Molecular Pharmacology Fast Forward. Published on November 30, 2012 as doi:10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #82958

$S1P_1$ Receptor Upregulation and Amelioration of Experimental Autoimmune Encephalomyelitis by an $S1P_1$ Antagonist

Stuart M. Cahalan, Pedro J. Gonzalez-Cabrera, Nhan Nguyen, Miguel Guerrero, Elizabeth A. George Cisar, Nora B. Leaf, Steven J. Brown, Edward Roberts, and Hugh Rosen

Departments of Chemical Physiology (S.M.C., P.J.G.C., N.N., E.A.G.C., N.L., S.J.B., and H.R.) and Chemistry (M.G., E.R.), The Scripps Research Institute, La Jolla, CA 92037

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: $S1P_1$ antagonist reverses EAE independent of CNS $S1P_1$

Address correspondence to:

Dr. Hugh Rosen, Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037. E-mail: hrosen@scripps.edu

Text Pages: 23 (Including Title Pages & References)

Tables: 1

Figures: 4 + 2 Supplemental Figures

References: 30

Words in Abstract: 128

Words in Introduction: 490

Words in Results/Discussion (Combined for Accelerated Communication): 951

Abbreviations

CNS: Central Nervous System

EAE: Experimental Autoimmune Encephalomyelitis

Ex26: 1-(5'-((1-(4-chloro-3-methylphenyl)ethyl)amino)-2'-fluoro-3,5-dimethyl-[1,1'-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid

FTY720: 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol

MOG_{33-55}: Peptide consisting of residues 33-55 of myelin oligodendrocyte glycoprotein

MS: Multiple Sclerosis

PTX: Pertussus toxin

S1P: Sphingosine 1-Phosphate

$S1P_{1-5}$: Sphingosine 1-Phosphate Receptor 1-5

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Sphingosine 1-phosphate receptor 1 is a G protein-coupled receptor that is critical for proper lymphocyte development and recirculation. Agonists to $S1P_1$ are currently in use clinically for the treatment of multiple sclerosis, and these drugs may act both on $S1P_1$ expressed on lymphocytes and on $S1P_1$ expressed within the central nervous system. Agonists to $S1P_1$ or deficiency in $S1P_1$ both cause lymphocyte sequestration in the lymph nodes. Here we show that $S1P_1$ antagonism induces lymphocyte sequestration in the lymph nodes similar to that observed with $S1P_1$ agonists while upregulating $S1P_1$ on lymphocytes and endothelial cells. Additionally, we show that $S1P_1$ antagonism reverses experimental autoimmune encephalomyelitis in mice without acting on $S1P_1$ expressed within the central nervous system, demonstrating that lymphocyte sequestration via $S1P_1$ antagonism is sufficient to alleviate autoimmune pathology.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Sphingosine 1-phosphate receptor 1 ($S1P_1$) plays an important role in many physiological systems, including vascular development, lymphocyte development, and lymphocyte recirculation(Allende et al., 2004; Allende et al., 2003; Cyster and Schwab, 2012; Liu et al., 2000; Matloubian et al., 2004). $S1P_1$ is required on developing lymphocytes to mature beyond a semi-mature $CD69^{hi}$ $CD62L^{lo}$ state, rendering the blood and lymph of mice lacking $S1P_1$ on developing lymphocytes largely devoid of T cells. When $S1P_1^{-/-}$ thymocytes are transferred into recipient mice, they are also retained from blood and lymphatic circulation. $S1P_1$ became a relevant drug target in the treatment of autoimmune disease following the discovery that FTY720 (fingolimod, Gilenya), which was known to inhibit lymphocyte recirculation, is a S1P receptor prodrug that is phosphorylated *in vivo* to yield a potent agonist of $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ (Mandala et al., 2002). $S1P_1$ selective agonists demonstrated that FTY720 acted via $S1P_1$ to induce lymphocyte sequestration(Sanna et al., 2004). The ability of FTY720-P and other $S1P_1$ agonists to induce sustained internalization and/or degradation of $S1P_1$ (Gonzalez-Cabrera et al., 2007; Gonzalez-Cabrera et al., 2008; Graler and Goetzl, 2004), combined with the deficient egress of $S1P_1$-deficient lymphocytes, has led to the hypothesis that $S1P_1$ agonists act as functional antagonists (Graler and Goetzl, 2004). Several $S1P_1$-selective antagonists have also been generated, which inhibit agonist-dependent effects *in vitro*, stabilize the $S1P_1$ receptor allowing for its structural determination, induce pulmonary edema *in vivo*, and initial antagonists could reverse agonist-induced lymphocyte sequestration while being unable to induce lymphocyte sequestration themselves (Foss et al., 2005; Hanson et al., 2012; Sanna et al., 2006; Wei et al., 2005). Recent work has shown that $S1P_1$ antagonists can indeed induce lymphocyte sequestration at high plasma concentrations (Tarrason et al., 2011), and one such $S1P_1$

