Fingolimod Reduces Hemorrhagic Transformation Associated With Delayed Tissue Plasminogen Activator Treatment in a Mouse Thromboembolic Model

Francisco Campos, PhD; Tao Qin; José Castillo, MD, PhD; Ji Hae Seo, PhD; Ken Arai, PhD; Eng H. Lo, PhD; Christian Waeber, PhD

Background and Purpose—The sphingosine 1-phosphate receptor agonist fingolimod reduces infarct size in rodent models of stroke and enhances blood–brain barrier integrity. Based on these observations, we hypothesized that combination of fingolimod with tissue plasminogen activator (tPA) would reduce the risk of hemorrhagic transformation associated with delayed administration of tPA.

Methods—We evaluated the effects of fingolimod in a mouse model of thromboembolic stroke, in which both the beneficial effect of reperfusion associated with early tPA treatment and hemorrhagic transformation associated with delayed administration mimic clinical observations in humans.

Results—Our results demonstrate that fingolimod treatment attenuates the neurological deficit and reduces infarct volume after in situ thromboembolic occlusion of the middle cerebral artery. Combination of fingolimod and tPA improves the neurological outcome of the thrombolytic therapy and reduces the risk of hemorrhagic transformation associated with delayed administration of tPA.

Conclusion—This study confirms the protective efficacy of fingolimod as a treatment against ischemic stroke in another rodent model of stroke (thromboembolic occlusion), and suggests that fingolimod could potentially be used in combination with tPA to reduce the risk of brain hemorrhage. *(Stroke. 2013;44:XXX-XXX.)*

Key Words: fingolimod ▪ hemorrhage ▪ stroke ▪ tPA ▪ thromboembolic model

A cute cerebral ischemia is a major cause of mortality and disability worldwide, but no successful pharmacotherapy has been established that can benefit patients beyond the time window of thrombolysis with recombinant tissue-type plasminogen activator (tPA).1 Because of risks related to hemorrhage, this window is within 3 to 4.5 hours after the onset of symptoms.2 The tPA has also shown neurotoxicity in experimental models of cerebral ischemia.3 Therefore, a combination treatment that would reduce the deleterious effects of tPA, while maintaining the benefits of recanalization, might extend the usability and efficacy of tPA.

Fingolimod (FTY720, Gilenya) is a sphingosine analog that, when phosphorylated, acts on sphingosine1-phosphate receptors, regulating cellular and physiological mechanisms, including proliferation, apoptosis, adhesion, migration, differentiation/morphogenesis, inflammation, or blood–brain barrier (BBB) integrity.4,5 Fingolimod has emerged as a new treatment for multiple sclerosis.6,7 The effectiveness of fingolimod has also been shown by several groups in rodent models of ischemic brain injury.8–12 These studies suggest that antiinflammatory mechanisms and vasculoprotection possibly underlie the beneficial effects of fingolimod after stroke.13 We therefore hypothesized that combining fingolimod with tPA might reduce hemorrhagic transformation associated with delayed administration of tPA.

In this study, we evaluated the effect of fingolimod in a mouse model of thromboembolic stroke, in which the beneficial effect of tPA-induced reperfusion and hemorrhagic transformation associated with delayed administration are similar to those occurring in humans.

Material and Methods

Animals

C57BL/6 male mice (25–30 g, Charles River Laboratory) were maintained on a 12/12 hours light/dark cycle and fed ad libitum. Experiments were conducted according to protocols approved by the Stroke Research Laboratory, Department of Radiology, Massachusetts General Hospital, Charlestown, MA (F.C., T.Q., C.W.); Clinical Neurosciences Research Laboratory, Department of Neurology, University Clinical Hospital, University of Santiago de Compostela, IDIS, Santiago de Compostela, Spain (F.C., J.C.); and Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital, Charlestown, MA (J.H.S., K.A., E.H.L.).

Correspondence to Christian Waeber, PhD, Room 1.26, Cavanagh Pharmacy Building, College Road, University College Cork, Cork, Ireland. E-mail c.waeber@ucc.ie

© 2013 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org

Received October 3, 2012; final revision accepted October 25, 2012; accepted October 30, 2012.

