Deconstructing the pharmacological contribution of sphingosine-1 phosphate receptors to mouse models of multiple sclerosis using the species selectivity of ozanimod, a dual modulator of human sphingosine-1 phosphate receptor subtypes 1 and 5

Julie V. Selkirk, Kevin C. Dines, Yingzhuo Grace Yan, Nathan Ching, Deepak Dalvie, Shameek Biswas, Andrea Bortolato, Jeffrey M. Schkeryantz, Carlos Lopez, Iliana Ruiz, Richard Hargreaves

Primary laboratory of origin: San Diego Science Park Research & Early Development Laboratories

JVK, KCD, YGY, NC, DD, SB, AB, JS, CL, IR, RH: Bristol Myers Squibb, Princeton, NJ

Running title: Defining S1PR roles in models of multiple sclerosis using ozanimod

Corresponding author:
Julie Selkirk, PhD
Bristol Myers Squibb
3401 Princeton Pike
Lawrenceville, NJ 08648
Phone: (908) 673-9000
Email: Julie.Selkirk@BMS.com

Number of text pages: 25
Number of tables: 6
Number of figures: 8
Number of references: 52
Word count of abstract: 250
Word count of introduction: 763
Word count of discussion: 1569

List of nonstandard abbreviations:
ALCs, absolute lymphocyte counts
B\textsubscript{max}, maximum number of binding sites for a ligand
CDNAs, complementary deoxyribonucleic acids
CFA, complete Freund’s Adjuvant
CHO, Chinese hamster ovary
CNS, central nervous system
CPZ, cuprizone
Cyno, cynomolgus monkey
DMSO, dimethyl sulfoxide
EAE, experimental autoimmune encephalomyelitis
FEP+, free energy perturbation method
GPCR, G protein-coupled receptors
K\textsubscript{D}, dissociation constant
LC-MS/MS, liquid chromatography–mass spectrometry
MOG\textsubscript{35-55}, myelin oligodendrocyte glycoprotein 35 - 55
MRI, magnetic resonance imaging
MS, multiple sclerosis
NFL, neurofilament light
NSB, non-specific binding
OPC, oligodendrocytes precursor cell
PLP, proteolipid protein
Rapa, rapamycin
S1P, sphingosine-1 phosphate
S1P\textsubscript{1}, S1P receptor subtype1
S1P\textsubscript{5}, S1P receptor subtype 5

Section assignment: Drug Discovery and Translational Medicine
Abstract

Ozanimod, a sphingosine-1 phosphate (S1P) receptor modulator that binds with high affinity selectively to S1P receptor subtypes 1 (S1P₁) and 5 (S1P₅), is approved for the treatment of relapsing multiple sclerosis (MS) in multiple countries. Ozanimod profiling revealed a species difference in its potency for S1P₅ in mouse, rat, and canine compared with that for human and monkey. Site-directed mutagenesis identified amino acid alanine at position 120 to be responsible for loss of activity for mouse, rat, and canine S1P₅ and mutation back to threonine as in human/monkey S1P₅ restored activity. Radioligand binding analysis performed with mouse S1P₅ confirmed the potency loss is a consequence of a loss of affinity of ozanimod for mouse S1P₅ and was restored with mutation of alanine 120 to threonine. Study of ozanimod in preclinical mouse models of MS can now determine the S1P receptor(s) responsible for observed efficacies with receptor engagement as measured using pharmacokinetic exposures of free drug. Hence, in the experimental autoimmune encephalomyelitis model, ozanimod exposures sufficient to engage S1P₁, but not S1P₅, resulted in reduced circulating lymphocytes, disease scores, and body weight loss; reduced inflammation, demyelination, and apoptotic cell counts in the spinal cord; and reduced circulating levels of the neuronal degeneration marker, neurofilament light. In the demyelinating cuprizone model, ozanimod prevented axonal degradation and myelin loss during toxin challenge but did not facilitate enhanced remyelination post-intoxication. Since free drug levels in this model only engaged S1P₁, we concluded that S1P₁ activation is neuroprotective but does not appear to affect remyelination.

Significance statement:

Ozanimod, a selective human S1P₁/₅ modulator, displays reduced potency for rodent and dog S1P₅ compared with human, which results from mutation of threonine to alanine at position 120. Ozanimod can thus be used as a selective S1P₁ agonist in mouse models of multiple sclerosis to define efficacies driven by S1P₁ but not S1P₅. Based on readouts for experimental
autoimmune encephalomyelitis and cuprizone intoxication, S1P₁ modulation is neuroprotective but S1P₅ activity may be required for remyelination.
Introduction

Sphingosine-1 phosphate (S1P) and its cognate family of receptors play a key role in the immunomodulatory and direct central nervous system (CNS) effects associated with multiple sclerosis (MS) (Cohan et al., 2020). Multiple sclerosis is an autoimmune disease where B and T lymphocytes from the periphery enter the CNS and attack the myelin sheath that insulates and protects neuronal axons, resulting in decreased nerve conduction (Stassart et al., 2018; Stadelmann et al., 2019). The family of S1P receptors is comprised of five G protein-coupled receptors (GPCR) of the class A family of seven transmembrane domain GPCRs, designated S1P subtypes 1-5 (S1P\textsubscript{1-5}) (Rosen et al., 2013; Kihara et al., 2014), with S1P\textsubscript{1} and S1P\textsubscript{5} expressed on key cell types that contribute to MS disease pathology (Rothhammer et al., 2017; Groves et al., 2018; Kim et al., 2018;).

The predominant S1P receptor involved in lymphocyte trafficking in MS is S1P\textsubscript{1}, which is expressed on B and T cells, and following exposure to agonist is rapidly down modulated from the plasma membrane with internalization to the intracellular compartment (Sanna et al., 2004; Scott et al., 2016). This internalization of S1P\textsubscript{1} prevents specific subsets of B and T cells in the peripheral lymphoid tissue from sensing the S1P concentration gradient that exists between tissues and the systemic circulation and thus blocks traffic out of the lymphoid tissue into the circulation. As a result, these B and T cells are retained in the lymphoid tissue and significantly reduce the numbers of circulating lymphocytes in patients with MS (Cohen et al., 2019; Comi et al., 2019). The retention of B and T cells in lymphoid tissue and the lowering of absolute lymphocyte counts (ALCs) in the circulation limits their ability to traffic into the CNS, reducing subsequent damage to the myelin sheaths (Uher et al., 2020). S1P\textsubscript{1} is also expressed by astrocyte and microglial cells within the CNS (Choi et al., 2011; Noda et al., 2013; Musella et al., 2020), and down modulation of S1P\textsubscript{1} in these cell types attenuates their activation state which may abrogate the local pro-inflammatory environment that exists during an MS relapse.
In contrast to S1P1, S1P5 expression is more restricted and, within the CNS, is limited to oligodendrocytes, the myelinating cells. Indeed, S1P5 is expressed at all stages of oligodendrocyte development: oligodendrocyte precursor cells (OPCs), pre-oligodendrocytes, and fully differentiated mature cells capable of depositing myelin (Jaillard et al., 2005). Activation of S1P5 increases the survival of OPCs in vitro (Miron et al., 2008) and causes the initial process retraction followed by robust re-extension of pre-oligodendrocytes that then mature into complex myelinating cells (Jaillard et al., 2005). Hence, it is postulated that agonism of S1P5 may play a role in myelin repair in MS.

Ozanimod (Zeposia®), an S1P receptor modulator that binds with high affinity selectively to S1P1 and S1P5, is approved in multiple countries for relapsing forms of MS to include clinically isolated syndrome, relapsing-remitting disease, and active secondary progressive disease in adults (Scott et al., 2016; Cohen et al., 2019; Comi et al., 2019; FDA 2020). Compared with other S1P modulators, ozanimod has superior S1P receptor selectivity and does not require first dose heart rate monitoring compared with fingolimod (FTY-720, Gilenya®; Brinkmann et al., 2002; FDA, 2010), and a lack of predominant CYP2C9 metabolism negates the need for pre-dose genetic testing compared with siponimod (BAF-312, Mayzent®, Gardin et al., 2018; FDA 2019).

During the development of ozanimod, S1P1-5 were cloned from multiple pre-clinical species and the activity of ozanimod and its metabolites characterized to understand the potencies relative to the human receptor homologues. In so doing, we discovered a species variation in the activity of ozanimod and its major metabolite in mouse, RP101075, for S1P5, whereas activity for S1P1 remained consistent across all species tested. This finding does not affect the clinical profile of ozanimod in humans where the potency is in the low nanomolar range for both S1P1 and S1P5; however, it has provided a deeper understanding of the role of S1P1 and S1P5 in mouse models.
of MS since the potency of ozanimod and RP101075 were observed to be significantly reduced for mouse S1P$_5$.

In the current studies we report on the identification of ozanimod/RP101075’s reduced potency for S1P$_5$ in mouse, rat, and dog compared with human and cynomolgus monkey (cyno) and identify the amino acid directly involved that is not conserved across all species. Moreover, we apply this knowledge to measurable efficacy observed in multiple MS mouse models, and by measuring the achieved drug exposure, we can hypothesize the S1P receptor subtype(s) responsible for the different efficacy readouts investigated.

Materials and Methods

Compounds

Ozanimod, RP101075, and A971432 were synthesized at Celgene/BMS Science Park (San Diego, CA). FTY720-phosphate (FTY720-p) was obtained from Toronto Research (North York, Canada). Siponimod was acquired from Fisher Scientific (Pittsburgh, PA). The radioligands, tritium-labeled ozanimod ([$^3$H]-ozanimod) and tritium-labeled S1P$_5$ selective ligand A971432 ([$^3$H]-A971432) were custom synthesized by Novandi Chemistry (Södertälje, Sweden).