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

antagonists can alleviate animal models of autoimmune arthritis (Fujii et al., 2012), cardiac allograft rejection (Angst et al., 2012), and multiple sclerosis (Quancard et al., 2012).

S1P receptor agonists have come of age with FTY720's FDA approval for the treatment of relapsing-remitting multiple sclerosis. The efficacy of FTY720 is not solely dependent on its ability to cause full lymphocyte sequestration via $S1P_1$, as it is effective at doses that maintain ~50% lymphopenia. This efficacy probably involves both $S1P_1$ and other S1P receptors within the central nervous system (CNS) (Cohen and Chun, 2011; Hla and Brinkmann, 2011). $S1P_1$ agonists that can efficiently penetrate the CNS can induce receptor signaling and degradation of $S1P_1$ expressed on neurons and astrocytes (Gonzalez-Cabrera et al., 2012), and require lymphocyte sequestration for only a third of a dosing interval in order to reverse EAE in mice. Additionally, mice lacking $S1P_1$ on astrocytes are refractory to developing EAE and are suggested to be important targets of FTY720 (Choi et al., 2011). Several other S1P receptors are expressed within the CNS, and the activation and/or degradation of these receptors by FTY720 may also play important roles in reversing the immunopathology of multiple sclerosis (Miron et al., 2008; Miron et al., 2010).

Here we demonstrate that $S1P_1$ antagonism sequesters lymphocytes in the peripheral lymph nodes but not the spleen, similar to that observed with $S1P_1$ agonists. $S1P_1$ antagonism also causes significant upregulation of $S1P_1$ expression on peripheral lymphocytes, mature thymocytes, and lung endothelial cells. Additionally, $S1P_1$ antagonism can alleviate EAE in mice despite the inability of the antagonist used to penetrate the CNS. Thus, lymphocyte sequestration induced by $S1P_1$ antagonists is sufficient to ameliorate the autoimmune pathology observed in

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

EAE, and does not require antagonism of $S1P_1$ expressed on neurons or astrocytes within the CNS.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Compounds and *in vitro* Assays

Example 26 (Ex26, 1-(5'-((1-(4-chloro-3-methylphenyl)ethyl)amino)-2'-fluoro-3,5-dimethyl-[1,1'-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid) was synthesized as a racemic mixture according to its published synthesis in the patent literature (Angst et al.). RP-001 was synthesized as previously described (Cahalan et al., 2011). FTY720 was purchased from Cayman Chemicals. Ex26 and RP-001 were solubilized in 50 mM Na_2CO_3, while FTY720 was solubilized in H_2O. *In vitro* assays for S1P receptor function were performed using the following cell lines: $S1P_1$, $S1P_4$, $S1P_5$ – Tango U2OS cells (Invitrogen) expressing the indicated receptor; $S1P_2$ – CHO cells expressing $S1P_2$ coupled to a CRE-Bla reporter; $S1P_3$ – CHO cells expressing $S1P_3$ coupled to a NFAT-Bla reporter through Gα16. $S1P_1$ internalization and polyubiquitinylation were evaluated using HEK cells expressing $S1P_1$-eGFP as previously described (Gonzalez-Cabrera et al., 2007), pretreating cells for 1 h with Ex26.

Evaluation of Lymphocyte Sequestration, Pulmonary Edema

8 week old male C57Bl/6J mice were purchased from the TSRI mouse breeding facility for evaluation of lymphocyte sequestration and pulmonary edema. Mice were injected i.p with Ex26 or 50 mM Na_2CO_3 vehicle, and blood was removed from the heart following euthanasia. Blood was lysed in 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, washed with PBS containing 2% FBS, 1 mM EDTA, 0.1% NaN_3, counted using a ViCell-XR counter (Beckman), stained with antibodies and analyzed by flow cytometry. To evaluate pulmonary edema, mice were perfused with 15 mL of PBS through the right ventricle, then the lungs were removed, blotted dry to

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

remove excess PBS, and weighed. All mouse experiments were performed using protocols approved by the Institutional Animal Care and Usage Committee.

