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 O_2/N_2O. Body temperature was maintained at 36.5-37ºC 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 mm^2 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 (mm^3) 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 (mm^3) 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 mm^3 of infarct (mm^3/mm^3).[6]

Neurological deficit evaluation
Neurological deficit was evaluated after ischemia using the grid and cylinder tests, as described[7]. 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[8]. As for hemorrhage analysis, extravasated dye content was divided by the corresponding stroke lesion volumes to normalize values (ng/mm^3).[6]

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 [^{14}C]iodoantipyrine autoradiography technique described previously in mice [9]. In animals with permanent occlusion, [^{14}C]iodoantipyrine was injected 4 hours after thrombin injection, and in animals treated with tPA (30 min or 3 after thrombin), [^{14}C]iodoantipyrine was injected 4 hours after thrombolytic treatment.

Supplemental Table

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.

Groups	Drug treatment	Total number	Mortality	Animals excluded			Animals included
				CSD	Unsuccessful occlusion	Unsuccessful reperfusion	
Sham		6	0	1	0	0	5
Thrombin alone	*Vehicle*	13	1	0	2	0	10
	Fingolimod	12	0	2	0	0	10
Thrombin 30min tPA	*Vehicle*	14	1	1	1	1	10
	Fingolimod	13	0	2	0	1	10
Thrombin 3hr tPA	*Vehicle*	14	3	0	0	2	9
	Fingolimod	13	1	1	1	1	9

