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Research Article

Pharmacological Actions of Adapalene on Platelet Function and Thrombosis through the PI3K/AKT Signaling Pathway

Liyuan Zhu[1,4], Congqin Chen[1], Yingnan Liao[1,2,3*]

[1]Xiamen Key Laboratory of Cardiovascular Disease, Xiamen Cardiovascular Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen, China.

[2]Sichuan Provincial Key Laboratory for Human Disease Gene Study and the Center for Medical Genetics, Department of Laboratory Medicine, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China.

[3]Research Unit for Blindness Prevention, Chinese Academy of Medical Sciences (2019RU026), Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, China.

[4]Center of Clinical Pharmacology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China.

[*]**Corresponding author:** Yingnan Liao, Xiamen Cardiovascular Hospital, Xiamen University, 2999 Jinshan Road, Xiamen 361000, Fujian Province, People's Republic of China.

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Abstract

Background and aims: Given the global impact of Cardiovascular Disease (CVD) linked to abnormal platelet activation, there is an urgent demand for the development of safer and more effective antiplatelet agents. Adapalene, an FDA-approved anti-acne medication, has recently been repurposed as a third-generation synthetic retinoid for tumor treatment. The exploration of novel clinical applications represents a focused approach in the realm of drug discovery and development. Our research aimed to elucidate the impact of adapalene on platelet functionality and the associated molecular mechanisms. **Method:** Mouse or human platelets were thoroughly washed and then pretreated with adapalene or Dimethyl Sulfoxide (DMSO). Subsequently, their platelet aggregation capacities were assessed using an aggregometer. Flow cytometry was used to evaluate granule secretion and integrin αIIbβ3 activation, while platelet spreading on fibrinogen was visualized via the staining of FITC-phalloidin. To evaluate the *in vivo* antithrombotic effects of adapalene, a mesenteric artery thrombus formation model induced by FeCl3 was utilized. Changes in critical signaling pathways were detected through Western blotting analysis. **Results:** Adapalene exhibited a concentration-dependent inhibition of thrombin- and ADP (adenosine diphosphate)-induced platelet aggregation. This was accompanied by reductions in the membrane translocation of CD62P and LAMP-1, as well as decreased ATP release. Furthermore, adapalene effectively attenuated integrin αIIbβ3-mediated inside-out and outside-in signaling processes, impacting platelet adhesion and spreading. Key signaling pathways including Phosphoinositide 3 Kinase (PI3K)/Protein Kinase B (AKT)/Glycogen Synthase Kinase (GSK3β), SRC proto-oncogene, nonreceptor Tyrosine Kinase (Src)/Spleen Tyrosine Kinase (Syk)/Phospholipase C Gamma 2 (PLCγ2), and Mitogen-Activated Protein Kinase (MAPK) were notably counteracted by adapalene. Intravenous administration of adapalene significantly prolonged mesenteric artery occlusion times in mice without a concurrent increase in tail bleeding events. **Conclusions:** Our findings uncovered a novel pharmacological mechanism of action of adapalene in suppressing platelet activity and thrombosis formation through the inhibition of agonist-induced platelet signal transduction pathways, specifically targeting the PI3K/AKT/GSK3β, SRC/Syk/PLCγ2, and MAPK pathways. These results suggest that adapalene holds promise as a prospective therapeutic agent for the management and prophylaxis of thrombotic cardiovascular disorders.

Keywords: Adapalene; Thrombosis; Platelet; Aggregation; PI3K/AKT.

