Selective Sphingosine 1-Phosphate Receptor 1 Agonist Is Protective Against Ischemia/Reperfusion in Mice

Vanessa H. Braït, PhD; Gema Tarrasón, PhD; Amadeu Gavaldà, PhD; Núria Godessart, PhD; Anna M. Planas, PhD

Background and Purpose—Growing evidence supports that the immunomodulatory drug fingolimod is protective in stroke. Fingolimod binds to 4 of 5 sphingosine-1-phosphate (S1P) receptors and, among other actions, it induces lymphopenia. In this study, we investigated whether a selective S1P1 agonist is protective in experimental stroke.

Methods—Drug selectivity was studied in vitro in cells overexpressing the human S1P receptors. Mice (n=54) received different doses of LASW1238 (3 or 10 mg/kg), fingolimod (1 mg/kg), or the vehicle intraperitoneal, and lymphopenia was studied at different time points. After intraluminal middle cerebral artery occlusion for 45 minutes and immediately after reperfusion, mice (n=56) received the drug treatment. At 24 hours, a neurological test was performed and infarct volume was measured. Treatment and all the analyses were performed in a blind fashion.

Results—In vitro functional assays showed that LASW1238 is a selective agonist of the S1P1 receptor. At 10 mg/kg, this compound induced sustained lymphopenia in mice comparable with fingolimod, whereas at 3 mg/kg it induced short-lasting lymphopenia. After ischemia, both LASW1238 (10 mg/kg) and fingolimod reduced infarct volume, but only LASW1238 (10 mg/kg) showed statistically significant differences versus the vehicle. The neurological function and plasma cytokine levels were not different between groups.

Conclusions—The selective S1P1 agonist LASW1238 reduces infarct volume after ischemia/reperfusion in mice, but only when lymphopenia is sustained for at least 24 hours. S1P1 and lymphocytes are potential targets for drug treatment in stroke. Defining the best drug dosing regimen to control the extent and duration of lymphopenia is critical to achieve the desired effects. *(Stroke. 2016;47:3053-3056. DOI: 10.1161/STROKEAHA.116.015371.)*

Key Words: brain ▪ fingolimod ▪ lymphopenia ▪ rodentia ▪ sphingosine 1-phosphate ▪ stroke

Fingolimod is an oral drug approved for the treatment of relapsing-remitting multiple sclerosis. Several studies reported beneficial effects of fingolimod in experimental models of brain ischemia and in ischemic stroke patients. Fingolimod is the prodrug of fingolimod phosphate (fingolimod-P), an agonist of 4 of the 5 sphingosine-1-phosphate (S1P) receptors (all except S1P2). S1P receptors are G-protein-coupled receptors regulating diverse cellular functions, including survival, proliferation, and migration. S1P regulates lymphocyte migration from lymphoid tissue to blood. Synthetic S1P1 agonists behave as functional antagonists by inducing lymphocyte receptor internalization, leading to receptor unavailability at the cellular membrane. Therefore, cells are retained in lymphoid organs causing lymphopenia.

Fingolimod penetrates and accumulates in the brain, where S1P receptors are expressed. The contribution of S1P receptors other than S1P1 on the beneficial effects of fingolimod in stroke is not completely understood. We addressed this question and the mechanism behind the efficacy by studying the effects of a selective S1P1 agonist in mouse ischemic stroke.

Methods

See the online-only Data Supplement for further details.

Animals

Animal work was conducted in adult male C57BL/6J mice (Charles River) after the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and with approval from the Animal Experimentation Ethical Committee of Almirall (Barcelona, Spain) or the University of Barcelona (CEEA).

Chemicals and Reagents

LASW1238 and fingolimod were synthesized by the Department of Medicinal Chemistry (Almirall R&D Center, Barcelona, Spain).
Drugs were suspended in vehicle (0.5% carboxymethyl cellulose, 0.1% Tween-80 in purified water) and were administered intraperitoneally.

Target Selectivity Profile

Assays were performed on membranes of Chinese hamster ovary (CHO) cells transfected with human S1P receptors. Agonist potencies were measured by [35S]-GTPγS binding after drug stimulation of the receptors (n=5). Potential off-target effects of LASW1238 (10 µmol/L) were studied on a panel of G-protein–coupled receptors and enzymes.

