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S1P₁ Receptor Upregulation and Amelioration of Experimental Autoimmune Encephalomyelitis
by an S1P₁ Antagonist

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Running Title: S1P₁ antagonist reverses EAE independent of CNS S1P₁

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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

MOG₃₃₋₅₅: Peptide consisting of residues 33-55 of myelin oligodendrocyte glycoprotein

MS: Multiple Sclerosis

PTX: Pertussis toxin

S1P: Sphingosine 1-Phosphate

S1P₁₋₅: Sphingosine 1-Phosphate Receptor 1-5

Abstract

Sphingosine 1-phosphate receptor 1 is a G protein-coupled receptor that is critical for proper lymphocyte development and recirculation. Agonists to S1P₁ are currently in use clinically for the treatment of multiple sclerosis, and these drugs may act both on S1P₁ expressed on lymphocytes and on S1P₁ expressed within the central nervous system. Agonists to S1P₁ or deficiency in S1P₁ both cause lymphocyte sequestration in the lymph nodes. Here we show that S1P₁ antagonism induces lymphocyte sequestration in the lymph nodes similar to that observed with S1P₁ agonists while upregulating S1P₁ on lymphocytes and endothelial cells. Additionally, we show that S1P₁ antagonism reverses experimental autoimmune encephalomyelitis in mice without acting on S1P₁ expressed within the central nervous system, demonstrating that lymphocyte sequestration via S1P₁ antagonism is sufficient to alleviate autoimmune pathology.

Introduction

Sphingosine 1-phosphate receptor 1 (S1P₁) 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). S1P₁ is required on developing lymphocytes to mature beyond a semi-mature CD69^{hi} CD62L^{lo} state, rendering the blood and lymph of mice lacking S1P₁ on developing lymphocytes largely devoid of T cells. When S1P₁^{-/-} thymocytes are transferred into recipient mice, they are also retained from blood and lymphatic circulation. S1P₁ 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 S1P₁, S1P₃, S1P₄, and S1P₅ (Mandala et al., 2002). S1P₁ selective agonists demonstrated that FTY720 acted via S1P₁ to induce lymphocyte sequestration (Sanna et al., 2004). The ability of FTY720-P and other S1P₁ agonists to induce sustained internalization and/or degradation of S1P₁ (Gonzalez-Cabrera et al., 2007; Gonzalez-Cabrera et al., 2008; Graler and Goetzl, 2004), combined with the deficient egress of S1P₁-deficient lymphocytes, has led to the hypothesis that S1P₁ agonists act as functional antagonists (Graler and Goetzl, 2004). Several S1P₁-selective antagonists have also been generated, which inhibit agonist-dependent effects *in vitro*, stabilize the S1P₁ 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 S1P₁ antagonists can indeed induce lymphocyte sequestration at high plasma concentrations (Tarrason et al., 2011), and one such S1P₁

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 S1P₁, as it is effective at doses that maintain ~50% lymphopenia. This efficacy probably involves both S1P₁ and other S1P receptors within the central nervous system (CNS) (Cohen and Chun, 2011; Hla and Brinkmann, 2011). S1P₁ agonists that can efficiently penetrate the CNS can induce receptor signaling and degradation of S1P₁ 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 S1P₁ 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 S1P₁ antagonism sequesters lymphocytes in the peripheral lymph nodes but not the spleen, similar to that observed with S1P₁ agonists. S1P₁ antagonism also causes significant upregulation of S1P₁ expression on peripheral lymphocytes, mature thymocytes, and lung endothelial cells. Additionally, S1P₁ antagonism can alleviate EAE in mice despite the inability of the antagonist used to penetrate the CNS. Thus, lymphocyte sequestration induced by S1P₁ antagonists is sufficient to ameliorate the autoimmune pathology observed in

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EAE, and does not require antagonism of S1P₁ expressed on neurons or astrocytes within the CNS.

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 Na₂CO₃, while FTY720 was solubilized in H₂O. *In vitro* assays for S1P receptor function were performed using the following cell lines: S1P₁, S1P₄, S1P₅ – Tango U2OS cells (Invitrogen) expressing the indicated receptor; S1P₂ – CHO cells expressing S1P₂ coupled to a CRE-Bla reporter; S1P₃ – CHO cells expressing S1P₃ coupled to a NFAT-Bla reporter through Gα16. S1P₁ internalization and polyubiquitinylation were evaluated using HEK cells expressing S1P₁-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 Na₂CO₃ vehicle, and blood was removed from the heart following euthanasia. Blood was lysed in 150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, washed with PBS containing 2% FBS, 1 mM EDTA, 0.1% NaN₃, 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

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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 S1P₁ Antagonist

6-week-old S1P₁-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 Na₂CO₃ 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, S1P₁ 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

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obtained from BD. Data was collected using an LSRII flow cytometer (BD) and analyzed using FlowJo (Treestar). S1P₁ expression by flow cytometry was measured using S1P₁-eGFP knockin mice (Cahalan et al., 2011). S1P₁ expression in the CNS in EAE experiments was evaluated using a C-terminal specific S1P₁ 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 Na₂CO₃ vehicle, 30 mg/kg Ex26 or 10 mg/kg FTY720 in a volume of 10 μ L per gram weight of mouse beginning the first day on which clinical signs were observed in that mouse.

