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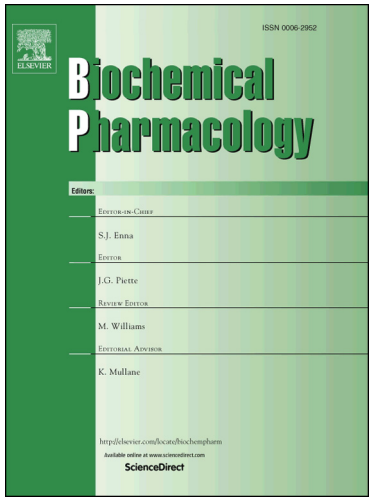

Anti-thrombotic efficacy of S007-867: pre-clinical evaluation in experimental models of thrombosis in vivo and in vitro

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PII: S0006-2952(18)30013-3
DOI: https://doi.org/10.1016/j.bcp.2018.01.013
Reference: BCP 13007

To appear in: *Biochemical Pharmacology*

Received Date: 28 October 2017
Accepted Date: 3 January 2018

Please cite this article as: A. Misra, P. Prakash, H. Aggarwal, P. Dhankani, S. Kumar, C.P. Pandey, N. Pugh, D. Bihan, M.K. Barthwal, R.W. Farndale, D.K. Dikshit, M. Dikshit, Anti-thrombotic efficacy of S007-867: pre-clinical evaluation in experimental models of thrombosis in vivo and in vitro, *Biochemical Pharmacology* (2018), doi: https://doi.org/10.1016/j.bcp.2018.01.013

Title: Anti-thrombotic efficacy of S007-867: pre-clinical evaluation in experimental models of thrombosis in vivo and in vitro

Short/Running Title: S007-867 inhibits intravascular thrombosis.

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Abstract

Pharmacological inhibition of platelet collagen interaction is a promising therapeutic strategy to treat intra-vascular thrombosis. S007-867 is a novel synthetic inhibitor of collagen-induced platelet aggregation. It has shown better antithrombotic protection than aspirin and clopidogrel with minimal bleeding tendency in mice. The present study is aimed to systematically investigate the antithrombotic efficacy of S007-867 in comparison to aspirin and clopidogrel in vivo and to delineate its mechanism of action in vitro. Aspirin, clopidogrel, and S007-867 significantly reduced thrombus weight in arterio-venous (AV) shunt model in rats. In mice, following ferric chloride induced thrombosis in either carotid or mesenteric artery; S007-867 significantly prolonged the vessel occlusion time (1.2 fold) and maintained a sustained blood flow velocity for >30 minutes. Comparatively, clopidogrel showed significant prolongation in TTO (1.3 fold) while aspirin remained ineffective. Both S007-867 and aspirin did not alter bleeding time in either kidney or spleen injury models, and thus maintained haemostasis, while clopidogrel showed significant increase in spleen bleeding time (1.7 fold). The coagulation parameters namely thrombin time, prothrombin time or activated partial thromboplastin time remained unaffected even at high concentration of S007-867 (300μM), thus implying its antithrombotic effect to be primarily platelet mediated. S007-867 significantly inhibited collagen-mediated platelet adhesion and aggregation in mice ex-vivo. Moreover, when blood was perfused over a highly thrombogenic combination of collagen mimicking peptides like CRP-GFOGER-VWF-III, S007-867 significantly reduced total thrombus volume or ZV_{50} (53.4±5.7%). Mechanistically, S007-867 (10-300μM) inhibited collagen-induced ATP release, thromboxane A_2 (TxA_2) generation, intra-platelet [Ca^{+2}] flux and global tyrosine phosphorylation including PLCγ2. Collectively the present study highlights that S007-867 is

a novel synthetic inhibitor of collagen induced platelet activation, that effectively maintains blood flow velocity and delays vascular occlusion. It inhibits thrombogenesis without compromising hemostasis. Therefore, S007-867 may be further developed for the treatment of thrombotic disorders in clinical settings.

Keywords: Blood flow velocity, Collagen, Hemostasis, Platelet activation, Thrombosis

1. Introduction

Arterial thrombosis is the root cause of acute myocardial infarction and cerebral stroke in which platelets play a leading role [1]. Although the present antithrombotic strategy has been clinically successful [2], it suffers from major limitations such as enhanced incidence of bleeding and recurrence of thrombotic episodes. Additionally, the inconvenient intravenous route of administration of drugs like integrin $\alpha_{IIb}\beta_3$ antagonists coupled with their inadequacy to cure cerebral stroke warrants development of new antithrombotic drugs.

Platelet collagen receptors play a critical role in the pathogenesis of athero-thrombosis [3, 4], while their absence produces only a modest prolongation in bleeding time. It is noteworthy that collagen is abundant in atherosclerotic plaques where it contributes to lesion growth and arterial narrowing. Moreover the over-expression of collagen receptors is correlated with the incidence of stroke and myocardial infarction [5, 6]. GPVI deficient mice subjected to transient middle cerebral artery occlusion showed significant protection with respect to reduction in brain infarct along with minimal bleeding phenotype [7]. It is anticipated that inhibitors blocking platelet–collagen interaction may provide better efficacy and safety than existing drugs. However, at present not a single inhibitor- synthetic or natural - targeting platelet collagen interaction is in clinical use.

A number of unique strategies of collagen antagonism have been developed such as recombinant fusion proteins (Revacept), small peptides, proteins isolated from natural products and recombinant monoclonal antibodies. Few promising entities are in different stages of pre-clinical and clinical testing. Revacept [8], is presently undergoing Phase III trial on patients with symptomatic carotid artery stenosis, TIAs, or stroke [9]. Among small molecule inhibitors, Losartan inhibits platelet activation by blocking GPVI clustering [10]. Similarly, caffeic acid phenethyl ester (CAPE) [11], its analogue CAPE-NO_2 [12], Honokiol

and Hinokitiol [13-15] are bioactive compounds derived from plant or other natural sources known to inhibit collagen-induced platelet activation. Recently, a synthetic platelet GPVI antagonist, S002-333 belonging to a new class of 2,3-disubstituted tetrahydropyridoindoles showed promising antithrombotic efficacy in animal and in vitro models [16]. Additionally, a number of alternative strategies targeting the regulation of expression and functions of collagen receptors have also been proposed. Few such targets include collagen induced signalling (SYK inhibitors) [17, 18], GPVI dimerization and clustering [19], or receptor shedding from the platelet surface [20]. However, there is still a wide gap between efficacy and safety of such anti-platelet compounds. An early clinical trial regarding proof of principle/ concept may assist in screening potent and efficacious molecules.

