ACCELERATED COMMUNICATION

Sphingosine 1-Phosphate Receptor 1 (S1P1) Upregulation and Amelioration of Experimental Autoimmune Encephalomyelitis by an S1P1 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.B.L., S.J.B., H.R.) and Chemistry (M.G., E.R.), The Scripps Research Institute, La Jolla, California

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ABSTRACT

Sphingosine 1-phosphate receptor 1 (S1P1) 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 on both S1P1 expressed on lymphocytes and S1P1 expressed within the central nervous system. Agonists to S1P1 and deficiency in S1P1 both cause lymphocyte sequestration in the lymph nodes. In the present study, 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 physiologic systems, including vascular development, lymphocyte development, and lymphocyte recirculation (Liu et al., 2000; Allende et al., 2003, 2004; Matloubian et al., 2004; Cyster and Schwab, 2012). S1P1 is required on developing lymphocytes to mature beyond a semimature CD69lo, CD62Lhi 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 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, mono dihydrogen phosphate ester (FTY720-P), 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-deficient lymphocytes, has led to the hypothesis that S1P1 agonists act as functional antagonists (Graler and Goetzl, 2004). Several S1P1-selective agonists have also been generated, which inhibit agonist-dependent effects in vitro; stabilize the S1P1 receptor, allowing for its structural determination; and induce pulmonary edema in vivo. In addition, initial antagonists could reverse agonist-induced lymphocyte sequestration while being unable to induce lymphocyte sequestration themselves (Foss et al., 2005; Wei et al., 2005; Sanna et al., 2006; Hanson et al., 2012). Recent work has shown 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.

ABBREVIATIONS: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; Ex26, 1-(5′-[(1-(4-chloro-3-methylphenyl)ethyl)(l-aminomethyl)-2-fluoro-3,5-dimethyl-1′-biphenyl]-4-ylcarboxamidocyclopropane-carboxylic acid; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol; PBS, phosphate-buffered saline; S1P, sphingosine 1-phosphate; S1P1-eGRP, S1P1-enhanced green fluorescent protein; RP-001, 3-(4-(5-(3-cyano-4-isopropoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-ylamino)propanoic acid.

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SIP1 antagonists can indeed induce lymphocyte sequestration at high plasma concentrations (Tarrason et al., 2011) and SIP1 antagonists can alleviate animal models of autoimmune arthritis (Fujii et al., 2012), cardiac allograft rejection (Angst et al., 2012), and multiple sclerosis (Quandcard et al., 2012).

SIP receptor agonists have come of age with the Food and Drug Administration’s approval of FTY720 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 SIP1, as it is effective at doses that maintain ~50% lymphopenia. This efficacy probably involves both SIP1 and other SIP receptors within the central nervous system (CNS) (Cohen and Chun, 2011; Hla and Brinkmann, 2011). SIP1 antagonists that can efficiently penetrate the CNS can induce receptor signaling and degradation of SIP1 expressed on neurons and astrocytes (Gonzalez-Cabrera et al., 2012), and require lymphocyte sequestration for only one-third of a dosing interval to reverse experimental autoimmune encephalomyelitis (EAE) in mice. Additionally, mice lacking SIP1 on astrocytes are refractory to developing EAE, and are suggested to be important targets of FTY720 (Choi et al., 2011). Several other SIP 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, 2010).

In the present study, we demonstrate that SIP1 antagonism sequesters lymphocytes in the peripheral lymph nodes but not the spleen, similar to that observed with SIP1 agonists. SIP1 antagonism also causes significant upregulation of SIP1 expression on peripheral lymphocytes, mature thymocytes, and lung endothelial cells. Additionally, SIP1 antagonism can alleviate EAE in mice despite the inability of the antagonist to penetrate the CNS. Thus, lymphocyte sequestration induced by SIP1 antagonists is sufficient to ameliorate the autoimmune pathology observed in EAE, and does not require antagonism of SIP1 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., 2009). RP-001 (3-(4-(5-(3-cyano-biphenyl)-4-ylcarboxamido)cyclopropanecarboxylic acid) was synthesized as previously described ((1-(4-chloro-3-methylphenyl)ethyl)amino)-2