Supplemental figures

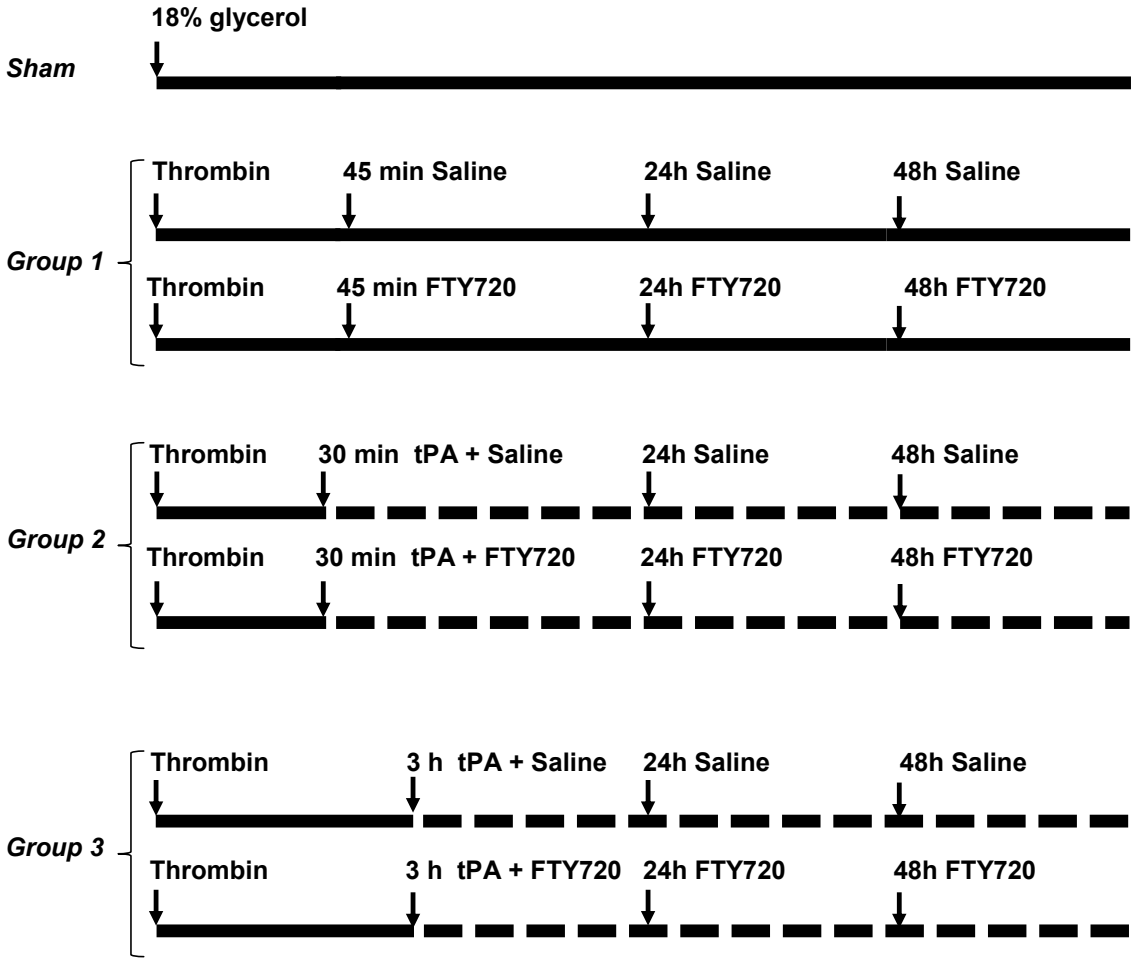

Figure S1. Experimental protocol.
Sham mice were generated by injecting 18% glycerol (thrombin solution medium) into the MCA following the protocol used for thrombin.
Group 1 represents the animals with permanent occlusion induced by injection of thrombin into the MCA. Vehicle (saline) or fingolimod (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.