From the Stroke Research Laboratory, Department of Radiology, Massachusetts General Hospital, Charlestown, MA (F.C., T.Q., C.W.); Clinical Neurosciences Research Laboratory, Department of Neurology, University Clinical Hospital, University of Santiago de Compostela, IDIS, Santiago de Compostela, Spain (F.C., J.C.); and Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital, Charlestown, MA (J.H.S., K.A., E.H.L.).

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.112.679043/-/DC1.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.112.679043/-/DC1.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.112.679043/-/DC1.

DOI: 10.1161/STROKEAHA.112.679043
Experimental Groups

Three cohorts were studied (Figure I in the online-only Data Supplement): (1) middle cerebral artery occlusion (MCAO) not treated with tPA (permanent occlusion); in this group, animals were treated (i.p.) with 0.5 mg/kg fingolimod or saline 45 minutes, 24 and 48 hours after occlusion. (2) MCAO-early tPA, in which tPA was administered intravenously 30 minutes after thrombin injection (transient occlusion); in this group, animals received fingolimod or saline 30 minutes (together with tPA), 24 and 48 hours after occlusion. (3) MCAO+delayed tPA, in which tPA was administered intravenously 3 hours after thrombin injection (transient occlusion); fingolimod or saline was administered 3 hours (together with tPA), 24 and 48 hours after occlusion.

Fingolimod and phosphofingolimod (generous gifts from Dr. Volker Brinkmann, Novartis Institutes for Biomedical Research, Basel) were dissolved in saline and stored at 4°C for <48 hours. Brinkmann, Novartis Institutes for Biomedical Research, Basel) were dissolved in saline and stored at 4°C for <48 hours. Thrombin (Hematologic Technologies Inc) dissolved in 18% glycerol/saline, was placed in a micromanipulator and 0.5 µL of thrombin solution or vehicle was injected into the lumen of the artery bifurcation to induce the formation of a clot. A micropipette (tip size: 30–50 µm), filled with 2 UI/µL mouse α-thrombin (Hematologic Technologies Inc) dissolved in 18% glycerol/saline, was placed in a micromanipulator and 0.5 µL of thrombin solution or vehicle was injected into the lumen of the artery bifurcation to induce the formation of a clot. A micropipette was removed 15 minutes later, when the clot had stabilized. Artery occlusion was considered successful when Laser Speckle Flowmetry showed a rapid and drastic fall of brain perfusion that remained stable during 80 minutes (mean reduction of 70% to 80%; Figure IIA in the online-only Data Supplement). To induce reperfusion, tPA (10 mg/kg; Alteplase, Activase) was administered i.v. (200 µL, 10% bolus, 90% perfusion during 40 minutes), 30 minutes (early recanalization), or 3 hours (delayed recanalization) after the injection of thrombin. In mice with early recanalization, we defined effective reperfusion when blood flow recovered to at least 75% of basal values (Figure IIB in the online-only Data Supplement).

Results

Effect of Thrombin and tPA Administration on Cerebral Blood Flow

Blood pressure and blood gases were stable in all groups under both basal and ischemic conditions (Table). No significant differences in these parameters were observed between treated and nontreated mice.

In all groups, local injection of thrombin into the MCA caused an immediate drop (to ≈10% of baseline) of cerebral blood flow. In animals not treated with tPA (permanent occlusion), the occlusion was stable during 80 minutes (as assessed by laser speckle flowmetry; Figure 1A) and for at least 4 hours (assessed by [125I]iodoantipyrine autoradiography in a separate cohort of 4 mice; Figure 1B).

Treatment with tPA 30 minutes after thrombin injection induced a gradual reperfusion starting around 30 minutes after its administration (Figure 1A). No significant differences in reperfusion time were observed between animals treated and nontreated with fingolimod. In mice receiving tPA after...
3 hours, the cerebral flow was only measured during the first 80 minutes of the procedure. The occlusion was stable during this period.

The effectiveness of reperfusion in mice with transient occlusion (see Material and Methods) was confirmed by \(^{14}\)C iodoantipyrine autoradiography. \(^{14}\)C iodoantipyrine autoradiography of brains obtained 4 hours after tPA administration confirmed that the clot was completely dissolved in both transient ischemic groups.

In the sham group, local administration of thrombin vehicle (18% glycerol) did not alter cerebral blood flow. Mice in which baseline cerebral flow was altered as result of the craniotomy were not included in the study (Table I in the online-only Data Supplement). No persistent cortical spreading depression events were observed after thrombin injection in any of the 3 groups studied.