Generation of stable recombinant S1P$_5$ expressing cell lines

Human, mouse, and rat S1P$_5$ complementary deoxyribonucleic acids (cDNAs) (UniProt accession numbers, Q9H228, Q91X56, and Q9JKM5) and codon optimized cyno and canis familiaris (dog) S1P$_5$ cDNAs (UniProt accession numbers G7PZ80 and E2RDP1) were cloned into pcDNA3.1 (+) vector (Thermo Fisher Scientific, Santa Clara, CA) with a hemagglutinin (HA) tag added to the N-terminal. Chinese hamster ovary (CHO) K1 cells were purchased from the American Type Culture Collection (Manassas, VA). Stable cell lines of individual species S1P$_5$
receptors were generated by transfecting the expression construct of each species to CHO K1 cells. After selection with Geneticin (G418 sulfate), the positive clones were identified by cyclic adenosine monophosphate (cAMP) assay using a Lance Ultra cAMP kit (Perkin Elmer, cat# TRF0264, Waltham, MA) and flow cytometry using an anti-HA–Alexa Fluor™ 647 conjugated antibody (Cell Signaling, Boston, MA). The final clone was selected and verified using [35S]-GTPγS binding assays as described below.

**Site-directed mutagenesis of S1P₅**

To identify the amino acid(s) responsible for the ozanimod potency shift observed in mouse S1P₅, two rounds of site-directed mutagenesis were performed using pcDNA3.1(+) -mS1P₅ wild type plasmid as the template using a whole plasmid mutagenesis method. Complementary primers containing the desired mutation were used to amplify the entire plasmid, which was then used to transform Escherichia coli. Clones containing the desired mutation were then selected and sequence-confirmed by polymerase chain reaction (performed by Genewiz, La Jolla, CA).

In the first round, mS1P₅_G229R, mS1P₅_L369S, and mS1P₅_G229R_L369S expression constructs were generated. In the second round, mS1P₅_A120T, mS1P₅_L202V, and mS1P₅_A120T_L202V expression constructs were generated. Later, rat S1P₅_A120T and dog S1P₅_A120T expression constructs were also generated using the same methodology, using wild type rat and dog S1P₅ in pcDNA 3.1(+) as the respective templates.

**Transient transfection of mutated S1P₅ constructs**

Mutated S1P₅ expression constructs were transiently transfected into CHO K1 cells using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, MA). Cells were plated at a density of 11 million cells per 500 cm² culture tray the day before transfection. Transfection was performed using 120 µg of each respective expression construct plasmid DNA per 500 cm²
culture tray and cells were harvested 24 hours post-transfection and cell membranes prepared as described below.

**Cell membrane preparations**

Membranes were prepared from stable or transient S1P$_5$ expressing cells following adherent culture in 500 cm$^2$ culture trays. Cells were detached with cell-lifting buffer (10 mM HEPES, 154 mM NaCl, 6.85 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4) and pelleted by centrifugation for 5 minutes at 1000 rpm. Cell pellets were then re-suspended and homogenized in membrane preparation buffer (10 mM HEPES and 10 mM EDTA, pH 7.4) using a Polytron PT 1200E homogenizer (Kinematica, Luzern, Switzerland). Cells homogenates were centrifuged at 48,000 × g at 4°C for 30 minutes to collect the membrane pellet. The supernatant was discarded, and the pellet was re-homogenized and re-centrifuged as described above in membrane preparation buffer. The final pellet was collected and homogenized in ice cold re-suspension buffer (10 mM HEPES and 0.1 mM EDTA, pH 7.4). Aliquots were stored at -80°C until required for radioligand binding or [$^{35}$S]-GTP$_{\gamma}$S binding assays.

**Saturation radioligand binding**

Saturation binding analysis was performed with [$^3$H]-ozanimod and [$^3$H]-A971432 using 96-well non-binding surface plates (Corning, cat# 3604, Corning, NY) with a final volume of 200 μL. One assay plate was prepared for each of the following membrane preparations: human S1P$_5$, mouse S1P$_5$, mouse S1P$_5$-A120T, and CHO K1 parental cells. For determination of total binding, half of the assay plate was prepared with 60 μL/well of 0.33% dimethyl sulfoxide (DMSO) vehicle, and the other half of the plate was prepared for non-specific binding (NSB) measurement, with 60 μL/well of 33.33 μM unlabeled ozanimod or A971432. Serial 2-fold dilutions of [$^3$H]-ozanimod and [$^3$H]-A971432, from 200 to 0.39 nM, were prepared in assay
buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 0.1% fatty acid free bovine serum albumin, and 30 μg/mL saponin, pH7.4) in glass vials. Each concentration of radioligand (40 μL/well) was then added in triplicate to both the total binding and NSB wells. The reaction was then started with the addition of 100 μL of 48 μg/mL membrane preparations to all wells. Plates were sealed and incubated at room temperature with gentle agitation for 60 minutes before assay termination by filtration. Filter plates (Millipore, cat# MAHFC1H60, Burlington, MA) were prepared by incubating with 80 μL/well of 0.3% PEI for 60 minutes at room temperature, before washing with 150 mL/plate of filtration buffer (50 mM Tris HCl, 5 mM MgCl₂, and 1 mM EDTA, pH7.4) on a Filtermate-96 Harvester (Perkin Elmer, Waltham, MA). After filtration, the excess unbound radioligand was washed with 10 x 200 μL/well wash cycles using filtration buffer plus an additional prolonged wash with 500 mL total of filtration buffer per plate. Filter plates were then allowed to air dry before the addition of 50 μL/well Microscint-20 cocktail (Perkin Elmer, cat# 6013621, Waltham, MA), plate sealing and then reading on a MicroBeta2 microplate scintillation counter (PerkinElmer, Waltham, MA).

**Competition radioligand binding**

Competition radioligand binding assays with [³⁵H]-A971432 were also performed in 96-well nonbinding surface plates with a final volume of 200 μL. Test compounds were serial diluted in DMSO directly to the assay plate using the Tecan D300E digital printer (Tecan, Männedorf, Switzerland) in a total volume of 0.4 μL followed by either 60 μL/well assay buffer or 60 μL/well of 3.33X (3 μM final) unlabeled A971432 to define NSB. Next, 40 μL/well of 5X [³⁵H]-A971432 (5 nM final) was added before the experiment was initiated by the addition of 100 μL membranes to all wells to yield a final protein concentration of 4.8 μg/well. Assay plates were sealed and incubated at room temperature with gentle agitation for 60 minutes before filtration, washing and counting as detailed above for the saturation radioligand binding.
**[35S]-GTPγS binding assay**

Assays to quantitate receptor activation and G-protein coupling were performed using [35S]-GTPγS binding assays. All assays were performed in 96-well non-binding surface plates in a final volume of 200 μL. Test compounds were serial diluted in DMSO directly to the assay plate using the Tecan D300E digital printer in a total volume of 0.4 μL. The endogenous ligand, S1P, was used as a normalization control and was prepared separately to produce a 400 μM stock solution prepared from a 100 nmol pellet of S1P in 10 mM Na₂CO₃ with 2% β-cyclodextrin. The serial dilution of S1P was performed by hand in assay buffer and 40 μL/well was transferred to wells containing 0.4 μL DMSO vehicle. All the wells were brought to a total volume of 40 μL with assay buffer. The reaction was initiated by the addition of 120 μL/well of assay buffer containing a mixture of 40 μg/mL S1P receptor membranes, 16.67 μM guanosine diphosphate (Sigma Aldrich, cat# G7127, St. Louis, MO), and 2.5 mg/mL of WGA PVT SPA beads (Perkin Elmer, cat# RPNQ0001). Assay plates were then sealed and incubated at room temperature with gentle agitation for 30 minutes before the addition of 40 μL/well of 5X [35S]-GTPγS (200 pM final) (PerkinElmer, cat# NEG030X250UC, Waltham, MA) made up in basic assay buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, and 1 mM EDTA, pH7.4), resealing of the plates and an additional 40-minute incubation at room temperature with gentle agitation. The experiment was terminated by centrifugation of the plates at 1000 rpm for 3 minutes using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) and reading on a MicroBeta2 microplate scintillation counter (PerkinElmer, Waltham, MA).

**MOG₃₅-₅₅ Experimental Autoimmune Encephalomyelitis Model**

Experimental autoimmune encephalomyelitis (EAE) was induced in 10-week-old female C57BL/6 mice (Taconic Biosciences, USA) by subcutaneous (SC) immunization with an emulsion of myelin oligodendrocyte glycoprotein 35 - 55 (MOG₃₅-₅₅) in Complete Freund’s Adjuvant (CFA) followed by intraperitoneal (IP) injections of pertussis toxin 2 and 24 hours later.
Mice received two SC injections, one in the upper and one in the lower back, of 0.1 mL MOG35-55/CFA emulsion per site and both IP injections of pertussis toxin were 100 ng/dose at a volume of 0.1 mL/dose. The study was performed at Hooke Laboratories (Lawrence, MA, USA) using Hooke Kit™ MOG35-55/CFA Emulsion PTX number EK-2110. Female mice were selected for EAE experimentation since more females than males suffer clinically with MS as well as other autoimmune diseases (Voskuhl, 2011). EAE is an immune-driven pre-clinical model of MS and female mice are reported to experience greater severity of disease (Papenfuss et al., 2004; Rahn et al., 2014).

