SUPPLEMENTAL MATERIAL

Innovative thrombolytic strategy using a heterodimer diabody against TAFI and PAI-1 in mouse models of thrombosis and stroke


SUPPLEMENTAL METHODS

Materials
Db-TCK26D6x33H1F7 was designed (supplemental Figure S1) and produced as previously described.\(^1\) MA-RT36A3F5\(^2\) (raised against rat TAFI and cross-reacting with mouse TAFI), MA-RT82F12\(^2\) (raised against rat TAFI and cross-reacting with mouse TAFI), MA-TCK32G12\(^3\) (raised against human TAFI-ACIYQ and cross-reacting with human and mouse TAFI), recombinant mouse TAFI\(^4\) and recombinant mouse PAI-1\(^5\) were generated and purified as described before. tPA (Actilyse\(^*)\) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Thromboplastin (Innovin\(^*\), Dade) was used to induce thromboembolism.

Ethical statement
Experiments were performed in accordance with local ethical laws and the local ethical committees (P112-2012 and P081-2014 KU Leuven, Leuven, Belgium; act no. 87–848; Ministère de l’Agriculture et de la Forêt; authorisation code CENOMEXA 0113-03) and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals. All efforts were made to limit animal suffering. In the thrombin- and FeCl\(_3\)-induced MCAo models, none of the experimental procedures induced animal mortality. In the monofilament-mediated MCAo model, a mortality rate of 10 % occurred in all groups. After stroke surgery, mice were returned into cages with half-fresh bedding material and any sign of pain or distress (e.g. vocalizations, absence of
grooming, prostration, locomotion, interaction with conspecifics) was checked regularly. In the thrombin- and FeCl₃-induced MCAo models, no animal had to be euthanized because of suffering and no analgesic had to be employed. In the monofilament-mediated MCAo model, animals received an administration of buprenorphine (Ecuphar, 0.15 mg/kg sc) prior to surgery and were hydrated with a total of 1 ml isotonic (0.9%) saline divided over several subcutaneous injections upon reperfusion. All experiments were performed following the ARRIVE guidelines (www.nc3rs.org.uk), including randomization of treatment as well as surgery and analysis blind to the treatment.

Venous thromboembolism model

**Fibrin quantification in lungs**

Fibrin deposition in lungs was quantified as described previously.³ Mice were anesthetized by pentobarbital (Nembutal, 60 mg/kg IP) and 15 min post thromboplastin challenge lungs were perfused with 10 IU/ml heparine (heparin LEO). Left lungs were immediately isolated and ribolyzed for 45 seconds in PBS. PBS-washed lung homogenate containing insoluble fibrin was incubated with 2 µmol/L microplasmin (a gift from Thrombogenics, Leuven, Belgium) in order to convert fibrin into solubilized fibrin degradation products (FDPs). FDPs were subsequently quantified using a mouse fibrinogen ELISA with cross-reactivity towards FDPs (Immunology Consultants Laboratory, Portland, OR, USA). Fibrin deposition in lungs was expressed as fibrinogen equivalents (µg/mL). Baseline levels were obtained from lungs of healthy mice (not exposed to thrombotic challenge).

**Quantification of TAFI levels**

TAFI levels in plasma samples were determined by ELISA MA-RT36A3F5/MA-RT82F12-HRP, described previously.⁴ Wells of polystyrene microtiter plates were incubated with 200 µl MA-RT36A3F5 in PBS (pH 7.4; 4 µg/ml) for 72 hours at 4°C, emptied and treated for two hours with PBS supplemented with 1% (m/v) bovine serum albumin. After washing, serial two-fold dilutions (180 µl) of plasma samples were added to the wells and incubated overnight at 4°C. Then, the wells were washed and
incubated with 170 µl MA-RT82F12-HRP for 2 hours at room temperature. All washing steps were performed with PBS containing Tween 80 (0.002%) and dilutions were made in PBS containing Tween 80 (0.002%) and bovine serum albumin (0.1% m/v). The ELISA was developed using 150 µl of 0.1 mol/L citrate-0.2 mol/L sodium phosphate buffer, pH 5.0, containing 300 µg/mL o-phenylenediamine and 0.01% hydrogen peroxide. After 30 min at room temperature the peroxidase reaction was stopped with 50 µl 4 mol/L H₂SO₄. The absorbance was measured at 492 nm. Mouse TAFI was used as calibrator.