**Evaluation of Lymphocyte Sequestration, Pulmonary Edema.** Eight-week-old male C57Bl/6J mice were purchased from the The Scripps Research Institute mouse breeding facility (La Jolla, CA) 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, and 0.1 mM EDTA; washed with phosphate-buffered saline (PBS) containing 2%

**Fig. 1.** Ex26 is a potent, selective SIP1 antagonist. (A) Dose response in vitro of Ex26 on SIP1-expressing cells in the presence of 5 nM S1P. The 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) and (C) having four mice per group per experiment. Graphs are plotted as the mean ± S.E.M.

**TABLE 1**

Selectivity of Ex26 on SIP receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Antagonist EC50 nM</th>
<th>Agonist EC50 nM</th>
</tr>
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<tbody>
<tr>
<td>SIP1</td>
<td>0.93</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>SIP2</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>SIP3</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>SIP4</td>
<td>4900</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>SIP5</td>
<td>3100</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Ex26, 1-(5’-((1-(4-chloro-3-methylphenyl)ethyl)amino)-2’-fluoro-3,5-dimethyl-[1,1’-biphenyl]-4-ylcarboxamido)cyclopropanecarboxylic acid; SIP, sphingosine 1-phosphate.
determined by disruption of brain tissue in water by probe sonication, followed by extraction with acetonitrile and filtration through MultiScreen hydrophilic polytetrafluoroethylene 0.45 μm filters (EMD Millipore, Billerica, MA). Filtrates were analyzed using a API 4000 liquid chromatography-tandem mass spectrometer (AbSciex, Framingham, MA) and quantified using a positive-ion multiple reaction monitoring method (495.1/242.1, m/z).

Continuous Administration of S1P1 Antagonist. Six-week-old S1P1-eGFP mice were anesthetized with isoflurane, and their backs were shaved, cleaned with 70% ethanol 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; Alzet, Cupertino, CA) containing either 50 mM 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 (San Diego, CA). Fluorescently labeled antibodies specific to CD19, CD31, CD45.2, CD62L, and CD69 were obtained from Beckton-Dickinson (San Diego, CA). Data were collected using an LSRII flow cytometer (Beckton-Dickinson) and analyzed using FlowJo (Treestar, Ashland, OR). 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 analyses were performed using GraphPad Prism Software (GraphPad, La Jolla, CA).

EAE Induction and Scoring. EAE was induced in female 10-week-old C57Bl/6J mice purchased from Jackson Laboratories (Bar Harbor, ME). EAE was induced using a Hooke Laboratories EAE induction kit (Lawrence, MA; EK-0114 for EAE, CK-0114 for control) according to the 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), and 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 μl per gram weight of mouse beginning the first day on which clinical signs were observed in that mouse.
Results and Discussion

Ex26 is an S1P1 Antagonist that Inhibits Lymphocyte Egress. Most existing S1P1 antagonists are S1P analogs with IC50 values in the double-digit nanomolar range that possess relatively short half-lives. Recently, new S1P1 antagonists have been described, including a series of biaryl benzylamines by Novartis (Angst et al. 2009). We synthesized and characterized one of these compounds, Ex26, and confirmed it to be a potent and selective antagonist of S1P1 (Fig. 1A; 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). Similar to other previously described S1P1 antagonists, Ex26 induced dose-dependent and time-dependent pulmonary edema in vivo (Fig. 1, B and 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 (Sanna et al., 2006; Foss et al., 2007). Recent work has found that W146 induces transient lymphocyte sequestration at high doses (Tarrason et al., 2011), which we replicated (unpublished data). Ex26 induced lymphocyte sequestration at low doses, possessing an ED50 of ∼0.06 mg/kg when examined 2 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 in T and B cells within the spleen, similar to S1P1 agonists (Fig. 2, C and D). Continuous administration of Ex26 also led to thymic retention of mature CD62LHi single-positive 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 the inhibition of lymphocyte and thymocyte egress.

