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

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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 S1P1 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 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.

SIGNIFICANCE STATEMENT
Ozanimod, a selective modulator of human sphingisone 1-phosphate receptor subtypes 1 and 5 (S1P1/5), 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 comprises five G protein–coupled receptors of the class A family of seven transmembrane domain G protein–coupled receptors, designated S1P subtypes 1–5 (S1P1–5) (Rosen...
et al., 2013; Kihara et al., 2014), with S1P1 and S1P5 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 S1P1, which is expressed on B and T cells, and after exposure to agonist is rapidly downmodulated from the plasma membrane with internalization to the intracellular compartment (Sanna et al., 2004; Scott et al., 2016). This internalization of S1P1 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., 2021). S1P1 is also expressed by astrocyte and microglial cells within the CNS (Choi et al., 2011; Noda et al., 2013; Musella et al., 2020), and downmodulation of S1P1 in these cell types attenuates their activation state, which may abrogate the local proinflammatory 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), preoligodendrocytes, 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 preoligodendrocytes 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, including 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; Zeposia package insert, 2020). Compared with other S1P modulators, ozanimod has superior S1P receptor selectivity and does not require the need for predose genetic testing compared with siponimod (BAF-312, Mayzent) (Gardin et al., 2019; Mayzent package insert, 2019). Compared with other S1P modulators, ozanimod has superior S1P receptor selectivity and does not require first dose heart rate monitoring compared with fingolimod (FTY720, Gilenya) (Brinkmann et al., 2002; Gilenya package insert, 2010), and a lack of predominant CYP2C9 metabolism negates the need for predose genetic testing compared with siponimod (BAP-312, Mayzent) (Gardin et al., 2019; Mayzent package insert, 2019).

During the development of ozanimod, S1P1-5 were cloned from multiple preclinical species and the activity of ozanimod and its metabolites characterized to understand the potencies relative to the human receptor homologs. In so doing, we discovered a species variation in the activity of ozanimod and its 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 was observed to be significantly reduced for mouse S1P5.

In the current studies we report on the identification of ozanimod/RP101075’s reduced potency for S1P5 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 (3H-ozanimod) and tritium-labeled S1P5 selective ligand A971432 ([3H]-A971432) were custom synthesized by Novandi Chemistry (Södertälje, Sweden).

#### Generation of Stable Recombinant S1P5 Expressing Cell Lines

Human, mouse, and rat S1P5 cDNAs (UniProt accession numbers, Q9H228, Q91X56, and Q8JKM5) and codon optimized cyno and Canis familiaris (dog) S1P5 cDNAs (UniProt accession numbers GT7280 and E2RDP1) were cloned into pcDNA3.1(+) vector (Thermo Fisher Scientific, Waltham, MA) with a hemagglutinin 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 S1P5 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 (TRF0264, Perkin Elmer, Waltham, MA) and flow cytometry using an anti-hemagglutinin-Alexa Fluor 647–conjugated antibody (Cell Signaling, Boston, MA). The final clone was selected and verified using guanosine 5′-O-(3-[35S]thio)triphosphate ([35S]-GTP-S) 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 Gene-wiz, 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_L260V, and mS1P5_A120T_L260V 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). 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 after transfection and cell membranes prepared as described below.