**Mechanical transient MCAo (tMCAo)**

**Surgical procedures and treatments**

Mechanical transient MCAo (tMCAo) was performed as described previously. After a midline skin incision in the neck of anaesthetized mice (induction by inhalation of 5% isoflurane/oxygen mixture and maintenance by inhalation of 2% isoflurane/oxygen mixture), the proximal common carotid artery and the external carotid artery were ligated, and a standardized silicon rubber–coated 6.0 nylon monofilament (6021; Doccol Corp, Redlands, CA) was inserted and advanced via the right internal carotid artery to occlude the origin of the right MCA. Operation time per animal did not exceed 15 min. The intraluminal suture was left in situ during 60 min. Then animals were reanesthetized, and the occluding monofilament was withdrawn to allow reperfusion. Immediately after reperfusion, diabody (0.8mg/kg) or vehicle (PBS) was administered IV.

**Neurological tests**

24 hours post occlusion, mice were subjected to the modified Bederson test and the grip test to assess global neurological function and motor function, respectively. The modified Bederson test uses the following scoring system: 0, no deficit; 1, forelimb flexion; 2, decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement. The grip test was performed in which a mouse was placed on a wooden bar (3 mm diameter, 40 cm long) attached to 2
vertical supports 40 cm above a flat surface. When placing the mouse on the bar midway between the supports, the experiment was rated according to the following system: 0, falls off; 1, hangs onto bar by 2 forepaws; 2, same as for 1, but attempts to climb onto bar; 3, hangs onto bar by 2 forepaws plus 1 or both hind paws; 4, hangs onto bar by all 4 paws plus tail wrapped around bar; 5, escape (mouse able to reach one of the supports).

**Lesion quantification**

24 hours post occlusion, mice were euthanized to quantify lesion volumes through staining with 2% 2,3,5-triphenyl-tetrazolium chloride. Brains were quickly harvested and cut into 2-mm-thick coronal sections using a mouse brain slice matrix. The presence of cerebral hemorrhages was assessed visually. The slices were stained with 2% 2,3,5-triphenyl-tetrazolium chloride (Sigma-Aldrich, St. Louis, MO) in PBS to distinguish healthy tissue from unstained infarctions. Stained slices were photographed with a digital Nikon D70 camera, and infarct areas (white) were measured using Image J software (National Institutes of Health, Bethesda, MD).

**Protein extraction and Western blot analysis**

Ischemic tissue including the cortex and basal ganglia was dissected from formalin-fixed TTC-stained brain slices and homogenized in RIPA buffer (25mmol/L Tris pH 7.4, 150mmol/L NaCl, 1% NP40) containing 0.1% SDS and 0.25% protease inhibitor cocktail (Roche) as previously described with slight modifications. A Western blot analysis was performed to quantify fibrinogen and actin. Samples were homogenized using a CLI12 mixer followed by incubation at 4°C for 20 min and subsequent sonication on ice. Then tissue lysates were centrifuged at 15,000 xg for 20 min at 4°C and supernatants were subjected to Western blot analysis as follows. 30 µg of total protein was loaded, electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking for 1h with blocking buffer (5% nonfat dry milk, 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20) membranes were incubated with either anti-fibrinogen polyclonal antibody (AP00766PU-N, Acris; diluted 1:500) or anti-actin MA (MAB1501, Millipore; diluted 1:500) at 4°C
overnight or for 1 hour, respectively. Then membranes were washed followed by incubation with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; diluted 1:14000) (fibrinogen) or goat anti-mouse IgG (Dako; diluted 1:2000) (actin) for 60 min at room temperature. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and signal was detected with the LAS-4000 mini imager (GE Healthcare).

**FeCl₃-induced MCAo**

**Surgical procedure and treatments**

Deeply anesthetized mice by inhalation of 5% isoflurane/oxygen mixture were placed on a stereotaxic device and were maintained under anesthesia by inhalation of 2% isoflurane/oxygen mixture during surgical procedures. Body temperature was maintained at 37°C with a thermostat-controlled heating pad throughout the whole procedure. Right middle cerebral artery (MCA) was exposed by craniectomy. *In situ* occlusion of the MCA was performed by application of a filter paper saturated with FeCl₃¹⁰ (Sigma Aldrich, France). In order to promote occlusive thrombosis, 20% FeCl₃ was used and applied during 5 min on the intact dura mater.¹¹ Diabody (Db) or vehicle (PBS) was injected IV via a tail vein catheter 15 min post clot onset as determined by Laser Doppler flowmetry. Five min after, tPA (10 mg/kg) or saline was administered IV (10% as bolus and 90% infused over 40 min).

**Cerebral blood flow (CBF)**

In mice under anesthesia, regional blood flow was monitored up to one hour after initial MCAo by positioning a Laser Doppler flowmetry probe downstream of the MCAo. Laser Doppler data was analyzed by LabScribe2 V 2.349000 to calculate remaining blood flow 60 min post occlusion.