Lymphopenia

Mice were randomized to receive the vehicle, LASW1238 (3 or 10 mg/kg) or fingolimod (1 mg/kg; n=54), and blood cell counts were obtained in a hemocytometer.

Focal Brain Ischemia/Reperfusion

The mouse right middle cerebral artery was intraluminally occluded for 45 minutes followed by reperfusion (n=56). Cerebral blood flow (CBF) was measured with laser Doppler flowmetry (Perimed AB, Järfälla, Sweden). CBF data were used as criteria to exclude animals (n=16) from the study if they did not show: (1) a drop in CBF after middle cerebral artery occlusion >75% from basal values; (2) recovery in CBF at reperfusion >70% of basal. The mice were randomized, and treatments were given after reperfusion in a blinded fashion. At 24 hours, a neurological score was performed, and brain infarction was studied with the 2,3,5-triphenyltetrazolium chloride technique.

Statistical Analyses

One-way ANOVA or the Kruskal–Wallis test followed by post hoc analyses were performed using GraphPad Prism software.

Results

In Vitro Studies

Evaluation of drug affinity for human S1P receptors showed that LASW1238 is a highly selective S1P1 agonist compared with S1P3 and S1P5 and had no effect on S1P2 (Table). LASW1238 did not show cytotoxicity in Chinese hamster ovary cells at doses of up to 40 µmol/L, whereas fingolimod showed cytotoxicity at 8.5 µmol/L. Moreover, LASW1238 (10 µmol/L) did not show activity on any of the >100 different targets tested, except for a 55% inhibition of binding to the M2 muscarinic receptor (not shown).

In Vivo Pharmacology: Lymphopenia

All the treatments reduced the numbers of circulating lymphocytes by ~80% at 4 hours (Figure 1). Lymphopenia was sustained for at least 24 hours with LASW1238 (10 mg/kg) and fingolimod (1 mg/kg). Dose differences between these drugs needed for this effect agree with the 10-fold difference in their affinities for S1P1. The low-dose LASW1238 (3 mg/kg) induced transient lymphopenia at 4 hours that was not sustained at 24 hours.

LASW1238 Reduces Infarct Volume

Mice of all groups showed similar CBF changes after middle cerebral artery occlusion (Figure 2A). High-dose (10 mg/kg) LASW1238 produced a smaller infarct volume at 24 hours (P<0.05; Figure 2B and 2C) than both the vehicle and the low-dose (3 mg/kg) LASW1238. Fingolimod (1 mg/kg) tended to produce a smaller infarct volume, but this did not reach statistical significance (Figure 2C). Edema volume (Figure I in the online-only Data Supplement) and the neurological score (Figure 2D) did not differ between groups. There was no mortality in any of the groups.

Discussion

This study shows that the selective S1P1 agonist LASW1238 reduced infarct volume after brain ischemia/reperfusion in mice, suggesting that S1P1 is a relevant target in ischemic stroke rather than other S1P receptors that are also targets of fingolimod. This finding is in agreement with a previous study, showing that another selective S1P1 agonist was protective when given after ischemia/reperfusion in rats and suggested that the drug acted on neuronal S1P1. Although actions of S1P1 agonists on brain or vascular cells cannot be ruled out, our study suggests that lymphopenia is important for the beneficial effect of the S1P1 agonist because LASW1238 reduced infarct volume only at the dose able to sustain lymphopenia for 24 hours, but not at a lower dose causing short-lasting lymphopenia. In line with our results, fingolimod was not protective after ischemia/reperfusion in lymphocyte-deficient mice. The current results show that drug-induced lymphopenia needs to be sustained for at least 24 hours to translate into benefits after brain ischemia.

Bradycardia is observed in multiple sclerosis patients treated with fingolimod, and it can cause atrioventricular blockade because of S1P1 direct agonistic action on cardiomyocytes. This side effect requires patient monitoring for 6 hours after the first drug administration to avoid complications, whereas full lymphopenia is only achieved hours later. Although a quick onset of lymphopenia is not required in a chronic disease, such as multiple sclerosis, it is currently unknown how fast after stroke onset and for how long lymphopenia should