**Cell Membrane Preparations**
Membranes were prepared from stable or transient S1P₅ expressing cells after adherent culture in 500 cm² culture trays. Cells were detached with cell-lifting buffer (10 mM HEPES, 154 mM NaCl, 6.85 mM EDTA, pH 7.4) and pelleted by centrifugation for 5 minutes at 1000 rpm. Cell pellets were then resuspended 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 rehomogenized and recentrifuged as described above in membrane preparation buffer. The final pellet was collected and homogenized in ice cold resuspension buffer (10 mM HEPES, 1 mM EDTA, 100 mM NaCl, 1 mM MgCl₂, 0.1% fatty acid free bovine serum albumin, and 30 μg/ml saponin, pH 7.4) 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 40 μl per well of 0.33% DMSO vehicle, and the other half of the plate was prepared for nonspecific binding (NSB) measurement, with 60 μl per well of 33.3 μM unlabeled ozanimod or A971432. Serial 2-fold dilutions of [³H]-ozanimod and [³H]-A971432 using 96-well nonscuffing surface plates (3604, Corning, Corning, NY) with a final volume of 200 μl. One assay plate was prepared for each of the following membrane preparations: human S1P₅, mouse S1P₅, mouse S1P₅/Δ120T, and CHO K1 parental cells. For determination of total binding, half of the assay plate was prepared with 60 μl per well of 0.33% DMSO vehicle, and the other half of the plate was prepared for nonspecific binding (NSB) measurement, with 60 μl per well of 33.3 μM unlabeled ozanimod or A971432. Serial 2-fold dilutions of [³H]-ozanimod and [³H]-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, pH 7.4) 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 (MAHFC1H60, Millipore, Burlington, MA) were prepared by incubating with 80 μl per well of 500 ml total of filtration buffer per plate. Filter plates were then 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.

**Saturation Radioligand Binding**
Saturation binding analysis was performed with [³H]-ozanimod and [³H]-A971432 using 96-well nonscuffing surface plates (3604, Corning, Corning, NY) with a final volume of 200 μl. One assay plate was prepared for each of the following membrane preparations: human S1P₅, mouse S1P₅, mouse S1P₅/Δ120T, and CHO K1 parental cells. For determination of total binding, half of the assay plate was prepared with 60 μl per well of 0.33% DMSO vehicle, and the other half of the plate was prepared for nonspecific binding (NSB) measurement, with 60 μl per well of 33.3 μM unlabeled ozanimod or A971432. Serial 2-fold dilutions of [³H]-ozanimod and [³H]-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, pH 7.4) 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 (MAHFC1H60, Millipore, Burlington, MA) were prepared by incubating with 80 μl per well of 500 ml total of filtration buffer per plate. Filter plates were then 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]-GTP₅ Binding Assay**
Assays to quantitate receptor activation and G protein coupling were performed using [³⁵S]-GTP₅/S binding assays. All assays were performed in 96-well nonbinding 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 per 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 per well of assay buffer containing a mixture of 40 μg/ml S1P receptor membranes, 16.67 μM guanosine diphosphate (G7127, Sigma Aldrich, St. Louis, MO), and 2.5 mg/ml of WGA PTV SPA beads (RNQ0001, Perkin Elmer). Assay plates were then sealed and incubated at room temperature with gentle agitation for 30 minutes before the addition of 40 μl per well of 5x [³⁵S]-GTP₅/S (200 pm final) (NEG030X250UC, Perkin Elmer) made up in basic assay buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, and 1 mM EDTA, pH 7.4), 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).

**MOG₃₅-₅₅ Experimental Autoimmune Encephalomyelitis Model**
Experimental autoimmune encephalomyelitis (EAE) was induced in 10-week-old female C57BL/6 mice (Taconic Biosciences, Rensselaer, NY) by subcutaneous immunization with an emulsion of myelin oligodendrocyte glycoprotein 35-55 (MOG₃₅-₅₅) in complete Freund’s adjuvant (CFA) followed by intraperitoneal injections of pertussis toxin 2 and 24 hours later. Mice received two subcutaneous injections, one in the upper and one in the lower back, of 0.1 ml MOG₃₅/CFA emulsion per site, and both intraperitoneal injections of pertussis toxin were 100 ng per dose at a volume of 0.1 ml per dose. The study was performed at Hooke Laboratories (Lawrence, MA) 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 preclinical 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) using the Simoa NF-light Advantage kit (102258). Mice were anesthetized and 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. Histologic 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, Bar Harbor, ME) 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 intraperitoneal 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 intraperitoneal 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). 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 and 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 after 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 intraperitoneal 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. 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, magnetization transfer-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, whereas 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% paraformaldehyde mix for at least 12 hours. A small piece of corpus callosum was identified by specific morphologic 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 30µm thick-free-floating sections; two sections per animal were stained with either SMI-32 (nonphosphorylated 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) after 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 MOG35–55 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 preclinical studies where dosing is initiated straight away with the dose to be assessed without titration. Brains were homogenized in acetoneitrile at a 1:3 (w/v) ratio using a Biospec Bead Beater-16 with 1 mm glass beads and proteins precipitated further with a 10:1 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 bioanalytical run using a Kinetex C18 2.6µm 30 × 3 mm column (Phenomenex Inc., Torrance, CA), 0.1% formic acid in deionized 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 [35S]-GTPγS binding assay buffer. Serum protein binding was