**Magnetic resonance imaging (MRI) analysis**

MRI was performed at 24 h post occlusion on a Pharmascan 7 T/12 cm system using surface coils (Bruker Biospin, Wissembourg, France). Brain lesion volume was determined by T2-weighted MRI
(Multi-Slice Multi-Echo (MSME) sequence: TR/TE 2500 ms/51 ms\textsuperscript{11}), the angiographic scoring of the MCA (0= occlusion, 1= partial recanalization and 2= complete recanalization) was determined by MR angiography (MRA) (2D-TOF sequences \textsuperscript{12}) and cerebral hemorrhages were detected by T2*-weighted MRI (Fast Low Angle Shot sequence: TR/TE 350 ms/ 6 ms). Five mice with a lesion volume <3 mm\textsuperscript{3} were excluded from FeCl\textsubscript{3}-induced MCA\textsubscript{o} experiments: 2 in PBS group, 1 in tPA group and 2 in Db 1.6 mg/kg group).

**Tail bleeding assay**

Mouse tail vein bleeding times were determined with a tail-clipping assay, as described previously.\textsuperscript{13} Mice were administered with PBS, diabody, tPA as a single administration or diabody 5 min prior to tPA as a co-administration via proximal tail vein injection. Five min post (the last) injection, a distal 3 mm segment of the tail was clipped and the amputated tail was immersed immediately in 0.9% isotonic saline at 37°C. Bleeding time was monitored until initial cessation of bleeding (i.e. no rebleeding within 30 s). Accumulative hemoglobin loss was determined over a period of 60 min after tail-clipping. Subsequent to centrifugation (10 min at 2000 × g), blood cells were resuspended in 1 mL isotonic saline, and the hemoglobin content was measured on a Cell-Dyn 3500R counter (Abbott, Diegem, Belgium).

**Determination of circulating half-life of diabody**

**Sample collection**

Db-TCK26D6x33H1F7 (0.8 mg/kg) was administered IV via tail vein injection in mice. 24 hours prior to the experiment, 10 µl blood was withdrawn via retro-orbital puncture on 3.8% trisodium citrate (1:10 volume) (=pre-sample, n=6). Post injection, 10 µl blood was withdrawn on 3.8% trisodium citrate (1:10 volume) at several time points: 5 min, 45 min, 3 h and 6 h. Per time point, triplicate plasma
samples were obtained and per mouse, a blood sample at maximally two time points was obtained. At 24h, an additional blood sample was obtained after which the animal was euthanized.

**ELISA detection**

Diabody concentrations in plasma samples were determined by an ELISA based on the simultaneous binding of the diabody towards PAI-1 and TAFI, similar to the one previously described.\(^1\) Wells of polystyrene microtiter plates were incubated with 200 µl recombinant mouse PAI-1 in PBS (pH 7.4; 4 µg/ml) for 72 hours at 4°C, emptied and treated for two hours with PBS supplemented with 1% (m/v) bovine serum albumin. After washing, serial two-fold dilutions (180 µl) of plasma samples were added to the wells and incubated overnight at 4°C. Then, the wells were washed and incubated with 170 µl mouse TAFI (0.1 µg/ml) for 2 hours at room temperature. Subsequently, plates were washed and 160 µl HRP-conjugated MA-TCK32G12 (directed against TAFI) was added to the wells followed by incubation for 2 hours at room temperature. All washing steps were performed with PBS containing Tween 80 (0.002%) and dilutions were made in PBS containing Tween 80 (0.002%) and bovine serum albumin (0.1% m/v). The ELISA was developed using 150 µl of 0.1 mol/L citrate-0.2 mol/L sodium phosphate buffer, pH 5.0, containing 300 µg/mL o-phenylenediamine and 0.01% hydrogen peroxide. After 30 min at room temperature the peroxidase reaction was stopped with 50 µl 4 mol/L H\(_2\)SO\(_4\). The absorbance was measured at 492 nm. Db-TCK26D6x33H1F7 was used as calibrator. Circulating half-life of the diabody was retrieved after nonlinear fitting of plasma levels plotted against time (Graphpad Prism Version 5).

**Neurotoxicity**

Neuronal cultures were prepared from Swiss mouse embryos (embryonic day 14) as described earlier.\(^1\)\(^4\) Cortices were dissected and dissociated in DMEM, and plated on 24-well plates coated with poly-D-lysine (0.1 mg/ml) and laminin (0.02 mg/ml). Cells were cultured in DMEM supplemented with 5% foetal bovine serum, 5% horse serum (both from Invitrogen, Cergy Pontoise, France) and 2...
mM glutamine. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. To inhibit glial proliferation, cytosine β-D-arabinoside (10 μM) was added after 3 days in vitro (DIV) to the cortical cultures.

