sodium dodecyl-sulfate polyacrylamide gel electrophoresis

Conflicts of Interest
The authors declare that they have no conflict of interests

Ethics Approval and/or Participant Consent
This research protocol did not require ethics approval or participant consent as no experiment was conducted. For experimentation, ethics approval will be obtained from the University of British Columbia's Animal Care Committee.

Authors' Contributions
KKR: made substantial contributions to the study design, contributed to study planning and drafting, critically revised the manuscript and gave approval of the final version.
ISD: made substantial contributions to the study design, contributed to study planning and drafting, critically revised the manuscript and gave approval of the final version.
GKN: made substantial contributions to the study design, contributed to study planning and drafting, critically revised the manuscript and gave approval of the final version.

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