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ORIGINAL ARTICLE

Inhibition of adrenaline and adenosine diphosphate induced platelet aggregation by Lansberg's hognose pit viper (*Porthidium lansbergii hutmanni*) venom

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Abstract The haemostatic components of venom from the genus *Porthidium* has been poorly studied, although it is known that severe manifestations occur when humans are envenomed, which include invasive oedema and disseminated ecchymosis. The effects of venom on blood platelets are commonly studied and are normally carried out with platelet-rich plasma (PRP). A series of crude venom dilutions was used to determine the effects of adenosine diphosphate (2 μM) and adrenaline (11 μM) induced platelet aggregation. Venom of *Porthidium lansbergii hutmanni* was fractioned by anionic exchange chromatography, and the fractions were also used to determine the 50% inhibition of adenosine diphosphate (ADP) and adrenaline-induced platelet aggregating dose (AD50). Crude venom has more effect in inhibiting adrenaline-induced aggregation (AD50=0.0043 μg) followed by the adenosine diphosphate (AD50=17 μg). Peaks I and II obtained by chromatography also inhibited adrenaline-induced platelet aggregation with an AD50 of 3.2 and 0.013 μg, respectively, and both peaks inhibited ADP-induced platelet aggregation with an AD50 of 10 μg. The main purpose of this work was to characterise the in vitro effects caused by *P. lansbergii hutmanni* crude venom and its fractions on the platelet aggregation mediated by adrenaline and ADP agonists.

Keywords Adenosine diphosphate · Adrenaline · Platelet aggregation · *Porthidium lansbergii hutmanni* · Snake venom

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Introduction

Platelets play a significant role in the recognition of injured vessels, formation of haemostatic plugs, clot retraction, wound healing and prevention of bleeding. However, they can initiate events leading to clinical complications associated with cardiovascular disorders when they develop severe dysfunctions. ADP and adrenaline take part in a decisive role in platelet activation [1, 2], and their receptors are potential targets for anti-thrombosis drugs [3].

It is documented that venoms of Viperidae snakes cause severe manifestations through diverse pharmacological activities on the haemostasis, one being platelet aggregation dysfunctions [4–7]. The identification of certain proteins present in the snake venoms, which selectively act on vascular proteins of mammals, such as GPIb-IX-V, von Willebrand factor and integrins $\alpha 2\beta 1$ and $\alpha_{IIb}\beta 3$ has been reported [8]. Lansberg's hognose pit viper (*Porthidium* sp.) venom components have been poorly studied; however, it has been reported that humans bitten by these snakes present severe manifestations such as invasive oedema and disseminated ecchymosis [9].

This study reports the inhibitory effects of ADP and adrenaline-induced platelet aggregation by venom and venom fractions of the Venezuelan snake *Porthidium lansbergii hutmanni*.

Table 1 Comparison of adrenaline-induced platelet aggregation inhibition (AD_{50}) of *P. lansbergii hutmanni* crude venom and its peaks I and II obtained from anion exchange chromatography with isolated fractions and crude venom of other reported reptile species

Species	AD_{50}	Mechanism	References
Echis carinatus	13.8 μg/ml	PLA_2-acid	[21]
Heloderma horridum	8 μg/ml	PLA_2-acid	[10]
Porthidium lansbergii hutmanni (Plh)	0.0043 μg[a]	N.D.	–
Plh Peak I/mono-Q1	3.2 μg	N.D.	–
Plh Peak II/mono-Q1	0.0130 μg[a]	N.D.	–

N.D. Not determined
[a] Calculated theoretical value

Materials and methods

Porthidium lansbergii hutmanni venom

Crude venom of *P. lansbergii hutmanni* (CV*Plh*) was obtained from 12 adult snakes captured in Juan Griego and Porlamar, Margarita Island, Venezuela. The snakes were maintained in captivity in the Serpentarium of the Laboratory of Investigations, Pharmacy Faculty of the Universidad Central de Venezuela. The venom was dehydrated in a vacuum desiccator maintained with calcium chloride at 4°C until total crystallisation.

Blood samples

Blood samples were collected from healthy donors at the Municipal Blood Bank of Caracas. The samples were collected in plastic tubes containing 3.8% sodium citrate (1:9 citrate/blood). Patients were chosen according to the Blood Bank norms, and they signed a consent form, which was required from each donor.

