CM-352 EFFICACY IN A MOUSE MODEL OF ANTICOAGULANT-ASSOCIATED INTRACRANIAL HAEMORRHAGE

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Abstract:

Background: Intracranial haemorrhage (ICH) is one of the major devastating complications of anticoagulation. Matrix metalloproteinases (MMPs) inhibition has been proposed as a novel pharmacological approach for ICH treatment.

Objectives: We evaluated the effects of CM-352 (MMPs-fibrinolysis inhibitor) in an experimental ICH model associated with oral anticoagulants as compared with clinically used prothrombin concentrate complex (PCC).

Methods: ICH was induced by collagenase injection into the striatum of WT (C57BL/6J) anticoagulated mice (warfarin or rivaroxaban) and Mmp10 -/- mice. Hematoma volume and neurological deficits were measured 24h later by diaminobenzidine staining and different behavioural test. Circulating plasminogen activator inhibitor-1 (PAI-1) activity and interleukin-6 (IL-6) were measured in plasma samples and local inflammation was assessed by neutrophil infiltration. Finally, fibrinolytic effects of MMP-10 and rivaroxaban were evaluated by thromboelastometry and thrombin-activatable fibrinolysis inhibitor (TAFI) activation assays.

Results: Only PCC reduced haemorrhage volume and improved functional outcome in warfarin-ICH, but both, PCC and CM-352 treatments, diminished haemorrhage volume (46%, p<0.01 and 64%, p<0.001, respectively) and ameliorated functional outcome in rivaroxaban-ICH. We further demonstrated that CM-352, but not PCC decreased neutrophil infiltration in the haemorrhage area at 24h. The effect of CM-352 could be related to MMP-10 inhibition since Mmp10 -/- mice showed lower haemorrhage volume, better neurological score, reduced IL-6 levels and neutrophil infiltration, and increased PAI-1 after experimental ICH. Finally, we found that CM-352 reduced MMP-10 and rivaroxaban-related fibrinolytic effects in thromboelastometry and TAFI activation.

Conclusions: CM-352 treatment, by diminishing MMPs and rivaroxaban-associated fibrinolytic effects, might be a novel anti-haemorrhagic strategy for rivaroxaban-associated ICH.

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SUPPLEMENTARY INFORMATION

CM-352 EFFICACY IN A MOUSE MODEL OF ANTICOAGULANT-ASSOCIATED INTRACRANIAL HAEMORRHAGE

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SUPPLEMENTARY MATERIAL AND METHODS

Oral anticoagulation

Warfarin anticoagulation (Non-direct anticoagulant)

Assuming that the regular water intake of C57BL/6J mice is 5 mL per day, a dose of 2 mg/Kg of warfarin (Sigma-Aldrich, Madrid, Spain) was dissolved in the drinking water and left for 24 h. The International Normalized Ratio (INR) was measured from a blood drop collected by submandibular vein puncture, using the CoaguCheck® (Roche, Barcelona, Spain) coagulometer system 30 min before the experimental models. Tail bleeding or the collagenase-induced ICH model were performed only in animals that showed an INR within the targeted supra-therapeutic range (INR 2.3 to 5) just before the procedure and randomly assigned to the different treatments. To confirm the reversal effects of PCC in warfarin anticoagulated animals, we also measured the INR before and 30 min after PCC administration in an additional group of animals (n=6).

Rivaroxaban anticoagulation (Direct anticoagulant)

Considering the low aqueous rivaroxaban solubility and that plasma concentration reached the peak 1 h after administration in mice, rivaroxaban was administered (3 and 10 mg/Kg, Selleckchem, Munich, Germany) by gastric gavage 1 h before the experimental models of haemorrhage.

A sample of citrated blood was collected from submandibular vein 30 min before the experimental ICH model to assure the anticoagulation levels by a modified anti-Xa activity assay (Sta-Liquid Anti-Xa®, Diagnostica Stago, Barcelona, Spain). Briefly, Xa activity was measured in 25 µL of citrated plasma (1/16 dilution) incubated with 50 µL of recombinant Xa (12.5 mIU) and 50 µL of substrate for 1 min, and read at 37 °C and 405 nm (Sunrise, Tecan, Männedorf, Switzerland) against a Xa standard curve prepared with recombinant Xa (0-12.5 mIU). Only animals with anti-Xa activity above 50% from basal were subjected to the collagenase-induced ICH model and randomly assigned to the different treatments. To evaluate the inhibition of Xa activity by rivaroxaban over time, an additional group of animals (n=5), 8 weeks of age C57BL/6J mice, were injected with rivaroxaban (10 mg/Kg) and citrated blood samples were collected before and after treatment (10 min, 30 min, 1 h, 3 h and 24 h).

Experimental models

Tail-bleeding model:

Bleeding assay was performed in WT C57BL/6J mice (n=50) under oral anticoagulation (warfarin: 2 mg/Kg or rivaroxaban 3 mg/Kg, supplemental Fig. 1A). Animals were anesthetized with a mixture of ketamine (100 mg/Kg, Imalgene, Merial laboratories, Barcelona, Spain) and xylazine (10 mg/Kg, Ronpun®, Bayer, Barcelona, Spain) by intraperitoneal injection. Mice were given 4-factor Prothrombin Complex Concentrate (PCC) (100 UI/Kg, 4F-PCC (Octaplex®, Octapharma, Vienna, Austria), CM-352 (1 mg/Kg) or saline by tail vein bolus injection. A distal 5 mm segment of the tail was
transected with a scalpel blade 5 minutes after the treatments. The tail was immediately immersed in a tube containing pre-warmed saline (37 °C in a water bath). Bleeding time was defined as the time elapsed until bleeding stops for a maximum of 30 min. Anesthetised animals were euthanized after the experiment by cervical dislocation.

Collagenase-induced ICH model:

Experimental ICH model was performed in anticoagulated (warfarin: 2 mg/Kg or rivaroxaban: 10 mg/Kg, supplemental Fig. 1B) WT C57BL/6J mice (n=85). Animals were deeply anesthetized with 2.5 % isoflurane in oxygen mixture (30% O₂) and placed in a stereotaxic base (WPI, Friedberg, Germany). A craniotomy was performed (0.5 mm anterior and 2 mm lateral to bregma) and a 32-gauge needle (7000.5 Hamilton, Bonaduz, Switzerland) was placed into the right striatum at 3.5 mm depth. Then, 0.2 μL of collagenase type-VII (0.075 U, Sigma) was injected during 5 min. After 10 min the needle was removed, the bore hole was sealed with wax and sutured. 30 min after the haemorrhage induction, mice received a bolus injection trough the tail vein of saline, CM-352 (1 mg/Kg) or 4-factor PCC (100 UI/Kg). As a control, the sham-operated animals were subjected to the same procedure without collagenase type-VII intracranial injection.

In addition, male MMP-10 deficient (Mmp10 -/-, C57BL/6J) mice 8-12 weeks of age and 25-30 g weight were subjected to the collagenase-induced ICH model (n=20).