Compound Concentrations in Plasma and Tissues

Ex26 plasma concentrations were determined using methanol extraction as previously described(Cahalan et al., 2011), detecting a m/z value of 495.2 for Ex26 using an Agilent 6410 triple quadrupole mass spectrometer coupled to an Agilent 1100LC system. Ex26 concentrations in the brain were determined by disruption of brain tissue in water by probe sonication, followed by extraction with acetonitrile and filtration through Millipore filters (MultiScreen Solvinert, hydrophilic, PTFE, 0.45 µm). Filtrates were analyzed by LC-MS/MS API4000 (AbSciex) and quantified using a positive ion MRM method (495.1/242.1, m/z).

Continuous Administration of $S1P_1$ Antagonist

6-week-old $S1P_1$-eGFP mice were anesthetized with isoflurane and their backs were shaved, cleaned with 70% EtOH to remove any excess hair, then wiped with povidone iodine. An incision was made on the lower back of the mice and micro-osmotic pumps (Alzet model 1003D) containing either 50mM Na_2CO_3 vehicle or 2 mg/mL Ex26 were implanted, yielding a dose of ~0.1 mg/kg per hour. Mice were given an i.p. dose of 3 mg/kg Ex26 or vehicle immediately following surgery.

Flow Cytometry, $S1P_1$ Expression, and Statistical Analysis

Fluorescently labeled antibodies specific to CD4 and CD8 were obtained from Biolegend. Fluorescently labeled antibodies specific to CD19, CD31, CD45.2, CD62L, and CD69 were

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

obtained from BD. Data was collected using an LSRII flow cytometer (BD) and analyzed using FlowJo (Treestar). $S1P_1$ expression by flow cytometry was measured using $S1P_1$-eGFP knockin mice (Cahalan et al., 2011). $S1P_1$ expression in the CNS in EAE experiments was evaluated using a C-terminal specific $S1P_1$ antibody (H-60, Santa Cruz Biotechnology, Santa Cruz, CA used at 1:500 dilution). All statistical analysis was performed using GraphPad Prism Software.

EAE Induction and Scoring

EAE was induced in female 10-week-old C57Bl/6J mice purchased from Jackson Labs. EAE was induced using Hooke Labs EAE induction kit (EK-0114 for EAE, CK-0114 for control) according to manufacturer's instructions. Mice were scored by the following criteria: 0.5: Weak tail; 1: Limp tail; 1.5: Weak tail + weak hind limbs; 2: Limp tail + weak hind limbs; 2.5: Limp tail + unilateral hind limb paralysis; 3: Limp tail + bilateral hind limb paralysis; 4: Limp tail + bilateral hind limb paralysis + partial front limb paralysis; 5: Moribund or dead. Mice scoring 4 for two consecutive days were euthanized and recorded as 5 for the remaining days of the experiment. Mice were injected i.p daily with 50 mM Na_2CO_3 vehicle, 30 mg/kg Ex26 or 10 mg/kg FTY720 in a volume of 10 μL per gram weight of mouse beginning the first day on which clinical signs were observed in that mouse.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Results and Discussion

Ex26 is a $S1P_1$ antagonist that inhibits lymphocyte egress

Most existing $S1P_1$ antagonists are S1P analogs with IC_{50} values in the double-digit nanomolar range and possessing relatively short half-lives. Recently, new $S1P_1$ antagonists have been described including a series of biaryl benzylamines by Novartis (Angst et al.). We synthesized and characterized one of these compounds, 1-(5'-((1-(4-chloro-3-methylphenyl)ethyl)amino)-2'-fluoro-3,5-dimethyl-[1,1'-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid (Ex26), and confirmed it to be a potent and selective antagonist of $S1P_1$ (Fig. 1A and Table 1), similar to a recently published antagonist (Quancard et al., 2012). Ex26 could inhibit RP-001-induced $S1P_1$ internalization and polyubiquitinylation *in vitro* (Supplemental Fig. 1). Like other previously described $S1P_1$ antagonists, Ex26 induced dose-dependent and time-dependent pulmonary edema *in vivo* (Fig. 1B-C), and had a relatively short *in vivo* half-life of approximately 73.5 minutes (Supplemental Fig. 1C).