Mice were assessed daily and upon the first emergence of signs of disease, randomized into treatment groups (n=12) on the basis of comparable group average values for time of EAE onset and disease score at the onset of treatment. Dosing was initiated on the first day of EAE disease via once daily oral gavage of vehicle (5% v/v DMSO, 5% v/v Tween20, 90% v/v Milli-Q water; 5 mL/kg) or ozanimod at doses of 0.05, 0.2 or 1 mg/kg for 14 consecutive days. Efficacy was evaluated by recording daily visual EAE disease scores as described previously by Scott et al., 2016, as well as body weight measurement three times per week. Approximately 24 hours after the final dose, a blood sample was collected in EDTA coagulant for the assessment of absolute numbers of circulating lymphocytes by differential count, and a separate plasma sample was processed and stored at -80°C for subsequent analysis of neurofilament light by Quanterix (Lexington, MA, USA) using the Simoa™ NF-light Advantage kit (product #102258).

Mice were anesthetized, perfused with phosphate buffered saline, and the spinal cords collected and stored in 10% buffered formalin for imaging analysis. For each mouse spinal cord, three hematoxylin and eosin sections were prepared and analyzed for the number of inflammatory foci (approximately 20 cells per foci), estimation of demyelinated area (scores of 0 – 5 representing <5%, 5% to 20%, 20% to 40%, 40% to 60%, 60% to 80%, and 80% to 100% demyelinated area, respectively and as defined by interruption of normal structure such as
pallor and vacuolation consistent with edema and demyelination, as well as dilated axons) and apoptotic cell counts. Histological analysis was performed by a pathologist blinded to the experimental design and readouts.

**Cuprizone/Rapamycin Demyelination Model: Neuroprotection and Remyelination**

Cuprizone/rapamycin-induced demyelination was initiated in 8-week-old male C57BL/6J mice (Jackson Laboratories, USA) by ad libitum access to normal rodent diet (Harlan Teklad, Madison, WI) containing cuprizone (0.3% w/w) for a period of 6 weeks with once daily IP injection of rapamycin. Rapamycin was prepared fresh daily at 10 mg/kg at a volume of 5 mL/kg in 5% v/v pure ethanol/5% v/v Tween 80/5% PEG1000, aqueous. Age-matched control mice had ad libitum access to the same diet not containing cuprizone and received daily IP injection with vehicle. Mice were group housed 4 to 5 per cage and fresh food was provided three times weekly. All mice had ad libitum access to reverse osmosis filtered, acidified palatable drinking water at a pH level of 2.5 to 3.0. The study was performed at Renovo Neural, Inc. (Cleveland, OH, USA). Male mice were chosen for the demyelination model since a number of studies have reported that females are more resistant to the toxin and hence more robust demyelination is observed in males (MacArthur & Papanikolaou, 2014).

After 2 weeks of acclimation, mice were randomly assigned to dose groups and received once-daily oral gavage administration of vehicle (5% v/v DMSO, 5% v/v Tween20, 90% v/v Milli-Q water; 5 mL/kg) or ozanimod 5 mg/kg following the dosing and sample collection/testing regimen depicted in Fig. 1. For the assessment of ozanimod on neuroprotection and demyelination, dosing was initiated on day 1 concurrent with cuprizone/rapamycin and continued daily for 6 weeks. For the assessment of ozanimod’s effect on remyelination, daily dosing was also initiated on day 1 but continued beyond the 6-week cuprizone/rapamycin challenge for a further 12-week period (weeks 7-18 of the study). Mice in the remyelination arms
of assessment were discontinued from cuprizone diet and daily IP rapamycin injection at the end of the 6-week challenge period and returned to normal rodent diet.

In vivo brain magnetic resonance imaging (MRI) was used to monitor the effects of the 6-week cuprizone/rapamycin treatment and after a further 12 weeks after the demyelination challenge (study weeks 6 and 18). Mice were imaged on a 7T/20 Bruker-Biospec system to acquire high quality three-dimensional MRI longitudinally in the same animals. Mice were sedated with 1% to 3% isoflurane with adjusted respiration rate of approximately 50 to 80 breaths per minute. Level of induction was constantly monitored during the MRI imaging. The heated bed of the system-maintained animals at 35°C for the duration of the experiment. At the end of the scan, isoflurane was discontinued, and the mouse was returned to its cage to recover. To quantify changes in myelin loss sensitive magnetization transfer ratio, MT-weighted MRI images were acquired. After outlier removal based on image quality and animal stability in the MRI machine, group sizes were 6 to 9 mice.

Mice were not treated on the day of termination. Twelve animals per group (six for age-matched controls) were euthanized after 6 weeks of cuprizone/rapamycin treatment while the remaining animals continued on treatment until study weeks 9, 12, and 18, at which point these animals were sacrificed and samples collected (n = 6 per group for study weeks 9 and 12, n = 12 per group for study week 18). Animals were perfused with phosphate buffered saline and the brains were removed and fixed in 4% paraformaldehyde overnight at 4°C. The brains were dissected using a custom brain-slicing mold and further trimmed to isolate the corpus callosum, which was then fixed in a 2.5% glutaraldehyde/4% PFA mix for at least 12 hours. A small piece of corpus callosum was identified by specific morphological landmarks then cut and embedded in Epon resin. The rostral and caudal part of the brain (either side of the slice) was placed in a cryoprotection solution at 4°C overnight. The rostral section was sectioned with a microtome to
generate 30 μm thick free-floating sections were two sections per animal were stained with either SMI-32 (non-phosphorylated neurofilament H) or myelin proteolipid protein (PLP) antibodies and visualized by 3,3′-diaminobenzidine. The SMI-32-stained sections were evaluated to assess axonal ovoids in the white matter (corpus callosum) and the PLP stained sections were evaluated to assess the extent of remyelination in the hippocampus and cortex.

**Pharmacokinetics**

The pharmacokinetic profiles of ozanimod and its primary active rodent metabolite, RP101075, are similar in male and female C57BL/6J mice and so were assessed in plasma and brains of 8-week-old male C57BL/6J mice (Jackson Laboratories, USA) following daily oral dosing with ozanimod for 7 consecutive days. Ozanimod was dosed at either 1 or 5 mg/kg in the same vehicle as used for the MOG_{35-55} EAE and cuprizone/rapamycin in vivo efficacy studies and terminal plasma and brain samples were collected 3, 6, and 24 hours after the seventh daily dose of ozanimod. Of note, in the clinical setting, the dosing of ozanimod involves a dose titration to avoid and potential risk of mechanism-based bradycardia but this is not adopted when assessing efficacy in pre-clinical studies where dosing is initiated straight away with the dose to be assessed without titration. Brains were homogenized in acetonitrile at a 1:3 (w:v) ratio using a Biospec Bead Beater-16 with 1 mm glass beads and proteins precipitated further with a 1:10 dilution in acetonitrile to 1:30 (w:v) final. Plasma proteins were precipitated with acetonitrile at a 1:3 ratio (v:v). Samples were centrifuged and supernatants were analyzed by liquid chromatography–mass spectrometry (LC-MS/MS). For the tissue analysis, a standard curve was prepared using homogenized brain samples from untreated animals. A 10 point standard curve of ozanimod or RP101075 spanning a range of 0.046 nM to 500 nM was included with each bio-analytical run using a Kinetex C18 2.6μ 30×3 mm column (Phenomenex Inc., Torrance, CA), 0.1% formic acid in DI H₂O mobile phase A and 0.1% formic acid in
acetonitrile mobile phase B. Data were collected and analyzed using Analyst software version 1.5.1.

**Protein Binding**

To determine the free fraction of ozanimod and RP101075, protein binding assessments were performed with plasma, brain tissue and $[^{35}\text{S}]$-GTP$\gamma$S binding assay buffer. Serum protein binding was assessed by equilibrium dialysis using the 96-well micro equilibrium dialysis device (HTDialysis, Gales Ferry, CT). Regenerated cellulose dialysis membranes (12K molecular weight cut off) were used with a matched set of Teflon bars with a Teflon base block and stainless-steel pressure plate. Serum pooled from male and female C57Bl/6 mice (Bioreclamation Inc. (now BioIVT), New York; Catalogue # MSERM-C57, LOT MSE196727), was spiked with ozanimod or RP101075 at 100 or 500 nM before 100 μL of PBS was added to the dialysate side of the chamber followed by 100 μL of compound-spiked plasma added to the other side of the dialysis chamber, n = 7-8 replicates. The dialysis device was incubated with shaking at 37°C for at least 4 hours. Following incubation, 40 μL from the dialysate (buffer) side, representing the free concentration and an equivalent volume from the serum side, representing the total concentration was transferred from the dialysis plate to a 96-well assay block. 40 μL of PBS was added to the plasma sample and 40 μL of untreated plasma was added to the PBS sample for matrix matching to the standard curve (an eight point standard curve of ozanimod or RP101075 spanning a range of 0.14 nM to 500 nM was included with each bioanalytical run). Protein precipitation was achieved by the addition of 300 μL of acetonitrile, followed by centrifugation at 3000 rpm for 10 minutes at 20°C. The supernatants were analyzed by LC-MS/MS.