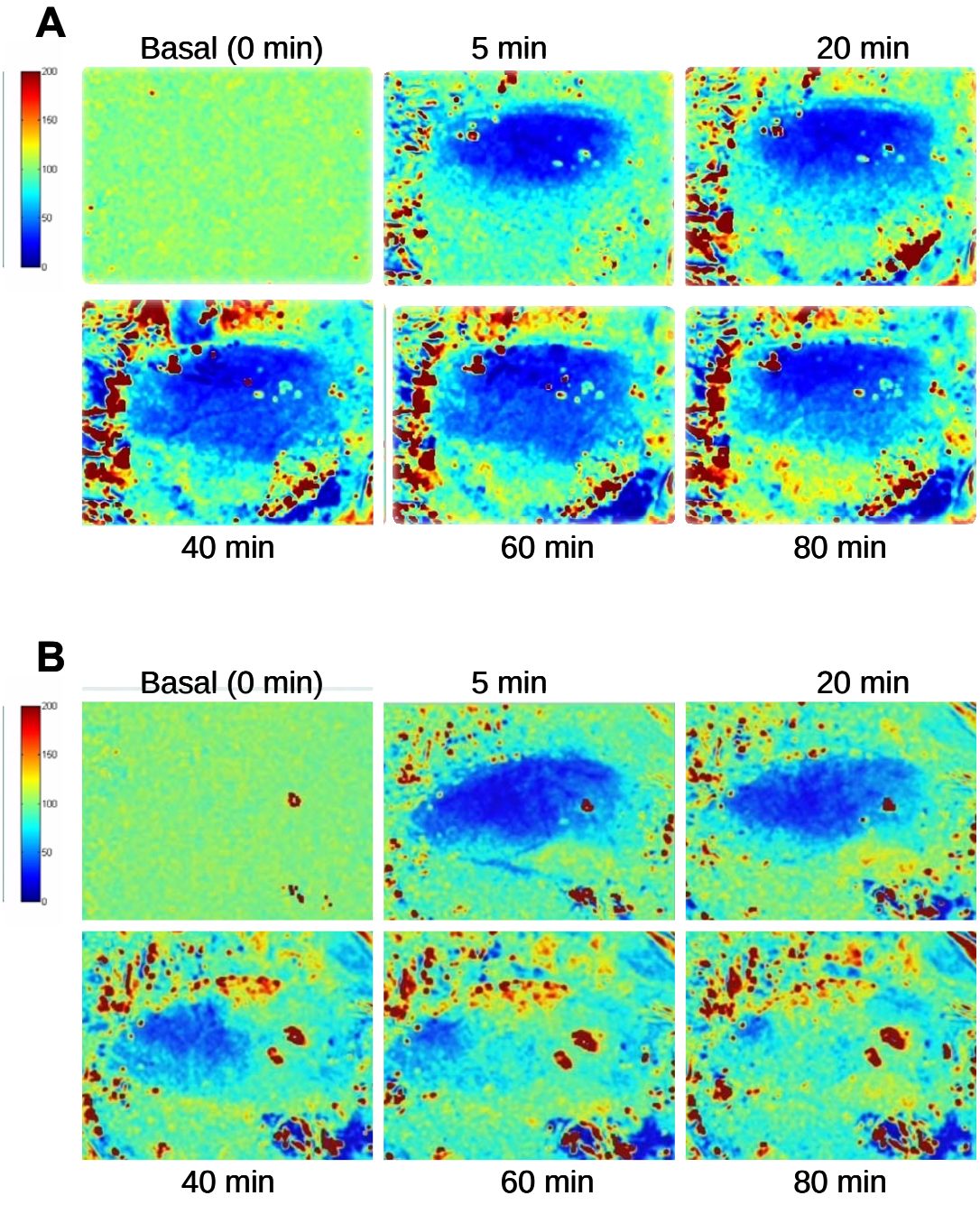

Figure S2. Pseudocolor images of regional cerebral blood flow (CBF) measured by laser speckle flowmetry (LSF). Basal images were acquired before thrombin injection. Animals with alteration of baseline after craniotomy were not considered in the study. Panel A shows the CBF of a representative animal 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.

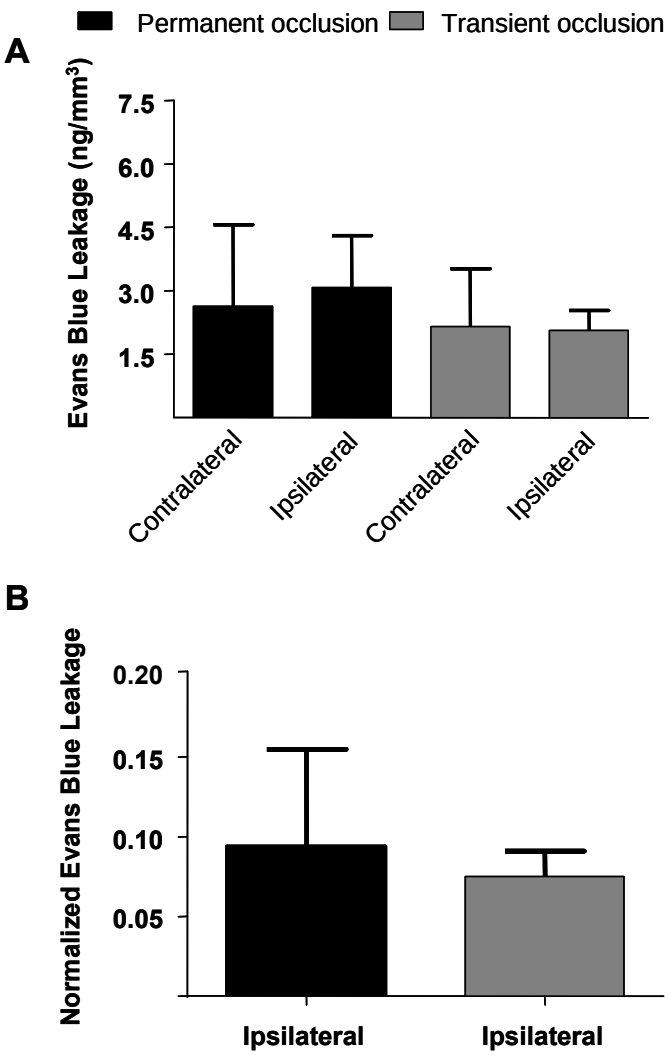

Figure S3. A: Analysis of Evans Blue extravasation in contralateral and ipsilateral hemisphere, in animals with permanent occlusion induced by injection of thrombin and in animals with transient occlusion performed with a vessel clip during 3 h. Evans Blue extravasation was determined 24 h after occlusion. B: Evans Blue extravasation data in ipsilateral hemisphere normalized for infarct volume. Data are means ± SD; n=6 ; *P<0.05.