Earlier work showed that the S1P-like $S1P_1$ antagonists W146 and VPC44116 reversed agonist-induced lymphocyte sequestration while not causing lymphocyte sequestration (Foss et al., 2007; Sanna et al., 2006). Recent work has found that W146 induces transient lymphocyte sequestration at high doses (Tarrason et al., 2011), which we replicated (Data not shown). Ex26 induced lymphocyte sequestration at low doses, possessing an ED_{50} of ~0.06 mg/kg when examined two hours following treatment (Fig. 2A). Lymphocyte sequestration by Ex26 resolved with similar kinetics as did Ex26-evoked pulmonary edema (Fig. 2B). To examine the effects of extended antagonist treatment, we implanted mice with micro-osmotic pumps to continuously deliver Ex26 at a dose of 0.1 mg/kg per hour for 3 days following a loading dose of 3 mg/kg.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Extended treatment with Ex26 led to significant retention of T and B cells within the lymph nodes and significant decreases of T and B cells within the spleen, similar to $S1P_1$ agonists (Fig. 2C-D). Continuous administration of Ex26 also led to thymic retention of mature $CD62L^{Hi}$ SP thymocytes, also similar to the effects induced by $S1P_1$ agonists (Fig 2E). These data demonstrate that disruption of $S1P_1$ signaling by $S1P_1$ antagonism leads to inhibition of lymphocyte and thymocyte egress.

$S1P_1$ Antagonism Upregulates $S1P_1$ Expression

Since $S1P_1$ agonists downregulate $S1P_1$, we wanted to determine whether $S1P_1$ antagonism could conversely upregulate $S1P_1$. Continuous $S1P_1$ antagonism in mice expressing $S1P_1$-eGFP from the $S1P_1$ locus (Cahalan et al., 2011) for 3 days by micro-osmotic pumps caused significant upregulation of $S1P_1$-eGFP on lymphocytes within the lymph node (Fig. 3A). This suggests that the low concentration of S1P within the lymph node (Schwab et al., 2005) under normal physiological conditions is sufficient to suppress the expression of $S1P_1$. We observed similar upregulation within the spleen (Data not shown), and a modest upregulation of $S1P_1$-eGFP on fully mature $CD62L^{hi}$ SP thymocytes (Fig. 3B). $S1P_1$ agonists cause a loss of surface expression of CD69 on mature thymocytes (Alfonso et al., 2006). In contrast to the effects seen with agonists, continuous Ex26 treatment led to significant upregulation of CD69 (Fig. 3B), indicating that $S1P_1$ signaling, not only expression of $S1P_1$ (Bankovich et al., 2010), is critical for suppressing the surface expression of CD69; thus, downregulation of CD69 by $S1P_1$ agonists is a measure of agonism, not functional antagonism. Upregulation of $S1P_1$-eGFP was not limited to lymphocytes, as blood endothelial cells within the lung significantly upregulated $S1P_1$-eGFP expression (Fig. 3C). Unlike many $S1P_1$ agonists including FTY720-P, Ex26 did not cause any

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

changes in the expression of $S1P_1$-eGFP within the brain (Fig. 3D), due to the fact that Ex26 was almost undetectable within the CNS (Plasma: 6.8 ± 0.3 μM, Brain: 0.01 ± 0.005 μM, 2/3 animals below level of detection, mean ± S.E.M.).