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![Fig. 1](https://example.com/fig1.png)  
**Fig. 1.** Study design to assess the effect of ozanimod on cuprizone (0.3% in diet)/rapamycin (10 mg/kg, i.p., once daily)–induced axonal degeneration and demyelination (weeks 0–6) as well as subsequent remyelination (weeks 6–18).
assessed by equilibrium dialysis using the 96-well micro equilibrium dialysis device (HTDialysis, Gales Ferry, CT). Regenerated cellulose dialysis membranes (12 K molecular weight cut-off) were used with a matched set of Teflon bars with a Teflon base block and stainless-steel price plate. Serum pooled from male and female C57BL6 mice (MSERM-C57, lot M5SE196727, Bioreclamation Inc., now BioIVT, Westbury, New York), 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 = 7-9 replicates). The dialysis device was incubated with shaking at 37°C for at least 4 hours. After 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. Phosphate buffered saline (40 μl) was added to the plasma sample, and 40 μl of untreated plasma was added to the phosphate buffered saline 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 [35S]-GTP binding assay medium protein binding assay were assessed by equilibrium dialysis using the Rapid Equilibrium Dialysis device (Thermo Fisher Scientific). The single-use Rapid Equilibrium Dialysis device plate contains made of two side-by-side chambers separated by a vertical cylinder of dialysis membrane (8 K molecular weight cutoff) validated for minimal non-specific binding. Brain from serum perfused male C57BL6 mice was homogenized in PBS at a 1:19 (w/v) ratio using a Biospec Bead Beater-16 with 1 mm glass beads. Brain homogenate or [35S]-GTP binding assay medium were spiked with either ozanimod or RP101075 at 1 and 5 μM and triplicate before 200 μl of compound-spiked brain homogenate or medium was 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. After incubation, 50 μl aliquots were removed from the respective brain homogenate[35S]-GTP-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[35S]-GTP-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 was analyzed by liquid chromatography–mass spectrometry (LC-MS/MS). The Kalvaas equation (Kalvaas 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 [3H]-ozanimod or [3H]-A971432 to the various S1P receptors expressing membranes was determined by subtracting out the binding to parental CHO membranes. The total binding was thus the total binding to S1P$_r$-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 S1P$_r$-expressing membranes minus the NSB to parental CHO membranes. The specific binding of [3H]-ozanimod or [3H]-A971432 to S1P$_r$ 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 S1P$_r$. For competitive radioligand binding with [3H]-A971432, raw data were 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 nonlinear regression using one site fit to generate K_D having entered the K_D for [3H]-A971432 using GraphPad Prism (version 8.0.0).

[35S]-GTP-S 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 SIP 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 (EC50), 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 three-dimensional coordinates of the human S1P$_r$ receptor were downloaded as described by Burley et al. (2019) using the Protein Data Bank identifier 3V2Y (Hanson et al., 2012). The human S1P$_r$ receptor protein sequence was downloaded from UniProt (identifier: Q8H228). The homology model of the human S1P$_r$ receptor was created in Prime using methods described by Jacobson et al. (2002) with human S1P$_r$, as template. Siponimod, FTY720-p, and ozanimod chemical structures were drawn in Maestro 2020-1 (Schrödinger, LLC, New York, NY) and prepared for docking using LigPrep (Schrödinger). 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-glycerol-3-phosphocholine) membrane bilayer, which 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 300 K 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 for membrane systems plus an additional 50 ns molecular dynamics simulation (NVT ensemble at 300 K temperature). For each docked ligandreceptor 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 with the simulation time extended to 10 ns per lambda window.