**Effect of Fingolimod on Infarct Volume and Neuronal Deficit**

Permanent occlusion caused a cortical infarct of 27.3±6.1 mm\(^3\) after 3 days. Ischemia was associated with neurological deficits evaluated using the cylinder and grid walking tests on day 3. In the absence of tPA treatment, infarct volume was significantly reduced (17.5±9.1 mm\(^3\), \(P<0.05\)) when animals received 3 injections of fingolimod, 45 minutes, 24 and 48 hours after thrombin (Figure 2). Reduction of infarct volume by fingolimod was paralleled by better functional outcome (Figure 3).

Early vessel recanalization with tPA reduced infarct volume (18.0±5.5 mm\(^3\)) and improved functional outcome, compared with the permanent ischemic group. Fingolimod administered in combination with tPA, and 24 and 48 hours after occlusion further improved the neuronal deficit in both behavioral tests, but did not decrease lesion volume beyond the effect of tPA alone (15.7±6.7 mm\(^3\)).
Delayed administration of tPA had no beneficial effect on infarct (29.0±4.7 mm³) and neuronal deficit. Both parameters were similar to those seen in animals with permanent occlusion. Administration of fingolimod in combination with tPA and 24 and 48 hours after occlusion reduced infarct volume (22.0±5.9 mm³, \( P<0.05 \)), and improved both functional outcome measures.

Effect of Fingolimod on Hemorrhagic Transformation and Blood Barrier Breakdown
Permanent occlusion induced by thrombin and early recanalization were associated with only minor hemorrhages in the ischemic region at 3 days (total volume: 0.27±0.33 mm³ and 0.17±0.38 mm³ in the permanent and 30 minutes tPA groups).

Figure 3. Effect of vehicle and fingolimod (FTY720, 0.5 mg/kg) on functional deficit, evaluated by the grid-walking test in the 3 experimental groups tested. Panels A and B show the percentage of footslips for the left and right forepaws. The grid-walking test was performed 3 days after occlusion. C. Shows the effect of vehicle and fingolimod (FTY720, 0.5 mg/kg) on functional deficit, evaluated by the cylinder test in the 3 experimental groups tested. This test was performed before surgery (baseline) and 3 days after occlusion. Animals treated with FTY720 show a behavior close to baseline. Data are means±SD; n=9 to 10; *\( P<0.05 \).

Delayed recanalization caused a significant increase in hemorrhage volume (1.10±0.62 mm³) compared with animals not treated with tPA (\( P<0.05 \)) and animals treated with tPA 30 minutes (\( P<0.05 \)) after occlusion. Hemorrhagic transformation was significantly reduced (0.32±0.44 mm³) when tPA was administered in combination with fingolimod (\( P<0.05 \)). The same effects were observed after normalization of these data by infarct volumes.

Figure 4. A. Representative 3, 3’-diaminobenzidine (DAB)-stained sections illustrating the effect of vehicle or fingolimod (0.5 mg/kg FTY720) on hemorrhage volume in animals with permanent occlusion, and animals with early or delayed recanalization. B. The volume of hemorrhages (small arrows in A) was analyzed 3 days after occlusion. Panel C represents the ischemic region normalized for infarct volumes. Data are means±SD; n=9 to 10; *\( P<0.05 \).
To further examine the effect of fingolimod on vascular integrity, we examined Evans Blue extravasation 24 hours after occlusion in mice receiving delayed recanalization determined in contralateral and ipsilateral hemisphere of control and treated group. B. Shows Evans Blue extravasation in ischemic region normalized for infarct volume. Data are means±SD; n=6; *P<0.05.

The extent of Evans Blue leakage 3 and 48 hours after tPA administration was similar to that observed at 24 hours (data not shown), and the effect of fingolimod was only investigated at 24 hours.

To demonstrate that the increased Evans Blue leakage was caused by tPA and was not because of ischemic damage or reperfusion injury, we assessed Evans Blue leakage in mice with permanent thrombin-induced occlusion, and in mice in which transient occlusion (3 hours) was induced using a vessel clip; but we did not observe an ipsilateral increase in Evans Blue extravasation, and therefore did not study the effect of fingolimod in either cohort (Figure III in the online-only Data Supplement). No differences in the pattern of Evans Blue leakage were observed after normalizing these data for infarct volume.