S1P1 Antagonism Uregulates 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 physiologic conditions is sufficient to suppress the expression of S1P1. We observed similar upregulation within the spleen (unpublished data) and a modest upregulation of S1P1-eGFP on fully mature CD62Lhi 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 ± 0.3 µM, brain: 0.01 ± 0.005 µM; 2/3 animals below the level of detection; mean ± S.E.M.).

**S1P1 Antagonism Ameliorates EAE.** Because Ex26 did not enter the CNS or 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. 2, A and B).

To examine whether S1P1 antagonism could ameliorate EAE similar to S1P1 agonism, we induced disease using the myelin oligodendrocyte glycoprotein residues 33–55 peptide model, and, upon development of clinical signs of disease, treated mice i.p. once daily with 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 (unpublished data). 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 with 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 (unpublished data), suggesting that treatment with pertussis toxin 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 the 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 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 brains of mice euthanized at the end of the experiment that were treated daily with 30 mg/kg Ex26 compared with 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

![Fig. 4. S1P1 antagonism by Ex26 alleviates EAE. (A) Average EAE scores from myelin oligodendrocyte glycoprotein residues 33-55-induced mice injected daily i.p. with vehicle, 30 mg/kg Ex26, or 10 mg/kg FTY720 following the onset of symptoms. ***P < 0.0001 compared with vehicle as calculated by one-way repeated measures analysis of variance with Bonferroni’s multiple comparison post test. The graph represents two separate experiments as the 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 S1P1 on the brains of mice with EAE treated daily with vehicle (50 mM Na2CO3), 30 mg/kg Ex26, or 10 mg/kg FTY720 following the onset of symptoms. The graph represents S1P1 expression as determined by densitometry. a.u., arbitrary units; n.d., not detectable.](molpharm.aspetjournals.org)
organists 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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Authorship Contributions

Participated in research design: Cahalan, Gonzalez-Cabrerab, Rosen.
Conducted experiments: Cahalan, Gonzalez-Cabrera, Nguyen, Cisar, Leaf, Brown.
Contributed new reagents or analytic tools: Guerrero, Roberts. Performed data analysis: Cahalan, Gonzalez-Cabrerab, Cisar, Brown, Rosen.
Wrote or contributed to the writing of the manuscript: Cahalan, Gonzalez-Cabrerab, Rosen.

References


Supplemental Figure 1: Ex26 inhibits S1P1 internalization and polyubiquitinylation in vitro and exhibits a short half-life in vivo. (A) Ex26 inhibits S1P1 internalization induced by the S1P1 agonist RP-001 in vitro. HEK cells expressing S1P1-eGFP were pretreated with the indicated concentrations of Ex26 for 1 hour, and then were incubated in the presence of 100 nM RP-001 for an additional hour. (B) Ex26 inhibits RP-001-induced polyubiquitinylation. Cells were treated as in (A), but were incubated with 50 nM RP-001. Lysates were immunoprecipitated with an antibody specific to GFP, then blotted for P4D1 to detect ubiquitin. (C) Ex26 has a relatively short half-life following treatment with 3 mg/kg i.p. All data are representative of at least two experiments, with (C) utilizing 4 mice per group per experiment.
Supplemental Figure 2: High dose Ex26 can cause sustained lymphocyte sequestration and pulmonary edema. (A) CD4+ T cell counts from the blood of mice treated i.p. 24 hours previously with the indicated doses of Ex26. (B) Lung wet weights of mice treated i.p. 24 hours previously with the indicated doses of Ex26. Graphs are representative of two experiments, with 4 mice per group per experiment and are plotted as mean ± S.E.M. * p<0.05, *** p<0.001 as determined by one-way ANOVA with Bonferroni’s multiple comparison post-test.