Platelet-rich plasma

Platelet-rich plasma (PRP) was obtained by slow (90–120 g) centrifugation (Sorval RC3, model rotor HG4L) for 15 min and incubated at 37°C before aggregation studies.

Platelet-poor plasma

Platelet-poor plasma (PPP) was obtained by centrifugation at 120 g for 10 min, and then the plasma without cells was extracted and incubated at 37°C before aggregation studies.

Platelet-rich plasma quantification

The quantification of the platelets was carried out in a Coulter model T-890. The final concentration was adjusted at 3×10^8 platelets/ml diluted with PPP [5].

The effects of *Porthidium lansbergii hutmanni* crude venom and its fractions on platelet aggregation

A modified method of Huang and Chiang [10] was used to test platelet aggregation on a Chrono-Log aggregometer (Chrono-Log Pennsylvania, USA). Briefly, the inhibition of platelet aggregation was carried out by mixing 440 μl of PRP (3×10^8 cells/ml) +50 μl of crude venom or fractions +ADP (2 μM, 10 μl) or adrenaline (11 μM, 20 μl). All aggregation curves were registered for 5 min. Platelet-aggregating controls of ADP and adrenaline were run in the absence of venom samples. The percent inhibition of platelet aggregation with venom and fractions was compared to the ADP and adrenaline controls. The AD50 is defined as the dose of venom (μg) that was able to reduce inhibition by 50% compared to the controls. A mean of three trials (n=3) were reported for each sample.

Table 2 Comparison of ADP induced platelet aggregation inhibition (AD_{50}) of *P. lansbergii hutmanni* crude venom and its peaks I and II obtained from anion exchange chromatography with isolated fractions and crude venom of other reported reptile species

Species	AD_{50}	Mechanism	References
Trimeresurus gramineus	50 μg/ml[a]	5′nucleotidase	[22]
Agkistrodon acutus	100 μg/ml[a] (31% inhib)	5′nucleotidase	[23]
Agkistrodon rhodostoma	13.4 μg/ml[a]	Dimeric protein	[24]
Echis carinatus	>20 μg/450 μl PRP[a]	PLA_2-acid	[21]
Lachesis muta	>30 μg/ml[a]	PLA_2-acid	[6]
Agkistrodon piscivorus piscivorus	103 nM[a]	Piscivostatin dimeric disintegrin (GPIIb/IIIa)	[25]
Porthidium lansbergii hutmanni (Plh)	17 μg	N.D.	–
Plh Peak I/mono-Q1	10 μg (53.4%)	N.D.	–
Plh Peak II/mono-Q1	10 μg (43.8%)	N.D.	–

[a] IC_{50}: The amount of protein concentration that will inhibit platelet aggregation by 50%.

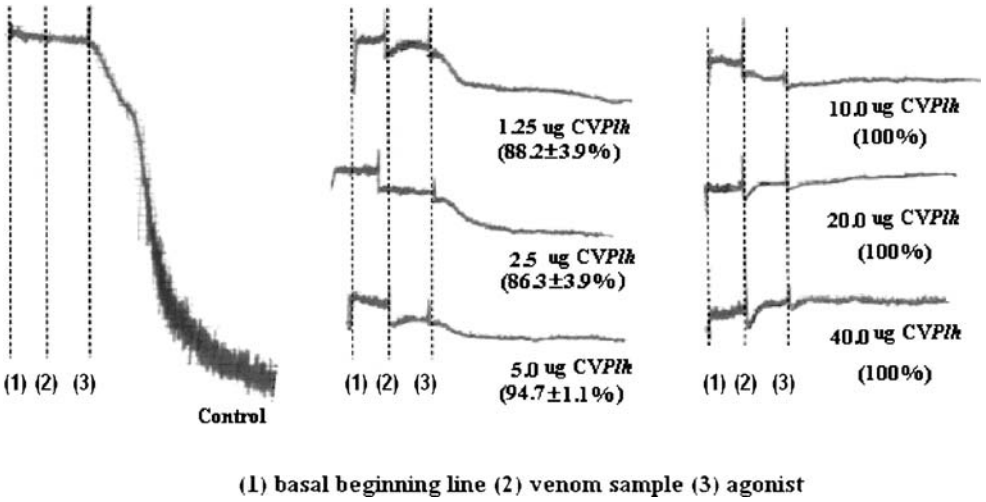

Fig. 1 Curves of platelet aggregation mediated by adrenaline (0.385 μM) and inhibited by *P. lansbergii hutmanni* crude venom