**In Vitro Analysis Interaction of Fingolimod/P-Fingolimod and tPA Activity**

In order to expand on the observation that fingolimod treatment did not affect the reperfusion time course of thrombolytic treatment compared with vehicle group (Figure 1), we used an in vitro assay to confirm that fingolimod or P-fingolimod did not interfere with the enzymatic activity of tPA. Fingolimod and P-fingolimod, tested in a range of concentrations between 0.01 µmol/L and 100 µmol/L, did not modify the activity of tPA, at variance with leupeptin, used as a positive control (Figure IV in the online-only Data Supplement).

**Discussion**

This study shows that fingolimod attenuates the neurological deficit and reduces infarct volume in a model of thromboembolic MCA occlusion. In this model, in combination with thrombolytic therapy, fingolimod improves the neurological outcome with early tPA administration, whereas with delayed administration of tPA, fingolimod both improves neurological outcome and reduces the infarct volume.

Fingolimod has been recently suggested to be a potential new treatment for ischemic brain injury, based on several studies using rodent models of brain ischemia9–12 (1 study, however, failed to detect an effect,16 suggesting variations possibly because of experimental conditions). When protective effects by fingolimod were observed, the mechanisms of action were not clearly established. Thus, when fingolimod was tested in a rat model of transient ischemia by filament occlusion of the MCA, it reduced neuronal injury and improved behavior by activation of Akt (protein kinase B) and ERK (extracellular signal-regulated kinase) via S1P, receptors, preventing neuronal apoptosis.10 In a mouse study, the protective efficacy of fingolimod was associated with a reduction of inflammatory response and a direct neuroprotective effect mediated by inhibition of apoptotic death, but preservation of BBB was not observed.9 We have recently described that in a transient mouse model, fingolimod reduced infarct size, neurological deficit, and edema.12 This protective effect was accompanied by decreased inflammation. However, at variance with the neuroprotective effect previously described,9,10 fingolimod did not protect primary neurons against glutamate excitotoxicity or hydrogen peroxide, but it decreased intercellular adhesion molecule 1 (ICAM-1) expression in brain endothelial cells stimulated by tumor necrosis factor-α. The findings of the later study suggest that anti-inflammatory mechanisms, and possibly vasculoprotection, rather than direct effects on neurons, are the most plausible mechanisms that underlie the beneficial effects of fingolimod.12

In the 3 treatment paradigms used in the present study, fingolimod improved neurological deficit, but did not achieve a significant reduction of infarct volume when administered in combination with tPA 30 minutes after occlusion (Figure 2). Previous clinical studies have observed that tPA treatment administered within 3 hours of symptom onset decreases the neuroinflammation that follows stroke.17 Although further studies are necessary to confirm this hypothesis, we speculate that the reduction of inflammatory response induced by early tPA administration might have masked the beneficial effect of fingolimod on infarct size.
Vasculoprotection and preservation of BBB integrity are other important processes associated with the activation of S1P receptors, and have been implicated in the effects of fingolimod in studies unrelated to stroke. S1P receptor activation stimulates the recruitment of endothelial proteins that form adherens junctions, thereby creating a tighter contact between endothelial cells, enhancing BBB integrity.

Early tPA administration is the optimal therapeutic strategy to rescue still viable ischemic tissue, and to improve the outcome in patients with acute ischemic stroke. But studies based on magnetic resonance perfusion/diffusion imaging (mismatch concept) suggest that there might be patients who could benefit from thrombolysis beyond 3 hours, even if they present a higher risk of BBB breakdown and hemorrhagic transformation. In agreement with these clinical data and also with previous experimental studies in rats and mice, we observed that tPA administered 30 minutes after thrombin injection (early administration) reduced ischemic injury without BBB damage, whereas BBB breakdown and hemorrhagic transformation appeared when tPA was given 3 hours after occlusion (delayed administration).