<table>
<thead>
<tr>
<th>Compounds</th>
<th>S1P1</th>
<th>S1P2</th>
<th>S1P3</th>
<th>S1P4</th>
<th>S1P5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P</td>
<td>5.6±0.72</td>
<td>5.8±1.1</td>
<td>1.3±0.1</td>
<td>17.3±5.2</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>Fingolimod-P</td>
<td>1.7±0.57</td>
<td>&gt;10000</td>
<td>10.4±1.8</td>
<td>2.8±1.4</td>
<td>0.48±0.15</td>
</tr>
<tr>
<td>LASW1238</td>
<td>18.8±3.2</td>
<td>&gt;10000</td>
<td>14023±4500</td>
<td>85±13</td>
<td>197±39</td>
</tr>
</tbody>
</table>

CHO indicates Chinese hamster ovary, and S1P, sphingosine-1-phosphate.
be maintained. Also, any potential adverse effects of lymphopenia should be investigated, for instance regarding the risk of infection associated with the naturally occurring stroke-induced immunodepression. Effective drug doses and time window would need careful assessment in stroke patients to minimize cardiac issues or infections.

**Conclusions**

We postulate that S1P1 is a candidate target for drug treatment in acute stroke. Prevention of lymphocyte migration to the central nervous system potentially underlies the beneficial effect of S1P1 agonists, although additional effects on S1P1 in other compartments cannot be excluded. Translation of these effects into clinical settings will require further investigation.

**Figure 1.** Drug effect on blood leukocyte counts. Percentage of the reduction of lymphocytes versus vehicle at different time points after administration of LASW1238 (3 or 10 mg/kg) or fingolimod (1 mg/kg). Mean±SEM (n=6 per time point and group). Two-way ANOVA with Bonferroni post hoc test of treatment groups versus vehicle. ***P<0.001.

**Figure 2.**

A. LASW1238 reduces infarct volume after brain ischemia. Mice received fingolimod (1 mg/kg; n=10), LASW1238 (10 mg/kg; n=8), or (3 mg/kg; n=10), or the vehicle (n=12) intraperitoneally after reperfusion after middle cerebral artery occlusion. Treatment was given blindly, and the identity of the codes was only revealed after the statistical analysis was completed. A, The blood flow drop during ischemia and recovery at reperfusion was comparable between groups. B, Infarct volume was assessed at 24 hours by 2,3,5-triphenyl-tetrazolium chloride staining as illustrated with representative brain sections per group. C, Infarct volume was smaller in the LASW1238 (10 mg/kg) group (*P<0.05 vs vehicle and LASW1238 3 mg/kg, 1-way ANOVA with the Bonferroni test). D, The neurological score was not significantly different between groups. Results (C, D) are shown as box and whiskers going from the minimum to the maximum values. rCBF indicates regional blood flow.
results into human stroke therapy would require establishing the adequate timing and extent of drug-induced lymphopenia needed for efficacy, as well as determining for how long lymphopenia should be maintained after stroke onset to ensure benefits minimizing any potential adverse effects.

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We acknowledge the contribution of Teresa Domènech for compound characterization and the significant technical support of Luis Boix and Jose Luis Gómez (Almirall) and Alba Hernández (Institut d’Investigacions Biomèdiques August Pi i Sunyer).

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**Disclosures**

Drs Tarrasón, Gavaldà, and Godessart are full time employees of Almirall. The other authors report no conflicts.

**References**

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SUPPLEMENTAL MATERIAL
A selective S1P1 agonist is protective against ischemia/reperfusion in mice. Brait et al.

Extended Materials and Methods

Animals
Male C57BL/6J mice weighing from 20-24g were supplied by Charles River (St. Cugat del Vallès, Spain; or Lyon, France). They were housed (for at least 5 days before use) in groups of four animals in Macrolon cages and kept in a room with controlled light (lights on from 07:00 to 19:00 hours), air cycle (10 per hour), temperature (22 ± 2ºC) and humidity (55 ± 15%), until the start of the experiment. Standard mice chow and tap water were available ad libitum. All experiments were carried out with the approval and authorization of the Animal Experimentation Ethical Committee of Almirall (Barcelona, Spain) or the Ethical Committee of the University of Barcelona (CEEA), and in accordance with EU Directive 2010/63/EU and the Spanish and autonomous Catalan laws (Real Decreto 53/2013 and Decreto 214/1997).