Abbreviations

ADP: Adenosine diphosphate

AKT: Protein kinase B

CDK: Cell cycle independent kinase

CHD: Coronary heart disease

COX-1: Cyclooxygenase-1

CVD: Cardiovascular disease

ERK: Extracellular signal-regulated kinase

FAK: Focal adhesion kinase

GSK3β: Glycogen synthase kinase

ITAM: Immunoreceptor tyrosine-based activation motif

MAPK: Mitogen-activated protein kinase

PI3K: Phosphoinositide 3 kinase

PLCγ2: Phospholipase C gamma 2

PT: Prothrombin time

RAR: Retinoid acid receptor

Src: SRC proto-oncogene, nonreceptor tyrosine kinase

Syk: Spleen tyrosine kinase

TT: Thrombin time

TXA2: Thromboxane A2

Introduction

Cardiovascular Disease (CVD) poses serious threats to human life and health and has become a leading cause of mortality worldwide. It is well documented that hyper or irregular platelet activities are involved in the development of CVDs, such as Coronary Heart Disease (CHD), Ischemic Stroke (IS) and acute myocardial infarction [1-3]. Under physiological conditions, the most prominent function of platelets is hemostasis and thrombosis; however, in the context of vascular injury or atherosclerosis [4], hyperactivation of platelets can result in hemostatic plugs or pathologic thrombus formation [1]. Currently, antiplatelet medications remain cornerstone therapies for treating CVD [5,6], and the most frequently used groups of antiplatelet drugs are aspirin, clopidogrel and prasugrel. However, a number of antiplatelet agents have certain disadvantages or side effects, which lead to under- or overtreatment in individuals. For instance, aspirin has been extensively applied for treating cardio-cerebrovascular diseases, especially in the prevention of arterial thrombosis. It is also used in the primary and secondary prevention of cardio- and cerebrovascular arterial thrombosis. Low-dose aspirin irreversibly inhibits the synthesis of Thromboxane A2 (TXA2) by acetylating the serine residue at position 529 of Cyclooxygenase-1 (COX-1) in platelets [7]. However, high-dose aspirin has obvious adverse effects (e.g., gastrointestinal tract adverse reactions and susceptibility to gastric ulceration). Clinical resistance and biochemical resistance to aspirin may coexist; although aspirin is administered at recommended dosages in human subjects, platelet activation is still persistent. Clopidogrel and prasugrel are P2Y12 receptor inhibitors with strong selectivity. Both require cytochrome P450 enzyme preactivation *in vivo* before binding to the P2Y12 receptor and play roles in antiaggregatory effects [8]. Clopidogrel has at least three defects: one is the time-lagged effect; the second is the high individual variation in response; and the third is the irreversible and strong blockage of P2Y12 receptors [9]. Prasugrel causes increased bleeding risks or life-threating hemorrhage. Hence, the development of novel medications with effective antiplatelet activity and fewer side effects remains a major subject of vast clinical implications, and it has become an important cutting-edge research topic.

Adapalene (Ada) is a Food and Drug Administration-approved third-generation retinoic acid that binds to the retinoic acid nuclear receptor and has become a front-line drug for acne [10] (Figure 1A). It is known that it has established roles, including anti-acne, anti-inflammatory [11], anti-proliferation [12,13] and immunomodulatory effects [14]. Adapalene has recently been discovered to be potent for skin diseases [15] and photoaging [16] beyond its efficacy in the treatment of acne vulgaris. Currently, adapalene may be a valuable therapeutic reagent for the treatment of multiple types of cancer, such as colorectal cancer [17], ovarian cancer [18] and prostate cancer [19]. Recent findings indicate that nanoparticles encapsulated with adapalene can sustain biological activity in the central nervous system for at least 24 hours [20]. It was also reported that adapalene modulates polymorphonuclear leukocyte activities by inhibiting leukotriene production, the lipoxygenase pathway, and oxygen free radical release [21,22]. Although adapalene displays diverse biological functions and pharmacological benefits, the mechanism of action and structure–activity relationship studies show that the biological potential of adapalene is currently underutilized [23]. Its potential roles in regulating thrombotic-related CAD are completely unknown. Therefore, based on the concept of a "conventional drug in new use", the present study aimed to evaluate the pharmacological actions of adapalene in platelet function and thrombosis *in vitro* and *in vivo*, as well as to discover the underlying molecular mechanism.