Of note, the dose of 10 mg/kg tPA used in the current study is higher than the clinical dose (0.9 mg/kg). The fibrinolytic system in rodents has been known for 30 years to be about 10 times less sensitive to tPA than in humans. The vast majority of stroke studies in rodents have therefore been performed with 10 mg/kg tPA. In our study, significant hemorrhages were not seen with early tPA. We therefore assume that the hemorrhages we observed were the consequence of the delayed administration rather than of the high dose of tPA, and we believe that fingolimod-induced prevention of BBB damage caused by delayed tPA might indeed be clinically significant. Similarly, mice were treated with rather high doses of fingolimod (0.5 mg/kg bodyweight/d), but it is a dose commonly used to treat mice with experimental autoimmune encephalomyelitis (a model of multiple sclerosis).

It is possible that reduced hemorrhagic transformation and Evans Blue extravasation could be the mere consequence of reduced infarct volume in fingolimod-treated mice after delayed rtPA, as a direct effect of fingolimod on BBB damage was not demonstrated. But this is unlikely, as normalized data per infarct volume (Figures 4 and 5) or lack of Evans Blue leakage in mice with permanent occlusion or mice with transient occlusion (3 hours) induced using a vessel clip (Figure III in the online-only Data Supplement) suggest that BBB damage was not associated with infarct size and was mediated by delayed tPA administration, confirming the vasculoprotective effect of fingolimod.

Several treatments, some promising, have been tested in combination with tPA (see review) to reduce its neurotoxicity, risk of hemorrhage, and reperfusion injury, or to increase neuroprotection and increase therapeutic time window. One of the advantages of fingolimod with respect to other treatments is that, in addition to reducing the risk of hemorrhage, fingolimod is itself protective, which is likely to synergize with the benefits of thrombolytic therapy, as we have observed in our study.

In conclusion, in this study, we have confirmed the protective efficacy of fingolimod as a treatment against ischemic stroke in a novel model thromboembolic occlusion model, and we have shown the potential applicability of this treatment in combination with tPA to reduce the risk of brain hemorrhage.

Sources of Funding
This work was supported by NIH grants (NS049263 and NS055104) to CW and grant P30NS045776 (Interdepartmental Neuroscience Center). Spanish Ministry of Science and Innovation SAF2011-30517 AND RENEVAS (RD06/0026/000). Furthermore, FC is a recipient of a fellowship from the Conselleria de Industria, Xunta de Galicia (Programa Angeles Alvaríto).

Disclosures
None.

References


Fingolimod Reduces Hemorrhagic Transformation Associated With Delayed Tissue Plasminogen Activator Treatment in a Mouse Thromboembolic Model
Francisco Campos, Tao Qin, José Castillo, Ji Hae Seo, Ken Arai, Eng H. Lo and Christian Waeber

Stroke. published online January 3, 2013;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2013/01/03/STROKEAHA.112.679043

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2013/01/03/STROKEAHA.112.679043.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
ONLINE SUPPLEMENT

Fingolimod Reduces Hemorrhagic Transformation Associated With Delayed tPA Treatment In A Mouse Thromboembolic Model

Francisco Campos, Ph.D., Tao Qin, José Castillo, M.D, PhD., Ji Hae Seo, Ph.D., Ken Arai, Ph.D., Eng H. Lo, Ph.D., Christian Waeber, Ph.D.
SUPPLEMENTAL METHODS

Middle cerebral artery (MCA) occlusion
Experimental ischemia was carried out as described in 1-2. Mice were anesthetized with 2.5% and maintained at 1.5-2% isoflurane in a 30%/70% mixture of O₂/N₂O. Body temperature was maintained at 36.5-37°C using a feedback-controlled heating blanket. A catheter was inserted into the femoral artery to measure blood gases and blood pressure. Mice were placed in a stereotaxic frame, the skin between the right ear and eye was cut, the temporal muscle retracted and the temporal and parietal bones exposed. A small craniotomy was performed over the artery bifurcation, the meninges were cut and the MCA, with its parietal and frontal branches, was exposed.