Results

In Vitro. Potency and intrinsic activity assessment of ozanimod and RP101075 for S1P$_1$ and S1P$_5$ across human and preclinical species revealed that whereas the potency for S1P$_1$ was maintained across all species, the potency for S1P$_5$ was significantly reduced for the mouse, rat, and canine homologs compared with that of human and cyno (Table 1). Compared with ozanimod/RP101075’s potency for human S1P$_5$, the potency for cyno S1P$_5$ was within 2-fold; however, the potency for mouse, rat, and canine S1P$_5$ 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 siponimod 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 S1P$_5$ 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 S1P$_5$ 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 S1P3 was only observed with ozanimod/RP101075 and not with other S1P receptor modulators, FTY720-p (fingolimod) or siponimod, which retained their potency for S1P3 in the low nanomolar range across all species tested (Table 1).

To probe this finding, we first aligned the primary amino acid sequences for S1P3 across species (Fig. 2) and observed that the percent conservation of human S1P3 was highest with cyto 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]-GTPγS binding assays to measure the functional coupling of mouse S1P3 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 mS1P3 to 6.7 ± 0.3 nM for mS1P3_A120T to closely mirror the EC50 for hS1P3 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 for S1P3. An S1P3-selective agonist (Hobson et al., 2015). Saturation binding analysis determined that the Ki of ozanimod S1P3 was 6.56 nM for the human homolog (Fig. 4), and yet we were unable to measure any specific binding with up to 40 nM of [3H]-ozanimod at wild type mS1P3, suggesting the possibility of a species difference. The cellular membranes assessed in the radioligand binding assays were the same as those used for [35S]-GTPγS binding, indicating that mS1P3 was expressed at levels that should be detected with radioligand binding. To confirm this, we also ran a saturation analysis using an alternate S1P3 agonist radioligand [3H]-A971432 bound to wild type mS1P3 revealed adequate receptor expression with a Bmax of 5.69 pmol/mg protein and a dissociation constant of 8.75 nM confirming the membranes were reliable and mS1P3 expression was sufficient. Thus, it appeared that the binding affinity of ozanimod for mS1P3 was affected. We then performed a saturation analysis with [3H]-ozanimod using membranes expressing mS1P3_A120T and found that binding was restored yielding a KD of 7.35 nM, very similar to that observed with hS1P3.

Using [3H]-A971432 we performed competition radioligand binding to determine the inhibition constant (Ki) for ozanimod as well as FTY720-p, siponimod, and A971432 across hS1P5, mS1P3, and mS1P3_A120T. Confirming the data achieved using [35S]-GTPγS binding and saturation binding with [3H]-A971432, the Ks values determined for FTY720-p, siponimod, and A971432 were consistent between human and mouse S1P3 homologs, but the Ks for ozanimod was 30-fold weaker for mS1P3 compared with that of hS1P3 (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 S1P3 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, whereas the other two ligands were 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

### TABLE 1

| Receptor | Ozanimod; RP101075 | FTY720-p | Siponimod |
|----------|-------------------|----------|-----------|
| Human    | 92.3 ± 2.1        | 58.1 ± 0.7 | 102.3 ± 2.2 |
| S1P3     | 8.6 ± 0.6         | 74.8 ± 6.4 | 106.9 ± 4.9 |
| Cyto     | 15.9 ± 4.0        | 93.7 ± 3.7 | 91.1 ± 5.2 |
| Mouse    | 95.7 ± 203.7      | 63.9 ± 2.7 | 85.6 ± 9.3 |
| Rat      | 203.2 ± 191.2     | 74.8 ± 1.1 | 100.2 ± 0.5 |
| Canine   | 166.0 ± 164.92    | 69.6 ± 1.9 | 100.1 ± 0.8 |
| S1P1     | 382.3 ± 10.86     | 61.8 ± 1.2 |           |

Data shown are means ± S.E.M. for n = 3–6 independent experiments each performed in duplicate. IA indicates intrinsic activity, which is the maximal response achieved relative to the endogenous ligand, S1P, which was taken to be 100%.
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 hS1P5 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 transmembrane 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 subpocket 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 transmembrane domains 3 and 6 required for receptor activation.