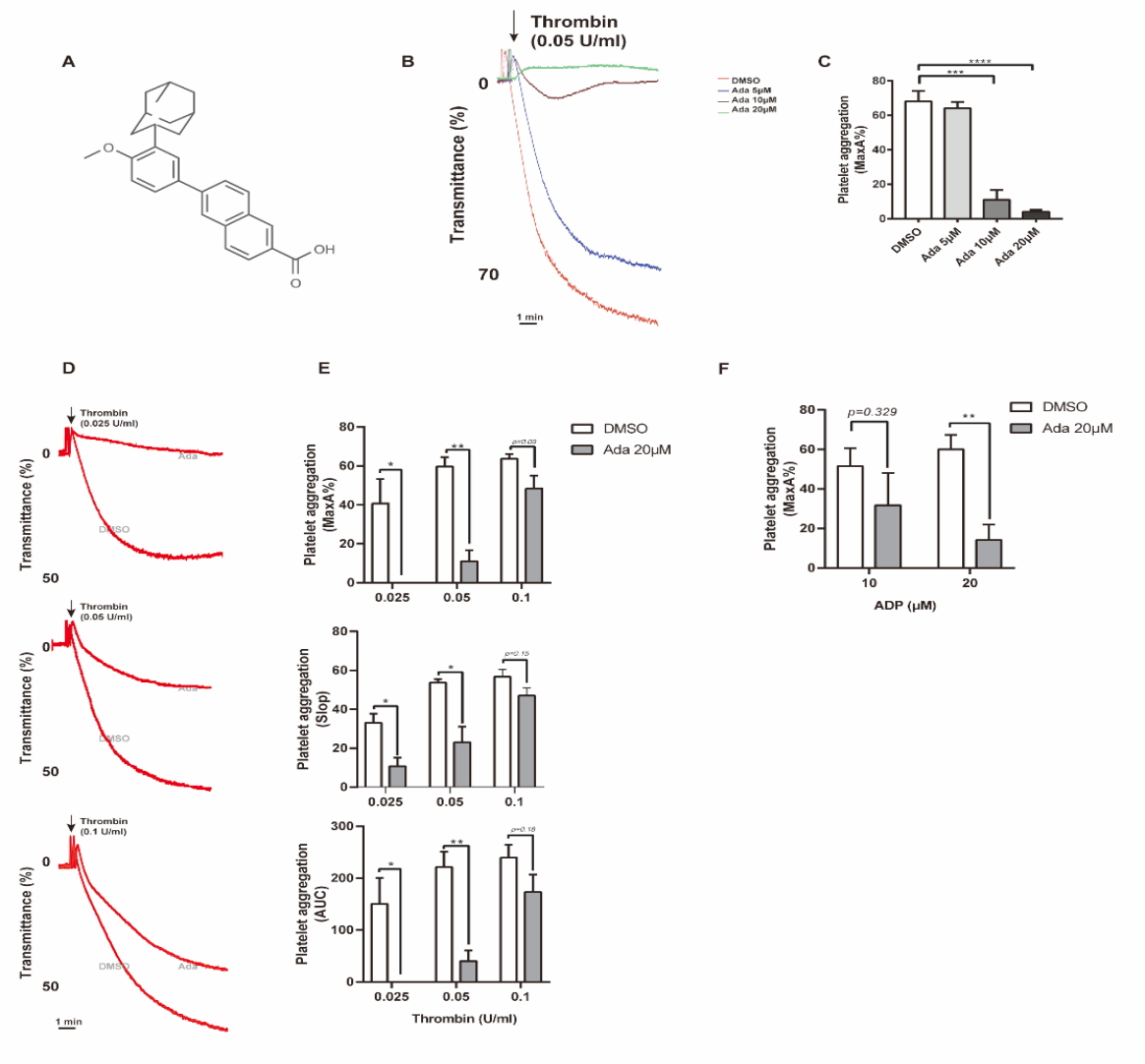


Figure 1: Effects of adapalene on platelet aggregation.

Washed mouse platelets were preincubated with adapalene or solvent (0.1% DMSO) for 10 min and subjected to an aggregation assay stimulated with the indicated agonists. (A) Chemical structure of Adapalene (Ada). (B&C) Representative curves showing the inhibitory effects of Ada (5, 10, 20 μM) on thrombin (0.05 U/mL)-triggered aggregation and statistical evaluation. (D & E) The responses of 20 μM Ada to increasing doses of thrombin-induced aggregation as well as evaluations by the max aggregation rate, slope and Area Under the Curve (AUC). (F) Bar graph showing the reactions of Ada (20 μM) on ADP (10 and 20 μM)-stimulated aggregation. All the data were obtained from at least three independent experiments, $^{**}p<0.01$ and $^{***}p<0.001$ vs. control.

Materials and Methods

Chemicals and Reagents

Adapalene (Targetmol, Catalog No. T1093), prostaglandin E1 (Sigma, Catalog No. P5515), APC-conjugated anti-CD62P (P-selectin) antibody and PE-conjugated anti-JonA (αIIbβ3) antibody were purchased from Biolegend. Anti-phospho-PI3K (Ser1070), anti-PI3K, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-GSK3β (Ser9), antiGSK3β, anti-phoshpo-PLCγ2 (Tyr1217), anti-PLCγ2, anti-β3, anti-phospho-β3 (Tyr474), anti-Syk, anti-phospho-Syk (Tyr525/526), anti-SRC and anti-phospho-SRC (Tyr525/526) antibodies were purchased from Cell Signaling Technology.

Platelet Preparation

This study was conducted in compliance with all the applicable institutional ethical guidelines for the care, welfare and use of animal. In our SPF, animal health was monitored once every one month. Animals were euthanized by intravenous injection of sodium pentobarbitone (240 mg/kg).

The specific criteria used to determine when animals should be euthanized as follows:

- The animal is near death or immobile, or no response after gentle stimulation;
- Dyspnea: typical symptoms are salivation and (or) cyanosis;
- Diarrhea or incontinence;
- Weight loss (20% of the pre-experiment weight);
- Inability to eat or drink;
- Symptoms that include obvious anxiety, restlessness or tumor weight exceeds 10% of the animal's own weight;
- Animal develops paralysis, perseverative epilepsy or stereotyped behaviors;
- Peripheral dermal injury accounts for over 30% of a whole body;
- Other situations where the veterinarian determines that a humane end point is required.

One hundred and twenty mice were used for platelet aggregation and ATP release assay, flow cytometric analysis and platelet adhesion and spreading assay (all mice were euthanized at the end of the experiment). After anesthesia with pentobarbital sodium (80 mg/kg intravenously), nearly 1 ml blood of C57BL6 mice (8-12 weeks) was slowly drawn from the inferior vena cava into a 1 mL syringe prefilled with 3.8% sodium citrate. Samples were mixed with equal amounts of prewarmed Tyrode's buffer containing 1 μM PGE1 and centrifuged at 1000 rpm for 10 min at room temperature to obtain Platelet-Rich Plasma (PRP). After a further centrifugation of PRP at 2100 rpm for an additional 15 min, platelet precipitates were collected and resuspended with an appropriate amount of Tyrode's buffer.

Platelet Aggregation and ATP Release Assay

For the aggregation assay, 250 μL of platelet suspension (3 × 108/mL) was preincubated with solvent (10% DMSO) or different concentrations of adapalene at 37°C for 10 min. The aggregation reaction was activated after adding $CaCl_2$ (1 mM) and agonists at the indicated doses. An aggregometer (Chronolog, model 700) dynamically recorded the platelet aggregation curve for 5 min with stirring (1200 rpm) at 37°C. Supernatants from the aggregation assay were harvested following 5 min of centrifugation at 3500 rpm at 4°C and further subjected to ATP release assessment by using a luciferase activity-based ATP content determination kit (Beyotime, Cat.S0026).