Neurological and functional evaluation

For all behavioral testing, observers were blinded to the treatment. Mice were group-housed in standard cages with bedding and nesting material under a 12-h light-dark cycle. Food and water were provided ad libitum.

Bederson´s score test

Mice were subjected to a modified version of the Bederson’s test. Bederson’s score test consisted of assessing 4 functional parameters: (a) spontaneous normal movement scored as 3, slight disability to walk normally scored as 2, disability to walk normally scored as 1 and no movement scored as 0. (b) Spontaneous rotation scored as 1 or 0 depending on its presence or not. (c) Inflexion of the torso and the forelimbs when suspended half of the animal or (d) the whole animal by the tail scored as 1 or 0. Maximum score of 6 was given to normal mice.

Pole test

Mice required a two-days training period prior the execution of this experiment. Mice were placed head-upward on the top of a vertical rough-surfaced pole. The time that the mice spend turning downwards (t-turn) and the time until it descends (t-total) were recorded with a maximum duration of 60 s. If the mouse fell, it was penalized with the maximum time 60s.
Coat-hanger test

This test was divided in two different consecutive parts. During the first, mice were suspended by the tail and let to grab the coat hanger only by their front limbs. During 5 s mice were recorded and several parameters were scored (5s-Score): (a) if the mouse fell, scored as 0 (yes) or 1 (no); (b) if the mouse was able to place another hind limb on the hanger scored as 1, the two hind limbs scored as 2 and the two hind limbs plus moving towards the edge of the hanger scored as 3. Maximum score of 4 was given to normal mice. During the second part, the latency to fall (t-total), time to reach one of the ends of the hanger (t-corner), as well as the distance moved (60s-Score) on the wire was measured in a single trial for 60s.

Histological, immunohistochemical and protein analysis

Haemorrhage volume:

To assess the collagenase-induced haemorrhage volume, brain sections were stained with diaminobenzidine (Liquid-DAB + Substrate chromogen system, Dako, Santa Clara, CA, USA). One section out of every 10 was stained covering the entire lesion. Stained sections were scanned (3200 Photo Scanner, Epson, Amsterdam, Netherlands) and the haemorrhage (stained area) was quantified using an image analysis system (Image-J, National Institutes of Health, Bethesda, MD, USA).

Neutrophils (NIMP-R14)

To evaluate the inflammation associated to the haemorrhage area, the number of infiltrated neutrophils were analysed by immunohistochemistry. As for haemorrhage volume, brain sections were immunostained for neutrophils (rat anti-neutrophil NIMP-R14, ab2557 Abcam, Cambridge, UK) followed by incubation with an anti-rat biotinylated secondary antibody (E0468, Dako) and TSA Cyanine 3 System (Perkin Elmer, Waltham, MA, USA). Slides were mounted with DAPI (4’,6-diamidino-2-phenylindole, Vectashield, Vector, Peterborough, UK). Stained sections were scanned at 10x (Vectra Polaris, Perkin Elmer) and positive red neutrophils in haemorrhagic area were quantified using an image analysis system (Image-J) and corrected by the size of haemorrhage in order to evaluate the density of neutrophils within the haemorrhage.

Netrophil Extracellular Traps (NETs)

Additionally, brain sections were stained for NETs using both rabbit anti-histone H3 (citrulline R2+R8+R17, ab5103, Abcam) and goat anti-myeloperoxidase (MPO, goat, AF3667, R&D Systems) followed by incubation with donkey anti-rabbit (Alexa-488, A31572, Invitrogen) and with donkey anti-goat (Alexa-555, A32814, Invitrogen) secondary antibodies. Slides were mounted with DAPI (4’,6-diamidino-2-phenylindole, Vectashield, Vector, Peterborough, UK). Stained sections were scanned at 10x (Vectra Polaris, Perkin Elmer) and double positive NETs in haemorrhagic area were quantified using an image analysis system (Image-J) and corrected by the size of haemorrhage to evaluate the density.
IL-6 western blot in brain tissue

Brain hemispheres (contralateral [CL] and ipsilateral [IL]) were collected separately from WT (n=2) and Mmp10−/− (n=3) and homogenized in cold RIPA buffer (Sigma-Aldrich). Samples were vortexed for 20 s, spun down, and the supernatants were collected. Protein concentration in the supernatants was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Munich, Germany). Samples (20 μg) were heated in loading buffer (Invitrogen) at 70 °C for 5 min, loaded and run in Bis-Tris 4–15% gels (Stain-Free Precast Gels, Bio-rad, Spain). After semi-dry transfer to nitrocellulose membranes (iBlot, Thermo Fisher, Darmstadt, Germany), blots were blocked for 1 h with 5% non-fat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, 0.01% Tween-20, pH = 7.6) at room temperature. To assess IL-6, membranes were incubated overnight at 4 °C with a rabbit polyclonal anti-IL-6 antibody (208113, Abcam) diluted 1:1000. Next, blots were incubated 1 h with secondary antibody goat anti-rabbit (Dako), washed and developed with HRP substrate TMA6 (Lumigen, Southfield, MI, USA). Immunoreactive bands were detected by chemiluminescence using an imaging system (Odyssey FC, LI-COR, Lincoln, NE, USA) and quantified by normalization against total protein on nitrocellulose membrane using Image Studio software (LI-COR).

Haemostatic and inflammatory parameters

Thrombin Activatable Fibrinolysis Inhibitor activation

Thrombin Activatable Fibrinolysis Inhibitor (TAFI) activation was measured in a purified system, using a commercial chromogenic assay (STA-Stachrom® TAFI kit, Stago) according to manufacturer’s conditions. Shortly, recombinant TAFI (30 nmol/L) incubated 10 min with Rivaroxaban (4 nmol, Selleckchem), CM-352 (4 nmol) or recombinant MMP-10 (4 nmol/L) at 37 °C was activated by thrombin/thrombomodulin (kit reagent) before substrate addition. The mixtures were measured and quantified at 405 nm for 15 min using a plate reader.

MMP-10 and Xa activities

Recombinant MMP-10 (0.2 nmol/L) and Xa (0.2 nmol/L, Enzyme Research Lab) activities were assayed with a fluorogenic peptide for stromelysins (10 μmol/L, ES002, Fluorogenic Peptide Substrate II, R&D Systems, Madrid, Spain) in the presence of CM-352 (40 nmol/L and 2.5 nmol/L respectively), warfarin (40 nmol/L and 2.5 nmol/L respectively, Sigma-Aldrich) and rivaroxaban (40 nmol/L and 2.5 nmol/L respectively, Deltaclon). Samples were monitored for 1 h at 37 °C with a spectrofluorometer at 320-405 nm excitation/emission (SpectraMAX GeminisXS, Molecular Devices, Wokingham, UK). Activity slopes were obtained and analysed.

