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[1]. 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[2], 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% O_2) 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[3]. 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.

Neutrophil 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, spinned 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 ºC 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 37ºC and 5% CO_2.

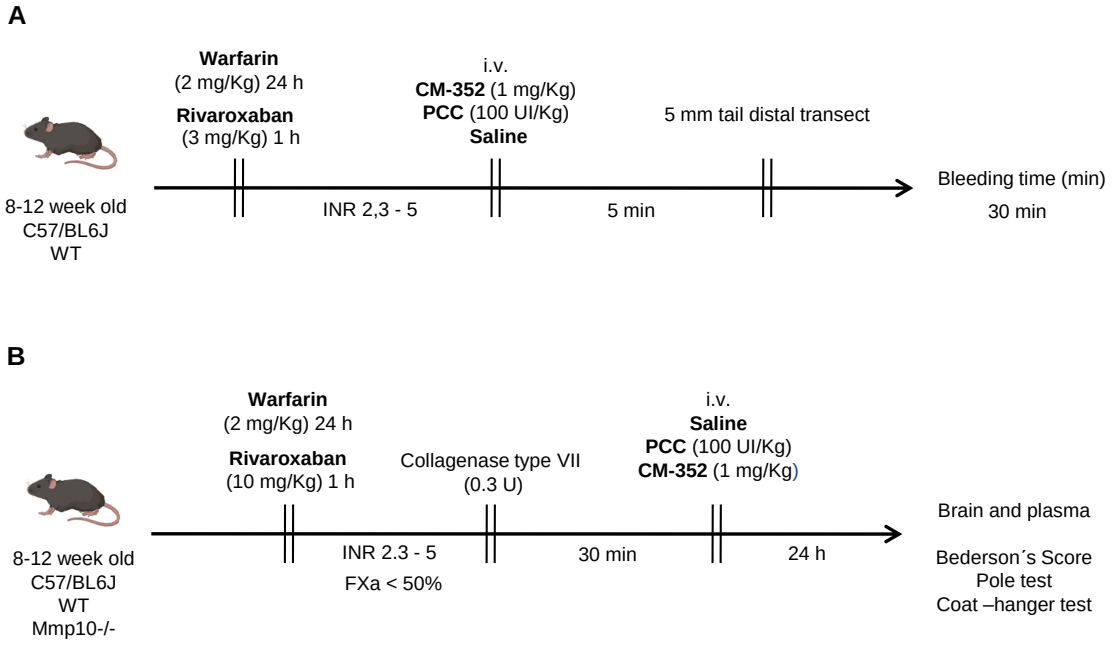
Cells ($4x10^6$) were transferred into culture flask with 5 mL of 10.000 sterilized micro carrier beads (Cytodex 3, GE Healthcare Bio-Sciences, Uppsala, Sweden) and incubated 4 h with gently agitation. After sedimentation, supernatant was discarded to remove non-adherent cells and cellular debris three times and complete medium (1:1 bead: medium volume ratio) was added and kept at 37ºC 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.

<u>EC-micro carrier visualization:</u>

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).

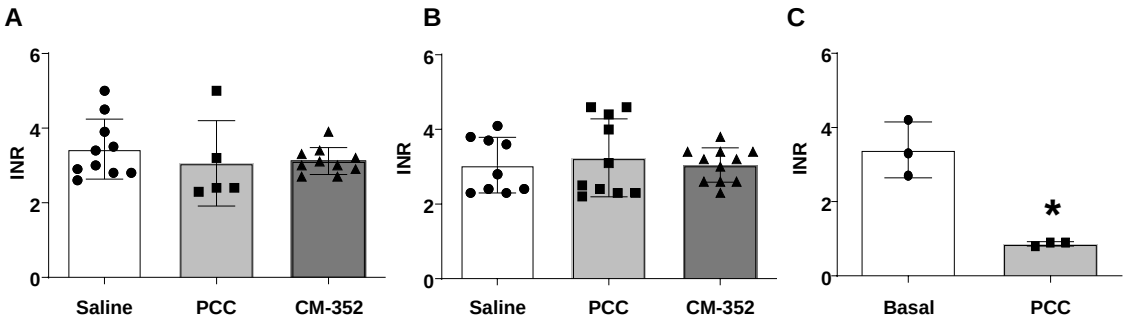
SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