**In Vivo.** The efficacy of ozanimod 0.2 and 0.6 mg/kg in the mouse MOG35–55 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 after immunization with disease scores of 1.08 ± 0.18, 1.13 ± 0.18, 1.13 ± 0.18, and 1.08 ± 0.16 (mean ± S.E.M.) 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. 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.
(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 neurofilament light (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. The 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).

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 S1P1 and S1P2 EC50 values calculated at three different time points (Table 5). Of note, the functional EC50 values were also corrected for [35S]-GTPγS assay medium protein binding, which was determined to be 32%, and so 68% of drug was free. This adjusted the ozanimod EC50 for mouse S1P1 from 0.90 nM to 0.61 nM and the mouse S1P5 EC50 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 after dose, respectively, compared with 25%, 28%, and 47% of RP101075 at 3, 6, and 24 hours after 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 after dose, respectively, compared with 35%, 30%, and 62% of RP101075 at 3, 6, and 24 hours after 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

Table 2

| Receptor | EC50 nM | IA % | Fold human S1P1 |
|----------|---------|------|-----------------|
| Human S1P1 | 8.6 ± 0.6 | 92.3 ± 2.1 | 1 |
| Cynomolgus monkey S1P5 | 15.9 ± 0.4 | 93.7 ± 3.7 | 1.8 |
| Mouse S1P5 | 957.5 ± 203.7 | 63.9 ± 2.7 | 111.6 |
| Rat S1P1 | 2032.7 ± 191.2 | 74.8 ± 1.1 | 236.9 |
| Canine S1P5 | 1662.0 ± 164.92 | 69.6 ± 1.9 | 194.6 |
| Mouse S1P5_A120T | 6.7 ± 0.3 | 95.9 ± 6.3 | 0.8 |
| Rat S1P5_A120T | 6.6 ± 1.2 | 82.1 ± 3.4 | 0.8 |
| Canine S1P5_A120T | 14.0 ± 1.5 | 74.2 ± 1.4 | 1.6 |

Data shown are means ± S.E.M. for n = 3 independent experiments each performed in duplicate. IA indicates intrinsic activity, which is the maximal response relative to the endogenous ligand, S1P, which was taken to be 100%.
used in the EAE study, free drug concentrations in the plasma were above the ozanimod mouse $S_1P_1$ 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 $S_1P_5$ EC$_{50}$ levels in either the plasma nor in the brain; in fact, the closest they came were 0.01-fold of the mouse $S_1P_5$ EC$_{50}$ in the plasma at 3 and 6 hours after dose. Likewise for the 5 mg/kg dose used in the cuprizone study, EC$_{50}$ coverage was achieved for $S_1P_1$ in both the plasma and the brain for the full 24 hours after dose in both compartments, yet $S_1P_5$ EC$_{50}$ levels were not achieved, with the highest exposure being 0.06-fold for the mouse $S_1P_5$ EC$_{50}$ in the plasma 3 hours after dose. The exposures relative to ozanimod EC$_{50}$ 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 $S_1P_5$ 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 $S_1P_5$ that supports this observation. The hydroxyl group of T120 creates a favorable polar

**TABLE 3**

| Receptor         | Ozanimod $K_i$ nM | FTY720-p $K_i$ nM | Siponimod $K_i$ nM | A971432 $K_i$ nM |
|------------------|-------------------|-------------------|--------------------|------------------|
| Human $S_1P_5$   | 2.0 ± 0.1         | 0.3 ± 0.01        | 0.2 ± 0.02         | 3.8 ± 0.4        |
| Mouse $S_1P_5$   | 59.9 ± 8.8        | 0.2 ± 0.01        | 0.3 ± 0.02         | 5.1 ± 0.4        |
| Mouse $S_1P_5$ A120T | 5.6 ± 1.2       | 0.3 ± 0.03        | 0.4 ± 0.03         | 6.0 ± 0.5        |