A micropipette (tip size: 30-50 µm), made with hematologic glass capillaries (World Precision Instruments, Inc. USA) using a puller (Sutter Instruments), was pneumatically filled with 2 UI/μl mouse α-thrombin (Haematologic Technologies Inc., USA) dissolved in 18% glycerol/saline. The micropipette was placed in a micromanipulator and 0.5 μl of thrombin solution or vehicle (18% glycerol/saline; sham group) was injected against the flow into the lumen of the artery bifurcation to induce the formation of a clot (Figure 2SC). The micropipette was removed 15 minutes later, when the clot had stabilized. To confirm the occlusion of the MCA and rule out potential effects of cortical spreading depression (CSD) induced by surgical trauma, spatiotemporal changes in cerebral blood flow were measured in all animals by laser speckle flowmetry (LSF). Artery occlusion was considered successful when LSF showed a rapid and drastic fall of brain perfusion that remained stable during 80 min (mean reduction of 70-80%). To dissolve the clot and induce reperfusion, tPA (10 mg/kg; Alteplase, Activase®) was administered i.v. (200 µL, 10% bolus, 90% perfusion during 40 min), either 30 min (early recanalization) or 3 hours (delayed recanalization) after the injection of thrombin. In mice with early recanalization, we defined effective reperfusion when blood flow recovered to at least 75% of basal values (Figure S2B). Our past experience with mouse stroke models has shown that extended duration of anesthesia increases mortality; therefore, in animals treated with tPA after 3 hours, cerebral flow was only measured during the first 80 min of the procedure, after which animals woke up, and were re-anesthetized 100 min later for tPA administration. Because cerebral blood flow cannot be recorded by LSF once mice are removed from the stereotaxic frame (lack of baseline), vessel recanalization had to be assessed by visual inspection in these animals.

Laser Speckle Flowmetry
Laser speckle flowmetry (LSF) was used to study the spatiotemporal characteristics of cerebral blood flow (CBF) changes during middle cerebral artery (MCA). The technique for LSF in mice has been described in detail elsewhere 3-4. Briefly, a charge-coupled device (CCD) camera (Cohu, San Diego, CA, USA) was positioned above the head, and a laser diode (780 nm) was used to illuminate the intact skull. Raw speckle images were used to compute speckle contrast, a measure of speckle visibility inversely related to the velocity of the scattering particles, and therefore CBF. The speckle contrast is defined as the ratio of the standard deviation of pixel intensities to the mean pixel intensity in a small region of the image 5. Consecutive raw speckle images were acquired at 15 Hz, processed by computing the speckle contrast using a sliding grid of 7 x 7 pixels, and averaged to improve the signal to noise ratio. Laser speckle perfusion images were obtained every 7.5 secs. Speckle contrast images were converted to images of correlation
time values, which represent the decay time of the light intensity autocorrelation function. The correlation time is inversely and linearly proportional to the mean blood velocity.

Two sets of laser speckle flowmetry images were obtained before the craniotomy. Once the craniotomy procedure ended, laser speckle flowmetry recording was started 1 min before thrombin MCA occlusion (base line) and continued for up to 80 min during occlusion. Mice in which baseline cerebral flow was altered as result of the craniotomy were not included in the study. Relative CBF images (percentage of baseline) were calculated by computing the ratio of subsequent images to the base line images.

CBF measurements were made using a region of interest (ROI) of 0.25 by 0.25 mm² placed over medial cerebral artery bifurcation where thrombin has been injected.

Assessment of lesion volume and histology
Three days after ischemia, mice (n=9-10 per group) were euthanized with an overdose of isoflurane and perfused transcardially with saline. Brains were frozen in isopentane chilled to -40ºC, cut into 20 µm thick coronal slices (separated by 0.5 mm) and stained with Hematoxylin/Eosin (H&E). Lesion volumes (mm³) were measured using a computerized image analysis system (MCID Elite, InterFocus Imaging, Cambridge, UK). Briefly, a second set of sections was stained using diaminobenzidine (DAB, which reacts with peroxidases in red blood cells, enabling the precise identification of hemorrhage). Hemorrhage volume (mm³) was calculated by adding the areas of bleeding in all sections and multiplying by 0.5 mm. In addition, since the extent of the hemorrhage transformation is influenced by the size of the ischemic lesion, hemorrhage volume data were normalized per mm³ of infarct (mm³/mm³).

Neurological deficit evaluation
Neurological deficit was evaluated after ischemia using the grid and cylinder tests, as described. Cylinder test was performed before surgery (baseline) and 3 days after ischemia while grid walking test was performed at day 3 after ischemia.