Flow Cytometric Analysis

After solvent or adapalene pretreatment, the platelet suspension (100 μL, 3 × 107/mL) was stained with antibody mixtures of BV510-CD41 with PE-CD62P, APC-LAMP1 or PE-JONA antibody and stimulated with thrombin (0.025 U/mL) at 37°C for 10 min. The reaction was terminated by the addition of 1 mL PBS and further subjected to FACS analysis with a BD LSR II Flow Cytometer (BD Biosciences, USA).

Platelet Adhesion and Spreading Assay

Coverslips were precoated with fibrinogen (50 μg/mL) at 4C overnight. After being washed with PBS, the coated wells were blocked with PBS containing 1% BSA for another 1 hour. Platelet suspensions (250 μL, 3 × 107/ml) were preincubated with DMSO or adapalene at the indicated concentrations at 37°C for 10 min and then allowed to spread on fibrinogen matrix for different time periods at 37°C with stimulation with 0.025 U/mL thrombin and 1 mM $CaCl_2$. All the samples were transferred into the well. After 15 min, 30 min and 60 min in an incubator, nonadherent platelets were carefully washed away, and adherent platelets were fixed for 15 min in 4% paraformaldehyde. The samples were washed three times with PBS, stained with TRITC phalloidin (final concentration of 1 μg/ml) and incubated at room temperature for 1 h in the dark. Samples were viewed and photographed by fluorescence. Five visual fields were randomly captured under the microscope and quantified by Image J software.

Immunoblot Analysis

Platelet lysates were prepared with 4 × RIPA lysis buffer (containing protease inhibitor and phosphatase inhibitor). Once transparent, the solution was placed on ice for 30 min and centrifuged at 4°C and 12000 rpm for 15 min, and the supernatant was taken as the protein. A 1/4 volume of 5 × SDS–PAGE sample buffer was added, and the protein was denatured by boiling in a metal bath at 98°C for 10 minutes. After gel preparation, protein electrophoresis, transfer, and blocking, the membranes were incubated overnight with the indicated primary antibodies followed by incubation with HRP-conjugated secondary antibodies. Electrochemical Luminescence (ECL) was used to detect the protein gel blot signals on an exposure machine (ChemiDoc MP system, Bio-Rad, USA).

Ferric chloride-induced mesenteric arterial thrombosis model

Twenty-five mice were used for $FeCl_3$-induced mesenteric vessel injury (all mice were euthanized at the end of the experiment). The mice were anesthetized using 5% inhaled isoflurane and placed on 37°C warming pads, and an incision of approximately 2 cm was made in the ventral midline of the abdomen to expose the mesentery. Then, it was removed and placed on cotton containing physiological saline. Next, a piece of filter paper (1×1 mm^2) saturated with 10% $FeCl_3$ solution was placed on the surface of the mesenteric arteriole for 5 minutes, and the blood flow signals were monitored by a Laser Speckle Blood Monitor (PERIMED, PeriCam PSI Z, Sweden). The vascular occlusion time was recorded for 15 minutes, and the vascular occlusion time was defined as the time without blood flow for 5 minutes. Animal death was confirmed when there were no indications of life (no ups and downs in the chest, white eyelids, no visual response, etc.).

Tail bleeding assay

Male C57 mice weighing 20-25 g were assigned to the solvent control group and the adapalene group (5 mg/kg). Twenty-two mice for tail bleeding (all mice were alive and kept feeding at the end of the study). After intravenous administration, the mice were allowed to move freely for 30 minutes, anesthetized mice with 4% isoflurane were placed on a heating pad before cutting 3 mm of the distal tip of the tail, and the tail was immediately put into preheated PBS at 37°C. Mice were maintained with oxygen and anesthetics (1–2% isoflurane). Bleeding time was recorded as the sum of bleeding within 20 minutes, including rebleeding.

Coagulation function test

Blood was collected from healthy human volunteers and centrifuged at 4000 rpm for 15 min to obtain Platelet-Poor Plasma (PPP). The PPP was incubated with adapalene (20 μM) or DMSO at 37°C for 30 min. After incubation, the Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and Thrombin Time (TT) of the samples were measured using an automated blood coagulation analyzer.

Statistical Analysis

All experimental data were analyzed by GraphPad Prism 7.0 software and are presented as the means ± SEM. The mean between the two groups was compared by unpaired t test, and multiple groups were compared by one-way ANOVA followed by Tukey's post-hoc test was used. Differences with $P \leq 0.05$ were considered statistically significant.