Binding affinity to human S1P receptors
The binding of an agonist to a GPCR induces a change in the receptor conformation state that promotes the exchange of GDP for GTP. The assays were carried out using membranes obtained in-house from CHO cells transfected with the human sphingophospholipid receptors S1P1, S1P2, S1P3, S1P4 or S1P5 measuring the 35S-GTPg binding after compound stimulation of the receptors. 35S-GTPgS was obtained from Perkin Elmer (#NEG 030X250UC), S1P was from Sigma (#S9666). Membranes (2-4µg) were incubated in a 200µl total volume containing 20mM HEPES pH 7.4, 100mM NaCl, 10mM MgCl2, 10µl GDP, 50µg/ml saponin and 0.2% fatty acid free BSAS (Sigma #A6003) at various compound concentrations, in the presence of 0.1nM 35S-GTPgS (NEN; specific activity 1250µCi/mmol). The assay was incubated for 90 min at room temperature with gentle mixing. After incubation, 150µl of the assay mix were transferred to 96 GF/C filter plates previously treated for 2h with 200ul wash buffer (25mM phosphate buffer pH7.4). Bound and free radioligand were separated by rapid vacuum filtration in a manifold and washed 4 times with 200µl ice-cold wash buffer. After drying for 30 min, 30µl of Optiphase Hisafe II were added to each well and radioactivity quantified using a Trilux microplate scintillation counter from Perkin Elmer. Raw data results were normalized using S1P at 1µM as maximal efficacy (100%). EC50s were calculated using Activity Base software from IDBS (www.idbs.com) and the four parameters-log equation.

Target Selectivity profile
The potential effect of LASW1238 on GPCRs was investigated in various receptor-binding assays in vitro. Functional profiling in 55 GPCRs was performed at a concentration of 10µM. The different GPCRs included: A) Non-peptide receptors such as adenosine A1, A2A and A3; adrenergic a1, a2, b1 and b2; cannabinoid; dopamine D1 and D2S; GABA; BDZ; Cl channel; histamine H1 and H2; muscarinic M1, M2 and M3; prostanoid, and serotonin 5HT1A, 1B, 2A, 2B, 5HT3, 5HT5A, 5HT6, and 5HT7. B) Peptide receptors such as angiotensin II, bradykinin, chemokines, cholecystokinin, endothelin, galanin, melancortin, neurokinin, neuropeptide Y1 and Y2, neurotensin, opioid and opioid-like, somatostatin, vasoactive intestinal peptide, and vasopressin. C) Ion channels such as Ca2+ channel (L-type, verapamil site) (phenylalkylamines), K’V channel, SK’Ca channel, Na+ channel (site 2) and Cl- channel. D) Amine transporters such as NE transporter and DA transporter.

Human Kinases
The potential inhibitory effect of LASW1238 on several enzymes was investigated in a selected panel of over 50 human kinase assays (Millipore) in vitro. Profiling was performed at a concentration of 10µM, and included Abl, AMPK a, Aurora-A, Bmx, BRK, CaMKIV, CDK1 cyclinB,
CDK2 CynclinA, CDK2 CyclinE, CHK1, CHK2, CK2, CSK, c-RAF, cSRC, EGFR, EphB4, FAK, Fes, FGFR1, FGFR3, Fit1, Flt3, GSK3beta, IKK beta, IR, JAK3, JNK1 alpha1, JNK2 alpha2, KDR, Lck, Lyn, MAPK2, MAPKAP-K2, MEK1, Met, p70S6K, PAK2, PDGFR beta, PDK1, PKA, PKB alpha, PKC alpha, PRK2, ROCK-II, RsK2, SAPK2a, SAPK3, Syk, TrkA, TrkB and ZAP-70.

In vitro cytotoxicity assessment
Cytotoxicity was evaluated as the damage induced to the mitochondrial respiratory chain of CHO cells and measured as changes in the ATP levels by ATP-lite test (Perkin Elmer), upon 24h incubation with LASW1238. For comparison purposes phosphorylated fingolimod was also evaluated.