Crude venom fractionations by ion interchange chromatography

A total of 20 mg/ml of venom was fractioned by an anionic exchange mono-Q1 (Biorad, USA) column using a Biologic work station (Biorad). A 50 mM Tris–HCl pH 8.3 with KCl saline gradient from 0 to 0.5 M with a constant pH was used. The proteins were monitored at 280 nm at a flow rate of 1 ml/min. Collected venom fractions were dialysed against distilled water for 48 h using a 2 kDa molecular weight cut-off membrane (Sigma, USA), then were lyophilised and stored at 4°C until use.

Determination of protein concentration

Protein concentrations of crude venom and fractions were measured by the Lowry method [11].

Results

CV*Plh* in vitro effect on the platelet aggregation

CV*Plh* was able to inhibit ADP and adrenaline-induced platelet aggregation in a dose-dependent manner using PRP. Tables 1 and 2 show the comparison of the AD_{50}s of CV*Plh* and its anionic peaks I and II mediated by adrenaline and ADP, respectively, to other reported reptile venoms. The AD_{50} of CV*Plh* (0.0043 μg) in Table 1 is a theoretical value calculated based on the control, as it was only possible to inhibit 84% with the lowest dose of venom used.

Figure 1 shows the inhibition of adrenaline (0.385 μM final concentration) induced platelet aggregation by varying doses of CV*Plh*. Significant inhibition of platelet aggregation was still present at the lowest dose of venom

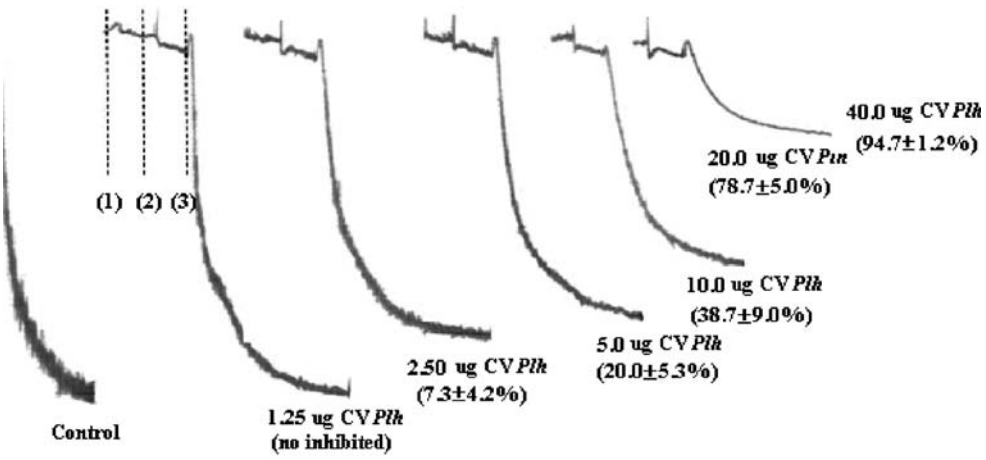

Fig. 2 Curves of platelet aggregation mediated by ADP [0.035 μM] and inhibited by *P. lansbergii hutmanni* crude venom