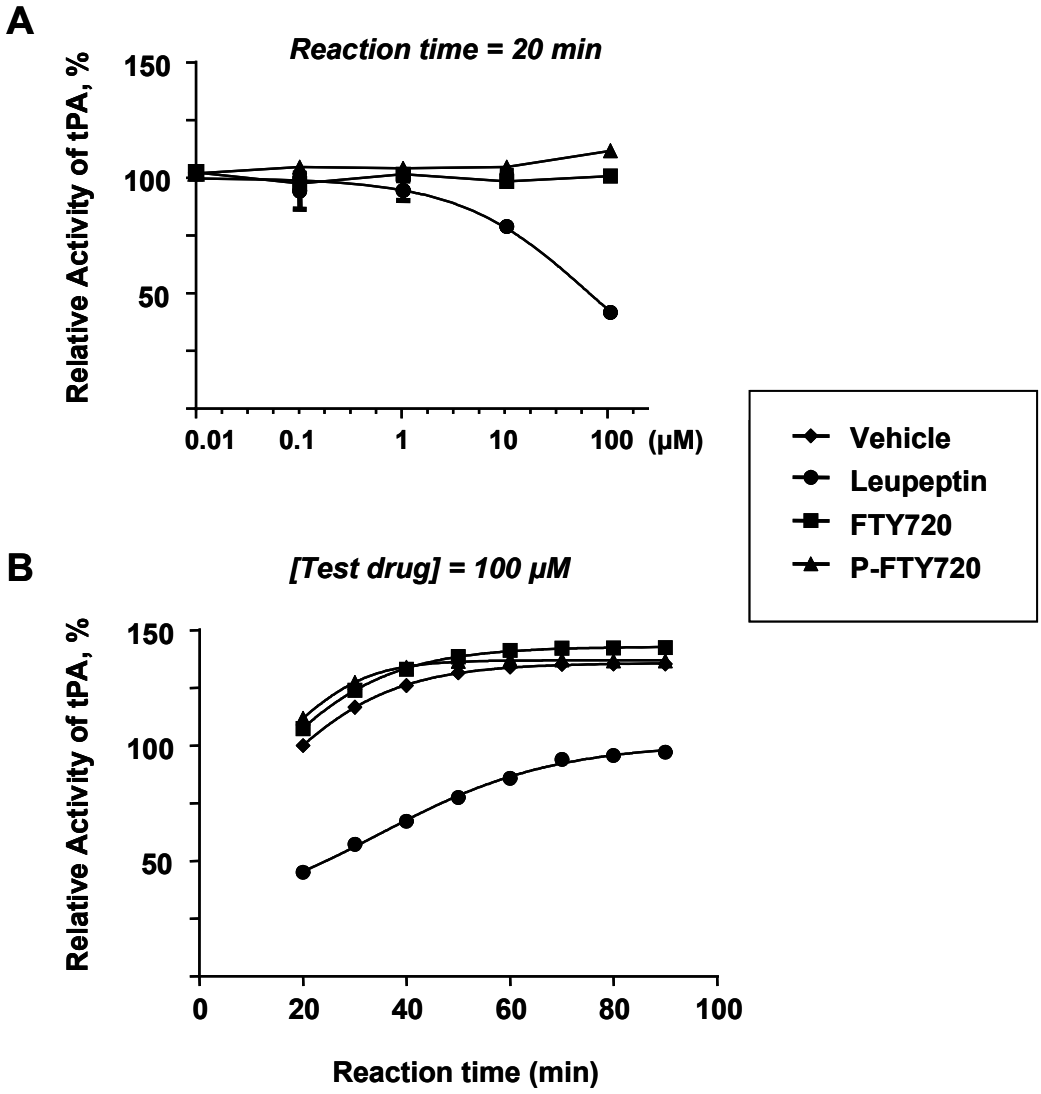

Figure S4. Interaction assay between tPA and FTY720 or P-FTY720. Enzyme activity was determined in the presence or absence of FTY720 or P-FTY720, tested between 0.01 μM and 100 μM over 90 min. Panel A shows tPA activity 20 min after starting the reaction and panel B represents the reaction time curve 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

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