Figure S1



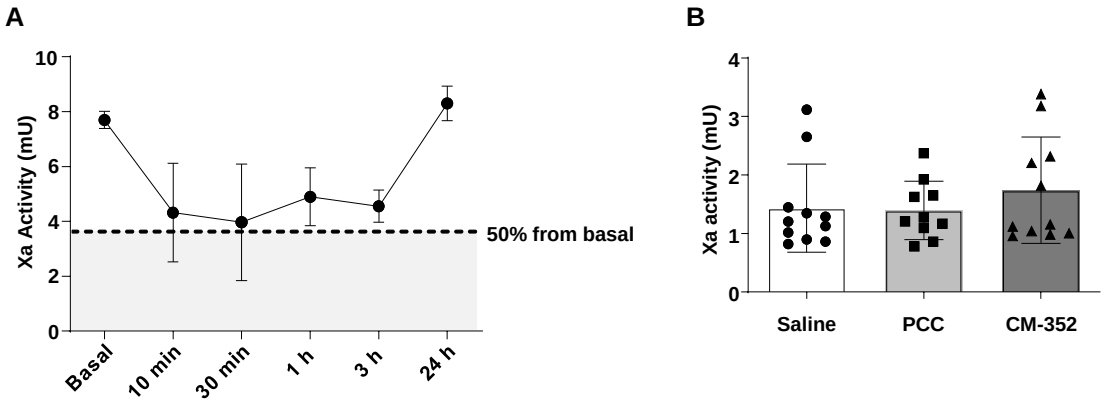
Supplementary Fig. S1. Schematic layout of the experimental models. (**A**) Tail-bleeding model and (**B**) collagenase-induced ICH model associated with oral anticoagulants (warfarin and rivaroxaban).

Figure S2



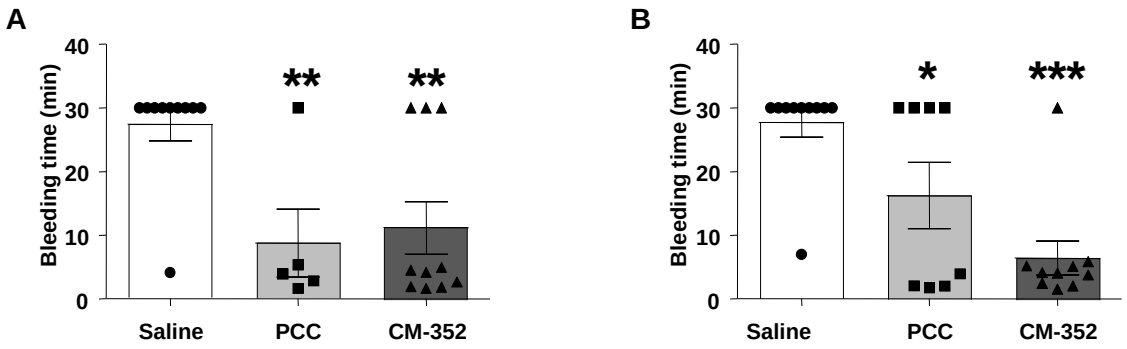
Supplementary Fig. S2. INR values of warfarin anticoagulated mice. INR values of mice included in (**A**) tail-bleeding and (**B**) collagenase-induced ICH experimental models. (**C**) Warfarin reversion, INR values 30 minutes after PCC administration. Treatments: PCC (100 UI/Kg) and CM-352 (1 mg/Kg). Mean±SD, *p< 0.05 vs. basal, using Mann-Whitney U test, n ≥ 3 /group.

Figure S3



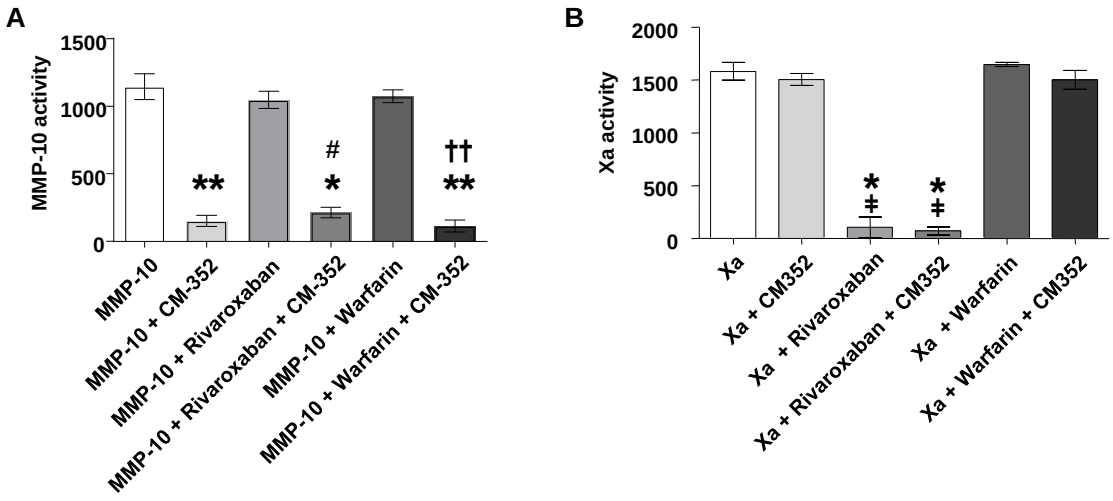
Supplementary Fig. S3. Plasma Xa activity in rivaroxaban (10 mg/Kg) anticoagulated mice. (**A**) Xa activity measured at basal, 10, 30 min, 1, 3 and 24 h after rivaroxaban (10 mg/Kg) administration, n ≥ 3 /group. Only animals with anti-Xa activity above 50% from basal (3.84 mIU) at 30 min were subjected 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.