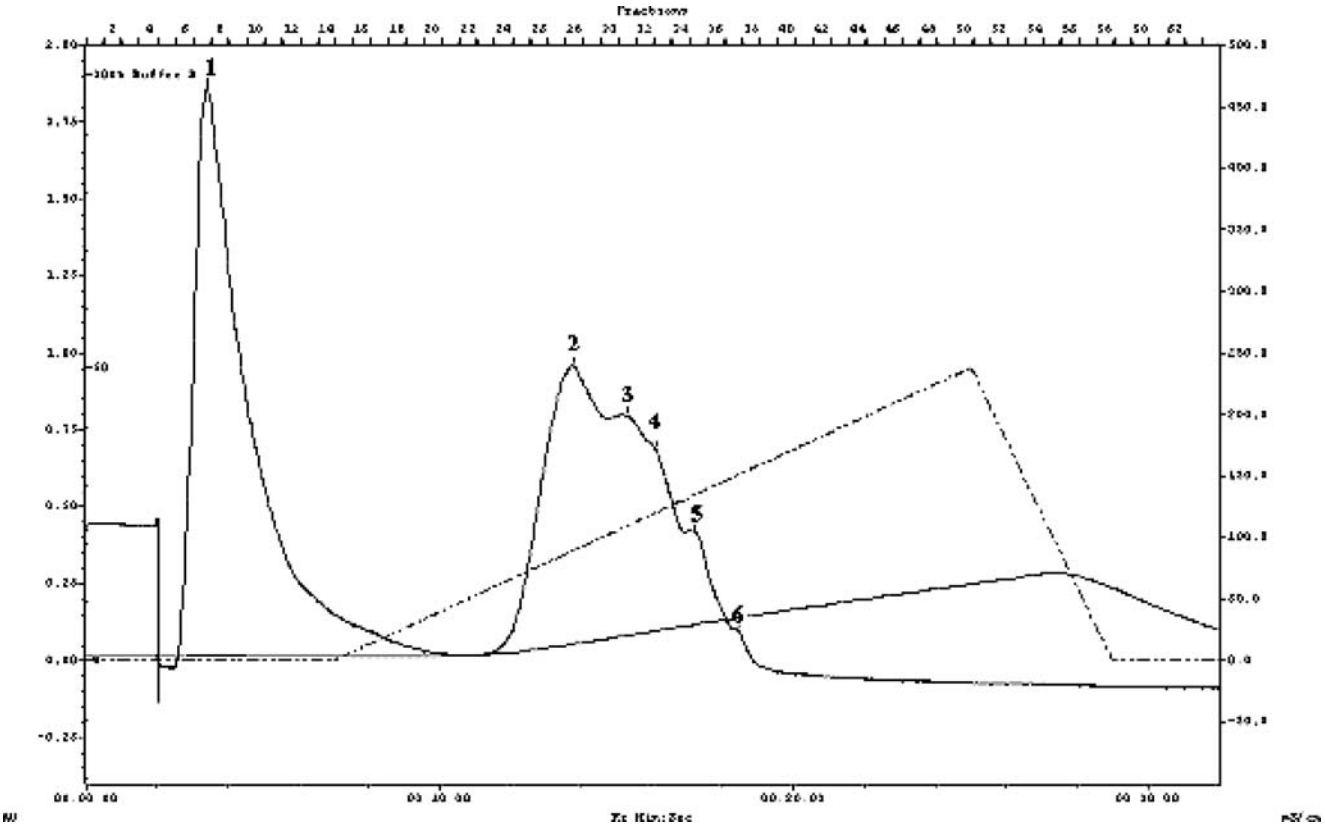

Fig. 3 Crude *P. lansbergii hutmanni* venom anion exchange (mono-Q1) chromatography using a 50 mM Tris–HCl, pH 8.3/0.5M KCl buffer

used (1.25 μg). Figure 2 shows the inhibition of ADP (0.035 μM final concentration) induced platelet aggregation by varying doses of CV*Plh*. The AD_{50} CV*Plh* was calculated based on the adrenaline and ADP controls.

Ionic interchange chromatography

Figure 3 shows CV*Plh* fractions collected with a Mono-Q1 (Biorad) chromatography column. Peak I was eluted in the void volume and peak II was eluted after 10 min during the salt gradient. Each peak was immediately dialysed, lyophilised and stored at 4°C until further use.

The effects of adrenaline and ADP-induced platelet aggregation by *Porthidium lansbergii hutmanni* venom peaks I and II

Figure 4a and b are the curves showing the inhibition of adrenaline induced platelet aggregation by peaks I and II. The AD_{50} for peaks I and II were 3.2 and 0.0130 μg, respectively.

Figure 5a and b are the curves showing the inhibition of ADP-induced platelet aggregation by peaks I and II. The AD_{50} for peaks I and II were ~10.0 μg for both.

Discussion

The impact of snake venoms on the haemostatic system and their ability to alter platelet functions remains well known [12]. A critical dysfunction in platelets associated with snake envenoming may result in abnormal platelet aggregation that could contribute to the risk of severe thrombosis.