$S1P_1$ Antagonism Ameliorates EAE

Since Ex26 did not enter the CNS nor cause any change in $S1P_1$ expression within the CNS, it allowed us to determine whether lymphocyte sequestration alone was able to reverse EAE. Whereas 3 mg/kg Ex26 induced relatively short-duration lymphocyte sequestration, we found that a single dose of 30 mg/kg caused lymphocyte sequestration and pulmonary edema that lasted 24 hours in naïve mice (Supplemental Fig. 2A-B). To examine whether $S1P_1$ antagonism could ameliorate EAE similar to $S1P_1$ agonism, we induced disease using the MOG_{33-55} peptide model, and, upon development of clinical signs of disease, treated mice i.p. once daily with either 30 mg/kg Ex26, 10 mg/kg FTY720, or 50 mM Na_2CO_3 vehicle, which we found to be indistinguishable from water, the usual vehicle for FTY720 (Data not shown). We found that treatment of mice with 30 mg/kg Ex26 daily significantly reduced the severity of EAE as assessed by examining clinical signs (Fig. 4A). We observed significant lymphocyte sequestration 3 hours following the last treatment of both 30 mg/kg Ex26 and 10 mg/kg FTY720, however, unlike its effect in naïve mice, 30 mg/kg Ex26 did not cause lymphocyte sequestration that lasted a full 24 hours in mice with EAE, whereas 10 mg/kg FTY720 did (Data not shown), suggesting that treatment with PTX used in the induction of EAE, or repeated dosing of Ex26, reduced the efficacy of Ex26, potentially by upregulating $S1P_1$ expression on lymphocytes. The reduction in severity of EAE was seen in the spinal cord, as 30 mg/kg Ex26 inhibited both lymphocyte infiltration and destruction of the white matter in the spinal cord of mice euthanized

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

at the end of the experiment (Fig. 4B). Consistent with the lack of CNS penetration of Ex26, we did not observe any changes in $S1P_1$ expression within the brain from mice euthanized at the end of the experiment that were treated daily with 30 mg/kg Ex26 compared to those treated daily with vehicle, whereas mice treated daily with 10 mg/kg FTY720 exhibited a complete loss in $S1P_1$ within the brain (Fig. 4C). This indicates that antagonism of $S1P_1$ expressed on neurons or astrocytes within the CNS is not required for the amelioration of EAE by $S1P_1$ antagonists, implying that lymphocyte sequestration by $S1P_1$ antagonists is sufficient to reverse the pathology of EAE, in keeping with the efficacy of lymphocyte migration inhibitory agents such as natalizumab that successfully treat multiple sclerosis.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

We thank Bill Webb and Mike Cameron for aid with mass spectrometry. We thank Margie Chadwell for help with histology.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Author Contributions

Participated in research design: Cahalan, Gonzalez-Cabrera, and Rosen

Conducted experiments: Cahalan, Gonzalez-Cabrera, Nguyen, Cisar, Leaf, and Brown

Contributed new reagents: Guerrero and Roberts

Performed data analysis: Cahalan, Gonzalez-Cabrera, Cisar, Brown, and Rosen

Wrote or contributed to the writing of the manuscript: Cahalan, Gonzalez-Cabrera, and Rosen

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

References

Alfonso, C., McHeyzer-Williams, M.G., and Rosen, H. (2006). CD69 down-modulation and inhibition of thymic egress by short- and long-term selective chemical agonism of sphingosine 1-phosphate receptors. European journal of immunology *36*, 149-159.

Allende, M.L., Dreier, J.L., Mandala, S., and Proia, R.L. (2004). Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. The Journal of biological chemistry *279*, 15396-15401.

Allende, M.L., Yamashita, T., and Proia, R.L. (2003). G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. Blood *102*, 3665-3667.

Angst, D., Bollbuck, P., Janser, P., and Quancard, J. Patent Number WO 072712 A1: Biaryl Benzylamine Derivatives. World Intellectual Property Organization, Submission Date 21 December 2009.

Angst, D., Janser, P., Quancard, J., Buehlmayer, P., Berst, F., Oberer, L., Beerli, C., Streiff, M., Pally, C., Hersperger, R., *et al.* (2012). An Oral Sphingosine 1-Phosphate Receptor 1 (S1P(1)) Antagonist Prodrug with Efficacy in Vivo: Discovery, Synthesis, and Evaluation. Journal of medicinal chemistry.

Bankovich, A.J., Shiow, L.R., and Cyster, J.G. (2010). CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. The Journal of biological chemistry *285*, 22328-22337.

Cahalan, S.M., Gonzalez-Cabrera, P.J., Sarkisyan, G., Nguyen, N., Schaeffer, M.T., Huang, L., Yeager, A., Clemons, B., Scott, F., and Rosen, H. (2011). Actions of a picomolar short-acting S1P agonist in S1P-eGFP knock-in mice. Nature chemical biology *7*, 254-256.