In a previous study, we identified a novel synthetic anti-platelet compound S007-867 (tert-butyl ((S)-1-(4-methyl-benyl)-5-oxopyrrolidine-2-carbonyl) piperidin-3-yl) methylcarbamate), that showed better antithrombotic activity than aspirin and clopidogrel with minimal bleeding tendency in mice [21, 22]. The subsequent pharmacokinetic studies revealed its rapid absorption and fast onset of action in mice after oral administration [22-24]. Although our preliminary study reported that S007-867 inhibits collagen induced platelet aggregation (IC_{50}=6μM) [21, 22], the efficacy of compound on different thrombosis models, coagulation parameters, different platelet functions and underlying signalling mechanisms is still not known.

Therefore, the present study is aimed to systematically elucidate a detailed pharmacological profile of S007-867 in mice, rats and human, so as to advance it for drug development and clinical use. We evaluated antithrombotic efficacy of S007-867 in different animal models of thrombosis in comparison to aspirin and clopidogrel. Furthermore, using human blood we have also delineated that its antithrombotic mechanism is platelet mediated without involving coagulation cascade. S007-867 inhibits collagen induced platelet activation and deregulates

subsequent signalling mechanisms namely platelet aggregation, ATP release from granules, thromboxane A_2 generation via cycloxygenase 1, intra-platelet calcium mobilization and tyrosine phosphorylation of multiple proteins including PLCγ2.

2. Materials and Methods:

2.1 Chemicals and reagents:

S007-867 (purity ≥ 99%) was synthesized and characterized in the Medicinal and Process Chemistry Division, CDRI, as described previously [22]. Aspirin was purchased from Sigma, USA and clopidogrel from Matrix Laboratories Limited, India. Adenosine Diphosphate (ADP), Thrombin, Arachidonic Acid, Collagen (fibrillar equine tendon type I) and Chronolume reagent were purchased from Chronolog corp (Pennsylvania, USA). The collagen related peptide (CRP) and other collagen-mimetic peptides were synthesized in Richard W Farndale's laboratory at Department of Biochemistry, Cambridge University as described earlier [25]. All peptides are triple-helical: CRP contains the sequence $[GPO]_{10}$, whilst the α2β1 recognition motif, GFOGER, is flanked by $[GPP]_5$ sequences, as is GPRGQOGVMGFO, the VWF-binding peptide from collagen III, designated here as VWF-III. Coagulation kits such as STA thrombin reagents, Neoplastin CI plus, Fibri-Prest, CK Prest were from Diagnostica Stago (France). BioTrak thromboxane B_2 EIA kit was from Cayman Chemicals (Ann Arbor, USA). Ketamine was from Themis Medicare Private Limited (India). P-nitrophenyl phosphate was from Sisco Research Laboratories (India). Prostaglandin I2, Ferric Chloride, Urethane, Xylazine, FITC labelled Dextran, Aprotinin, Leupeptin, Fura 2-AM, $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide), Heparin, Hirudin, Indomethacin and Phalloidin TRITC were purchased from Sigma (USA). PPACK (Phe-Pro-Arg-chloromethylketone) was from Calbiochem, USA. Calcein Red Orange-AM was purchased from Invitrogen Thermo Fisher (USA). The following antibodies were used: 4G10® Platinum, Anti-Phosphotyrosine Antibody (mouse monoclonal cocktail IgG2b, Upstate Biotechnology, CA, USA); anti-PLC-γ2 (Q-20) rabbit polyclonal IgG (Santa Cruz,

TX, USA), and Horseradish peroxidase (HRP) conjugated mouse anti-rabbit immunoglobulin G (Sigma, MO,USA)

2.2 Animal Experiments

Swiss albino mice (male, 25–30 g) and Sprague Dawly rats (male, 150–160 g) were obtained from Laboratory Animal Division, CSIR-CDRI. Animals were maintained at ambient temperature (23–25° C), relative humidity of 50–70% and 12hour/12 hour light/dark cycle. All the experimental protocols were approved by Institutional Animal Ethics Committee, CSIR-CDRI. All the animals were divided into individual groups each consisting of 6-8 animals. Urethane (1.25g/kg, i.p.) was used to anaesthetize rats for AV shunt model. A mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg) (i.p.) was administered to mice prior to surgery for $FeCl_3$ induced thrombosis and injury models. S007-867 was administered orally 1 hour prior to experiment, and the oral dose was formulated in 0.5% carboxymethyl cellulose suspension, that was also used as vehicle.

2.3. Arterio-venous shunt model in rats

A cervical incision was made and carotid artery and its contra lateral jugular vein was exposed. Two 7-cm siliconized polyethylene tubes (0.5/1.0mm inner/outer diameter) were linked to a central 6 cm silicon tube (1.0/1.5mm, inner/outer diameter) containing a 5 cm silk thread (pre-weighed) and were filled with saline. The shunt assembly was cannulated between the jugular vein and contra-lateral carotid artery and blood was allowed to circulate through the shunt. Blood flow through the shunt was maintained for 10 minutes, subsequently the central part of the shunt was removed and silk thread with thrombus was weighed. The thrombus adhered/deposited on thread was calculated by subtracting the wet weight of the silk thread. [26]**.**

2.4. $FeCl_3$-induced thrombosis in mice carotid artery using Doppler probe:

The carotid artery was dissected and a pulsed Doppler probe (DBF-120A- CPx, CBI-8000, Crystal Biotech, USA) was attached to record the blood flow and patency (BIOPAC Systems, USA) as described previously[27]. After stabilizing for 20 minutes, Whatman paper discs (0.5x1mm) pre-soaked in 10% $FeCl_3$ solution were placed on exposed artery for 5 minutes. Blood flow was recorded until complete cessation, and represent time to occlusion (TTO). If the blood flow did not cease within 120 minutes TTO was assigned as >120 minutes [27].

2.5. $FeCl_3$ induced thrombosis in mice mesenteric artery using intravital microscopy

2.5.1. Preparation of platelets:

Blood was isolated from anesthetized mice and diluted with tri sodium citrate (2.5% w/v, pH 6.4) in 1:9 ratio. Platelet-rich-plasma (PRP) was obtained by centrifugation at 100x g for 5 min. The PRP and buffy coat containing some RBCs was gently transferred to fresh polypropylene tubes and re-centrifuged at 100x g for 5 min. PRP was incubated with prostaglandin I_2 (PGI_2, 20 ng/mL) at 37°C for 5 minutes. PRP was further centrifuged at 600x g for 5 min and pellet obtained was resuspended in 1 mL modified Tyrode-HEPES buffer (137 mM NaCl, 0.3 mM Na_2HPO4, 2 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.2) containing PGI_2 (20 ng/mL) for 5 minutes at 37°C. Platelets were washed twice and finally resuspended in modified Tyrode-HEPES buffer and was fluorescently labeled with calcein red-orange, AM (2.5 µg/mL) for 20 min at room temperature.