Results

The phenomenon of agonist-induced platelet aggregation plays a crucial role in the formation of thrombosis. In order to evaluate the impact of adapalene on platelet aggregation, washed mouse platelets were pre-treated with adapalene or a control solvent for a duration of 10 minutes. Subsequently, an aggregation assay was performed using thrombin and ADP as triggering agents. Our findings demonstrate that adapalene exhibits a concentration-dependent reduction in thrombin (0.05 U/mL)-induced platelet aggregation (Figure 1B, C). Furthermore, at a dosage of 20 μM, adapalene significantly inhibits the aggregation induced by various doses of thrombin and almost entirely suppresses the platelet response to a lower concentration of thrombin (0.025 U/mL) in comparison to the solvent control group (Figure 1D, E). Additionally, 20 μM adapalene exhibits inhibitory effects on ADP-induced platelet aggregation (Figure 1F). These results provide evidence for the dose-dependent inhibitory effects of adapalene on agonist-induced platelet aggregation. To assess the potential cytotoxicity of adapalene on platelets and to ensure that the anti-aggregative actions of adapalene were not impeded by toxic effects within the tested dose range, an Alamar Blue assay was conducted. Our data, as depicted in (Figure S1A, B, C), illustrate that the platelet viabilities following treatment with 10 μM, 20 μM, and 50 μM adapalene for a duration of 4 hours were comparable to those of solvent-treated platelets. Consequently, we conclude that the anti-aggregative actions of adapalene are not dependent on toxic effects, at least within the range of doses that were tested.

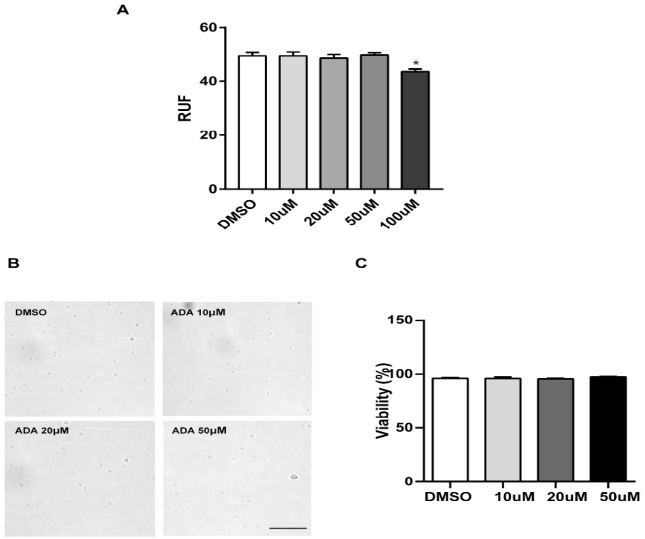


Figure S1: Effect of adapalene on platelet toxicity.

After preincubation with adapalene (10, 20, 50, 100 μM) or solvent for 10 min, washed mouse platelets were incubated with Alamar Blue (A) for 4 hours, and the Relative Fluorescence Units (RFU) were detected by a microplate reader. Cell viability images and data analysis as monitored by trypan blue staining (B&C). Bar graphs summarize data from three independent experiments, and the data are expressed as the mean ± SEM. $^{*}p<0.05$ *vs.* control.

Platelets undergo significant morphological changes after activation, as previously described [24,25]. To investigate the impact of Adenosine Deaminase (ADA) on platelet morphology at

rest, a hemogram analysis was conducted, revealing no significant changes in platelet parameters including platelet count, Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), and Thrombocrit (PCT) upon ADA treatment (Figure S2A-D). Notably, ADA was observed to markedly impede platelet morphological extension on Fibronectin (FN), as depicted by a higher proportion of ADA-treated platelets remaining in the initial spreading phase (Figure S2D & F). This observation suggests that ADA plays a role in modulating dynamic platelet morphological alterations, potentially underpinning the mechanisms of ADA-induced antiplatelet effects.