In this study, *P. lansbergii hutmanni* crude venom and its fractions were observed for their effects on platelet aggregation induced by adrenaline and ADP. *P. lansbergii hutmanni* venom and fractions exhibited a potent platelet aggregation inhibitory activity induced by both of the agonists. Nevertheless, the effectiveness of inhibition was different with each sample. Inhibition of aggregation of washed platelets was not the same as PRP. The presence of plasma phospholipids, which probably act as substrates to phospholipases present in the *Plh* venom, may contain components that contribute to the inhibition of platelet aggregation [13].

With regard to agonists, adrenaline-induced rat platelet aggregation through alpha2-adrenergic receptors, as is the case with human platelets [14] and ADP, were recognised as factors resulting from erythrocytes that allowed platelets to adhere to glass [1], and thus inducing platelet aggrega-

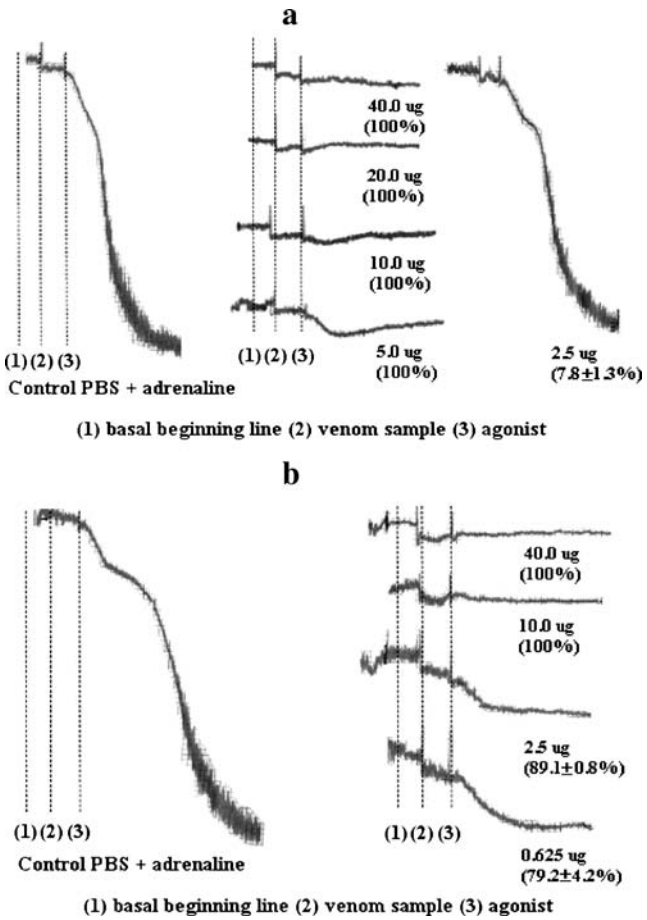

Fig. 4 Curves of platelet aggregation mediated by adrenaline (0.385 μM) and inhibited by peaks I (**a**) and II (**b**) mono-Q1 from *P. lansbergii hutmanni* crude venom

tion [15]. These alpha2-adrenergic receptors are abundant in platelets [16], and their critical role in physiological haemostasis has been documented [17]. ADP effects are mediated by three separate P2 receptors on blood platelets. These receptors play selective roles in platelet activation and thrombus formation, which means that they are desirable targets for anti-platelet drugs [18].

The inhibition observed when CV*Plh* suggests that the sum of inhibitors might be acting directly on the platelets. The CV*Plh* clearly inhibited in a dose-dependent manner the platelet aggregation mediated by adrenaline and ADP, resulting in adrenaline-induced aggregation being more inhibited than ADP-induced platelet aggregation.

The inhibitory activity of platelet aggregation by *P. lansbergii hutmanni* peaks was different. Peak II was more effective in inhibiting adrenaline-induced platelet aggregation than peak I (Fig. 4a,b). However, the inhibitory effect of peak I at a low dose of 5 μg as compared to a higher dose of 10 μg for peak II could be due to the presence of inhibitors in the venom, probably acidic proteins (phospholipases), which are more concentrated in peak I than in peak II.