Choi, J.W., Gardell, S.E., Herr, D.R., Rivera, R., Lee, C.W., Noguchi, K., Teo, S.T., Yung, Y.C., Lu, M., Kennedy, G., *et al.* (2011). FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. Proceedings of the National Academy of Sciences of the United States of America *108*, 751-756.

Cohen, J.A., and Chun, J. (2011). Mechanisms of fingolimod's efficacy and adverse effects in multiple sclerosis. Annals of neurology *69*, 759-777.

Cyster, J.G., and Schwab, S.R. (2012). Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Annual review of immunology *30*, 69-94.

Foss, F.W., Jr., Clemens, J.J., Davis, M.D., Snyder, A.H., Zigler, M.A., Lynch, K.R., and Macdonald, T.L. (2005). Synthesis, stability, and implications of phosphothioate agonists of sphingosine-1-phosphate receptors. Bioorg Med Chem Lett *15*, 4470-4474.

Foss, F.W., Jr., Snyder, A.H., Davis, M.D., Rouse, M., Okusa, M.D., Lynch, K.R., and Macdonald, T.L. (2007). Synthesis and biological evaluation of gamma-aminophosphonates as

potent, subtype-selective sphingosine 1-phosphate receptor agonists and antagonists. Bioorg Med Chem *15*, 663-677.

Fujii, Y., Hirayama, T., Ohtake, H., Ono, N., Inoue, T., Sakurai, T., Takayama, T., Matsumoto, K., Tsukahara, N., Hidano, S., *et al.* (2012). Amelioration of collagen-induced arthritis by a novel S1P1 antagonist with immunomodulatory activities. J Immunol *188*, 206-215.

Gonzalez-Cabrera, P.J., Cahalan, S.M., Nguyen, N., Sarkisyan, G., Leaf, N.B., Cameron, M.D., Kago, T., and Rosen, H. (2012). S1P(1) receptor modulation with cyclical recovery from lymphopenia ameliorates mouse model of multiple sclerosis. Molecular pharmacology *81*, 166-174.

Gonzalez-Cabrera, P.J., Hla, T., and Rosen, H. (2007). Mapping pathways downstream of sphingosine 1-phosphate subtype 1 by differential chemical perturbation and proteomics. The Journal of biological chemistry *282*, 7254-7264.

Gonzalez-Cabrera, P.J., Jo, E., Sanna, M.G., Brown, S., Leaf, N., Marsolais, D., Schaeffer, M.T., Chapman, J., Cameron, M., Guerrero, M., *et al.* (2008). Full pharmacological efficacy of a novel S1P1 agonist that does not require S1P-like headgroup interactions. Molecular pharmacology *74*, 1308-1318.

Graler, M.H., and Goetzl, E.J. (2004). The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. FASEB J *18*, 551-553.

Hanson, M.A., Roth, C.B., Jo, E., Griffith, M.T., Scott, F.L., Reinhart, G., Desale, H., Clemons, B., Cahalan, S.M., Schuerer, S.C., *et al.* (2012). Crystal structure of a lipid G protein-coupled receptor. Science *335*, 851-855.

Hla, T., and Brinkmann, V. (2011). Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation. Neurology *76*, S3-8.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., *et al.* (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. The Journal of clinical investigation *106*, 951-961.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C., *et al.* (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science *296*, 346-349.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

Miron, V.E., Jung, C.G., Kim, H.J., Kennedy, T.E., Soliven, B., and Antel, J.P. (2008). FTY720 modulates human oligodendrocyte progenitor process extension and survival. Annals of neurology *63*, 61-71.

Miron, V.E., Ludwin, S.K., Darlington, P.J., Jarjour, A.A., Soliven, B., Kennedy, T.E., and Antel, J.P. (2010). Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. The American journal of pathology *176*, 2682-2694.

Quancard, J., Bollbuck, B., Janser, P., Angst, D., Berst, F., Buehlmayer, P., Streiff, M., Beerli, C., Brinkmann, V., Guerini, D., *et al.* (2012). A Potent and Selective S1P(1) Antagonist with Efficacy in Experimental Autoimmune Encephalomyelitis. Chemistry & biology *19*, 1142-1151.

Sanna, M.G., Liao, J., Jo, E., Alfonso, C., Ahn, M.Y., Peterson, M.S., Webb, B., Lefebvre, S., Chun, J., Gray, N., *et al.* (2004). Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. The Journal of biological chemistry *279*, 13839-13848.