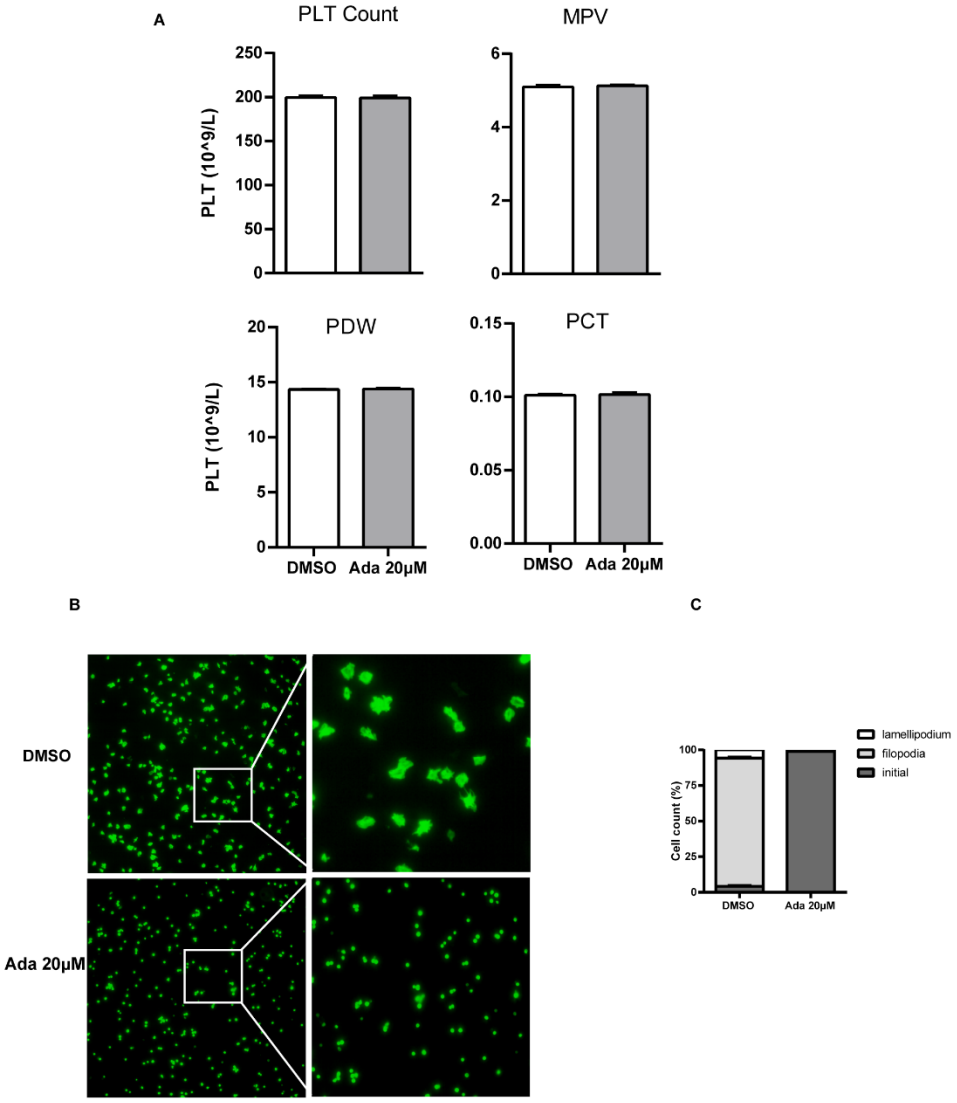


Figure S2: Effect of adapalene on platelet parameters and the number of platelets bound to fibrinogen.

(A) Platelet count, Mean Platelet Volume (MPV), Platelet Distribution Width (PDW) and Plateletcrit (PCT) determined by an automatic blood analyzer (mean ± SEM). (B) Quantitative assessment of the number of platelets bound to fibrinogen after DMSO and Ada treatment. Bar graphs summarize data from three independent experiments, and the data are expressed as the mean ± SEM. $^{*}p < 0.05$ *vs.* control.

Adapalene inhibits platelet granular content secretion

Platelet granules contain α-granules, dense granules and lysosomes, which play unique roles in regulating platelet recruitment, adhesion and aggregation. Platelet surface P-selectin is also termed CD62P and is expressed on the platelet α granule membrane. When platelets are activated, P-selectin is shed from the α granule membrane and translocated to the platelet membrane and fused with it. An additional portion is released into plasma [26]. The expression of P-selectin serves as a pivotal indicator of activated platelets and signifies the release of platelet alpha-granules. In order to assess the influence of adapalene on alpha-granule release, flow cytometry was utilized to measure the surface levels of P-selectin in platelets subsequent to thrombin stimulation. In accordance with findings obtained through platelet aggregometry, the presence of adapalene led to a notable reduction in thrombin (0.05 U/ml)-induced translocation of P-selectin to the cell membrane, decreasing from 33.4% in solvent-treated platelets to 16.6% and 7.7% in platelets treated with 10 μM or 20 μM of adapalene, respectively (Figure 2A, B). Similarly, platelets treated with adapalene exhibited a decrease in lysosomal degranulation as evidenced by the surface expression of LAMP-1 (18.1% in solvent versus 11.7% in adapalene-treated platelets, Figure 2C). Furthermore, the impact of adapalene on dense granule secretion was explored through the assessment of extracellular ATP release. As depicted in Figure 2D, treatment with adapalene at a concentration of 20 μM resulted in a noticeable reduction in the quantity of ATP released from thrombin-stimulated platelets. Taken together, these findings indicate that adapalene exerts a broad inhibitory effect on thrombin-induced degranulation processes.