2.5.2 Intravital microscopy

FITC-labelled Dextran (2mg/mice) and calcein red orange labelled platelets ($1.25x10^9$/kg) were injected through retro-orbital plexus to visualize the blood vessels and platelets

respectively as described before. An incision was made through abdominal wall, mesenteric artery was exposed and Whatmann filter paper (2mmx0.5mm) saturated with 10% $FeCl_3$ solution was applied topically for 3 minutes and then removed. Thrombus formation was monitored in real time under fluorescent microscope [Olympus FV1200 (BX61Wi), Japan] coupled to a charge-coupled device (CCD) camera. Time to vascular occlusion by thrombus was determined. The end point of the experiment was recorded when blood flow has completely stopped or 40 minutes in case occlusion is not observed. Blood flow velocity was calculated as the displacement of the fluorescently labelled platelets between two consecutive frames divided by the time delay between the two captures using Image J software [28-30].

2.6. Liver and Spleen haemorrhagic model:

The liver and spleen were exposed via a midline incision. A paraffin film was placed under the left anterior liver lobe. After incision, filter paper pieces (4-5 mm long and 3-4 mm wide) were placed over cut surface at 5 sec interval until complete cessation. The bleeding time was taken as the total time elapsed between the incision and the end point.Similarly filter paper pieces were placed over cut surface of spleen until the end point was reached, and total time was recorded as described previously [31-33].

2.7. Ex vivo studies:

2.7.1. Blood collection and platelet isolation

Blood was drawn by cardiac puncture from anaesthetized mice and mixed with 2.5% tri-sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 180 x *g* for 20 minutes at room temperature. Washed platelets were prepared as described in section 2.4.1 [34].

2.7.2. Platelet adhesion and aggregation

Glass cover slips were coated with equine tendon native fibrillar type I collagen (Chrono-par, Chrono-log Corp., USA), and subsequently blocked with 5mg/ml BSA at room temperature for 1 hour, and finally washed once with PBS. Human or murine platelets, were added to the cover slips (10^7/well) for 1 h [16]. After washing thrice, the adhered platelets were fixed with 2% paraformaldehyde for 15 minutes and then stained for 1hour with phalloidin-TRITC. Platelets were viewed on a fluorescent microscope (Leica FW 4000) at 40X magnification. The fluorescence images were recorded from at least five different microscope fields and the results are expressed as average number of cells adhered for all the areas [35].

Platelet aggregation was performed using a four channel-aggregometer (Chronolog-corp, Havertown, USA). Murine platelet rich plasma(1×10^8 platelets/ml, 0.45 ml) was pre-warmed to 37°C for 2 minutes before addition of various agonists [2µg/ml Collagen (Chrono-log, Corp.),10µM ADP (Sigma), 0.25mM Arachidonic Acid (Chrono-log Corp)]. The reactions were allowed to proceed for at least 5 minutes, and the extent of aggregation was expressed in light-transmission units using Aggrolink software [22].

2.8. In vitro studies:

2.8.1. Blood collection and platelet isolation

The present study was approved by the Institutional Ethics committee of CSIR-CDRI, Lucknow, India and King George Medical University, Lucknow, India. The study has conformed to the guidelines of Helsinki Declaration. Blood was drawn by venipuncture from healthy human volunteers in citrate-phosphate-dextrose or PPACK (for whole blood perfusion assay). All donors have provided their written informed consent. Dimethyl

sulfoxide (DMSO) was used to dissolve S007-867 and hence it was used at the same final concentration as vehicle/solvent in all experiments.

2.8.2. Whole Blood Perfusion assay

Whole blood was incubated with 3, 3'dihexyloxacarbocyanine iodide (DiOC6, 1µM) for 15 minutes and the perfusion studies were performed as described earlier [36, 37]. Glass coverslips were coated with collagen or other peptides overnight at 4°C, and blocked with 1% BSA. The coverslip, in a 125µm-deep flow chamber, was mounted on an FV300 laser-scanning confocal microscope (Olympus) and washed for 1 minute with HEPES buffer containing 100U/mL heparin. Blood was drawn through the chamber for 5 minutes using a syringe pump at a wall shear rate of $1000s^{-1}$.

2.8.3. Coagulation parameters

Coagulation parameters, prothrombin time [21], activated partial thromboplastin time (aPTT) and thrombin time (TT) were measured in plasma as per manufacturer's instructions using a Semi automated Coagulometer (Start4, Young Instruments, Stago, France)[38].

2.8.4. Intracellular calcium mobilization

Platelet rich plasma (PRP) was incubated with 5µM Fura-2 AM for 60 minutes at 37°C. After washing, the Fura-2 fluorescence was measured in collagen stimulated platelets at an excitation wavelength of 340/380 nm and emission wavelength of 500 nm using a fluorescence spectrophotometer (VARIAN, Cary Eclipse) [34].

2.8.5. ATP release

ATP secretion was monitored in platelet rich plasma by adding Chronolume reagent (Chronolog). The luminescence generated by platelets upon collagen stimulation was compared with an ATP standard as reported earlier [16].

2.8.6. Thromboxane A_2 generation

As described previously, washed platelets were stimulated with collagen for 5 minutes at 37°C, and the reaction was terminated by addition of ice-cold EDTA solution (10mM) containing 10µM indomethacin. The samples were centrifuged and thromboxane B_2 (TxB_2), the stable metabolite of TxA_2, was measured using a correlate-EIA thromboxane B_2 kit [34].

2.8.7. Immunoprecipitation

Washed platelets were lysed in radio immuno precipitation assay (RIPA) buffer [PBS containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitors cocktail] for 15 minutes. The supernatant was pre-cleared with protein A/G agarose (Amersham biosciences, Upsala, Sweden) and incubated with 1µg of primary antibody (PLCγ2). After 2 hours, 20µl of protein A/G agarose was added and incubated for 1 hours. The beads were washed, re-suspended in Laemmelli buffer, denatured at 95^0C for 5 minutes, and subsequently analyzed by immunoblotting [34, 38].