Data shown are means ± S.E.M. 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 $[^3H]$-A971432 binding once corrected for the concentration of radioligand used and the dissociation constant of the radioligand.
TABLE 4

| Dose                        | Terminal body weight | Spinal cord inflammation | Spinal cord demyelination | Spinal cord apoptotic cells | Plasma NfL (pg/ml) |
|-----------------------------|----------------------|--------------------------|---------------------------|-----------------------------|-------------------|
| Vehicle (5% DMSO, 5% Tween 20, 90% water) | 86.4 ± 3.2            | 8.50 ± 1.21              | 2.00 ± 0.15               | 2.25 ± 0.53                 | 4.37 ± 0.89       |
| Ozanimod (0.05 mg/kg)       | 85.8 ± 2.7            | 5.00 ± 1.03              | 0.91 ± 0.21***            | 1.08 ± 0.23*                | 3.53 ± 0.46       |
| Ozanimod (0.2 mg/kg)        | 95.7 ± 3.1*           | 3.54 ± 0.49**            | 0.73 ± 0.14 ***           | 0.91 ± 0.28*                | 2.62 ± 0.46       |
| Ozanimod (1 mg/kg)          | 102.8 ± 1.8*          | 2.67 ± 0.56***           | 0.33 ± 0.14 ***           | 0.60 ± 0.19**               | 1.91 ± 0.34**     |

Data shown are means ± S.E.M. Statistical significance for terminal body weight from vehicle was determined using a two-tailed Student’s t test. Statistically significant reductions 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.

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

Fig. 5. Receptor modeling for the binding of S1P receptor modulators to human S1P5, and the predicted effect of mutating threonine 120 to 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 S1P5 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 an FEP simulation of the human S1P5 homology model where the T120 has been mutated in silico to alanine 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 means ± S.E.M. 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 with 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 versus vehicle; *P < 0.05, **P < 0.01, ***P < 0.001. (B) Absolute lymphocyte counts were quantitated by differential count from blood samples collected 24 hours after the last dose. The percent decrease compared with vehicle control is indicated above each bar, and statistically significant decreases in absolute lymphocyte counts compared with vehicle were determined using one-way ANOVA with Dunnett’s post hoc test; *P < 0.05, ***P < 0.001.
ozanimod an S1P<sub>1</sub>-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<sub>1</sub> are scarce, and those that are available, such as AUY954, SEW2871, or CYM-5442, display weak potency or demonstrate marginal selectivity for mouse S1P<sub>1</sub> over mouse S1P<sub>5</sub> (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 S1P<sub>1</sub> 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, and 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 EC<sub>50</sub> of ozanimod for mouse S1P<sub>1</sub> but do not meet the EC<sub>50</sub> for mouse S1P<sub>5</sub>. 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 EC_{50} for S1P_1 or S1P_5 in this compartment. Therefore, efficacies observed in this model are most likely driven by pharmacological engagement of S1P_1 in the periphery and that significant reduction of the numbers of circulating B and T cells, as is clinically observed in patients with relapsing-remitting multiple sclerosis taking ozanimod (Harris et al., 2020), is sufficient for preclinical efficacy. Indeed, we reported that early treatment with ozanimod (beginning day 4 after MOG_{35-55} 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 MHCI^+ monocytes and microglia present in the spinal cord tissue. S1P_1 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). Continual low-level activation of astrocyte or microglial S1P_1, 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, was seen with this dose.