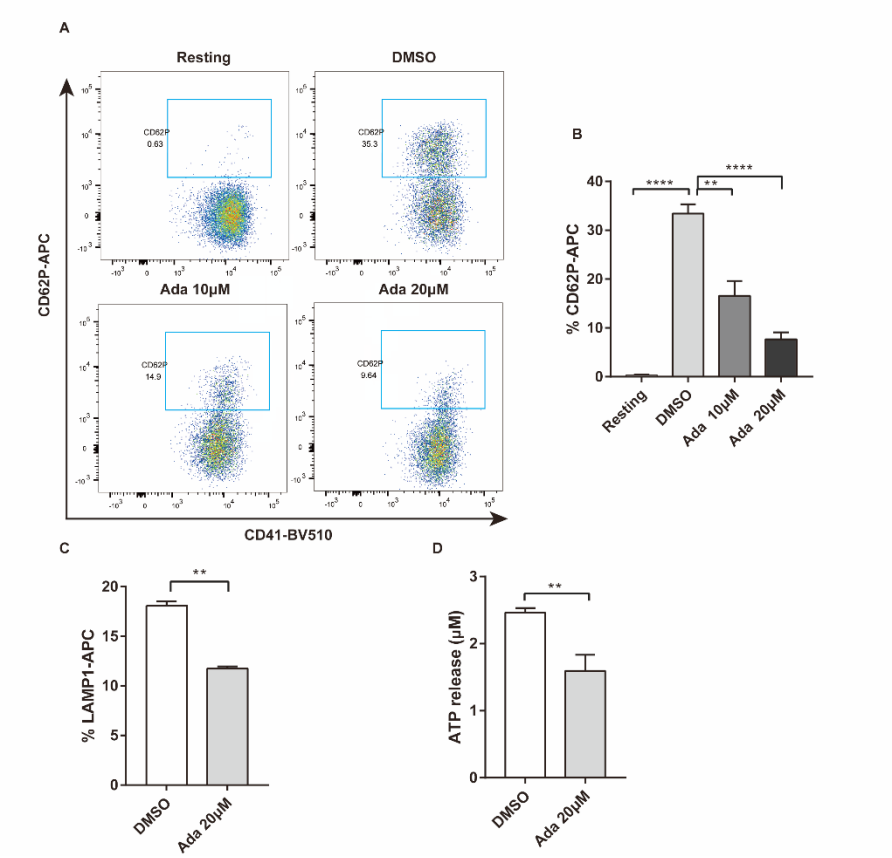


Figure 2: The consequences of adapalene treatment on thrombin-stimulated granule release.

Washed mouse platelets were preincubated for 10 min with DMSO (control) or adapalene (10, 20 μM) in the presence of the indicated primary antibodies and further stimulated with 0.025 U/mL thrombin for 10 min at 37°C. Representative Fluorescence-Activated Cell Sorting (FACS) plots showing (A) P-selectin expression and (B) quantification. (C) Statistical evaluation of LAMP1 expression. (D) Supernatants were harvested to determine ATP contents released from thrombin-stimulated platelets. Bar graphs summarize data from at least three independent experiments. $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* control.

Adapalene attenuates fibrinogen binding and spreading

Integrin αIIbβ3 activation serves as a crucial event in the cascade during platelet aggregation and thrombosis. To assess the impact of adapalene on integrin αIIbβ3 activation, we conducted flow cytometry analysis employing the conformation-sensitive antibody [JON/A] to detect the exposure of active αIIbβ3 on the cell surface. The results revealed that while platelets challenged with thrombin (0.025 U/mL) exhibited a notable integrin αIIbβ3 activation level of up to 60.3%, the response was significantly curtailed in the presence of adapalene, measuring 36.5% at 10 μM and 18.1% at 20 μM (depicted in Figure 3A, B). These findings indicate a suppressive effect of adapalene on platelet "inside-out" signaling transduction. The interaction between activated integrins and fibrinogen triggers an "outside-in" signaling pathway, ultimately leading to alterations in cytoskeletal reorganization and promoting late-stage thrombosis [27]. Platelet spreading and thrombus retraction are regulated by early and late αIIbβ3-dependent outside-in signaling pathways, respectively. Our initial investigation focused on platelet adhesion and spreading on a fibrinogen-coated surface following adapalene treatment, as illustrated in Figure 3C-E. Notably, adapalene demonstrated a significant inhibitory effect on both the number of adhered platelets and the total spread area. Upon integrin αIIbβ3 activation, the two NXXXY motifs located in the intracellular domain of integrin β3 were phosphorylated, leading to the subsequent activation of downstream signaling molecules such as Focal Adhesion Kinase (FAK), Syk, and SRC [28]. We lysed spreading platelets and detected the "outside-in" signal-related proteins. Compared with the solvent control, adapalene significantly reduced the phosphorylation levels of PLCγ2 and Syk (Figure 3F). It has been previously shown that FAK is required for cell adhesion, motility and migration. Phosphorylated FAK directly interacts with the SH2 part of the PI3K regulatory subunit p85, thereby inhibiting breast cancer cell apoptosis and promoting tumorigenesis [29]. In this study, we observed a notable decrease in FAK phosphorylation levels upon treatment with adapalene (Figure 3F). Considering the widespread inhibitory impact of ADA on the phosphorylation of Syk, PLC, and FAK during platelet spreading on Fibronectin (FN), we postulated that a shared upstream kinase influencing these proteins could be targeted by ADA. To explore this hypothesis, we examined Src activation, characterized by its autophosphorylation at tyrosine 419, and our results demonstrated a significant reduction in Src-419 phosphorylation in response to ADA treatment during platelet spreading on FN. The data presented above support an inhibitory effect of adapalene on αIIbβ3 outside-in signaling transduction.