2.8.8. Immunoblotting

Platelets were stimulated with collagen, and the reaction was stopped by adding 4X Laemmli buffer [39] (containing 10 µg/ml aprotinin, 1 mM PMSF, 10 µg/ml leupeptin, 10 mM NaF, 1 mM sodium orthovanadate). The lysates were boiled for 3 minutes [34, 38], and centrifuged at 12,000 ×g for 5 minutes. Samples containing 30-40 µg of protein were subjected to SDS-PAGE and immunoblotting as described previously [34, 38].

2.9. Statistical Analysis:

Graph Pad Prism 5.0 (Graph Pad Inc., La Jolla, CA, USA) was used for statistical analysis. All the results are expressed as the mean±standard deviation (SD). Student's t-test or one-way ANOVA followed by posthoc test was used and P values less than 0.05 were considered significant.

3. Results

3.1. In vivo Studies

3.1.1. Effect of S007-867 on arterio-venous shunt model in rats

When compared to vehicle, S007-867 significantly reduced wet thrombus weight from 51.2±12 mg to 31.2±2.5 mg, 31.2±7.2 mg, and 30.5±8.7 mg at doses of 10, 30, and 100 µmol/kg respectively **(Fig. 1A)**. Both aspirin and clopidogrel significantly reduced thrombus weight (25.4±3.3 mg, 24±3.3 mg respectively) in comparison to vehicle **(Fig. 1A)**.

3.1.2. Effect of S007-867 on ferric chloride-induced thrombosis in carotid artery in mice

Figure 1B shows that in vehicle-treated mice, $FeCl_3$ application led to complete occlusion of blood flow at 14±0.2 minutes, which was significantly prolonged to 20.2±2 min (1.3±0.1 fold) in mice pre-treated with clopidogrel. S007-867 (30, 100 & 300 µmol/kg) dose dependently prolonged the time to occlusion with significant elevation at 100 & 300 µmol/kg. It significantly delayed the growth of thrombi by 1.18±0.2 and 1.25±0.08 fold at 100 and 300µmol/kg respectively in comparison to vehicle treated mice. Aspirin displayed no significant antithrombotic protection in this model. **(Fig. 1B)**.

3.1.3. Effect of S007-867 on bleeding time in mice:

In vehicle treated mice, bleeding stopped after 3.5±0.7 minutes and 4.9±1.2 minutes in liver and spleen injury model respectively. Clopidogrel showed significant increase in bleeding in spleen (8.2±0.6 min), while there was no significant alteration in bleeding time in liver (3.0±0.8 min) in mice. Animals treated with aspirin (liver: 3.1±0.6 minutes; spleen: 5.9±1.8 minutes) or S007-867 (liver: 3.6±1.2 minutes; spleen: 5.5±1.9 minutes) did not show significant alteration in either of the injury models in comparison to vehicle treated mice

(Fig. 1C. D).

3.1.4. Effect of S007-867 on ferric chloride-induced thrombosis in mesenteric artery in mice

We further performed time-lapse analysis to reveal any changes in kinetics of thrombus formation after administration of S007-867 (30µmol/kg) under in vivo settings. Topical application of 10% $FeCl_3$ on mesenteric artery initiated thrombi formation within 3 minutes in vehicle treated mice **(Fig. 2 B, Video 1)**. The thrombi formed in S007-867 treated mice were initially smaller as compared to vehicle treated mice. Moreover the growth of thrombi was slower in S007-867 treated mice, while thrombi grew faster in vehicle treated mice. The blood flow velocity continuously decreased in control mice with complete cessation of blood flow at around 20 minutes whereas in S007-867 treated mice blood flow velocity was sustained even after 30 minutes of $FeCl_3$ injury **(Fig. 2 C, Video 2)**.Complete cessation of blood flow in vehicle treated mice was seen at an average vessel occlusion time of 21.2±6.2 minutes, while S007-867 treated mice exhibited an average time to occlusion of 42.13±10.3 minutes **(Fig. 2A)**. Thus, the compound S007-867 was able to delay the progression of thrombi and the time to occlusion as compared to control mice with sustained maintenance of vessel blood flow velocity.

3.1.5. Effect of S007-867 on collagen mediated platelet adhesion and aggregation in mice (ex vivo*):*

The aggregation profile of S007-867 in mice platelets exhibited significant reduction in collagen induced platelet aggregation in a dose dependent manner **(Fig. 3A)**. The mean percent aggregation following collagen stimulation at 30µmol/kg is 32±1.1% which was further reduced to 9.8±9% at 100µmol/kg and 2.8±2.7% at 300µmol/kg. The ADP- and

arachidonic acid-mediated platelet responses remained unaltered, thus confirming the anti-platelet mode of action of the compound to be mediated via collagen receptor **(Fig. 3A)**. S007-867 also evoked significant inhibition in platelet adhesion over collagen in a dose dependent manner (53.87±17% at 30 µmol/kg, 65.1±11.3% at 100µmol/kg and 79.2±8.6% at 300µmol/kg) **(Fig. 3B, C)**. Clopidogrel (100µmol/kg) also showed significant inhibition in the number of platelets adhered over collagen (67.3±7.4%), while Aspirin (100µmol/kg) did not show any effect.

3.2. In vitro Studies

3.2.1. Effect of S007-867 on whole blood perfusion assay:

Whole blood treated with S007-867 at a concentration of 100µM, did not show any inhibitory effect on surface coverage (indicating platelet adhesion), when perfused over collagen or other collagen mimetic peptides **(Fig. 4 A, B).** However S007-867 significantly reduced mean thrombus volume or ZV_{50} over CRP-GFOGER-VWF-III (CGV) surface from 3.7±0.38 mm to 1.7±0.17 mm **(Fig. 4 C, D).** S007-867 did not affect ZV_{50} on collagen, CRP-GFOGER or GFOGER-VWF-III surfaces. ZV_{50} measures the height of the centre of mass of thrombus. The height of the thrombus indicates build-up of thrombi due to platelet-platelet interaction and hence is considered to be a more direct measure of platelet activation.

3.2.2. Effect of S007-867 on coagulation parameters in human plasma:

No change was observed in Thrombin Time (TT), Prothrombin Time [21], and activated Partial Thromboplastin Time (aPTT) when compared to their isovolumetric vehicle control. Hirudin, a standard anticoagulant, significantly increased TT, PT and aPTT **(Fig. 5A, B, C)**.