Results and Discussion

Ex26 is a S1P₁ antagonist that inhibits lymphocyte egress

Most existing S1P₁ antagonists are S1P analogs with IC₅₀ values in the double-digit nanomolar range and possessing relatively short half-lives. Recently, new S1P₁ 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 S1P₁ (Fig. 1A and Table 1), similar to a recently published antagonist (Quancard et al., 2012). Ex26 could inhibit RP-001-induced S1P₁ internalization and polyubiquitinylation *in vitro* (Supplemental Fig. 1). Like other previously described S1P₁ 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 S1P₁ 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 ED₅₀ 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.

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 S1P₁ 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 S1P₁ agonists (Fig 2E). These data demonstrate that disruption of S1P₁ signaling by S1P₁ antagonism leads to inhibition of lymphocyte and thymocyte egress.

S1P₁ Antagonism Upregulates S1P₁ Expression

Since S1P₁ agonists downregulate S1P₁, we wanted to determine whether S1P₁ antagonism could conversely upregulate S1P₁. Continuous S1P₁ antagonism in mice expressing S1P₁-eGFP from the S1P₁ locus (Cahalan et al., 2011) for 3 days by micro-osmotic pumps caused significant upregulation of S1P₁-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 S1P₁. We observed similar upregulation within the spleen (Data not shown), and a modest upregulation of S1P₁-eGFP on fully mature CD62L^{hi} SP thymocytes (Fig. 3B). S1P₁ 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 S1P₁ signaling, not only expression of S1P₁ (Bankovich et al., 2010), is critical for suppressing the surface expression of CD69; thus, downregulation of CD69 by S1P₁ agonists is a measure of agonism, not functional antagonism. Upregulation of S1P₁-eGFP was not limited to lymphocytes, as blood endothelial cells within the lung significantly upregulated S1P₁-eGFP expression (Fig. 3C). Unlike many S1P₁ agonists including FTY720-P, Ex26 did not cause any

changes in the expression of S1P₁-eGFP within the brain (Fig. 3D), due to the fact that Ex26 was almost undetectable within the CNS (Plasma: $6.8 \pm 0.3 \mu\text{M}$, Brain: $0.01 \pm 0.005 \mu\text{M}$, 2/3 animals below level of detection, mean \pm S.E.M.).

S1P₁ Antagonism Ameliorates EAE

Since Ex26 did not enter the CNS nor cause any change in S1P₁ 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 S1P₁ antagonism could ameliorate EAE similar to S1P₁ agonism, we induced disease using the MOG₃₃₋₅₅ 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 Na₂CO₃ 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 S1P₁ 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

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at the end of the experiment (Fig. 4B). Consistent with the lack of CNS penetration of Ex26, we did not observe any changes in S1P₁ 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 S1P₁ within the brain (Fig. 4C). This indicates that antagonism of S1P₁ expressed on neurons or astrocytes within the CNS is not required for the amelioration of EAE by S1P₁ antagonists, implying that lymphocyte sequestration by S1P₁ 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.

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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

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Footnotes

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Figure Legends

Figure 1: Ex26 is a potent, selective S1P₁ antagonist. (A) Dose response *in vitro* of Ex26 on S1P₁ 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 \pm S.E.M.

Figure 2: S1P₁ 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 \pm S.E.M. * $p < 0.05$, ** $p < 0.01$ as determined by unpaired, two-tailed t-test.

Figure 3: S1P₁ antagonism by Ex26 upregulates S1P₁ and CD69, but not in the central nervous system. (A) S1P₁-eGFP expression on lymphocytes from lymph nodes from S1P₁-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

fluorescence intensity of S1P₁-eGFP on the indicated cell type. **(B)** Mean fluorescence intensity of S1P₁-eGFP (left) and CD69 (right) on CD4 SP thymocytes following continuous treatment with Ex26. **(C)** Mean fluorescence intensity of S1P₁-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 \pm S.E.M. ** $p < 0.01$, *** $p < 0.001$ as determined by unpaired, two-tailed t-test.

Figure 4: S1P₁ antagonism by Ex26 alleviates EAE. **(A)** Average EAE scores from MOG₃₃₋₅₅ 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 \pm 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 S1P₁ on brains of mice with EAE treated daily with either vehicle (50 mM Na₂CO₃), 30 mg/kg Ex26 or 10 mg/kg FTY720 following the onset of symptoms. Graph represents S1P₁ expression as determined by densitometry. n.d.: not detectable.

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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 S1P₁ over other S1P receptors. It also does not exhibit any detectable agonist activity on any S1P receptor.

Figure 1

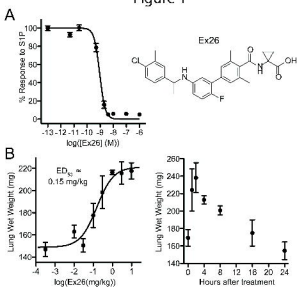


Figure 2

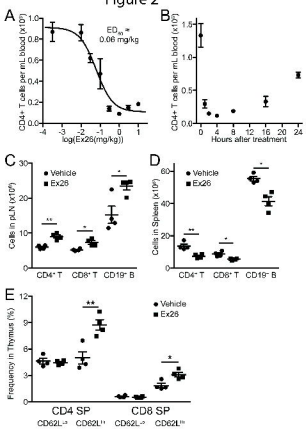


Figure 3