Interleukin-6 and Plasminogen Activator Inhibitor-1

To evaluate the systemic inflammation and fibrinolysis 24 h after the ICH experimental model, Interleukin-6 (IL-6, ELISA kit, ThermoFisher) and Plasminogen Activator Inhibitor-1 (PAI-1, Stachrom®, Stago) activity were measured in citrated plasma samples following manufacturer’s instructions.
Thromboelastometry (ROTEM®) with adherent endothelial cells

Human endothelial cells (Eahy926) were seeded onto micro beads to create transferable EC-micro carriers. Micro beads were then added to citrated whole blood in the measurement cup of a thromboelastometry device (ROTEM). EC-micro carries were prepared as previously described\(^4\). Briefly, Eahy926 cells were cultured in DMEM cultured media (ThermoFisher) supplemented with 10% of FBS at 37°C and 5% CO\(_2\).

Cells (4x10\(^6\)) were transferred into culture flask with 5 mL of 10,000 sterilized micro carrier beads (Cytodex 3, GE Healthcare Bio-Sciences, Uppsala, Sweden) and incubated 4 h with gently agitation. After sedimentation, supernatant was discarded to remove non-adherent cells and cellular debris three times and complete medium (1:1 bead: medium volume ratio) was added and kept at 37°C through the experiment. Thromboelastometry (ROTEM®, Delta, Werfen, Spain) experiments were performed using human citrated blood samples and different therapeutic concentrations of rivaroxaban (0, 58, 115, 230 and 460 mmol/L)\(^5\) and CM-352 (0, 0.4, 0.9, 1.8, and 3.7 µmol/L). 300 µL of blood and rivaroxaban were pipetted into the cuvette in combination with 50 µL EC-micro carriers and 1 µL of human tPA (150-250 U/mL, tPA, Actilyse, Boehringer Ingelheim, Ingelheim, Germany) or recombinant MMP-10 (200 nmol/L) to ensure clot lysis. Coagulation was initiated by re-calcification with 20 µL of CaCl\(_2\) (Star-TEM, 15 mmol/L, Delta). EC-micro carriers were added directly to re-calcified citrated blood and mixed gently. The analysed thromboelastometric parameters were clotting time (CT), representing the time in seconds from the start of the analysis to the initiation of clotting, and lysis time (LT), representing the time in seconds until complete clot lysis.

EC-micro carrier visualization:

Clots formed during thromboelastometric experiments were collected, fixated in PAF 4% and embedded in paraffin for histological analysis. Briefly, clot material was cut into 4 μm sections and incubated with a mouse anti-human CD31 antibody (JC70A, Dako). The following day, sections were incubated with a goat anti-mouse HRP antibody (K4001, Envision, Dako) followed by diaminobenzidine (Liquid-DAB + Substrate chromogen system, Dako). Representative images were obtained under light microscopy (Nikon Eclipse 80i, Amsterdam, Netherlands).
Supplementary Fig. S1. Schematic layout of the experimental models. (A) Tail-bleeding model and (B) collagenase-induced ICH model associated with oral anticoagulants (warfarin and rivaroxaban).
Supplementary Fig. S2. INR values of warfarin anticoagulated mice. INR values of mice included in (A) tail-bleeding and (B) collagenase-induced ICH experimental models. (C) Warfarin reversion, INR values 30 minutes after PCC administration. Treatments: PCC (100 UI/Kg) and CM-352 (1 mg/Kg). Mean±SD, *p< 0.05 vs. basal, using Mann-Whitney U test, n ≥ 3 /group.
Figure S3

Supplementary Fig. S3. Plasma Xa activity in rivaroxaban (10 mg/Kg) anticoagulated mice. (A) Xa activity measured at basal, 10, 30 min, 1, 3 and 24 h after rivaroxaban (10 mg/Kg) administration, n ≥ 3/group. Only animals with anti-Xa activity above 50% from basal (3.84 mIU) at 30 min were subjected to the collagenase-induced ICH model. (B) Xa activity levels of mice included in the collagenase-ICH model. Treatments: PCC (100 UI/Kg) and CM-352 (1 mg/Kg), n ≥ 10/group.
Supplementary Fig. S4. Tail-bleeding model on mice under oral anticoagulation. Bleeding time of mice anticoagulated with (A) warfarin (2 mg/Kg) and (B) rivaroxaban (3 mg/Kg). Mice were treated with saline (control), PCC (100 UI/Kg) and CM-352 (1 mg/Kg). Mean±SD, *p< 0.05, **p< 0.01 and ***p< 0.001 vs. saline, using Kruskal Wallis and Mann-Whitney U tests, n ≥ 5/group.
Supplementary Fig. S5. Enzymatic MMP-10 and Xa activities in the presence of oral anticoagulants. (A) MMP10 (0.2 nmol/L) activity in the presence of CM-352 (40 nmol/L) warfarin (40 nmol/L) or rivaroxaban (40 nmol/L). (B) Xa (0.2 nmol/L) activity in the presence of CM-352 (2.5 nmol/L), warfarin (2.5 nmol/L) or rivaroxaban (2.5 nmol/L). Mean±SD, *p< 0.05 and **p< 0.01 vs. MMP10 or Xa; #p< 0.05 vs. MMP10 + riva; ††p< 0.01 vs. MMP10a + warfarin; ‡p< 0.05 vs. Xa + CM-352, using Kruskal Wallis and Mann-Whitney U test, n ≥ 3 / group.
**Supplementary Fig. S6.** Local inflammation 24 h after experimental ICH. (A) Quantitative analysis of IL-6 expression normalized by total protein in contralateral (CL) and ipsilateral (IL) brain hemispheres of WT and Mmp10 -/- mice and representative western blot. (B) NETs formation in the haemorrhage area of rivaroxaban anticoagulated mice. (C) Representative immunofluorescence images showing NETs (co-localization of Citruline-H3 positive in red and MPO positive in green) and DAPI (blue) in the haemorrhage area of rivaroxaban treated mice 24 h after ICH. Scale = 20 µm. Mean±SD, *p<0.05 vs. saline, using Kruskal Wallis and Mann-Whitney U test, n ≥ 2 /group.
SUPPLEMENTAL REFERENCES


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ABSTRACT

Background: Intracranial haemorrhage (ICH) is one of the major devastating complications of anticoagulation. Matrix metalloproteinases (MMPs) inhibition has been proposed as a novel pharmacological approach for ICH treatment.
**Objectives:** We evaluated the effects of CM-352 (MMPs-fibrinolysis inhibitor) in an experimental ICH model associated with oral anticoagulants as compared with clinically used prothrombin concentrate complex (PCC).

**Methods:** ICH was induced by collagenase injection into the striatum of WT (C57BL/6J) anticoagulated mice (warfarin or rivaroxaban) and Mmp10-/- mice. Hematoma volume and neurological deficits were measured 24h later by diaminobenzidine staining and different behavioural test. Circulating plasminogen activator inhibitor-1 (PAI-1) activity and interleukin-6 (IL-6) were measured in plasma samples and local inflammation was assessed by neutrophil infiltration. Finally, fibrinolytic effects of MMP-10 and rivaroxaban were evaluated by thromboelastometry and thrombin-activatable fibrinolysis inhibitor (TAFI) activation assays.