3.2.3. Effect of S007-867 on collagen induced intra-platelet calcium flux:

Addition of collagen to human platelet suspension in the presence of 1 mM $CaCl_2$ resulted in an increase in $[Ca^{2+}]_i$ **(Fig. 5D)**. S007-867 significantly decreased the collagen-induced mobilization of intra-platelet $[Ca^{2+}]_i$ in a concentration dependent manner. The $[Ca^{+2}]_i$ levels were reduced to 78.2%±6.0%, 40.6%±15.4% and 28.02%±18.8% of control at concentration of 10, 30 and 100µM respectively **(Fig. 5D, E)**.

3.2.4. Effect of S007-867 on collagen induced ATP release from platelets:

The compound S007-867 attenuated collagen induced ATP release from platelets in a concentration-dependent manner. The vehicle (DMSO) treated platelets released 4.6±0.14nm of ATP, which was significantly reduced to 1.9±0.2nm (55±10.8%) and 1.6±0.1nm (63.1±5.4%) by S007-867 at concentrations 30 and 100µM respectively **(Fig. 5F)**. It showed maximum inhibition in ATP release at 300µM (80.5±6.8%).

3.2.5. Effect of S007-867 on thromboxane A_2 generation from platelets:

S007-867 exhibited 30±10% and 56±36% inhibition in TxB_2 levels at 30µM and 100µM respectively. Aspirin also displayed a concentration dependent inhibition in TxB_2 release with mean maximal inhibition of 48.4±28% at 100µM. Apparently the inhibitory potential of S007-867 was almost comparable to that of COX inhibitor Aspirin **(Fig. 5G)**.

3.2.6. Effect of S007-867 on collagen induced tyrosine phosphorylation of platelet proteins:

The activation of platelets by collagen contributes to the assembly and stabilization of various signaling complexes. When treated with S007-867 at various concentrations (10-300µM), the phosphorylated levels of a number of proteins were decreased compared to vehicle control. As shown in **Fig. 5H**, S007-867 exhibited concentration-dependent reduction in the phosphorylation levels of proteins lying approximately in the molecular weight range of 100-

130 kDa, 75-90 kDa, 45-60 kDa. Furthermore, S007-867 inhibited the phosphorylation of platelet PLCγ-2 in a concentration-dependent manner with a significant effect at 300μM **(Fig. 5I)**, thereby implicating that the compound disrupts platelet function via modulation of collagen induced signalling cascade.

4. Discussion

Platelet collagen interaction is a critical phenomenon underlying thrombosis. Blocking collagen induced platelet activation to prevent thrombosis is anticipated to be safer and efficacious, either alone or in combination with existing drugs. In a previous study, we have identified S007-867 as a novel synthetic antithrombotic compound that specifically inhibits collagen-induced platelet aggregation. The present study describes the pre-clinical pharmacological profile of S007-867 in detail in animal and in vitro models of thrombosis.

S007-867 (30 μmol/kg) in mice displayed a remarkable antithrombotic efficacy with minimal impact on tail bleeding time, in comparison to aspirin and clopidogrel [22]. In the present study, S007-867 showed significant reduction in thrombus weight even at a low dose of 10μmol/kg in rat model of arterio venous shunt. The thrombus in arterio-venous shunt model closely mimics arterial thrombus and is mainly composed of platelets and fibrin [40]. The antithrombotic efficacy of S007-867 was comparable to that of standard drug aspirin and clopidogrel in minimizing thrombosis.

Another model used in the present study is ferric chloride induced arterial injury, which is routinely used for pre-clinical testing of antithrombotic compounds, either using a Doppler flow probe to record time of vessel occlusion, or using intravital microscopy to directly visualize thrombosed blood vessels in vivo. We have used both techniques to assess antithrombotic efficacy of S007-867 in mice. Using Doppler flow probe in carotid artery, we observed that S007-867 and clopidogrel significantly prolonged the time to occlusion (TTO), while aspirin did not show any protective effects. Collagen Type I is exposed following endothelial injury due to ferric chloride and has been visualized in vivo using anti-collagen antibodies [41]. Our results are in agreement with previous study that showed reduced thrombus formation in the absence or blocking of platelet collagen receptor GPVI indicating

a significant participation of platelet collagen interaction in this model [42]. Intravital microscopy has been routinely used to study the progression and mechanisms of thrombosis in vivo in real time, and is a highly useful technique to check the validity of in vitro or ex vivo experimental observations in the live animal. We observed that platelet deposition and the onset of thrombus formation in mesenteric artery were delayed in S007-867 treated mice in comparison to control. None of the S007-867 treated mice showed blood flow cessation within 30 minutes. Thus, S007-867 significantly maintained the blood flow velocity for a longer duration and delayed vessel occlusion.

Perioperative bleeding episodes is one of the major concerns with the use of current anticoagulants and anti-platelet drugs. An ideal antithrombotic agent is anticipated to maintain an appropriate balance between haemostasis and thrombosis. Screening antithrombotic test compounds in different bleeding models can be a good predictor of its hemorrhagic potential, because vascular beds in different organs vary in their structural composition, local tissue factor generation and other surface-dependent processes at the site of injury. We evaluated S007-867 in liver and spleen hemorrhagic model at its efficacy dose (30µmol/kg). The bleeding time was not significantly altered in either aspirin or S007-867 treated mice, while clopidogrel showed significant increase in spleen bleeding time. Therefore, S007-867 might offer some improvement with respect to the critical disadvantage of bleeding encountered with current antithrombotics. Additionally, owing to its minimal impact on hemostasis, it may be further tested in combination with standard anti-platelet drug (like aspirin) and anticoagulants.

Previous studies from our lab showed that S007-867 specifically inhibited collagen induced platelet aggregation in human plasma (IC_{50}=6µM), whilst other platelet activation pathways remained unhindered [22]. However its effect on the coagulation cascade is not known. The present study confirmed that the mode of action of S007-867 is platelet-mediated, as it did

not exhibit any effect on either the extrinsic or intrinsic coagulation cascade in plasma even at very high concentrations.