Sanna, M.G., Wang, S.K., Gonzalez-Cabrera, P.J., Don, A., Marsolais, D., Matheu, M.P., Wei, S.H., Parker, I., Jo, E., Cheng, W.C., *et al.* (2006). Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P1 antagonist in vivo. Nature chemical biology *2*, 434-441.

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J.G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science *309*, 1735-1739.

Tarrason, G., Auli, M., Mustafa, S., Dolgachev, V., Domenech, M.T., Prats, N., Dominguez, M., Lopez, R., Aguilar, N., Calbet, M., *et al.* (2011). The sphingosine-1-phosphate receptor-1 antagonist, W146, causes early and short-lasting peripheral blood lymphopenia in mice. International immunopharmacology *11*, 1773-1779.

Wei, S.H., Rosen, H., Matheu, M.P., Sanna, M.G., Wang, S.K., Jo, E., Wong, C.H., Parker, I., and Cahalan, M.D. (2005). Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. Nature immunology *6*, 1228-1235.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was funded by National Institutes of Health [Grants AI05509, AI074564, MH084812]

Address reprint requests to: Dr. Hugh Rosen, Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037. E-mail: hrosen@scripps.edu

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1: Ex26 is a potent, selective $S1P_1$ antagonist. (**A)** Dose response *in vitro* of Ex26 on $S1P_1$ expressing cells in the presence of 5 nM S1P. Structure of Ex26 is depicted on the right (**B)** Ex26 induces dose-dependent pulmonary edema 2 hours following i.p. treatment. (**C)** Pulmonary edema induced by 3 mg/kg Ex26 i.p. resolves by 16-24 hours following treatment. All data are representative of at least two experiments, with (**B)-(C)** having 4 mice per group per experiment. Graphs are plotted as mean ± S.E.M.

Figure 2: $S1P_1$ antagonism by Ex26 induces lymphocyte sequestration in the lymph nodes and thymus. (A) Ex26 induces dose-dependent lymphocyte sequestration 2 hours following i.p. treatment. (**B)** Lymphopenia induced by 3 mg/kg Ex26 i.p. resolves by 24 hours following treatment. (**C-D)** Continuous administration of Ex26 in 6-week-old mice by micro-osmotic pumps sequesters T and B cells in the peripheral lymph nodes (**C**), leaving the spleen depleted of lymphocytes (**D)**. pLN cell numbers derive from combined inguinal, axillary, and brachial lymph nodes. (**E)** Ex26 leads to accumulation of mature $CD62L^{Hi}$, but not immature $CD62L^{Lo}$, SP thymocytes. All graphs are representative of three experiments, with 3-4 mice per group per experiment. Graphs are plotted as mean ± S.E.M. * $p<0.05$, ** $p<0.01$ as determined by unpaired, two-tailed t-test.

Figure 3: $S1P_1$ antagonism by Ex26 upregulates $S1P_1$ and CD69, but not in the central nervous system. (A) $S1P_1$-eGFP expression on lymphocytes from lymph nodes from $S1P_1$-eGFP knockin mice continuously administered Ex26 by micro-osmotic pump for 3 days. Gray shaded histograms represent background fluorescence in wild-type mice. Graph on right represents mean

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

fluorescence intensity of $S1P_1$-eGFP on the indicated cell type. (**B)** Mean fluorescence intensity of $S1P_1$-eGFP (left) and CD69 (right) on CD4 SP thymocytes following continuous treatment with Ex26. (**C)** Mean fluorescence intensity of $S1P_1$-eGFP on lung endothelial cells following continuous treatment with Ex26. (**D)** Fluorescent scan of SDS-PAGE gel from brains of mice following 3 days of treatment with Ex26. Graph on right is obtained by densitometric analysis of the gel on the left. All histograms and graphs are representative of three experiments, with 3-4 mice per group per experiment. Graphs are plotted as mean ± S.E.M. ** $p<0.01$, *** $p<0.001$ as determined by unpaired, two-tailed t-test.