Brain protein binding and $[^{35}\text{S}]$-GTP$\gamma$S binding assay medium protein binding were assessed by equilibrium dialysis using the Rapid Equilibrium Dialysis device (Thermo Fisher, Waltham, MA).
The single-use Rapid Equilibrium Dialysis device plate contains inserts made of two side-by-side chambers separated by a vertical cylinder of dialysis membrane (8K molecular weight cut off) validated for minimal non-specific binding. Brain from saline perfused male C57Bl/6 mice was homogenized in PBS buffer at a 1:19 (w:v) ratio using a Biospec Bead Beater-16 with 1 mm glass beads. Brain homogenate or $[^{35}S]$-GTP$\gamma$S binding assay medium were spiked with either ozanimod or RP101075 at 1 and 5 μM in triplicate before 200 μL of compound-spiked brain homogenate or medium were added to one side of the dialysis chamber and 350 μL phosphate buffer to the other side. The plate was then placed in a Dubnoff shaking water bath to equilibrate for 4 or 24 hours at 37°C. Following incubation, 50 μL aliquots were removed from the respective brain homogenate/$[^{35}S]$-GTP$\gamma$S binding assay medium and from the phosphate buffer sides from triplicate equilibrium dialysis chambers and transferred to a 96-well assay block. After the addition of 50 μL of buffer to the brain homogenate/$[^{35}S]$-GTP$\gamma$S binding assay medium samples or brain/medium to the phosphate buffer sample, 200 μL of cold 50:50 acetonitrile:methanol containing internal standard was added to each sample. The plates were vortexed, then centrifuged at 4000 rpm for 10 minutes and the supernatant analyzed by LC-MS/MS. The Kalvass equation (Kalvass et al., 2018) was used to correct the fraction unbound to the undiluted fraction unbound in the homogenized tissue or assay medium.

**Data analysis**

**Radioligand binding assays**

Raw counts per 60 seconds per well were collected from the MicroBeta2. For saturation binding analysis, the binding of each concentration of $[^{3}H]$-ozanimod or $[^{3}H]$-A971432 to the various S1P$_5$-expressing membranes was determined by subtracting out the binding to parental CHO membranes. The total binding was thus the total binding to S1P$_5$-expressing membranes minus the total binding to parental CHO membranes, and the NSB (the counts remaining in presence
of 10 μM of unlabeled ozanimod or A971432) was the NSB to S1P5-expressing membranes minus the NBS to parental CHO membranes. The specific binding of [3H]-ozanimod or [3H]-A971432 to S1P5 was then calculated by subtracting the NSB from the total binding for each membrane preparation to calculate the dissociation constant (K_D) and maximal number of binding sites (B_max) values for [3H]-ozanimod or [3H]-A971432 to S1P5. For competitive radioligand binding with [3H]-A971432, raw data was normalized to the DMSO vehicle and 3 μM A971432 which was taken to be 0% inhibition and 100% inhibition of [3H]-A971432 binding, respectively. Concentration response curves for the inhibition of [3H]-A971432 binding were analyzed by non-linear regression using one site fit to generate K_i values having entered the K_D for [3H]-A971432 using GraphPad Prism (version 8.0.0).

[^S]-GTPγS binding assays

Raw counts per 40 seconds per well were collected from the MicroBeta2. The raw counts were analyzed by nonlinear regression using GraphPad Prism (version 8.0.0) to generate concentration response curves. The data were normalized to the percent response relative to the internal S1P control with the maximal S1P response taken to be 100% and the S1P basal response taken to be 0%. The potency, measured as concentration required to elicit a 50% response (EC_{50}), as well as the magnitude of the test compound response, or intrinsic activity, was calculated as the difference between the maximum and the minimum of each independent agonist concentration response curve.

Ligand:receptor modeling

The 3D coordinates of the human S1P1 receptor were downloaded from the described by Burley et al., 2019 using the Protein Data Bank ID: 3V2Y (Hanson et al., 2012). The human S1P5 receptor protein sequence was downloaded from UniProt (ID: Q9H228). The homology model of the human S1P5 receptor was created in Prime using methods described by Jacobson et al.,
2002 using human S1P1 as template. Siponimod, FTY720-p, and ozanimod chemical structures have been drawn in Maestro 2020-1 (Schrödinger, LLC, New York, NY), prepared for docking using LigPrep (Schrödinger) and custom force field parameters were optimized using the Force Field Builder (Schrödinger). Ozanimod was docked in the homology model using Glide SP (Halgren et al., 2004). The resulting system was prepared for molecular dynamics simulation using System Builder in Maestro. The protocol embedded the complex in a POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) membrane bilayer, it added equilibrated water molecules in the simulation box, and neutralized the total charge adding the correct type and number of ions. The system was equilibrated using Desmond molecular dynamics software 2020 (Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY) using the default protocol for membrane systems plus an additional 50 ns molecular dynamics simulation (NVT ensemble at 300K temperature). After removal of ozanimod, the equilibrated system was used to dock siponimod and FTY720-p using Glide SP. For each docked ligand-receptor complex, T120 was mutated in silico using the free energy perturbation method (FEP+; Wang et al., 2015) for a total of three independent calculations. FEP+ was used with default settings but extending the simulation time to 10 ns per lambda window.

Results

In vitro

Potency and intrinsic activity assessment of ozanimod and RP101075 for S1P1 and S1P5 across human and preclinical species revealed that while the potency for S1P1 was maintained across all species, the potency for S1P5 was significantly reduced for the mouse, rat, and canine homologues compared with that of human and cyno (Table 1). Compared to ozanimod/RP101075’s potency for human S1P5, the potency for cyno S1P5 was within 2-fold; however, the potency for mouse, rat, and canine S1P5 was shifted 111-, 237-, and 194-fold, respectively for ozanimod and 85-, 183-, and 86-fold, respectively for RP101075. Likewise, the
relative intrinsic activity taken as a percentage of the maximal response achieved by the endogenous ligand S1P was similar for human and cyno at 92% to 93% for ozanimod, and 75% to 91% for RP101075, but was reduced for mouse, rat, and canine S1P₅ to 64%, 75%, and 70%, respectively for ozanimod, and 68%, 78% and 62%, respectively for RP101075. This may be a consequence of the reduced potency for S1P₅ and the inability to reach a true maximal response within the concentration range tested that went up to 10 μM. Interestingly this observed shift in potency for S1P₅ was only observed with ozanimod/RP101075 and not with other S1P receptor modulators, FTY720-p (fingolimod) or siponimod which retained their potency for S1P₅ in the low nanomolar range across all species tested (Table 1).

In order to probe this finding, we first aligned the primary amino acid sequences for S1P₅ across species (Fig. 2) and observed that the percent conservation of human S1P₅ was highest with cyno at 95%, and lower for mouse, rat, and canine at 84.6%, 85.9% and 85.7%, respectively. We then selected individual and pairs of amino acids for site directed mutagenesis to determine if we could identify the amino acid(s) responsible for the observed loss of ozanimod activity.

Using [³⁵S]-GTP₇S binding assays to measure the functional coupling of mouse S1P₅ to G proteins, we established that a single amino acid at position 120 was responsible for the observed rightward shift in potency. In the mouse sequence there is an alanine at position 120 whereas in human there is a threonine. Mutating the alanine in the mouse sequence back to threonine restored the potency of ozanimod, shifting the EC₅₀ from 958 ± 204 nM for mS1P₅ to 6.7 ± 0.3 nM for mS1P₅_A120T to closely mirror the EC₅₀ for hS1P₅ of 8.6 ± 0.6 nM (Fig. 3). Notably, the other mutations assessed, L202V, G229R and L369S, did not rectify the potency; however, L202V in combination with A120T did result in corrected potency.

Interestingly, the amino acid at position 120 is also an alanine in the rat and canine sequence. Upon mutation of alanine to threonine in these species S1P₅ homologues, the potency was also restored to within 2-fold of that of the hS1P₅ potency as shown in Table 2. This leftward shift in
potency with the A120T mutation was also accompanied by an increase in observed intrinsic activity.

To determine if the observed shift in potency was due to a reduction in the binding affinity of ozanimod for S1P5, we developed radioligand binding assays to probe the mouse receptor homologue using both [3H]-ozanimod and [3H]-A971432, an S1P5-selective agonist (Hobson et al., 2015). Saturation binding analysis determined that the K\textsubscript{D} of ozanimod S1P\textsubscript{5} was 6.56 nM for the human homologue (Fig. 4) and yet we were unable to measure any specific binding with up to 40 nM of [3H]-ozanimod at wild type mS1P\textsubscript{5} suggesting the possibility of a species difference. The cellular membranes assessed in the radioligand binding assays were the same as those used for [35S]-GTP\gammaS binding, indicating that mS1P\textsubscript{5} was expressed to levels that should be detected with radioligand binding. To confirm this, we also ran a saturation analysis using an alternate S1P\textsubscript{5} agonist radioligand [3H]-A971432. [3H]-A971432 bound to wild type mS1P\textsubscript{5} revealed adequate receptor expression with a B\textsubscript{max} of 5.69 pmol/mg protein and a dissociation constant of 8.75 nM confirming the membranes were reliable and mS1P\textsubscript{5} expression was sufficient. Thus, it appeared that the binding affinity of ozanimod for mS1P\textsubscript{5} was affected. We then performed a saturation analysis with [3H]-ozanimod using membranes expressing mS1P\textsubscript{5_A120T} and found that binding was restored yielding a K\textsubscript{D} of 7.35 nM, very similar to that observed with hS1P\textsubscript{5}.