Following endothelial injury or atherosclerotic plaque rupture, collagens I and III are the predominant species exposed to the circulating platelets. The multiple collagen receptors on platelet surface act in tight synergy with each other to initiate platelet adhesion and thrombus formation. Platelets are transiently attached to collagen via VWF and its receptor-GP1b/IX/V. This interaction is further strengthened by platelet integrin $\alpha_2\beta_1$, which arrests platelet rolling and also co-operate with platelet GPVI to bind collagen, finally resulting in firm platelet adhesion and thrombus growth [43]. Our major finding in ferric chloride induced thrombosis model was sustained maintenance of blood flow velocity in S007-867 treated mice. This observation was further corroborated by whole blood perfusion assay over collagen and collagen-mimetic peptides, CRP, GFOGER and vWF-III. We performed whole blood perfusion assay with an aim to resolve the discrepancy with respect to specific collagen receptor being targeted by S007-867. Specific peptides and their combinations are useful in defining the individual role of platelet collagen receptors during thrombus formation. S007-867 did not show inhibition in thrombus formation when blood was perfused over VWF-III or a mixture of VWF-III and GFOGER, thereby ruling out GP1b/IX/V or $\alpha_2\beta_1$ as the main target of S007-867. It is noteworthy that S007-867 did not inhibit primary platelet adhesion as evidenced by its minimal inhibitory effect on surface coverage or thrombus volume over either collagen or combination of peptides. Interestingly the compound showed significant inhibition of thrombus volume (ZV_{50}) formed over a mixture of CRP, GFOGER and VWF-III peptide coated surfaces. This effect was seen as a striking change in thrombus morphology, with abundant but much smaller thrombi remaining. Since ZV_{50} is an indicator of thrombus height defined by platelet-platelet interaction and hence denotes the extent of platelet activation, it is assumed that S007-867 might be acting by either inhibiting GPVI receptor

activation or its immediate downstream signalling pathways. However, with strong agonists like polymeric collagen and CRP, weak inhibition was observed with S007-867 suggesting that strong stimulation of GPVI activation/signalling pathway can survive blockade by S007-867 and promotes good adhesion or activation.

Interestingly, the pharmacologically effective concentrations to inhibit in vitro platelet adhesion and activation are high (ranging from 30-300μM), while the same compound showed low effective dose in animal models (30μmol/kg) and low IC_{50} in plasma [22]. Previous study regarding pharmacokinetic assessment in mice has shown that S007-867 is rapidly absorbed after oral dosing, and hence exhibits fast onset of action [24]. S007-867 at a dose of 30μmol/kg (~13mg/kg) achieved maximal plasma concentration (C_{max}) upto 1823.33 ng/mL within 15 minutes in mice [24], and showed significant antithrombotic efficacy in mice at such low concentration detected in plasma. However, S007-867 owing to its low log P value, is a highly hydrophobic compound. Since most of the *in vitro* experimental assays rely upon the use of aqueous buffers which may induce precipitation or incomplete binding of hydrophobic compounds during the course of an experiment. Hence S007-867 is required in high concentrations (30-300μM) and showed high IC_{50} when used in buffers to effectively inhibit collagen induced platelet activation. Although the accurate assessment of its in vitro potency against platelet activation was somewhat difficult owing to its high hydrophobicity, the present study shows that S007-867 specifically inhibits collagen induced platelet activation in a concentration dependent manner.

Collagen strongly activates intra-platelet signalling events, which can be assessed by mapping global protein tyrosine phosphorylation. Upon collagen stimulation, platelet Syk undergoes autophosphorylation and binds to immunoreceptor tyrosine based activation motif (ITAM) of FcR-γ. This initiates downstream signalling via PLCγ-2 and generates second messenger inositol 1,4,5-trisphosphate and DAG. Intra-platelet $[Ca^{2+}]i$ is increased and other

signalling pathways are activated. S007-867 inhibits tyrosine phosphorylation of many proteins including PLCγ-2. Furthermore it also blocked intra-platelet [Ca^{2+}] flux, ATP release from platelet granules and thromboxane A_2 generation via COX-1 activation.

In conclusion, the present study, using in vivo experimental models in rats and mice, confirms that S007-867 inhibits arterial thrombosis and delays vessel occlusion time by inhibiting collagen induced platelet activation. It significantly inhibits collagen mediated platelet activation and subsequently reduced the release of ATP from dense granules and thromboxane A_2 via COX1 activation. S007-867 also inhibits collagen induced calcium flux and tyrosine phosphorylation of various proteins including PLCγ2. S007-867, being an orally active synthetic compound did not induce or promote bleeding, and hence offers better benefit to risk balance in pre-clinical screening and efficacy models. Therefore, S007-867 may be further developed and clinically deployed for the treatment of coronary and cerebral artery diseases.

Acknowledgements

The present work was financially supported by the intramural grant from Council of Scientific and Industrial Research (CSIR)-Central Drug Research Institute (CDRI), Lucknow. Financial assistance from CSIR- Network project THUNDER (BSC0102) to M. Dikshit is duly acknowledged. The authors are thankful for the award of fellowship by CSIR, New Delhi, India to A. Misra, P. Prakash, H. Aggarwal, and P. Dhankani. Authors acknowledge the technical help of Ms. Smriti Srivastava and Mr. Pankaj Shukla in animal experimentation. The authors also acknowledge the Cambridge-Indo Partnership Fund (CIPF) for sponsoring whole blood perfusion studies at Department of Biochemistry, Cambridge, UK. RW Farndale, N Pugh and D Bihan were supported by grants from Wellcome Trust (094470/Z/10/Z) and British Heart Foundation (RG/09/003/27122).

Conflict of Interest

The authors declare no conflict of interest.

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Figure Captions:

Figure 1: S007-867 dose dependently inhibits thrombogenesis in rat and mice models without altering kidney and spleen bleeding time. Rats and mice were orally administered with vehicle (0.5% carboxymethylcellulose), Aspirin (170µmol/kg), Clopidogrel (50µmol/kg) or S007-867 (3-300µmol/kg) 1 hour before thrombus induction. (A) Arterio-venous shunt model in rats was established as described in methods. The bar graph represents thrombus weight in different treatment groups (B) 10% ferric chloride solution was topically applied over carotid artery to induce thrombogenesis in mice. Bar graph represents fold change in total time to occlusion (TTO) of carotid artery. (C, D) Anaesthetized mice were subjected to standardized protocols of liver and spleen incision to assess bleeding tendency. Bleeding time represents the total time required to completely stop bleeding. All results are expressed as mean±SD (no. of animals=8-10/group). *P <0.05, **P <0.01 ***P <0.001 compared with vehicle treated animals.

Figure 2: S007-867 (30µmol/kg) prolongs blood flow velocity and time to occlusion in murine model of ferric chloride induced thrombosis in mesenteric artery as observed under intravital fluorescent microscopy. Mice were orally administered with vehicle or S007-867 (30 µmol/kg) 1 hour prior to arterial injury. (**A)** Time to occlusion. **(B)** Blood flow velocity at the indicated time points after vascular injury. **(C)** Representative images of thrombi were taken at 5, 10, 15, 20 and 32 minutes. Red arrows indicate the assembly of tiny thrombi and red circle shows formation of stable thrombus that finally stopped the blood flow. All results are expressed as mean ± SD of 6experiments. ** $P < 0.01$, *** $P < 0.001$ compared with vehicle treated animals.