Figure 4: $S1P_1$ antagonism by Ex26 alleviates EAE. (**A)** Average EAE scores from MOG_{33-55} induced mice injected daily i.p. with either vehicle, 30 mg/kg Ex26 or 10 mg/kg FTY720 following the onset of symptoms. **** $p<0.0001$ compared to vehicle as calculated by one-way repeated measures ANOVA with Bonferroni's multiple comparison post-test. Graph is representative of two separate experiments as mean ± S.E.M with 9-10 mice per group. **(B)** Representative spinal cord sections stained with H&E from control mice without EAE (top left) or mice with EAE that had been treated daily as indicated following the onset of clinical signs. **(C)** Western blot for $S1P_1$ on brains of mice with EAE treated daily with either vehicle (50 mM Na_2CO_3), 30 mg/kg Ex26 or 10 mg/kg FTY720 following the onset of symptoms. Graph represents $S1P_1$ expression as determined by densitometry. n.d.: not detectable.

Molecular Pharmacology Fast Forward. Published on November 30, 2012 as DOI: 10.1124/mol.112.082958
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Selectivity of Ex26 on S1P receptors

Receptor	Antagonist IC_{50} (nM)	Agonist EC_{50} (nM)
$S1P_1$	0.93	>10,000
$S1P_2$	>10,000	>10,000
$S1P_3$	>10,000	>10,000
$S1P_4$	4900	>10,000
$S1P_5$	3100	>10,000

Ex26 displays excellent selectivity for $S1P_1$ over other S1P receptors. It also does not exhibit any detectable agonist activity on any S1P receptor.

Figure 1

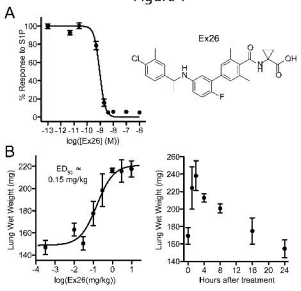
A
% Response to S1P
log([Ex26] (M))
Ex26
B
Lung Wet Weight (mg)
ED50 ≈
0.15 mg/kg
log(Ex26(mg/kg))
Hours after treatment

Figure 2

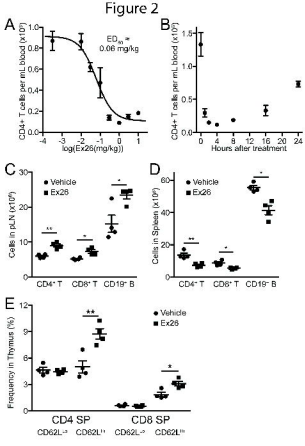
A
CD4+ T cells per mL blood (x10⁶)
ED50 = 0.06 mg/kg
log(Ex26(mg/kg))
B
CD4+ T cells per mL blood (x10⁶)
Hours after treatment
C
Vehicle
Ex26
Cells in nLN (x10⁶)
CD4+ T
CD8+ T
CD19+ B
D
Vehicle
Ex26
Cells in Spleen (x10⁶)
CD4+ T
CD8+ T
CD19+ B
E
Vehicle
Ex26
Frequency in Thymus (%)
CD4 SP
CD8 SP
CD62L-/+
CD62L+/+
CD62L-/-
CD62L+/-

Figure 3

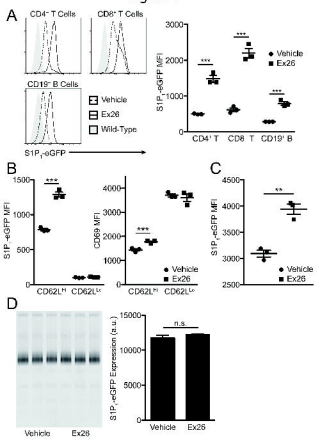
A
CD4+ T Cells
CD8+ T Cells
CD19+ B Cells
Vehicle
Ex26
Wild-Type
S1P1-eGFP
S1P1-eGFP MFI
CD4+ T
CD8+ T
CD19+ B
B
CD62L
CD69 MFI
C
S1P-eGFP MFI
D
S1P1-eGFP Expression (a.u.)
n.s.

Figure 4


A
30 mg/kg Ex26
10 mg/kg FTY720
Vehicle
Average EAE Score
Days Following EAE Induction
B
Control
Vehicle
30 mg/kg Ex26
10 mg/kg FTY720
C
30 mg/kg Ex26
10 mg/kg FTY720
Vehicle
S1P1
S1P Expression (a.u.)
n.d.
30 mg/kg Ex26
10 mg/kg FTY720
Vehicle