Using [3H]-A971432 we performed competition radioligand binding to determine the inhibition constant (K\textsubscript{i}) for ozanimod as well as FTY720-p, siponimod, and A971432 across hS1P\textsubscript{5}, mS1P\textsubscript{5} and mS1P\textsubscript{5_A120T}. Confirming the data achieved using [35S]-GTP\gammaS binding and saturation binding with [3H]-A971432, the K\textsubscript{i} values determined for FTY720-p, siponimod and A971432 were consistent between human and mouse S1P\textsubscript{5} homologues but the K\textsubscript{i} for ozanimod was 30-fold weaker for mS1P\textsubscript{5} compared with that of hS1P\textsubscript{5} (Table 3). Again, mutation of amino acid 120 from alanine to threonine corrected this shift in affinity.
To understand the reason for the shift with ozanimod but not with FTY720-p or siponimod, we created a homology model of the human S1P$_5$ receptor to better understand from a structural viewpoint the different effects of mutating the residue in position 3.37 (T120) on the in vitro activity of the agonists (Fig. 5). The lipophilic tail of ozanimod was predicted to bind in the pocket close to T120, while the other two ligands are not projected to bind as deep in the orthosteric binding site (Fig. 5B). As shown, the lipophilic tail of ozanimod interacts with T120 whereas the tails for the other two compounds are predicted to be 3-4 Å farther away. In light of the significant distance from T120, using the Free Energy Perturbation method, a T120 to alanine mutation in silico would not be expected to affect binding to FTY720-p or siponimod, which agrees with our in vitro experimental findings. However, due to the close proximity of ozanimod to T120 a mutation of this residue would be expected to significantly affect its activity as we observed (Fig. 5C). The model predicts that within hS1P$_5$ the nitrile group (Fleming et al., 2010) of ozanimod would accept a hydrogen bond interaction from residue 6.48 (W264) about 50% of the time, consequently affecting its rotameric state leading to conformational changes of trans-membrane domains 3 and 6. This conformational change, known as the toggle switch, is important for Family A G protein-coupled receptor activation (Tehan et al., 2014). The T120A mutation creates a more hydrophobic sub-pocket with ozanimod seemingly preferring to orient the isopropyl group toward W264 (Fig. 5D), which may affect the binding affinity by not favoring the conformational changes of trans-membrane domains 3 and 6 required for receptor activation.

**In vivo**

The efficacy of ozanimod 0.2 and 0.6 mg/kg in the mouse MOG$_{35-55}$ EAE model (Scott et al., 2016) reduces clinical disease scores and attenuates body weight loss, which coincides with reduced circulating ALCs. Here we expand upon those data both in dose range and efficacy readouts. All dose groups exhibited onset of disease around day 7 to 8 post-immunization with
disease scores of 1.08 ± 0.18, 1.13 ± 0.18, 1.13 ± 0.18, and 1.08 ± 0.16 (mean ± SEM) for vehicle, 0.05 mg/kg, 0.2 mg/kg, and 1 mg/kg ozanimod, respectively. Mice were dosed daily for 14 days and terminal disease scores were significantly attenuated with the 0.2 and 1 mg/kg doses (Fig. 6A). ALCs were significantly reduced in all dose groups (Fig. 6B). Significant increases in terminal body weight were observed in the 0.2 and 1 mg/kg dose groups (Table 4), which, together with the disease score and ALC data, confirmed the previous report. Additional efficacy readouts included histological evaluation of the spinal cord tissue where ozanimod was determined to significantly reduce spinal cord inflammation and demyelination, as well as attenuate the number of spinal cord apoptotic cells at all three doses assessed (Table 4). In addition, analysis of the plasma collected at termination of the study revealed that ozanimod significantly reduced the levels of circulating neurofilament light at the top dose of 1 mg/kg.

The effect of treatment with ozanimod was also assessed in a mouse cuprizone (CPZ) model of neuroprotection and remyelination (Fig. 1). After 6 weeks of concurrent treatment with CPZ and rapamycin (Rapa), one group of vehicle or ozanimod 5 mg/kg treated animals were first assessed for myelin content by MRI before sacrifice and assessment of the corpus callosum for neuronal breaks using SMI-32 immunohistochemical staining. As shown in Fig. 7A and Fig. 7B and consistent with reduced systemic levels of NfL, ozanimod significantly protected neuronal axons, preventing breakage and ovoid formation in the corpus callosum of CPZ/Rapa treated mice. In addition, ozanimod significantly attenuated the extent to which the corpus callosum demonstrated reduced myelin content as visualized by MRI (Fig. 7C). To assess the ability of ozanimod to enhance remyelination, additional groups of CPZ/Rapa treated mice were allowed to recover with concurrent dosing of either vehicle or ozanimod 5 mg/kg before sacrifice and assessment of myelin content using PLP staining following a further 3, 6, or 12 weeks of dosing after the 6 week CPZ/Rapa challenge. Ozanimod did not result in enhanced myelin content beyond that observed in the vehicle group in the cortex (Fig. 8A), corpus callosum (data not
shown), or hippocampus (data not shown) at any of the time points assessed with PLP staining, nor after a further 12 weeks of dosing as assessed by MRI of the corpus callosum (Fig. 8B).

In order to put these in vivo findings into context, the pharmacokinetics of the 1 mg/kg and 5 mg/kg doses of ozanimod that demonstrated robust efficacy in the EAE and CPZ/Rapa models, respectively, both total exposure and protein unbound exposure were measured in plasma and in brain tissue and the concentration multiples relative to mouse S1P$_1$ and S1P$_5$ EC$_{50}$ values calculated at three different time points (Table 5). Of note, the functional EC$_{50}$ values were also corrected for $[^{35}S]$-GTP$_\gamma$S assay medium protein binding which was determined to be 32%, and so 68% of drug was free. This adjusted the ozanimod EC$_{50}$ for mouse S1P$_1$ from 0.90 nM to 0.61 nM and the mouse S1P$_5$ EC$_{50}$ from 957.5 nM to 651.4 nM. Both the exposure of ozanimod and the major mouse active metabolite, RP101075, were measured and factored into the calculations. Ozanimod 1 mg/kg made up 74%, 71%, and 46% of the total agonist within the CNS at 3, 6, and 24 hours post-dose, respectively, compared with 25%, 28%, and 47% of RP101075 at 3, 6, and 24 hours post-dose, respectively. Ozanimod 5 mg/kg made up 64%, 69%, and 35% of the total agonist within the CNS at 3, 6, and 24 hours post-dose, respectively, compared with 35%, 30%, and 62% of RP101075 at 3, 6, and 24 hours post-dose, respectively. The remaining 1% to 7% of total agonist in the mice was comprised of other minor ozanimod metabolites, since the metabolism is quite different to that observed in humans (Surapaneni et al., 2021). The unbound fraction was calculated based on the fact that ozanimod is 92.5% bound to plasma proteins and 99.96% bound to brain proteins, and RP101075 is 95.3% bound to plasma proteins and 99.97% bound to brain proteins. At the 1 mg/kg dose used in the EAE study, free drug concentrations in the plasma were above the ozanimod mouse S1P$_1$ EC$_{50}$ for a full 24 hours but did not reach EC$_{50}$ levels in the brain at any time point assessed. Unbound drug levels did not come close to reaching S1P$_5$ EC$_{50}$ levels in either the plasma nor in the brain; in fact, the closest they came were 0.01-fold of the mouse S1P$_5$ EC$_{50}$ in the plasma at 3
and 6 hours post-dose. Likewise for the 5 mg/kg dose used in the cuprizone study, EC\textsubscript{50} coverage was achieved for S1P\textsubscript{1} in both the plasma and the brain for the full 24 hours post-dose in both compartments, yet S1P\textsubscript{5} EC\textsubscript{50} levels were not achieved, with the highest exposure being 0.06-fold for the mouse S1P\textsubscript{5} EC\textsubscript{50} in the plasma 3 hours post-dose. The exposures relative to ozanimod EC\textsubscript{50} were calculated (rather than relative to RP101075) to be the most conservative since ozanimod is slightly less potent than RP101075.