Figure 3: S007-867 inhibits collagen mediated platelet aggregation and adhesion in mice (ex vivo) in a dose dependent manner. (A) Platelet rich plasma from mice pre-treated (1hour, per oral) with vehicle, S007-867 (10-300µmol/kg), Clopidogrel (100µmol/kg) and Aspirin

(100µmol/kg) was stimulated with collagen (5µg/ml), ADP (5µM) and arachidonic acid (0.25mM) under continuous stirring to initiate aggregation. (B) Quantitative assessment of static adhesion of mice platelets on collagen as observed under fluorescence microscope. (C) Representative images of murine platelets adhered over collagen coated coverslips and stained with phalloidin TRITC from different treatment groups (i) vehicle (ii) Aspirin (100µmol/kg) (iii) Clopidogrel (100 µmol/kg) (iv) S007-867 (10µmol/kg), (v) S007-867 (30µmol/kg), (vi) S007-867 (100µmol/kg) treated platelets over collagen coated surface (n=5) Data are shown as Mean ±SD. *P <0.05, ***P <0.001 compared with murine platelets treated with vehicle.

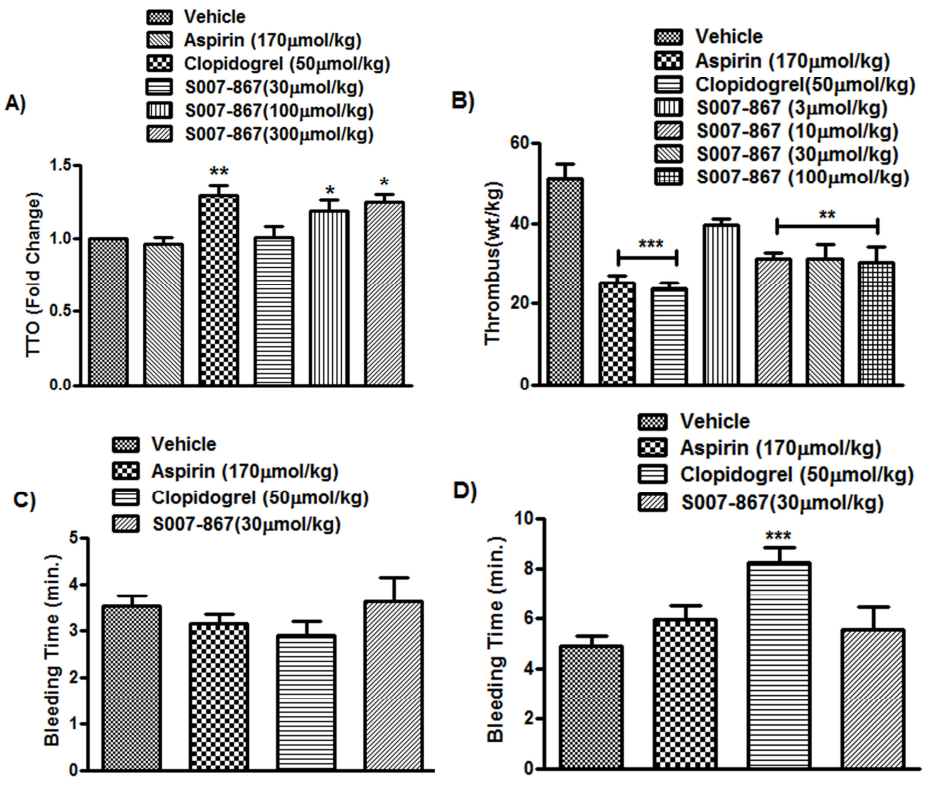
Figure 4: S007-867 inhibits platelet activation and thrombus formation when perfused over CRP-GFOGER-VWF-III coated surface. Glass Slides were coated with collagen (Coll I) or combinations of collagen peptides (CRP, GFOGER, VWF-III), and perfused with DiOC6-labeled whole blood (human), pre-treated with vehicle or S007-867 (100µM), for 5 minutes at $1000s^{-1}$shear rate. (A) Representative images of platelet thrombi formed following pretreatment with vehicle (upper panel) or S007-867 (100µM) (lower panel). Quantitative assessment of thrombus formed on different protein coated surfaces (B) Percent surface coverage, (C) Mean thrombus height (µM) (D) ZV_{50} (µM). Data are presented as the mean ± SD (n = 3). *P <0.05, compared with vehicle treated whole blood.

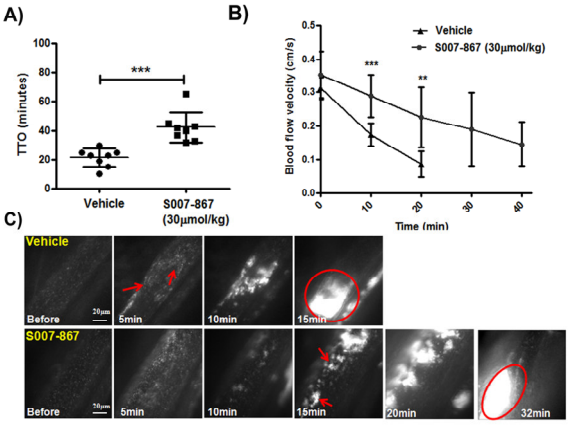
Figure 5: Effect of S007-867 on clotting parameters in human plasma, intra-platelet calcium flux, ATP secretion, thromboxane A_2 release, global tyrosine phosphorylation and phospholipase Cγ2 (PLCγ2) phosphorylation in collagen-stimulated platelets. **(A, B, C)** Platelet poor plasma was pre-incubated with S007-867 (30-300µM) or hirudin (1µg/ml) for 30 minutes followed by evaluation of Thrombin time (A) Prothrombin time (B) and