Lymphopenia in mice
Mice (n=54) were fasted for 4 hours with free access to water. On the day of the experiment, mice were weighed; randomly distributed into various treatment groups and administered i.p. with either vehicle, LASW1238 (3 or 10mg/kg) or fingolimod (1mg/kg). Two, 4 or 24 hours post-administration, mice were anesthetized with isofluorane and blood samples collected by retroorbital puncture (250µl for hematological counts, 0.4 mL for blood or plasma levels). Total and differential blood cell counts were performed with a hemocytometer Sysmex XT-2000i (Roche Diagnostics, Barcelona, Spain). At the end of the experiment animals were sacrificed with cervical dislocation prior to anesthesia recovery. Results are expressed as the mean percentage of reduction of circulating lymphocytes (lymphopenia) versus control group. The software used to establish the IC\textsubscript{70} was GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

Induction of Focal Brain Ischemia and Reperfusion
Focal brain ischemia was induced by intraluminal occlusion of the right middle cerebral artery (MCA) with reperfusion, as reported.\textsuperscript{1} Wild-type mice (n=56) were subjected to 45 minutes of ischemia followed by reperfusion. Briefly, mice were anesthetized with isofluorane in a mixture of O\textsubscript{2} and N\textsubscript{2}O (30:70). The common and external carotid arteries (CCA and ECA, respectively) were exposed and a branch of the ECA was cauterized. The ECA and CCA were ligated and a 6-0 monofilament with silicone-coated tip (Doccol Co., Redlands, CA, USA) was inserted via the ECA and then into the internal carotid artery (ICA; approximately 11-12 mm distal to the carotid bifurcation), leading to the occlusion of the middle cerebral artery (MCA) at its junction with the circle of Willis. The filament was maintained in position for 45 min, at which point the filament was removed, the ligature on the CCA untied, and the mouse sutured. The mice were then placed in a warm box until they regained consciousness, and were then returned to their cages. The drop in cerebral blood flow (CBF) during ischemia and the increase in CBF at reperfusion, at the region perfused by the MCA, were measured with laser Doppler flowmetry (Perimed AB, Järfälla, Sweden). Body temperature was maintained at 37°C during surgery. CBF data were used as criteria to exclude animals (n=16) from the study as follows: (1) mice that did not show a drop in CBF of greater than 75% from basal CBF values after introducing the MCA occlusion filament were excluded, as it was considered that ischemia was not successfully induced; (2) mice that did not show recovery in CBF of higher than 70% of the basal.

Fingolimod (1mg/kg), or LASW1238 at low dose (3mg/kg) or high dose (10mg/kg), and vehicle (0.5% carboxymethyl cellulose, 0.1% Tween-80 in purified water) were administered i.p. immediately after reperfusion. Treatment was carried out blindly and the mice were randomized to receive one of the four treatments.

Functional Outcome test
A neurological score modified from Orsini and colleagues\textsuperscript{2} ranging from 0 (no deficit) to 39 (maximal deficit) was performed at 24 h after MCAO. The scores represent the sum of the results of general deficits (5 categories) and focal deficits (8 categories).
Evaluation of Infarct Volume

Mice were anesthetized with isoflurane and killed by decapitation. The brain was removed and sliced in 1-mm thick coronal sections, which were stained with a 2% solution of 2,3,5-triphenyltetrazolium chloride for 5 mins at 37°C. Sections were then immersed overnight in 4% paraformaldehyde and then washed and stored at 4°C in 0.1mol/L phosphate buffer (pH 7.4). Images of the sections were scanned and analyzed with an image analysis system (ImageJ; NIH, Bethesda, MD, USA). Infarct volume was corrected for edema using the following formula: 

$\frac{\sum \text{contralateral hemisphere area} - \sum \text{ipsilateral non-infarct area} \times \text{section thickness}}{2}$

Edema volume was assessed as the sum of the difference in area between the ipsilateral and contralateral hemispheres.

Statistical Analyses

Comparisons between groups were made by one-way analysis of variance followed by Bonferroni’s post hoc analysis for data conforming normality. The extended neurological test data were analyzed using Kruskal–Wallis test followed by Dunn’s multiple comparison test. Statistical analyses were carried out with the GraphPad Prism software. The results of infarct volume and neurological score are presented as box and whiskers from minimum to maximum. The box extends from the 25th to 75th percentiles and the line in the middle of the box is plotted at the median.

References


Supplementary Fig. 1

Legend to Supplementary Fig. 1: Treatments did not change post-ischemic edema. Mice received fingolimod (1mg/Kg) (n=10), LASW1238 (10mg/Kg) (n=8) or (3mg/Kg) (n=10), or the vehicle (n=12) i.p. after reperfusion following MCAo. Treatment was given blindly and the identity of the codes was only revealed after the statistical analysis was completed. The volume of edema was not different between groups.