These anti-aggregating inhibitors found in these fractions are important and could be of medical effectiveness. For instance, reversible inhibition of platelets aggregation during cardiovascular surgery may be a striking tactic to guard platelets and stabilise postoperative bleeding times. Moreover, authors [19] reported that some disintegrins isolated from snake venoms, such as echistatin (*Echis*

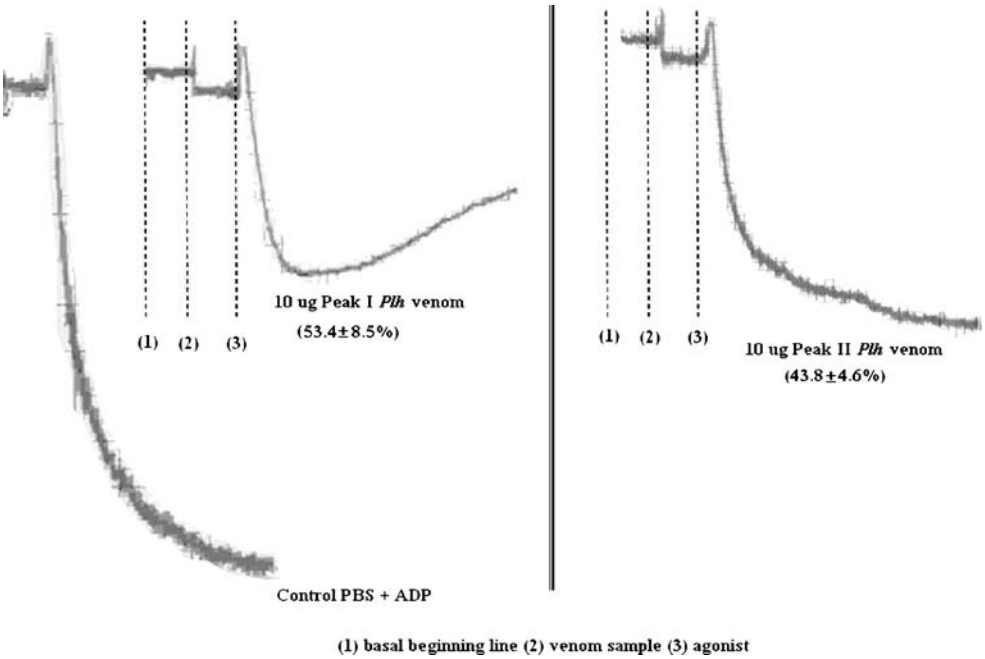

Fig. 5 Curves of platelet aggregation mediated by ADP (0.035 μM) and inhibited by peaks I (**a**) and II (**b**) (mono-Q1 from *P. l. hutmanni* crude venom)

carinatus), a 5 kDa disintegrin, which is a strong competitive inhibitor of platelet alpha (IIb) beta-3 binding to fibrinogen, reversibly inhibit platelets by different mechanisms. They proposed the hypothesis that reduced doses of echistatin provide platelet protection during simulated extracorporeal circulation and concluded that echistatin at doses that are incapable of producing clinical side effects entirely inhibit platelet activation and conserve platelet function during in vitro extracorporeal circulation.

Another potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom has been described [20]; triflavin is a single-chain polypeptide, with a molecular mass of 76 kDa, and in a dose-dependent manner inhibits ADP and adrenaline (including other agonists) induced human platelet aggregation. Its IC_{50} ranged from 38 to 84 nM, depending on the aggregation inducer used and the platelet preparation. Furthermore, triflavin blocked I^{25I}-labelled fibrinogen binding to ADP-activated platelets.

In the current results, CV*Plh* at varying doses from 1.25 to 40 μg showed different inhibitory effects on platelet aggregation caused by adrenalin and ADP and thus implying that the venom may not block a common step shared by these agonists (Figs. 1 and 2). It also points out that the site of action of inhibitors present is not at the receptor level. Results from peaks I and II also revealed that the inhibitors are present in both peaks although they differ in activity.

Overall, our results indicate that partially purified anti-aggregation factors from CV*Plh* may become relevant tools for anti-thrombosis strategies. Furthermore, anti-platelet therapy plays an important role in the treatment of atherosclerosis and its multifactorial clinical manifestations. Understanding the detailed interactions between platelets and the endothelium may lead to the progress of novel therapeutic strategies. These potential results permit the use of these compounds in established experimental thrombosis in higher animal models. Additional steps for this project will be to further purify these inhibitors and explore their properties on platelets to evaluate their potential as anti-thrombosis agents.

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