Materials and Methods

Preparation of washed human platelets

All studies involving human subjects have been reviewed and approved by the University of Michigan Institutional Review Board. A written informed consent was obtained from all healthy donors prior to the blood draws. Platelets were isolated as described previously.[1, 2]

Experimental Animals

All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee at the University of Michigan. C57BL/6 wild-type (WT) control mice and 12-LOX null (12-LOX$^{-/-}$) mice on C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in the research facility at the University of Michigan.

ML355 pre-treatments on experimental mice for in vivo *studies*

ML355 was specifically formulated in Polyethylene Glycol 300 (PEG 300) for oral gavage dosing in mice for *in vivo* thrombosis and hemostasis studies. For $FeCl_3$-induced mesenteric arteriole thrombosis model, WT mice (both recipient and platelet donor mice) were pre-treated with ML355 (15 mg/Kg and 30 mg/kg) formulated in PEG 300 solution or PEG 300 alone and 12-LOX$^{-/-}$ with or without ML355 treatment (30 mg/kg) as control via oral gavage 2 times per day for two days prior to intravital microscopy studies. For laser-induced cremaster arteriole thrombosis model, mice were treated with ML355 (1.88, 3.75, 7.5, 15, and 30 mg/kg based on *in vivo* pharmacokinetics) or with PEG 300 via oral administration as described above prior to intravital microscopy studies. The maximum plasma ML355 concentration and residual drug concentration/plasma clearance in mice administered ML355 were determined by pharmacokinetic studies. Aspirin (100 mg/Kg) was dissolved in DMSO and PBS and injected intravenously as a slow bolus (over 1 min).

Platelet aggregation

Platelets were prepared and aggregation was assayed as described previously.[1, 3, 4] Platelets were incubated with ML355 (25 μM, 50 μM, and 100 μM), 100 μM of aspirin or equivalent volume of DMSO control for 5 min and platelet aggregation was induced by various doses of thrombin (0.125 – 5 nM), PAR1-activating peptide (AP) (1 μM), PAR4-AP (50 μM) and collagen (0.5 μg/mL) as reported in previous studies.[5] Following platelet incubation with inhibitors, aggregation was measured in response to thrombin, PAR1-AP, PAR4-AP, or collagen with a lumi-aggregometer (Model 700D; Chrono-Log) under stirring conditions at 1100 rpm at 37°C.

Quantification of the effect of ML355 on oxylipin production from platelet 12-LOX pathway

Washed human platelets incubated with vehicle (DMSO), ML355, aspirin (100 μM), or both ML355 and aspirin were activated with thrombin and 12-HETE production was analyzed via UPLC-MS/MS as previously described.[3]

Ex vivo perfusion chamber

Perfusion assays were performed at arterial shear rate as previously described.[6, 7] Briefly, microfluidic perfusion chamber slides (μ-slide VI $^{0.1}$, ibidi, Martinsried, Germany) were coated with 100 μg/mL collagen type I (Kollagenreagens Horm; Nycomed) at 4°C overnight. Freshly drawn heparin-anticoagulated human whole blood samples were fluorescently labeled by incubating with DiOC6 (1 μM) for 15 min at 37°C. Human whole blood was pre-incubated with 25 -100 μM ML355, 100 μM aspirin, or both ML355 and aspirin, for 5 min prior to perfusion assays. Whole blood was then perfused over a collagen-coated surface at a controlled flow rate of 1800 seconds^{-1} for 4 min using a syringe pump (Harvard Apparatus, Holliston, MA) under Zeiss Axiovert 200M Inverted Fluorescent Microscope (40X W objective; Zeiss). Platelet adhesion, aggregation, and thrombus growth were recorded in real-time and quantitated offline by measuring the area of platelet surface coverage using the Slidebook program 6.0).

***In vivo* pharmacokinetics of ML355 following oral administration in mice**

Following the oral administration of ML355 (30 mg/kg), the drug concentration of plasma in mice (n=3) was monitored at 8 time points (0.25 h, 0.5, 1, 2, 4, 8, 12, 24) and assessed by PK analysis as described.[5]

Intravital microscopy thrombosis models

$FeCl_3$-induced mesenteric arteriole thrombosis model. $FeCl_3$-induced mesenteric arterial thrombosis was performed as previously described.[4, 8] Briefly, 3- to 4-week-old mice were injected with donor-matched fluorescently labeled gel-filtered platelets (5×10^6 platelets/g of mouse) via tail vein. Subsequently, the mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and the mesentery was externalized. Arteriole injury was induced by topical application of 30 μL of 250 mM $FeCl_3$ on a single mesenteric arteriole (100 - 120 μm diameter) in each mouse under Zeiss Axiovert 200M Inverted Fluorescent Microscope (25X oil objective; Zeiss). The dynamic process of thrombus formation and vessel occlusion in injured arterioles was recorded for up to 40 min using the Slidebook program.