Quantitative evaluation of Evans Blue extravasation
To evaluate the effect of fingolimod on blood-barrier damage, in an independent group of mice, vascular permeability was determined using fluorescent detection of extravasated Evans Blue dye (ng/mg of tissue) following the protocol described previously. As for hemorrhage analysis, extravasated dye content was divided by the corresponding stroke lesion volumes to normalize values (ng/mm³).

Absolute CBF measurement
To demonstrate that the clot occlusion was stable in mice with permanent occlusion and to confirm that tPA dissolved the clot, in another groups of animals (not treated with fingolimod) regional cerebral blood flow was visualized using the [¹⁴C]iodoantipyrine autoradiography technique described previously in mice. In animals with permanent occlusion, [¹⁴C]iodoantipyrine was injected 4 hours after thrombin injection, and in animals treated with tPA (30 min or 3 after thrombin), [¹⁴C]iodoantipyrine was injected 4 hours after thrombolytic treatment.
Table S1: Mortality during and after surgery and number of mice included and excluded from the study. Mortality represents animals which died during surgery and within the first 24 hrs after occlusion. Animals with partial occlusion of MCA after thrombin injection, with partial reperfusion after tPA administration or with cortical spreading depression (CSD) after craniotomy were not included in the study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drug treatment</th>
<th>Total number</th>
<th>Mortality</th>
<th>Animals excluded</th>
<th>Animals included</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CSD</td>
<td>Unsuccessful occlusion</td>
</tr>
<tr>
<td>Sham</td>
<td></td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin alone</td>
<td>Vehicle</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fingolimod</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin 30min tPA</td>
<td>Vehicle</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fingolimod</td>
<td>13</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin 3hr tPA</td>
<td>Vehicle</td>
<td>14</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fingolimod</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
**Figure S1.** Experimental protocol.
Sham mice were generated by injecting 18% glycerol (thrombin solution medium) into the MCA following the protocol used for thrombin.

Group 1 represents the animals with permanent occlusion induced by injection of thrombin into the MCA. Vehicle (saline) or fingolimod (FTY720) 0.5 mg/kg was administered (i.p.) 45 min, 24 and 48 h after occlusion. Animals were sacrificed 3 days after ischemia.

Group 2 represents animals with transient occlusion induced by injection of thrombin into the MCA and tPA 30 min after ischemia (early reperfusion). Vehicle (saline) or fingolimod (FTY720) 0.5 mg/kg was administered (i.p.) 30 min (with tPA), 24 and 48 h after occlusion. Animals were sacrificed 3 days after ischemia.

Group 3 represents animals with transient occlusion induced by injection of thrombin into the MCA and tPA 3 h after ischemia (delayed reperfusion). Vehicle (saline) or fingolimod (FTY720) 0.5 mg/kg was administered (i.p.) 3 (with tPA), 24 and 48 h after occlusion. Animals were sacrificed 3 days after ischemia.
Figure S2. Pseudocolor images of regional cerebral blood flow (CBF) measured by laser speckle flowmetry (LSF). Basal images were acquired before thrombin injection. Animals with alteration of baseline after craniotomy were not considered in the study. Panel A shows the CBF of a representative animal with a permanent occlusion. Only animals with a stable occlusion during 80 min were included in the study. Panel B shows the CBF of a representative mouse with a transient occlusion (reperfusion being induced by tPA 30 min after occlusion). Reperfusion was deemed effective when CBF recovery was in the range of 75-100% of basal values.
Figure S3. A: Analysis of Evans Blue extravasation in contralateral and ipsilateral hemisphere, in animals with permanent occlusion induced by injection of thrombin and in animals with transient occlusion performed with a vessel clip during 3 h. Evans Blue extravasation was determined 24 h after occlusion. B: Evans Blue extravasation data in ipsilateral hemisphere normalized for infarct volume. Data are means ± SD; n=6; *P<0.05.
Figure S4. Interaction assay between tPA and FTY720 or P-FTY720. Enzyme activity was determined in the presence or absence of FTY720 or P-FTY720, tested between 0.01 µM and 100 µM over 90 min. Panel A shows tPA activity 20 min after starting the reaction and panel B represents the reaction time curve of tPA with FTY720 (100 µM), P-FTY720 (100 µM) and leupeptin (100 µM). Leupeptin was used as a control tPA inhibitor. Data are means ± SD (n=3).
Supplemental bibliography