**Discussion**

The affinity and potency of ozanimod for mouse, rat, and canine S1P\textsubscript{5} is affected by a single amino acid substitution, an alanine in place of threonine at position 120 compared with human or cynomolgus monkey. This substitution results in a marked rightward shift in the binding affinity and functional potency of ozanimod but not for FTY720-p or siponimod in these species, and we provide a structural explanation based on homology modeling of S1P\textsubscript{5} that supports this observation. The hydroxyl group of T120 creates a favorable polar environment stabilizing a position of the nitrile compatible with a direct H-bond interaction with W264. The rotameric state of this tryptophan is important for the conformational change of the receptor upon activation. The simulation study suggests the interaction with W264 is created only when T120 is present. T120A mutation results in a more hydrophobic environment orienting the nitrile in a position unable to interact with W264. Threonine 120 substitution effectively renders ozanimod an S1P\textsubscript{1}-selective agonist in the noted species and provides a valuable tool to probe the S1P receptor involvement in commonly used mouse models of MS. Selective agonists for S1P\textsubscript{1} are scarce and those that are available, such as AUY954, SEW2871, or CYM-5442, display weak potency or demonstrate marginal selectivity for mouse S1P\textsubscript{1} over mouse S1P\textsubscript{5} (Table 6) or have poor pharmacokinetic exposure and pharmacodynamic effects upon oral dosing (Sanna et al., 2004; Pan et al., 2006; Gonzalez-Cabrera et al., 2008).
Ozanimod is highly active in the mouse EAE model with reduced spinal cord inflammation, attenuated demyelination, and decreased spinal cord apoptotic cell counts reaching statistical significance with doses as low as 0.05 mg/kg. This coincides with significant engagement of S1P1 pharmacology evidenced by the pharmacodynamic readout of reduced ALCs. Dose increases also reveal additional levels of statistical significance, further efficacy in lessening the magnitude of EAE disease scores and attenuating the body weight deficit that typifies EAE, as well as reduced circulating NfL. Examining the pharmacokinetic exposures of free, unbound levels of ozanimod (in conjunction with RP101075, its primary active metabolite in mouse) at the highest dose of 1 mg/kg assessed in the EAE model, calculated unbound drug levels in the plasma were sufficient to cover the EC50 of ozanimod for mouse S1P1 but do not meet the EC50 for mouse S1P5. The levels of free drug in the brain are lower than in the plasma despite higher levels of total drug due to the very high binding to brain proteins. As a result, the levels do not reach the EC50 for S1P1 or S1P5 in this compartment. Therefore, efficacies observed in this model are most likely driven by pharmacological engagement of S1P1 in the periphery and that significant reduction of the numbers of circulating B and T cells, as is clinically observed in relapsing-remitting multiple sclerosis patients taking ozanimod (Harris et al., 2020), is sufficient for preclinical efficacy. Indeed, we reported that early treatment with ozanimod (beginning day 4 post-MOG35-55 immunization) diminishes Th1 and Th17 cell expansion in the periphery and reduces the number of Th1 and Th17 cells that infiltrate the spinal cord (Guimond et al., 2017). Also observed was a concurrent reduction in MHCII+ monocytes and microglia present in the spinal cord tissue. S1P1 expression is upregulated in astrocytes and potentially microglia in the EAE model (Colombo et al., 2014; Liu et al., 2016; Noda et al., 2013) and within lesions in patients with MS (Van Doorn et al., 2010). Thus, it is also possible that functional antagonism, or agonist-mediated S1P1 internalization in CNS cells, may contribute to ozanimod’s efficacy. A role in astrocytes is strongly supported by reduced disease scores and loss of efficacy of FTY720 in EAE performed in mice with astrocyte-specific S1P1 knockout (Choi et al., 2011).
This is confirmed further through demonstration that astrocytes are the first CNS cells activated within the context of EAE using a c-fos driven green fluorescent protein reporter mouse and showing that this activation is S1P₁-dependent using both genetic ablation of S1P₁ and pharmacological inhibition with FTY720 (Groves et al., 2018). Continual low level activation of astrocyte or microglial S1P₁ achieved with 1 mg/kg ozanimod in this study might be beneficial since significant reduction in circulating levels of the marker of neuronal damage, NfL were seen with this dose.

Neuroprotective effects of ozanimod in the CPZ/Rapa model were consistent with the MOG EAE model, yet ozanimod did not positively impact the remyelination phase. Concurrent treatment of ozanimod with induction of CPZ/Rapa-mediated demyelination resulted in significant prevention of axonal breaks with ovoid formation and a reduction in the magnitude of myelin loss within the corpus callosum as quantitated with MRI imaging. CD8⁺ and CD4⁺ T cells do not play a significant role in CPZ intoxication models (McMahon et al., 2002) and hence the efficacy observed with ozanimod in this portion of the model is T cell-independent and likely centrally driven. Both plasma and brain levels exceeded the EC₅₀ concentrations for S1P₁ with the 5 mg/kg dose but still fell significantly short for S1P₅. Ozanimod’s effects in this second mouse model are likely mediated by S1P₁. Direct CNS effects with S1P modulators are being increasingly reported in the literature (Groves et al. 2013; Kipp 2020). S1P₁ is upregulated in activated astrocytes in a CPZ intoxication mouse model, and selective S1P₁ downregulation by CYM5422 significantly reduces oligodendrocyte apoptosis and activation of both astrocytes and microglia (Kim et al., 2018). Moreover, the nonselective S1P receptor modulator, FTY720, was also active, and S1P₁ modulation was necessary during the early phase of CPZ intoxication to suppress oligodendrocyte death, and that suppression in the production of pro-inflammatory mediators leading to reactive gliosis is a contributing factor to the survival of the oligodendrocytes. Within the context of EAE, bypassing the periphery and introducing S1P
modulators directly into the CNS via intracerebral ventricular (ICV) microinjection results in key CNS protective effects in the absence of reduced numbers of lymphocytes; astrogliosis, microgliosis and GABA-ergic neuronal degeneration within the striatum were attenuated with ICV administration of siponimod (Gentile et al., 2016); attenuation of EAE disease scores in the absence of lymphocyte reduction was also observed with ICV dosing with S1P₁-preferring, AUY954 (Musella et al., 2020). Moreover, acutely prepared brain slice cultures isolated from EAE mice treated with siponimod demonstrate restoration of cortical network functionality (Hundehege et al., 2019), and teasing out the receptor(s) via ozanimod, AUY954, and A971432, has shown specifically that correction of parameters of spontaneous-evoked postsynaptic potentials in medium spiny neurons, such as decay time and half width, are more sensitive to S1P₁ modulation than S1P₅ (Musella et al., 2020).

Remyelination following cessation of CPZ/Rapa more closely reflects oligodendrocyte involvement following demyelination and neuronal injury. Oligodendrocytes are reported to express S1P₅ at all stages of their development from precursors (OPCs) through pre-oligodendrocytes and to fully differentiated oligodendrocytes capable of depositing myelin (Jaillard et al., 2005). The activation of S1P₅ is reported to increase the survival of OPCs (Miron et al., 2008), induce initial process retraction of pre-oligodendrocytes followed by extensive process re-extension and maturation (Jaillard et al., 2005), and enhance myelination following lyssolecithin-induced demyelination in CNS cortical organoids (Jackson et al., 2011). Although one might expect enhanced remyelination with S1P₅ activation, we did not observe this with ozanimod treatment. However, this is easily explained by calculating the pharmacological coverage of S1P₅ relative to the reduced potency of ozanimod for mouse S1P₅, which made it apparent that we did not achieve CNS exposures sufficient to activate S1P₅. Our finding suggests that S1P₁ does not directly contribute to remyelination, even though it may contribute to oligodendrocyte survival. Indeed, increased remyelination in rat telencephalon organoids in
vitro is observed with FTY720 and siponimod, which, as we describe, maintain their potency for mouse S1P5, but that this increased myelination is not observed for the S1P1-preferring agonist, AUY954 (Jackson et al., 2011). In CPZ intoxication, siponimod exerts neuroprotective effects similar to ozanimod; in addition, siponimod appeared to enhance remyelination as visualized with detailed electron microscopy but notably not with immunohistochemistry, suggesting a subtle effect (Tiwari-Woodruff., 2016). Modest positive effects on remyelination have been reported with FTY720 in an acute model of CPZ-induced demyelination but not in a chronic model, suggesting a fine window of opportunity to observe enhanced myelination (Slowik et al., 2015).

In conclusion we have described and characterized a novel discovery of a species difference in the activity of ozanimod for S1P5 and have capitalized on this finding to probe and elucidate the S1P receptor(s) involved in commonly used mouse models of MS which, until now, have only been speculated to be either S1P1 or S1P5-mediated. Thus, using finely calculated drug exposures, we show that S1P1 is responsible for multiple efficacy readouts in the EAE model that may either be as a consequence of the reduction in circulating lymphocytes and/or of direct effects on neural cells such as astrocytes and microglia. In the CPZ/Rapa model, we believe S1P1 to mediate the observed neuroprotection and reduction in myelin loss during the intoxication period, but that enhanced remyelination post-intoxication is not a downstream event of S1P1 engagement and indeed may require S1P5 activation. While we were unable to demonstrate a remyelination effect of ozanimod in these studies, this was likely due to the reduced potency for the mouse homologue of S1P5, and yet, based on these findings and prior published evidence, ozanimod still has the potential to trigger remyelination in patients with MS due to its high potency agonist activity for human S1P5.
Acknowledgments

All authors contributed and approved the final manuscript. The authors thank Samantha Richardson for her contributions to the mouse plasma and brain protein binding analysis. Editorial assistance was provided by Peloton Advantage, an OPEN Health company and was funded by Bristol Myers Squibb.

Disclosures

JVS, KCD, YGY, NC, DD, SB, AB, JS, CL, IR, and RH are employees and shareholders of Bristol Myers Squibb.

Author Contributions

Participated in research design: JVS, KCD, SB, and RH.

Conducted experiments: YGY, NC, DD, CL, and IR.

Contributed new reagents or analytical tools: AB and JS.

Performed data analysis: JVS, KCD, YGY, NC, DD, AB, and JS.

Wrote or contributed to writing of the manuscript: JVS, KCD, AB, JS, and RH.
References


Jaillard C, Harrison S, Stankoff B, Aigrot MS, Calver AR, Duddy G, Walsh FS, Pangalos MN,


Figure Legends

**Fig. 1.** Study design to assess the effect of ozanimod on cuprizone/rapamycin-induced axonal degeneration and demyelination (weeks 0–6) as well as subsequent remyelination (weeks 6–18).

CPZ = 0.3% cuprizone in diet; Rapa = rapamycin (10 mg/kg, IP, QD); SMI-32 = non-phosphorylated neurofilament H; MRI = magnetic resonance imaging; PLP = proteolipid protein.