Laser-induced cremaster arteriole thrombosis model. Adult mice (10 - 12 weeks old) were anesthetized as described above and surgically prepared as described in detail,[3, 9] and a tracheal tube was inserted to facilitate breathing. The cremaster muscle was prepared and perfused with preheated bicarbonate-buffered saline throughout the experiment. DyLight 488-conjugated rat anti–mouse platelet GP1bβ antibody (0.1 μg/g; EMFRET Analytics) and Alexa Fluor 647- conjugated anti-fibrin (0.3 μg/g) or Alexa Flour 647 rat-anti mouse CD62P (3 μg/mouse) were administered by a jugular vein cannula prior to vascular injury. Multiple independent thrombi were induced in the arterioles (30-50 μm diameter) in each mouse by a laser ablation system (Ablate! photoablation system; Intelligent Imaging Innovations, Denver,

CO, USA). Images of thrombus formation at the site of injured arterioles were acquired in real-time under 63X water-immersion objective with a Zeiss Axio Examiner Z1 fluorescent microscope equipped with solid laser launch system (LaserStack; Intelligent Imaging Innovations) and high-speed sCMOS camera. All captured images were analyzed for the change of fluorescent intensity over the course of thrombus formation after subtracting fluorescent background defined on an uninjured section of the vessel using the Slidebook program.

Intravital microscopy hemostasis models

Platelet fibrin hemostatic plug formation in saphenous vein induced by laser ablation. Laser ablation saphenous vein hemostasis model was performed as described.[10] Briefly, adult mice (10 -12 weeks old) were anesthetized and administered antiplatelet and anti-fibrin antibodies as described above. The saphenous vein was surgically exposed under intravital microscopy and superfused throughout the experiment with preheated bicarbonate-buffered saline. Blood flow in the saphenous vein was visualized under 20X water immersion objective using Zeiss Axio Examiner Z1 fluorescent microscope. The saphenous vein was exposed to two maximum-strength 532-nm laser pulses (70 lJ; 100 Hz; for about 7 ns, 10ms intervals) to puncture a whole (48 to 65um in diameter) through the vessel wall, which resulted in bleeding visualized by escape of fluorescent platelets to extravascular space. The laser injury was performed at 30 seconds and repeated at 5 and 10 min intervals at the same site of injury to assess re-bleeding. Platelet accumulation and fibrin deposition within the hemostatic clot was monitored in real-time and recorded for 5 min and the dynamic accumulation of fluorescent platelets and formation of fibrin within the growing hemostatic clot were analyzed using Slidebook software as described above.

Plasma extravasation following the laser-induced rupture of cremaster microvascular wall. Mice were anesthetized and the cremaster arteriole was prepared as described.[3, 11] The mice were intravenously injected with DyLight 488-conjugated rat anti-mouse platelet GP1bβ antibody (0.1 μg/g; EMFRET Analytics). 50 μg of fluorescein isothiocyanate-dextran (10,000 MW) was infused right before each laser injury in order to visualize the blood flow within the cremaster microvascular vessel and plasma extravasation from ruptured arterioles or venules following the laser ablation under fluorescent intravital microscopy. The cremaster muscle arteriole or venule was exposed to a high intensity laser pulse to puncture a hole through the arteriole wall as visualized by the disruption of the vessel wall and extravasation of blood from the ruptured vessel. Following plasma extravasation, the hemostatic response to the vascular injury (platelet accumulation and fibrin formation) at the site of the ruptured vascular wall results in the cessation of plasma. Plasma extravasation from the vessel was quantified by measuring the time required for dextran-labeled plasma leakage to cease following injury. Leakage was measured using a 40X water-immersion objective under confocal (CSU-X1 A1) spinning disk mounted to Zeiss Axio Examiner Z1 fluorescent microscope system equipped with solid laser launch system as described above. The captured confocal images were recorded and analyzed using Slidebook 6. The required time to cessation of plasma extravasation following the rupture of vessel wall was determined by reviewing single frame still images of fluorescein dextran from the time of initial leakage to cessation of bleeding within the boundaries of the vessels.

Statistics: Unpaired, paired two-tailed student t-tests, and two-way analysis of variance (ANOVA) were used to compare between experimental groups with Prism 6.0 software (GraphPad). Where appropriate, the statistical test used is contained in the figure legend. Data represents mean values ± SEM.

Analysis of tail-bleeding time and blood loss

Adult mice were anesthetized as previously described and tail-bleeding assays were performed.[1] Briefly, 5mm of the distal end of the tail was transected and the tail was submerged in warm saline at 37°C. Time to secession of tail-bleeding was recorded and total red blood cell count in saline was quantitated using a hemocytometer for the blood loss under the microscope.

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