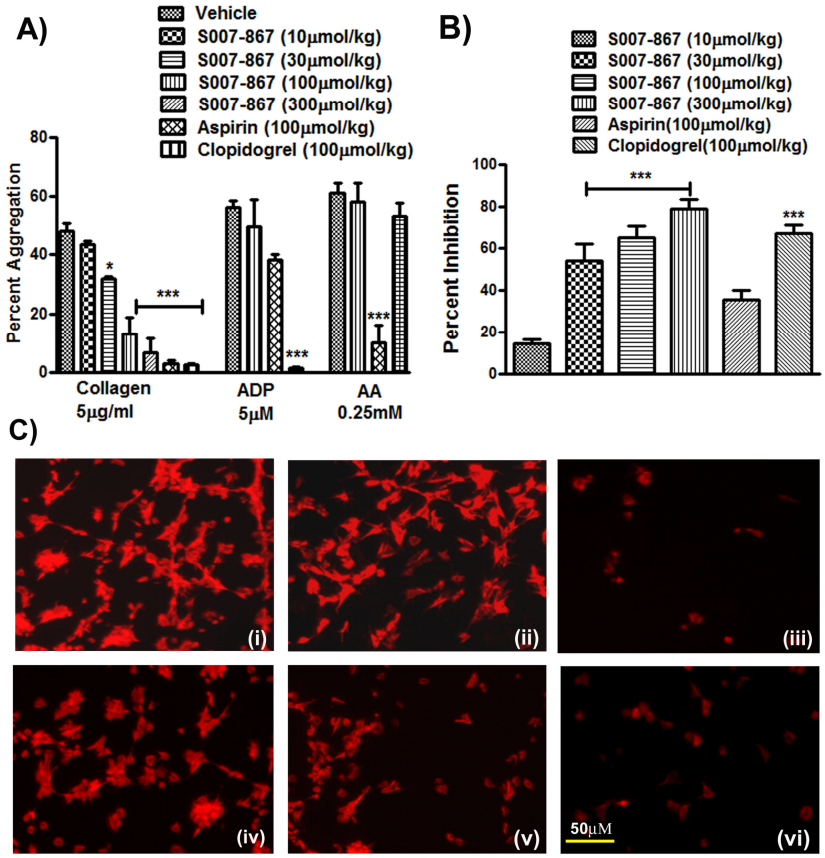
Activated partial thromboplastin time (C) in an automated coagulometer. For other experiments, washed platelets were pre-incubated with S007-867 (10-300μM) or 0.1% DMSO for 30 minutes, followed by stimulation with collagen (2μg/ml). Platelets were incubated with Fura 2 AM for 60 minutes followed by collagen stimulation to induce intra-platelet calcium mobilization. (D) Representative graph from 3 independent experiments. (E) Mean [Ca^{+2}] flux in treatment groups as a percentage of control. (F) Collagen induced release of ATP from dense granules was assessed by luciferin luciferase reaction in a lumiaggregometer. (G) The levels of TxB_2 were assayed in the supernatant of collagen stimulated washed platelet suspension. (H) collagen induced global tyrosine phosphorylation in platelets. Washed platelets pre-treated with S007-867 (10, 30, 100 and 300μM) or DMSO were stimulated with collagen (2μg/ml) and immunoblotted with anti-pTyr 4G10 or anti-actin (I) PLCγ2 was immunoprecipitated with anti-PLCγ2 and immunoblotted with anti-pTyr 4G10 or PLCγ2. The immunoblots are representative of three independent experiments under similar conditions. Upper panel in (I) represent the mean densitometric scans ±SD of three experiments. All the datasets are presented as mean ± SD (n = 3). *P <0.05, **P<0.01 and ***P < 0.001, compared with vehicle treated plasma or collagen stimulated platelets.

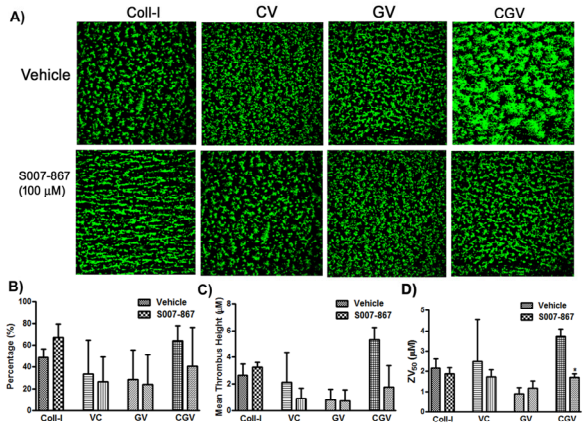
Video 1: Ferric chloride induced thrombus formation in mesenteric artery in vehicle treated mice. Fluorescence microscopy video shows assembly of platelet aggregates and formation of small thrombi, which gradually grows into stable thrombus and vessel occlusion in 15 minutes.

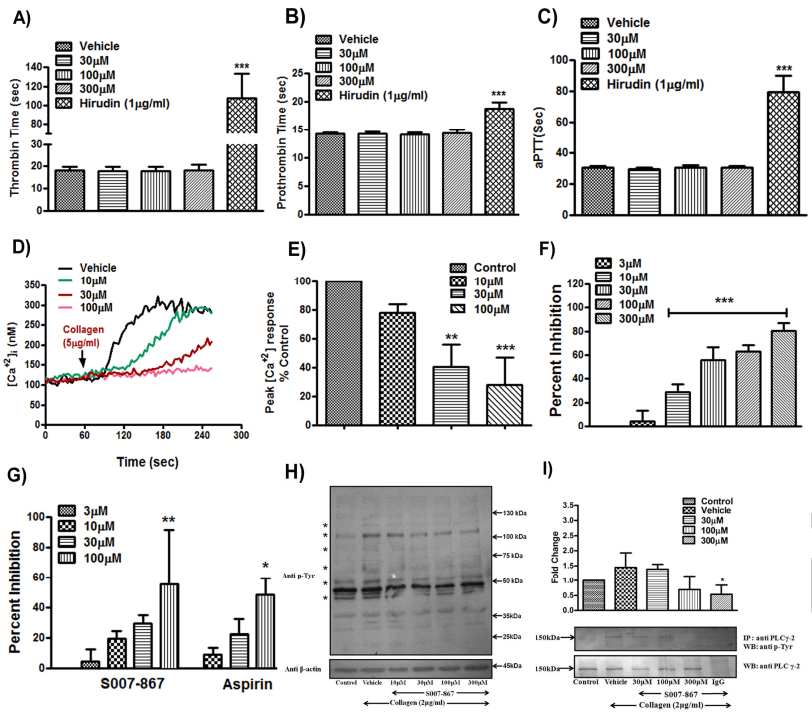
Video 2: Ferric chloride induced thrombus formation in mesenteric artery in S007-867 (30μmol/kg) treated mice. Fluorescence microscopy video shows sustained blood velocity upto 20 minutes and complete cessation of blood flow after 30 minutes.











A representative model of S007-867 mediated inhibition of collagen induced platelet activation, aggregation and intravascular thrombosis.