**Fig. 2.** Sequence alignment of human, cynomolgus monkey, mouse, rat, and canine S1P₅. The teal highlighted sections are the G-protein coupled receptor seven transmembrane domains and the amino acids (A120, L202, G229, L369) subjected to site-directed mutagenesis are indicated with * above. Alignment across all five species is denoted with * below. A colon (:) below indicates conservation between groups of strongly similar properties and a period (.) below indicates conservation between groups of weakly similar properties. S1P₅ = sphingosine-1 phosphate receptor subtype 5.

**Fig. 3.** [³⁵S]-GTPγS binding concentration response curves for ozanimod across wild type and mutated mouse S1P₅ performed in membranes prepared from transiently transfected CHO cells. Data shown are mean ± SEM for n = 3 or 4 independent experiments each performed in duplicate. EC₅₀ = the concentration of the test compound required to elicit a half maximal response. CHO = Chinese hamster ovary; S1P₅ = sphingosine-1 phosphate receptor subtype 5.

**Fig. 4.** Saturation binding analysis of [³H]-ozanimod to human S1P₅ (top left), mouse S1P₅ (top right), and mouse S1P₅_A120T (bottom left). Also shown is the saturation binding analysis for [³H]-A971432 to mouse S1P₅ (lower right). Key: ○ total binding; * non-specific binding; ● specific binding (= total binding minus non-specific binding).
*K_D and B_max undefined.

B_max = maximum number of binding sites; K_D = dissociation constant; S1P_5 = sphingosine-1 phosphate receptor subtype 5.

**Fig. 5.** Receptor modeling for the binding of S1P receptor modulators to human S1P_5 and the predicted effect of mutating threonine 120 to an alanine.

A Molecular structures of siponimod (top), FTY720-p (middle) and ozanimod (bottom). B Predicted binding mode of siponimod (green carbon atoms), FTY720-p (brown carbon atoms) and ozanimod (pink carbon atoms) to the human S1P_5 homology model. The receptor secondary structure is shown as tube color coded as rainbow from the N terminus to the C terminus. T120 is shown as a CPK space filling representation and generic residue numbers according to Isberg et al., 2015 are included as superscript. C Representative structure from a Free Energy Perturbation (FEP) simulation of the human S1P_5 homology model where the T120 has been mutated in silico to Ala in the presence of ozanimod (pink carbon atoms). D Representative FEP structure including the wild type T120. Labels for transmembrane helices 3 (TM3) and 6 (TM6) are included.

**Fig. 6.** Experimental autoimmune encephalomyelitis disease scores (A) and terminal absolute lymphocyte counts (B).

Data shown are mean ± SEM for C57/BL6 female mice randomized into treatment groups of n=12. Dosing was initiated on the first day of notable disease onset and continued for a further 14 days. A Disease scores were evaluated daily with scores ranging from 0.0 (no obvious changes in motor function compared to controls) to 5.0 (spontaneously rolling in the cage, found dead due to paralysis, or euthanized due to severe paralysis). Statistically significant improvements in disease end scores were determined using Wilcoxon’s nonparametric test vs Vehicle; *p < 0.05, ***p < 0.001. B Absolute lymphocyte counts were quantitated by differential
count from blood samples collected 24 hour post-last dose. The percent decrease compared to vehicle control is indicated above each bar and statistically significant decreases in absolute lymphocyte counts compared to vehicle were determined using one-way ANOVA with Dunnett’s post hoc test; *p < 0.05, **p < 0.001.

Fig. 7. Quantification of non-phosphorylated neurofilament H (SMI-32) staining (A) and immunohistochemistry in coronal sections of the corpus callosum (B), as well as MRI analysis of myelin content within the corpus callosum (C) following 6 weeks of cuprizone /rapamycin treatment.

Data shown in graph A are mean ± SEM of the number of SMI-32+ ovoids per 250,000 μm² section for n = 6 aged matched, n = 10 CPZ/Rapa vehicle and n = 11 CPZ/Rapa ozanimod treated mice. Statistically significant reduction in ovoid numbers were determined using one-way ANOVA with Dunnett’s multiple comparisons post-test; **p < 0.01. Image B shows representative sections of SMI-32 immunohistochemistry in the corpus callosum (20x). Data shown in graph C are mean ± SEM of the myelin MRI signal within the corpus callosum for n=8 age matched, n = 7 CPZ/Rapa vehicle and n=8 CPZ/Rapa ozanimod treated mice. Statistically significant difference in the myelin content between groups were determined using one-way ANOVA with Dunnett’s multiple comparisons post-test; *p < 0.05, **p < 0.01.

ANOVA = analysis of variance, CPZ = cuprizone, MRI = magnetic resonance imaging, Rapa = rapamycin.
Fig. 8. A Analysis of myelin content of the cortex 3 weeks post-cessation of 6 weeks of cuprizone/rapamycin treatment using PLP staining or B 12 weeks post-cessation using MRI.

Image A shows representative sections of PLP-staining in the cortex (2x). Data shown in graph B are mean ± SEM of the myelin MRI signal within the corpus callosum for n = 9 age matched, n = 8 CPZ/Rapa vehicle and n = 7 CPZ/Rapa ozanimod treated mice. Statistically significant difference in the myelin content between groups were determined using one-way ANOVA with Dunnett’s multiple comparisons post-test; *p < 0.05.

ANOVA = analysis of variance, CPZ = cuprizone, MRI = magnetic resonance imaging, Rapa = rapamycin.
Table 1. Potency and intrinsic activity of S1P receptor modulators for S1P<sub>5</sub> across species with [<sup>35</sup>S]-GTPγS binding performed using CHO cell membranes stably expressing the recombinant receptors. Data are also shown for ozanimod/RP101075 for S1P<sub>1</sub> across species.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ozanimod; RP101075</th>
<th>FTY720-p</th>
<th>Siponimod</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
<td>IA (% S1P)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
</tr>
<tr>
<td>Human S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.03 ± 0.16; 0.35 ± 0.01</td>
<td>91.9 ± 1.9; 85.6 ± 1.9</td>
<td>8.6 ± 0.6; 4.49 ± 0.67</td>
</tr>
<tr>
<td>Cynomolgus monkey S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.29 ± 0.05; 0.63 ± 0.02</td>
<td>93.3 ± 0.7; 86.8 ± 3.6</td>
<td>15.9 ± 0.4; 10.10 ± 0.91</td>
</tr>
<tr>
<td>Mouse S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.90 ± 0.21; 0.28 ± 0.02</td>
<td>92.0 ± 2.1; 85.8 ± 3.5</td>
<td>957.5 ± 203.7; 380.98 ± 125.24</td>
</tr>
<tr>
<td>Rat S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.02 ± 0.14; 0.31 ± 0.02</td>
<td>90.9 ± 2.7; 88.7 ± 6.1</td>
<td>2032.7 ± 191.2; 818.75 ± 86.51</td>
</tr>
<tr>
<td>Canine S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.61 ± 0.08; 0.51 ± 0.11</td>
<td>85.8 ± 1.7; 86.9 ± 7.2</td>
<td>1662.0 ± 164.92; 382.3 ± 10.86</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM for n=3-6 independent experiments each performed in duplicate. EC<sub>50</sub> = the concentration of the test compound required to elicit a half maximal response. IA = intrinsic activity which is the maximal response achieved relative to the endogenous ligand, S1P, which was taken to be 100%. S1P<sub>1</sub> = sphingosine-1 phosphate receptor subtype 1; S1P<sub>5</sub> = sphingosine-1 phosphate receptor subtype 5.
Table 2. Potency and intrinsic activity of ozanimod for wild type S1P$_5$ and S1P$_5$-A120T across species using [$^{35}$S]-GTP$\gamma$S binding performed using CHO cell membranes stably expressing the recombinant wild type receptors or transiently expressing the mutated A120T receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>EC$_{50}$ (nM)</th>
<th>IA (%)</th>
<th>Fold Human S1P$_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human S1P$_5$</td>
<td>8.6 ± 0.6</td>
<td>92.3 ± 2.1</td>
<td>1</td>
</tr>
<tr>
<td>Cynomolgus monkey S1P$_5$</td>
<td>15.9 ± 0.4</td>
<td>93.7 ± 3.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Mouse S1P$_5$</td>
<td>957.5 ± 203.7</td>
<td>63.9 ± 2.7</td>
<td>111.6</td>
</tr>
<tr>
<td>Rat S1P$_5$</td>
<td>2032.7 ± 191.2</td>
<td>74.8 ± 1.1</td>
<td>236.9</td>
</tr>
<tr>
<td>Canine S1P$_5$</td>
<td>1662.0 ± 164.92</td>
<td>69.6 ± 1.9</td>
<td>194.6</td>
</tr>
<tr>
<td>Mouse S1P$_5$-A120T</td>
<td>6.7 ± 0.3</td>
<td>95.9 ± 6.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Rat S1P$_5$-A120T</td>
<td>6.6 ± 1.2</td>
<td>82.1 ± 3.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Canine S1P$_5$-A120T</td>
<td>14.0 ± 1.5</td>
<td>74.2 ± 1.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM for n = 3 independent experiments each performed in duplicate.

EC$_{50}$ = the concentration of test compound required to elicit a half maximal response. IA = intrinsic activity which is the maximal response relative to the endogenous ligand, S1P, which was taken to be 100%. S1P$_5$ = sphingosine-1 phosphate receptor subtype 5.
Table 3. $[^{3}H]$-A971432 competition radioligand binding affinity assessment for human and mouse wild type S1P$_5$ and mouse S1P$_5$-A120T performed using CHO cell membranes stably expressing the recombinant wild type receptors or transiently expressing mouse S1P$_5$-A120T.