**Results:** Only PCC reduced haemorrhage volume and improved functional outcome in warfarin-ICH, but both, PCC and CM-352 treatments, diminished haemorrhage volume (46%, p<0.01 and 64%, p<0.001, respectively) and ameliorated functional outcome in rivaroxaban-ICH. We further demonstrated that CM-352, but not PCC decreased neutrophil infiltration in the haemorrhage area at 24h. The effect of CM-352 could be related to MMP-10 inhibition since Mmp10-/- mice showed lower haemorrhage volume, better neurological score, reduced IL-6 levels and neutrophil infiltration, and increased PAI-1 after experimental ICH. Finally, we found that CM-352 reduced MMP-10 and rivaroxaban-related fibrinolytic effects in thromboelastometry and TAFI activation.

**Conclusions:** CM-352 treatment, by diminishing MMPs and rivaroxaban-associated fibrinolytic effects, might be a novel antihaemorrhagic strategy for rivaroxaban-associated ICH.

**Key words:** Anticoagulants, Fibrinolysis, Hemorrhagic Stroke, Matrix Metalloproteinases, Thrombosis.

**INTRODUCTION**

Almost 2 million people suffer from intracranial haemorrhage (ICH) worldwide every year with a 30-day mortality rate of around 50%, however, its overall incidence has not diminished during the last 30 years1.

ICH is characterized by direct blood extravasation into the brain parenchyma2, thus, hematoma size and expansion are associated with poor outcome and neurological deterioration3. ICH is the most feared complication of oral anticoagulation since, accounting for nearly 15-20%, these patients present higher mortality rates and prolonged bleeding when compared to non-anticoagulated patients4.

In ICH patients under vitamin-k antagonists (VKAs), clinical guidelines strongly recommend the use of prothrombin complex concentrate (PCC) as the first option for rapid anticoagulation reversal5. Direct oral anticoagulants (DOACs), which specifically inhibit thrombin or Xa, have decreased the risk of ICH6. Because of the safety of DOACs and the recent approval of their specific antidotes7,8, their use will further
increase, multiplying the number of patients suffering from DOAC-ICH\textsuperscript{9}. The delay of specific antidotes approval\textsuperscript{10} has led to the off-label use of PCC for the reversal of oral Xa inhibitors but without showing beneficial effects on hematoma expansion\textsuperscript{11}. To date, no medical or surgical clinical trial has improved patient outcome after ICH\textsuperscript{12}, therefore, remaining a challenging unsolved clinical and public health problem\textsuperscript{13}.

Matrix metalloproteinases (MMPs) are endogenous zinc-endopeptidases that play a relevant role in vascular remodelling, neuroinflammatory processes, and blood brain barrier (BBB) disruption associated with the pathophysiology of ICH\textsuperscript{14}. Besides, ICH patients presented increased MMPs levels in the blood, cerebrospinal fluid, and perihematomal\textsuperscript{15–17}.

The potential of MMPs as pharmacological targets has not yet been fully identified\textsuperscript{18}. Experimentally, MMPs inhibition prevented haemorrhagic complications induced by tPA, via protection of BBB tight junctions\textsuperscript{19}. In addition, MMPs may play a role in thrombolysis, since the fibrinolytic system and MMPs cooperate in thrombus dissolution by directly targeting fibrin(ogen) or by collaborating with plasmin\textsuperscript{20,21}. Specifically, our group described the fibrinolytic role of MMP-10 by preventing the activation of thrombin-activatable fibrinolysis inhibitor (TAFI)\textsuperscript{22} in experimental models of stroke\textsuperscript{23,24}. Based on these results, we developed a potent MMPs inhibitor, CM-352, which inhibits MMP-10 and MMP-3 and fibrinolysis\textsuperscript{25}, and effectively reduces bleeding, hematoma expansion, and functional impairment in different experimental models of haemorrhage with no signs of thrombotic side effects\textsuperscript{26,27}.

In this study, we aimed to explore the antihaemorrhagic efficacy of CM-352 in a collagenase-induced ICH mouse model associated with oral anticoagulants (warfarin or rivaroxaban). We evaluated its anti-fibrinolytic and anti-inflammatory effects, compared to clinically used 4-factor PCC.

**MATERIALS AND METHODS**

**Human blood samples**

Citrated blood samples were obtained from healthy volunteers who provided informed consent in accordance with the Principles of Declaration of Helsinki on biomedical research involving human subjects.

**Animals and experimental models**

All animal experiments were performed in accordance with European Communities Council regulation (EU) 2019/1010 for the care and use of laboratory animals and were approved by the Universidad de Navarra Animal Research Review Committee (Ref/092-15). Experiments were performed in 8–12 weeks old, 25–30 g weight, wild-type (WT) male C57BL/6J mice (Envigo, Barcelona, Spain), and MMP-10 deficient mice (Mmp10\textsuperscript{-/-}, C57BL/6J).
Mice were orally anticoagulated before the tail-bleeding model with warfarin (2 mg/Kg, 24 h) or rivaroxaban (3 mg/Kg, 1 h) and the collagenase-induced ICH model with warfarin (2 mg/Kg, 24 h) or rivaroxaban (10 mg/Kg, 1 h). Animals were randomly assigned to receive an intravenous bolus injection of saline, CM-352 (1 mg/Kg) or 4-factor PCC (100 UI/Kg, Octaplex®, Octapharma, Vienna, Austria).

Anticoagulation levels were measured 30 min before the experimental models in citrated blood samples obtained by submandibular vein puncture. Animals with International Normalized Ratio (INR) between 2.3 to 5 or plasma anti-Xa activity above 50% were subjected to the ICH model. Investigators were blinded to treatment groups. For further details, see Supplementary Information and Supplementary Fig. S1A and 1B, available in the online version.

Neurological and functional evaluation

Behavioural assessments were performed in all mice before and 24 h after the collagenase-induced ICH. Three different behavioural tests were performed: Bederson’s, pole and coat-hanger test. See Supplementary information, for further details, available in the online version.

Sample collection and tissue preparation

Animals for immunohistochemical analysis were euthanized 24 h after collagenase-induced ICH using a CO₂ chamber and perfused with cold phosphate-buffered saline (PBS) and 4% paraformaldehyde (PAF, Sigma-Aldrich). Brains were removed, post-fixed in 4% PAF for 24 h, frozen in isopentane, and stored until use at -80ºC.

Animals for western blot analysis were euthanized under the same conditions, perfused with PBS, and brain tissue was frozen in liquid nitrogen, and stored at -80ºC until posterior protein analysis.

Citrated blood samples were collected after euthanasia, by cardiac puncture. Samples were centrifuged first at 2,500x g for 10 min, then at 13,000x g for 2 min at 4ºC, and finally stored at -80ºC.