Excitotoxicity was induced at 12-13 DIV by exposure to NMDA (10 μM) in serum-free DMEM supplemented with 10 μM of glycine for 24 hours. NMDA was applied alone or together with rtPA (20 μg/ml) and/or diabody (5 μg/ml). As a control, the diabody was added to the neuronal culture at 12-13 DIV at several concentrations (0.5 μg/ml – 50 μg/ml) in the absence of NMDA. After 24 hours, neuronal death was quantified by measurement of the activity of lactate dehydrogenase (LDH) released from damaged cells into the bathing medium (Roche Diagnostics, Mannheim, Germany). The LDH level corresponding to the maximal neuronal death (full kill, FK) was determined in sister cultures exposed to 500 μM NMDA. Background LDH levels were determined in sister cultures subjected to control washes. Experimental values were measured after subtracting LDH_{min} and then normalized to LDH_{max}-LDH_{min} to express the results in percentage of neuronal death.

**Statistical analysis**

All quantitative data are presented as mean and standard error of mean (SEM). Statistical analysis was performed with GraphPad Prism Version 5 (GraphPad Software). A chi-square test was performed to compare angiographic scores from different treatment groups. Outliers were excluded by performing the Grubb's test. Prior to statistical analysis, a D'Agostino and Pearson normality test was used to check data distribution. One-way ANOVA with Bonferroni's multiple comparison test was used for statistical comparison of lesion volumes and speckle contrast imaging data after FeCl₃-induced MCAo and an unpaired students t-test was used for statistical comparison of lesion volumes after mechanical tMCAo. Kruskal–Wallis ANOVA with Dunn’s multiple comparison test was used for statistical comparison of: (i) lung fibrinogen equivalents in the venous thromboembolism model; (ii) lesion volumes and speckle contrast imaging data in the Ila-induced MCAo model; (iii) laser Doppler
data in the FeCl$_3$-induced MCAo model and (iv) tail bleeding times and hemoglobin contents. A Mann-Whitney test was performed for statistical analysis of neurological/motor data, fibrinogen levels after mechanical tMCAo and *in vitro* neurotoxicity data. P-values less than 0.05 were considered significant.
Supplemental figure legends and figures:

Supplemental figure S1: **Schematic representation of DNA constructs containing antibody derivatives.**
L = AKTTPKLGG; SD (T7g10) = ribosome binding site or Shine Dalgarno sequence, T7g10-translational enhancer sequence; C-myc = c-myc epitope tag; His$_6$ = polyhistidine tag

![Diagram](image.png)
Supplemental figure S2: **Systemic pharmacokinetics of the diabody.**

Plasma levels (µg/ml) are plotted against time (min) after IV injection of diabody at 0.8 mg/kg (red arrow indicates circulating half-life= 121 min). Data are mean ± SD, n=3-6 mice per time point.
Supplemental figure S3: **Evaluation of the thrombolytic efficacy of the diabody (Db) on tPA-resistant platelet-rich clots in a model of FeCl₃-induced MCAo.**

(A) % cerebral blood flow (CBF) measured by laser Doppler probe 1 hour post stroke onset; (B) angiographic score (0= occlusion, 1= partial recanalization and 2= complete recanalization) measured by MRA 24h after stroke onset; (C) representative T2-weighted images 24 hours post stroke onset (dotted lines delineate stroke lesions); (D) lesion volume (mm³) 24 hours post occlusion of mice treated with vehicle, tPA (10 mg/kg) or Db (1.6 mg/kg or 3.6 mg/kg) at 20 min post occlusion. Data are represented as mean ± SEM (n= 8-15). *, p< 0.05. tPA indicates recombinant tissue-type plasminogen activator; Db, diabody.
Supplemental figure S4: Evaluation of the effect of the diabody (Db) on cortical neuronal death with or without NMDA-induced excitotoxicity.

(A) Cortical neurons were exposed to NMDA (as a full kill condition (FK); 500 μmol/L) or diabody (0.5 -50 μg/ml) alone; (B) cortical neurons were exposed to NMDA (500 μmol/L (full kill, FK) or 12.5 μmol/L), Db (5 μg/ml) alone or in combination with rtPA (20 μg/ml) during 24 hours before the measurement of neuronal death (N=2 independent cultures, n= 2-4, *p< 0.05; ns = not significant). tPA indicates recombinant tissue-type plasminogen activator; Db, diabody; NMDA, N-methyl-D-aspartate.
Supplemental references