<table>
<thead>
<tr>
<th></th>
<th>Ozanimod K$_i$ (nM)</th>
<th>FTY720-p K$_i$ (nM)</th>
<th>Siponimod K$_i$ (nM)</th>
<th>A971432 K$_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human S1P$_5$</td>
<td>2.0 ± 0.1</td>
<td>0.3 ± 0.01</td>
<td>0.2 ± 0.02</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Mouse S1P$_5$</td>
<td>59.9 ± 8.8</td>
<td>0.2 ± 0.01</td>
<td>0.3 ± 0.02</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Mouse S1P$_5$-A120T</td>
<td>5.6 ± 1.2</td>
<td>0.3 ± 0.03</td>
<td>0.4 ± 0.03</td>
<td>6.0 ± 0.5</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM for n = 3 independent experiments each performed in duplicate. K$_i$ is the binding affinity defined as the concentration required to elicit a 50% reduction in $[^{3}H]$-A971432 binding once corrected for the concentration of radioligand used and the dissociation constant of the radioligand. S1P$_5$ = sphingosine-1 phosphate receptor subtype 5.
Table 4. Summary of the additional efficacy endpoints in the mouse experimental autoimmune encephalomyelitis model.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Terminal Body Weight (% vs Day 1)</th>
<th>Spinal Cord Inflammation (Foci/20 cells)</th>
<th>Spinal Cord Demyelination (Score 0-5)</th>
<th>Spinal Cord Apoptotic Cells (Count/section)</th>
<th>Plasma NfL (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5% DMSO, 5% Tween 20, 90% water)</td>
<td>86.4 ± 3.2</td>
<td>8.50 ± 1.21</td>
<td>2.00 ± 0.15</td>
<td>2.25 ± 0.53</td>
<td>4.37 ± 0.89</td>
</tr>
<tr>
<td><strong>Ozanimod</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 mg/kg</td>
<td>85.8 ± 2.7</td>
<td>5.00 ± 1.03*</td>
<td>0.91 ± 0.21***</td>
<td>1.08 ± 0.23*</td>
<td>3.53 ± 0.46</td>
</tr>
<tr>
<td>0.2 mg/kg</td>
<td>95.7 ± 3.1*</td>
<td>3.54 ± 0.49***</td>
<td>0.73 ± 0.14 ***</td>
<td>0.91 ± 0.28*</td>
<td>2.62 ± 0.46</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>102.8 ± 1.8*</td>
<td>2.67 ± 0.56***</td>
<td>0.33 ± 0.14 ***</td>
<td>0.60 ± 0.19**</td>
<td>1.91 ± 0.34**</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM. Statistical significance for terminal body weight from vehicle was determined using a two-tailed Student’s t-test. Statistically significant reduction in spinal cord inflammation, spinal cord demyelination, spinal cord apoptotic cell count, and plasma NfL levels were determined using one-way ANOVA with Dunnett’s post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001. DMSO = dimethyl sulfoxide; NfL = neurofilament light.
Table 5. Total and unbound exposure of ozanimod and RP101075 in the plasma and the brains of C57Bl/6 mice relative to the ozanimod potency for mouse S1P\(_1\) and S1P\(_5\).

<table>
<thead>
<tr>
<th>Time post-final dose</th>
<th>Plasma Concentration (nM)</th>
<th>Brain Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (fold S1P(_1), fold S1P(_5)); Free (fold S1P(_1), fold S1P(_5))</td>
<td>Total (fold S1P(_1), fold S1P(_5)); Free (fold S1P(_1), fold S1P(_5))</td>
</tr>
<tr>
<td>3 hours</td>
<td>6 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>Ozanimod</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>138 (226, 0.21);</td>
<td>103 (168, 0.16);</td>
</tr>
<tr>
<td></td>
<td>9.27 (15.14, 0.01)</td>
<td>6.77 (11.06, 0.01)</td>
</tr>
<tr>
<td>Ozanimod</td>
<td>616 (1006, 0.95);</td>
<td>501 (819, 0.77);</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>41.13 (67.20, 0.06)</td>
<td>32.86 (53.69, 0.05)</td>
</tr>
</tbody>
</table>

Data shown are mean exposures of the sum of ozanimod and the major active rodent metabolite, RP101075, collected from \(n = 4\) mice per group. In order to calculate the free drug exposure, total exposure was multiplied by the fraction unbound as determined by plasma or brain protein binding analysis. The fold S1P\(_1\) and S1P\(_5\) receptor values in parentheses were calculated by dividing the total or free exposure by the functional potency of ozanimod for mouse S1P\(_1\) or mouse S1P\(_5\) once corrected for free drug fraction in the functional \[^{35}\text{S}]-\text{GTPyS}\) binding assay medium. Mice were dosed orally, once daily, for seven consecutive days with either 1 or 5 mg/kg ozanimod. S1P\(_1\) = sphingosine-1 phosphate receptor subtype 1; S1P\(_5\) = sphingosine-1 phosphate receptor subtype 5.
Table 6. Potency and intrinsic activity of S1P₁ agonists for wild type mouse S1P₁ and S1P₅ determined using [³⁵S]-GTPγS binding performed using CHO cell membranes stably expressing the recombinant wild type receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mouse S1P₁</th>
<th>Mouse S1P₅</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>IA (%)</td>
</tr>
<tr>
<td>AUY954</td>
<td>3.13 ± 0.02</td>
<td>95.2 ± 6.9</td>
</tr>
<tr>
<td>CYM-5442</td>
<td>6.75 ± 0.20</td>
<td>95.4 ± 3.8</td>
</tr>
<tr>
<td>SEW2871</td>
<td>121.1 ± 12.44</td>
<td>86.4 ± 5.0</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM for n=2 independent experiments each performed in duplicate.

EC₅₀ is defined as the concentration of test compound required to elicit a half maximal response. IA = intrinsic activity which is the maximal response relative to the endogenous ligand, S1P, which was taken to be 100%. CHO = Chinese hamster ovary; S1P₁ = sphingosine-1 phosphate receptor subtype 1; S1P₅ = sphingosine-1 phosphate receptor subtype 5.
Figure 1

This article has not been copyedited and formatted. The final version may differ from this version.
Table 1. 

<table>
<thead>
<tr>
<th>Mutant</th>
<th>EC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hS1P_{5}</td>
<td>8.6±0.6</td>
</tr>
<tr>
<td>mS1P_{5}</td>
<td>958±204</td>
</tr>
<tr>
<td>mS1P_{5}_G229R</td>
<td>1137±305</td>
</tr>
<tr>
<td>mS1P_{5}_L369S</td>
<td>429±33</td>
</tr>
<tr>
<td>mS1P_{5}_G229R_L369S</td>
<td>884±156</td>
</tr>
<tr>
<td>mS1P_{5}_A120T</td>
<td>6.7±0.3</td>
</tr>
<tr>
<td>mS1P_{5}_L202V</td>
<td>750±105</td>
</tr>
<tr>
<td>mS1P_{5}_A120T_L202V</td>
<td>9.8±1.0</td>
</tr>
</tbody>
</table>

Figure 3
Human Wild Type S1P<sub>5</sub>

$K_D = 6.56 \text{ nM}$

$B_{max} = 12.31 \text{ pmol/mg protein}$

Mouse Wild Type S1P<sub>5</sub>

$K_D = 8.75 \text{ nM}$

$B_{max} = 5.69 \text{ pmol/mg protein}$

Mouse S1P<sub>5</sub> A120T (transient)

$K_D = 7.35 \text{ nM}$

$B_{max} = 6.27 \text{ pmol/mg protein}$

Figure 4
Figure 5
Figure 6A-B

A

Day of Disease and Treatment

EAE Disease Score

Onset of Treatment

Vehicle
Ozanimod 0.05 mg/kg
Ozanimod 0.2 mg/kg
Ozanimod 1 mg/kg

B

Circulating ALCs (10^6 cells/mL)

Vehicle 0.05 0.2 0.1

63%
40%
35%
57%

Figure 6A-B
Figure 7A-C

A

** SM-32+ Ovoids (per 250,000 μm²)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-matched + Vehicle</td>
<td>**</td>
</tr>
<tr>
<td>CPZ/Rapa + Vehicle</td>
<td>**</td>
</tr>
<tr>
<td>CPZ/Rapa + Ozanimod</td>
<td></td>
</tr>
</tbody>
</table>

B

Age Matched

CPZ/Rapa Vehicle

CPZ/Rapa Ozanimod

C

Myelin MRI Corpus Callosum

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-matched + Vehicle</td>
<td>37% ↓</td>
</tr>
<tr>
<td>CPZ/Rapa + Vehicle</td>
<td>**</td>
</tr>
<tr>
<td>CPZ/Rapa + Ozanimod</td>
<td></td>
</tr>
</tbody>
</table>

** P < 0.01

* P < 0.05
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 8A-B

Panel A: Images of myelin MRI from different groups.

Panel B: Bar graph showing myelin MRI corpus callosum measurements for age-matched, CPZ/Rapa + Vehicle, and CPZ/Rapa + Ozanimod groups. The graph indicates a 16% decrease in the CPZ/Rapa + Vehicle group compared to the age-matched group and a 20% decrease in the CPZ/Rapa + Ozanimod group compared to the age-matched group. The CPZ/Rapa + Vehicle group shows a significant decrease (*) compared to the age-matched group, while the CPZ/Rapa + Ozanimod group shows a non-significant decrease (NS).