Histological, immunohistochemical and protein analysis

Frozen brains were cut into serial 20 μm thick coronal sections for histological (haemorrhage volume) and immunohistochemical analysis (neutrophil and Neutrophil Extracellular Traps [NETs]). Additionally, IL-6 was analyzed in frozen brain homogenates by western blot as described in detail in the Supplementary information, available in the online version.

Haemostatic and inflammatory parameters

To study the role of different haemostatic parameters, Thrombin Activatable Fibrinolysis Inhibitor (TAFI) activation and MMP-10 and Xa activities were measured in purified systems. Additionally, to evaluate systemic inflammation and fibrinolysis
h after the ICH experimental model, plasma levels of interleukin-6 (IL-6) and Plasminogen Activator Inhibitor-1 (PAI-1) activity were measured by ELISA and chromogenic assays, respectively. See Supplementary information, available in the online version, for further details.

**Thromboelastometry (ROTEM®) with adherent endothelial cells**

Human endothelial cells (Eahy926) were seeded onto microbeads to create transferable EC-micro carriers. Thromboelastometry (ROTEM®) experiments were performed using human citrated blood samples and different therapeutic concentrations of tPA, rivaroxaban, CM-352, and MMP-10. Clotting time (CT) and lysis time (LT) were analysed. See Supplementary information, available in the online version, for further details.

**Statistical analysis**

Data are presented as mean ± standard deviation of the mean (SD). Normality was assessed using the Kolmogorov-Smirnov test. Two independent samples were compared using the Mann-Whitney U two-tailed test and two related samples were compared using the Wilcoxon signed-rank test. The analysis for multiple observations was performed by the Kruskal Wallis test according to the data distribution. Statistical significance was established as p< 0.05. The statistical analysis was performed with SPSS (SPSS version 15.0 for Windows).

**RESULTS**

**Anticoagulant regimens**

No differences between INR values of mice anticoagulated with warfarin included in the tail bleeding and collagenase-ICH experimental models (Supplementary Fig. S2A, available in the online version) were found among the different treatments (Supplementary Fig. S2B, available in the online version). Furthermore, we demonstrated that the INR values returned to normal 30 min after PCC administration (3.4 ± 0.75 vs. 0.86 ± 0.05, p<0.05, Supplementary Fig. S2C, available in the online version).

A kinetic study was performed to determine the time-lapse of plasma Xa-inhibition by rivaroxaban (Supplementary Fig. S3A, available in the online version). WT mice were only included if anti-Xa activity was 50% above the mean basal value (3.84 mIU) 30 min after rivaroxaban administration. Plasma Xa activity was similar among the studied groups (Supplementary Fig. S3B, available in the online version). All mice under rivaroxaban anticoagulation were included in the tail-bleeding model.

**CM-352 effect on mice models of experimental hemorrhage**

Considering that CM-352 efficiently reduced haemorrhage volume in the collagenase-induced ICH model with rats, we first confirmed that CM-352 was effective in mice.
As shown in Fig. 1A and 1B, CM-352 reduced the hematoma volume of mice 24 h after collagenase induced-ICH when compared to saline (mm$^3$: 5.78 ± 1.46 saline vs. 3.79 ± 1.93 CM-352, p< 0.05) and prevented haemorrhage-related functional decline (p< 0.05 for saline, Fig. 1C). These results support the efficacy of CM-352 reducing hematoma volume and neurological deficit in rodent models of ICH.

To assess whether MMP-10 inhibition could be involved in the antihaemorrhagic effect of CM-352, we performed the collagenase-ICH experimental model in MMP-10 deficient mice (Mmp10 -/-). As shown in Fig. 1D, Mmp10 -/- mice presented smaller hematoma volume compared with WT mice (mm$^3$: 5.78 ± 1.46 WT vs. 3.77 ± 2.04 Mmp10 -/-, p<0.05) that was not further reduced upon CM-352 administration. Finally, Mmp10 -/- mice showed an improved score in the Bederson’s test when compared to WT 24 h after ICH (p<0.01, Fig. 1E), suggesting that the beneficial effects of CM-352 in ICH might be partially explained by MMP-10 inhibition.

Then, we assessed the anti-haemorrhagic efficacy of CM-352 and PCC in the tail-bleeding model associated with oral anticoagulants. Our results showed that CM-352 reduced the bleeding time as effectively as PCC in warfarin and rivaroxaban anticoagulated mice when compared with controls (Supplementary Fig. S4A and S4B respectively, available in the online version).

Once we confirmed that both CM-352 and PCC efficiently controlled acute bleeding in mice under oral anticoagulation, we tested the anti-haemorrhagic effects of CM-352 and PCC in the model of collagenase-induced ICH associated with oral anticoagulants.

In warfarin anticoagulated mice, PCC was able to significantly reduce the haemorrhage at 24 h when compared to saline (mm$^3$: 6.35 ± 2.89 saline vs. 3.88 ± 1.46 PCC, p<0.05, Fig. 2A), while the effect of CM-352 did not reach statistical significance. Moreover, treatment with PCC also preserved the functional outcome in the 5-second score test, whereas CM-352 and saline treatments did not (Fig. 2B).

In mice under rivaroxaban anticoagulation, CM-352 treated animals achieved a beneficial response, with a 64% reduction of the hematoma volume when compared with saline 24 h after ICH induction (mm$^3$: 5.76 ± 1.68 saline vs. 2.11 ± 1.63 CM-352, p<0.001). Likewise, PCC treated mice showed a 46% reduction in hematoma volume when compared with saline (mm$^3$: 5.76 ± 1.68 saline vs. 3.14 ± 1.65 PCC, p<0.01, Fig. 2C), with no further differences between CM-352 and PCC. Functional activity scores showed an improved 5-second score test in PCC and CM-352 groups when compared with the neurological deficits observed in the saline group (p<0.05 for saline, Fig. 2D). No differences in the rest of the neurological scores were found (data not shown). As anticipated, sham-operated mice did not develop cerebral injury (Fig. 2A and 2C). Together, these results suggest that only PCC is effective in controlling the hematoma expansion and neurological function in warfarin associated-ICH, while both CM-352 and PCC are effective in rivaroxaban associated-ICH.

CM-352 impact on MMP-10 and Xa in the presence of oral anticoagulants
Enzymatic activity assays were performed to exclude an interaction of warfarin or rivaroxaban on the anti-MMP10 activity of CM-352, or that of CM-352 on the inhibitory effect of rivaroxaban on Xa. As shown in Supplementary Fig. S5A, neither rivaroxaban nor warfarin affects the anti-MMP10 activity of CM-352. Similarly, CM-352 did not alter the inhibitory effect of rivaroxaban on Xa (Supplementary Fig. S5B, available in the online version).