Figure S4



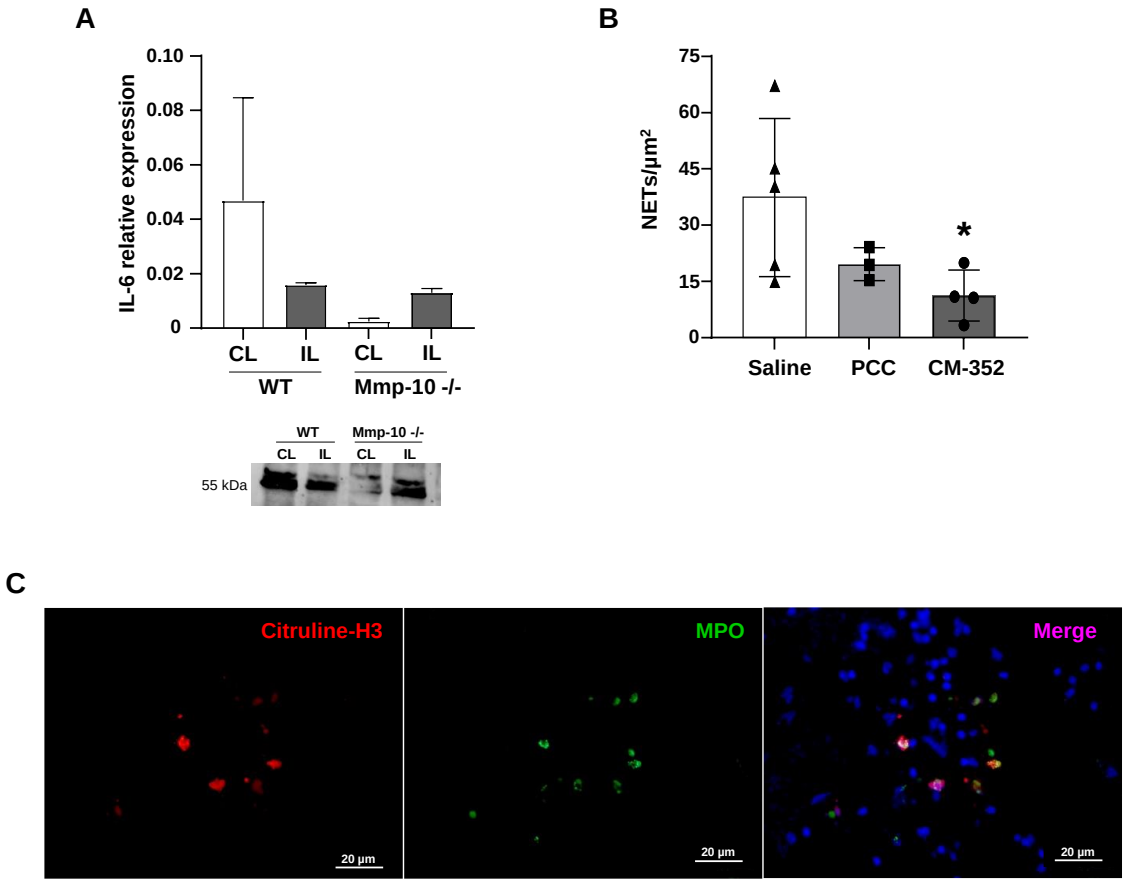
Supplementary Fig. S4. Tail-bleeding model on mice under oral anticoagulation. Bleeding time of mice anticoagulated with (**A**) warfarin (2 mg/Kg) and (**B**) rivaroxaban (3 mg/Kg). Mice were treated with saline (control), PCC (100 UI/Kg) and CM-352 (1 mg/Kg). Mean±SD, *p< 0.05, **p< 0.01 and ***p< 0.001 vs. saline, using Kruskal Wallis and Mann-Whitney U tests, n ≥ 5 /group.

Figure S5



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; ǂp< 0.05 vs. Xa + CM-352, using Kruskal Wallis and Mann-Whitney U test, n ≥ 3 /group.

Figure S6



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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