Neuroprotective effects of ozanimod in the CPZ/Rapa model were consistent with the MOG_{35-55} 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. 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 EC_{50} concentrations for S1P_1 with the 5 mg/kg dose but still fell significantly short for S1P_5. Ozanimod’s effects in this second mouse model are likely mediated by S1P_1. Direct CNS effects with S1P modulators are being increasingly reported in the literature (Grovés et al., 2013; Kipp 2020). S1P_1 is upregulated in activated astrocytes in a CPZ intoxication mouse model, and selective S1P_1 down-regulation by CYM-5422 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 S1P_1 modulation was necessary during the early phase of CPZ intoxication to suppress oligodendrocyte death, and that suppression in the production of proinflammatory 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 GABAergic 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 S1P_1-preferring AUY954 (Musella et al., 2020). Moreover, acutely prepared brain slice cultures isolated from EAE mice treated with siponimod demonstrated 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 S1P_1 modulation than S1P_5 (Musella et al., 2020).

Remyelination after cessation of CPZ/Rapa more closely reflects oligodendrocyte involvement after demyelination and neuronal injury. Oligodendrocytes are reported to express S1P_5 at all stages of their development from precursors (OPCs) through preoligodendrocytes and to fully differentiated oligodendrocytes capable of depositing myelin (Jaillard et al., 2005). The activation of S1P_5 is reported to increase the survival of OPCs (Miron et al., 2008), induce initial process retraction of preoligodendrocytes followed by extensive process re-extension and maturation (Jaillard et al., 2005), and enhance myelination after lyssolecithin-induced demyelination in CNS cortical organoids (Jackson et al., 2011). Although one

| Dose | Plasma concentration: nM | Brain concentration: nM |
|------|--------------------------|-------------------------|
|      | 3 hours after final dose | 6 hours after final dose | 24 hours after final dose | 3 hours after final dose | 6 hours after final dose | 24 hours after final dose |
|      | total (fold S1P_1, fold S1P_5) | free (fold S1P_1, fold S1P_5) | total (fold S1P_1, fold S1P_5) | free (fold S1P_1, fold S1P_5) |
| Ozanimod | 138 (226, 0.12) | 30 (49, 0.05) | 861 (957, 0.90) | 1001 (1112, 1.04) | 333 (370, 0.35) |
| (1 mg/kg) | 9.27 (15.14, 0.01) | 1.86 (3.04, 0.003) | 0.32 (0.53, 0.0005) | 0.37 (0.61, 0.0006) | 0.12 (0.19, 0.0002) |
| Ozanimod | 616 (1006, 0.95) | 1.92 (3.14, 0.003) | 5273 (8615, 8.09) | 6098 (9963, 9.36) | 2088 (3411, 3.20) |
| (5 mg/kg) | 41.13 (67.20, 0.06) | 32.86 (53.69, 0.05) | 2.25 (3.68, 0.003) | 2.5 (3.8, 0.003) | 0.7 (1.55, 0.001) |

Data shown are means exposures of the sum of ozanimod and the major active rodent metabolite, RP101075, collected from n = 4 mice per group. 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 [35S]-GTP\_S binding assay medium. Mice were dosed orally, once daily, for seven consecutive days with either 1 or 5 mg/kg ozanimod.
might expect enhanced remyelination with S1P1 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 this increased remyelination is not observed for the S1P1-prefering 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 et al., 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 remyelination (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 S1P3-mediated. Thus, using finely calculated drug exposures, we show that S1P1 is responsible for multiple efficacy readouts in the EAE model that may be 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 that S1P1 mediates the observed neuroprotection and reduction in myelin loss during the intoxication period but that enhanced remyelination after intoxication is not a downstream event of S1P1 engagement and indeed may require S1P3 activation. Although we were unable to demonstrate a remyelination effect of ozanimod in these studies, this was likely due to the reduced potency for the mouse homolog 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 S1P3.

**Author Contributions**

**Participated in research design:** Selkirk, Dines, Bortolato, Schkeryantz.

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**Contributed new reagents or analytical tools:** Bortolato, Schkeryantz.

**Performed data analysis:** Selkirk, Dines, Yan, Ching, Dalvie, Bortolato, Schkeryantz.

**Wrote or contributed to writing of the manuscript:** Selkirk, Dines, Bortolato, Schkeryantz, Hargreaves.

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