**CM-352 restores rivaroxaban and MMP-10 fibrinolytic effects in vitro**

Taking into account the potential fibrinolytic activity described for rivaroxaban, we analysed whether CM-352 could modulate it. Kinetics of clot formation and lysis (tPA-mediated) were analysed by thromboelastometry in presence of beads coated with endothelial cells to provide the system with cell membranes and thrombomodulin (Fig. 3A). As expected, coagulation time (CT) increased dose-dependently in presence of rivaroxaban (p<0.05, Fig. 3B). Furthermore, our results showed that rivaroxaban exhibited a fibrinolytic effect shortening the lysis time (LT) in a dose-dependent manner when compared with the control (p<0.01 for 460 nmol/L, Fig. 3C). Interestingly, CM-352 blocked the fibrinolytic effect induced by rivaroxaban (p<0.01 and p<0.05, for 1.8 and 3.7 μmol/L, Fig. 3E) without changes in CT (Fig. 3D).

Further, we tested the effects of CM-352 using rivaroxaban and MMP-10 as fibrinolytic agents. Rivaroxaban delayed the CT (p< 0.01) independently of MMP-10 and CM-352 (Fig. 3F). The LT was reduced by MMP-10 alone and was further accelerated when combined with rivaroxaban (p<0.05 and p< 0.01 respectively), while CM-352 reverted their effect (p< 0.05, Fig. 3G). These results suggest that CM-352 restores rivaroxaban and MMP-10 fibrinolytic effects.

**CM-352 prevents MMP-10-dependent TAFI inactivation**

Additionally, we assessed whether CM-352 could prevent MMP-10-induced TAFI inactivation. As shown in Fig. 4, MMP-10 alone or in combination with rivaroxaban reduced TAFI activation (p<0.05) that was restored by CM-352, also in the presence of rivaroxaban. These results suggest that the observed antifibrinolytic effects of CM-352 might depend on MMP-10 inhibition.

**MMP-10 inhibition contributes to reducing inflammation and fibrinolysis after experimental ICH.**

To assess whether PCC and CM-352 were able to modulate the systemic inflammatory status of the animals 24 h after the ICH induction, we measured circulating levels of IL-6 in untreated, warfarin and rivaroxaban anticoagulated mice (Fig. 5A-C), finding no differences in IL-6 plasma levels after ICH in any of the assessed experimental condition. However, Mmp10 -/- animals showed decreased IL-6 expression in plasma (pg/mL: 24.70 ± 9.66 saline Mmp10-/- and 22.85 ± 4.93 CM-352 Mmp10-/- vs. 33.84 ± 9.06 saline WT, p<0.05, Fig. 5A) as well as in brain tissue (Supplementary Fig. S6A) after ICH induction when compared with WT.
Additionally, we evaluated plasma PAI-1 activity 24 h after ICH as a marker of systemic inflammatory and haemostasis status. In mice under no oral anticoagulation, we found no differences in PAI-1 activity 24h after ICH except, for an increment observed only in CM-352-treated Mmp10 -/- animals (p<0.05 vs. saline WT; p< 0.01 vs. CM-352 WT and p< 0.01 vs. saline Mmp-10/-., Fig. 5D). In warfarin anticoagulated mice, we observed that PCC treated mice depicted lower PAI-1, but, on the other hand, in rivaroxaban anticoagulated mice, PCC treated mice showed higher PAI-1 activity when compared to controls after ICH (p<0.05 vs. saline, Fig. 5E and 5F respectively). We observed no changes in PAI-1 activity after CM-352 treatment in any of our anticoagulant experimental groups after ICH.

We also analysed the effect of PCC and CM-352 on local inflammation by examining neutrophil infiltration in the haemorrhage area at 24 h. Neutrophil infiltration into brain tissue after ICH was similar in untreated or warfarin anticoagulated mice, regardless of the treatment (saline, CM-352, or PCC., Fig. 6A and 6B). However, in mice anticoagulated with rivaroxaban, we found that only CM-352 reduced neutrophil infiltration when compared with controls (neutrophils/µm²: 93.30 ± 35.78 saline vs. 42.51 ± 27.50 CM-352, p<0.05, Fig. 6C). Additionally, in this group of mice, we observed that CM-352 diminished the density of NETs in the hemorrhage area as compared to controls (NETs/µm²: 37.39 ± 21.10 saline vs. 11.23 ± 6.79 CM-352, p< 0.05, Supplementary Fig. S6B and 6C).

These results suggest that CM-352 treatment might diminish local inflammation after experimental ICH associated with rivaroxaban anticoagulation. Moreover, we found that neutrophil infiltration in Mmp10 -/- animals was also decreased when compared to WT (neutrophils/µm²: 77.69 ± 18.92 WT vs. 46.41 ± 20.36 Mmp10-/-, p<0.01, Fig. 6D and 6E).

Altogether, our results suggest MMP-10 inhibition could help to reduce inflammation and control haemostasis after experimental ICH.

**DISCUSSION**

Here we reported that CM-352 effectively reduced hematoma volume and functional impairment in rivaroxaban-associated ICH. Furthermore, CM-352 prevented rivaroxaban and MMP-10-related fibrinolytic effects. Additionally, we reported that: (1) CM-352 and PCC effectively controlled experimental bleeding under oral anticoagulation with warfarin or rivaroxaban; (2) PCC reduced hematoma volume and functional decline in experimental ICH associated with warfarin or rivaroxaban; and (3) MMP-10 deficient animals showed smaller hematoma and better neurological function, suggesting that inhibition of MMP-10 by CM-352 could be related to the beneficial effects of CM-352 after experimental ICH.

MMPs inhibition has been hypothesized as a promising strategy for the treatment of ICH. In fact, our group previously demonstrated the effectiveness and safety of CM-352 after experimental collagenase-ICH in rats, and discarded any effect of CM-352 on
collagenase activity. In line with these results, the current study shows that CM-352 successfully reduces brain haemorrhage and prevents neurological decline in collagenase-induced ICH in mice. Particularly, we showed that MMP-10 deficient animals consistently displayed reduced hematoma volume and improved neurological function after experimental ICH while CM-352 did not present any additional advantage on these parameters. Taken together, these data indicate that the beneficial effects of CM-352 may partially depend on MMP-10 inhibition.

VKAs are widely prescribed effective anticoagulants for the prevention and treatment of thrombotic events, but the VKAs-related major bleeding complications (rate between 10-16%) must be taken into consideration. The risk of warfarin-associated ICH may reach 1 to 2% per year, and this risk increases up to 4.2% in older patients. In this context, CM-352 treatment does not control intracranial hemorrhage or improve motor activity in our experimental conditions. This result could be due to a lower efficacy of CM-352 on MMPs inhibition or fibrinolysis, although our data indicate that MMP-10 inhibition or PAI-1 activity are not modified by CM-352 in the presence of warfarin.

As stated in recent guidelines, PCC is recommended to decrease mortality and normalise the INR in ICH occurring under the use of VKAs. Clinical and experimental data showed that PCC improved hematoma expansion and outcome after warfarin-associated ICH. In line with these results, we observed that PCC reduced bleeding time as well as hematoma volume and functional decline after experimental warfarin-associated ICH. These data might be explained by INR normalization after PCC treatment and the subsequent reduction of fibrinolytic activity, which in turn could decrease PAI-1 activity. Some studies already reported 4-factor PCC treatment for acute warfarin reversal in adult patients, however, high-doses of PCC might led to overcorrection of thrombin generation and increase the risk of thrombotic complications.

PCC is also recommended (off-label) for the treatment of DOAC-associated ICH, when specific reversal antidotes are not available. A recent multicentre clinical study in factor Xa inhibitor-related ICH patients demonstrated that PCC, achieved excellent haemostasis with very low thrombotic events (4%) in patients treated with andexanet (specific-Xa antidote) compared with those treated with PCC (10.7 vs. 4.3%). Nevertheless, PCC still lacks the clinical efficacy required to improve patients outcome, thus, further studies are needed to answer this clinical need. Yet, experimentally Zhou et al. described that PCC treatment prevented hematoma expansion in a murine model of rivaroxaban-associated ICH. Our results confirm and extend these data showing an increase in PAI-1 activity that could be explained by an increased endogenous thrombin generation as already described in previous studies evaluating PCC treatment to reverse the anticoagulant effect of direct factor Xa inhibitors. However, absence of the PT restoration or incomplete correction of thrombin generation have also been described, so there remains significant uncertainty regarding the efficacy and potential harms associated with these agents.
Notably, we showed for the first time that CM-352 is as effective as PCC reducing hematoma and preventing functional impairment after rivaroxaban-associated ICH, suggesting that CM-352 can be a promising therapeutic approach in anticoagulant-associated ICH. Recently, some reports have described that Xa-DOACs, aside from their anticoagulant effects, enhance fibrinolysis by increasing urokinase plasminogen activator\(^{41}\), suggesting that these patients might specially benefit from antifibrinolytic compounds to prevent hemorrhage. In addition, the fibrinolytic activity of rivaroxaban has also been related to the reduction of TAFI activation since decreasing thrombin generation would reduce resistance to fibrinolysis\(^{29}\). Nevertheless, this effect might be dual since reduction of thrombin generation could also reduce fibrinolysis by lowering tPA release from endothelium\(^{42}\). Moreover, in experimental studies, the role of rivaroxaban as a cofactor of tPA inducing the fibrinolytic activity of FXa has been also described\(^{43}\). Therefore, we performed thromboelastometry experiments to assess the fibrinolytic effect of rivaroxaban and its modulation by CM-352. Interestingly, we found that rivaroxaban exhibited a fibrinolytic effect in blood of healthy volunteers, which was restored by CM-352 without altering coagulation. Our group previously described the profibrinolytic properties of MMP-10\(^{23,44}\), therefore, additional thromboelastometry experiments were performed in the presence of MMP-10. We showed that the profibrinolytic effect of MMP-10 was enhanced by rivaroxaban and blocked by CM-352, suggesting that CM-352 reverse the fibrinolytic activity of MMP-10 and rivaroxaban.

Like rivaroxaban, MMP-10 displays its fibrinolytic mechanism by cleaving TAFI and so preventing its activation\(^{22}\). Our data suggest that the antifibrinolytic effects of CM-352 might be mainly related to MMPs activity inhibition, since CM-352: 1) did not affect rivaroxaban-inactivation of FXa neither its anticoagulant activity, and, 2) it was able to preserve TAFI activation in presence of MMP-10 independently of rivaroxaban. Nevertheless, CM-352 might participate in other rivaroxaban-enhanced fibrinolysis mechanisms. Therefore, although CM-352 did not alter rivaroxaban inhibition of FXa, it may change the fibrinolytic effect of rivaroxaban-treated FXa.

Inflammatory cells and molecules localized in the lesion site and peripheral areas have been associated with secondary damage after ICH\(^{16}\). We have evaluated systemic and local inflammatory status by measuring, plasma and brain levels of IL-6, as well neutrophil infiltration and NETs formation in the haemorrhage area after experimental ICH.

Systemically, our results show that CM-352 have no apparent effect on inflammation regardless of the anticoagulant treatment. However, MMP-10 deficient animals treated with CM-352 present reduced levels of IL-6 in plasma and increased PAI-1 activity. Previous studies reported that IL-6 regulates PAI-1 expression\(^{42}\) and additionally that PAI-1 might be related to a reduction in MMPs activity\(^{45,46}\), suggesting that MMP-10 inhibition might contribute to control systemic inflammation and fibrinolysis and to the subsequent protective effect observed in the ICH experimental model.
Locally, in line with hematoma volume reduction and neurological outcome improvement, CM-352 diminishes neutrophil infiltration and NETs formation in rivaroxaban-associated ICH. Additionally, MMP-10 deficient animals present lower neutrophil infiltration and IL-6 after ICH. Likewise, experimental ICH studies have described that MMP-9 and -12 deficient mice exhibited diminished neutrophil infiltration in the lesion area associated with less brain damage. Altogether, our data suggest that the beneficial effects of MMP-10 inhibition in ICH might be partially related to reduce systemic and local inflammation.

We are aware of the limitations of our study and extrapolation of the results should be applied with caution. Age and sex are non-modifiable risk factors for ICH, and in this study, we used adult male animals, therefore, further experimental studies should address gender differences and the effect of aging when assessing its therapeutic potential for ICH. We have not examined the anti-Xa activity levels in mice anticoagulated with rivaroxaban and subjected to the tail-bleeding model. However, all the control animals bled for 30 min assuring effective anticoagulation. In addition, we have found that the beneficial effects of CM-352 might be related, at least in part, to MMP-10 inhibition, although CM-352 is a pan-MMPs inhibitor that also inhibits MMP-2, MMP-9, and MMP-12 activity in the nanomolar range, thus the reported benefits could also involve the modulation of other MMPs. Hence, further experiments using other MMP deficient animals should be performed to establish a cause-effect relationship.

CONCLUSIONS

CM-352 and PCC effectively control oral anticoagulant-associated acute bleeding. In anticoagulant associated ICH, only PCC reduces haemorrhage and improves functional outcome in warfarin anticoagulated mice, while both PCC and CM-352 prevent hematoma expansion and functional impairment in mice anticoagulated with rivaroxaban. The mechanism behind the effect of CM-352 in experimental ICH might depend on MMP-10 inhibition and its antifibrinolytic and anti-inflammatory effects. Additionally, CM-352 prevents rivaroxaban and MMP-10-related fibrinolytic effects in thromboelastometry, as well as in TAFI activation. Therefore, CM-352 has the potential to provide a paradigm shift for rivaroxaban-associated ICH.

What is known on this topic?

- Intracranial haemorrhage (ICH) is the most feared complication of oral anticoagulation.
- Matrix metalloproteinases (MMPs) inhibition has been proposed as a novel pharmacological approach for ICH treatment.

What does this paper add?
• Prothrombin concentrate complex (PCC) and CM-352 are effective treatments reducing haemorrhage volume and functional decline in rivaroxaban-associated ICH, while only PCC is effective in warfarin-associated ICH mouse model

• The effect of CM-352 could be related to MMP-10 inhibition, since Mmp10−/− mice showed lower haemorrhage volume, and inflammation, and better neurological score after experimental ICH

• CM-352 prevents and attenuates MMP-10 and rivaroxaban-related fibrinolytic effects.

Authors’ contributions

M. Navarro-Oviedo participated in the design of the project, experimental work, statistical analysis, and wrote, reviewed and edited the manuscript; J. Marta-Enguita participated in the analysis of data, and edited and reviewed the manuscript; C. Roncal participated in experimental work, data analysis and reviewed the manuscript; J. A. Rodriguez, B. Zandio, J. Hermida, J. Oyarzabal, J. A. Paramo and R. Muñoz participated in the design of the project and reviewed the manuscript; A. Pineda-Lucena, and R. Lecumberri have provided intellectual content and reviewed the manuscript; and J. Orbe was in charge of project design, supervised the work and wrote, edited and reviewed the manuscript.

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Conflict of interest

M.N.-O.: recipient of a PhD scholarship from the Asociación de Amigos de la Universidad de Navarra (ADA). J.Oy., J.A.R, J.A.P. and J.Or.: were involved in a granted patent family (EP 12382285) that was licensed to Hemostatics Pharmaceuticals S.L. The other authors declare no significant conflict of interest.

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**Figure legends**

**Fig. 1.** Non-anticoagulated mice 24 h after experimental ICH. (A) Haemorrhage volume and (B) representative DAB staining images showing haemorrhage volume of *wild-type* (WT) mice treated with saline and CM-352 under no anticoagulation. (C) Functional evaluation (pole test). (D) Haemorrhage volume and (E) functional evaluation (Bederson’s test) in WT and MMP-10 deficient (*Mmp10* -/-) mice. Treatment: CM-352 (1 mg/Kg). Mean±SD, *p< 0.05 vs. saline; #p< 0.05 vs. basal; ††p< 0.01 vs. WT, using Kruskal Wallis and Mann-Whitney U test, n ≥ 9 /group.

**Fig. 2.** Anticoagulated mice 24 h after experimental ICH. (A) Haemorrhage volume and (B) functional evaluation (coat hanger) in warfarin-anticoagulated mice. (C) Haemorrhage volume and (D) functional evaluation (coat hanger) in rivaroxaban-
anticoagulated mice. Treatments: warfarin (2 mg/Kg), rivaroxaban (10 mg/Kg), PCC (100 UI/Kg) and CM-352 (1 mg/Kg). Mean±SD, *p< 0.05, **p< 0.01 and ***p< 0.001 vs. saline; #p< 0.05 vs. basal, using Kruskal Wallis and Mann-Whitney U test, n ≥ 5 /group.

**Fig. 3.** Thromboelastometric analysis with adherent EC-microcarriers using human whole blood samples. (A) Representative image of CD31 positive cells (brown, black arrows) surrounding the surface of Cytodex 3 EC-microcarriers (white arrows) in a blood clot obtained by ROTEM. Scale bar= 100 µm. (B) Clotting time (CT) and (C) Lysis Time (LT) in the presence of 58, 115, 230, or 460 nmol/L of rivaroxaban. (D) CT and (E) LT in the presence of 0.4, 0.9, 1.8 or 3.7 µmol/L of CM-352 and 460 nmol/L of rivaroxaban. (F) CT and (G) LT in the presence of MMP-10 (200 nmol/L), rivaroxaban (460 nmol/L) and CM-352 (1.8 µmol/L). CT and LT times expressed in seconds (s) are presented in the graphs. Mean±SD, *p< 0.05 and **p< 0.01 vs. 0 nmol/L; #p< 0.05 and ##p< 0.01 vs. 0 µmol/L; †p< 0.05 and ††p< 0.01 vs. Ctrl; ‡‡p< 0.01 vs. MMP-10; ¥p< 0.05 vs. MMP-10 + Riva, using Kruskal Wallis and Mann-Whitney U test, n ≥ 3 /group.

**Fig. 4.** MMP-10-dependent TAFI activation. TAFI (30 nmol/L) activation measured in the presence of MMP-10 (4 nmol/L), rivaroxaban (4 nmol/L), and CM-352 (4 nmol/L). TAFI relative Activation (%) is shown. Mean±SD, *p< 0.05 vs. TAFI, using Kruskal Wallis and Mann-Whitney U test, n ≥ 3 /group.

**Fig. 5.** Systemic inflammation and fibrinolysis 24 h after experimental ICH. Plasma IL-6 levels of (A) non-anticoagulated wild-type (WT) and MMP-10 deficient (Mmp10 -/-) animals (B) warfarin, and (C) rivaroxaban anticoagulated mice. (D) PAI-1 activity in non-anticoagulated wild-type (WT) and MMP-10 deficient (Mmp10 -/-) animals, (E) warfarin, and (F) rivaroxaban anticoagulated mice. Mean±SD, *p< 0.05 vs. saline WT; ##p< 0.01 vs. CM-352 WT; ††p< 0.01 vs. saline Mmp10 -/-; using Kruskal Wallis and Mann-Whitney U test, n ≥ 6 /group.

**Fig. 6.** Local inflammation 24 h after experimental ICH. Neutrophil infiltration in the haemorrhage area of (A) non-anticoagulated (B) warfarin and (C) rivaroxaban anticoagulated mice. (D) Neutrophil infiltration in the haemorrhage area of wild-type (WT) and MMP-10 deficient (Mmp10 -/-) animals. (E) Representative immunofluorescence images showing neutrophils (red) and DAPI (blue) in the haemorrhage area (white dots) of WT and Mmp10 -/- mice. Scale = 200 µm.
Magnification images of selected areas (*). Scale = 20 μm. Mean±SD, *p< 0.05 vs. Saline and ##p< 0.01 vs. WT using Kruskal Wallis and Mann-Whitney U test, n ≥ 4 /group.
Figure 1

A

Haemorrhage volume (mm²)

Saline  CM-352

B

Saline  CM-352

C

T-turn (s)

Saline  CM-352

D

Haemorrhage volume (mm²)

Saline  CM-352

E

Bederson's test (Score)

WT  Mmp-10 −/−
Figure 2

A

B

C

D

Haemorrhage volume (mm$^2$)

Neurological outcome (Score 5s)

Haemorrhage volume (mm$^2$)

Neurological outcome (Score 5s)

Saline  PCC  CM-352  Sham

Saline  PCC  CM-352

Saline  PCC  CM-352

Basal  #  24 h  #

Basal  #  24 h
Figure 3
Figure 4
Figure 5

(A) IL-1β (pg/mL)

(B) IL-6 (pg/mL)

(C) IL-10 (pg/mL)

(D) P2X4 (mM)

(E) P2X5 (mM)

(F) P2Y2 (mM)
Figure 6