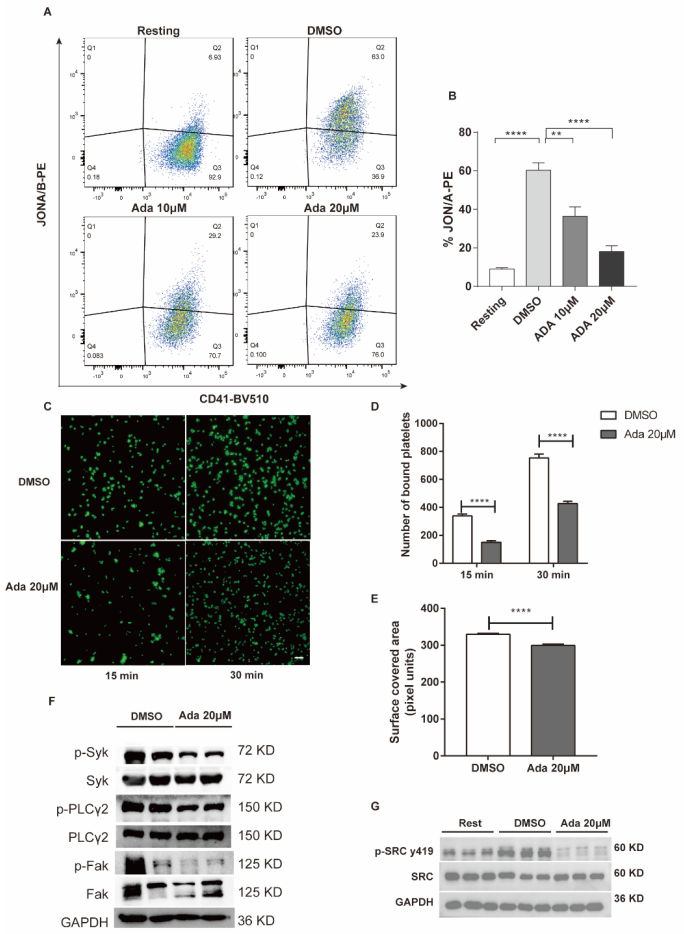


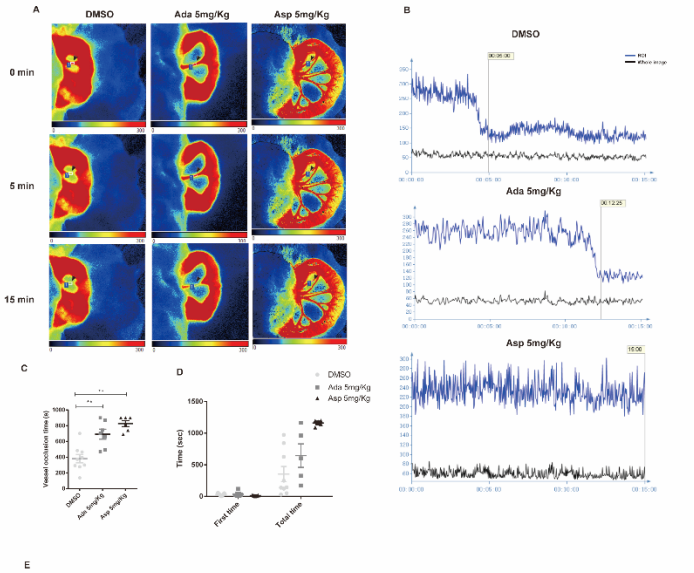
Figure 3: The effects of adapalene on Integrin αIIbβ3 activation and platelet outside-in signaling.

Washed mouse platelets were preincubated for 10 min with DMSO (control) or adapalene (10, 20 μM) in the presence of PE-conjugated JONA/B and further stimulated with 0.025 U/mL thrombin for 10 min at 37°C. Representative Fluorescence-Activated Cell Sorting (FACS) plots showing (A) the levels of active integrin αIIbβ3 and (B) corresponding quantification; platelets with or without pretreatment with 20 μM Ada were allowed to spread on fibrinogen matrix for 15 or 30 min. (C) Representative images

showing platelet morphology visualized by FITC-phalloidin staining and (D) statistical analysis for adherent platelet numbers and (E) averaged spreading area. (F&G) Western blotting images presenting the phosphorylation levels of SRC, Syk, PLCγ2, and Fak in platelets spreading on FN for 30 min. Data are expressed as the mean ± SEM (n>3), and $^{**}p<0.01$ and $^{***}p<0.001$ vs. control.

Adapalene protected mice from thrombosis *in vivo*

To evaluate the *in vivo* anti-thrombotic efficacy of adapalene, we employed an $FeCl_3$-induced mesenteric artery thrombus model and quantified blood flow changes using a Doppler flow probe. Mice were administered 5 mg/kg of adapalene via intravenous injection half an hour prior to model induction. Our findings revealed that the average occlusion time in the 5 mg/kg adapalene-treated group was 692 ± 63 s, whereas that in the aspirin-treated group was 826 ± 91 s, both significantly longer than the control group (380 ± 53 s) (Figure 4A, B, C). Notably, although the tail first bleeding time and total bleeding duration exhibited a slight increase in the adapalene group, the differences were not statistically significant (Figure 4D), indicating that adapalene at antithrombotic doses did not lead to significant bleeding events when compared to aspirin. Standard coagulation tests revealed that adapalene did not cause notable changes in PT, APTT, or TT (Figure 4E). As a whole, adapalene weakly influenced physiological hemostasis and coagulation.



Parameters	DMSO	Ada 20μM	Reference range	*p*-Value
TT (s)	20±0.07	20.08±0.17	14-21	0.34
PT (s)	11.12±0.04	10.6±0.09	9-13	9.15E-07
APTT (s)	31.88±0.04	32.52±0.90	25-33	0.15

Figure 4: The impacts of adapalene on FeCl3-induced thrombus formation in mouse mesenteric arteries.

Mice were i.v. administered saline (0.1% DMSO), Ada (5 mg/kg), or Asp (5 mg/kg) 30 min before $FeCl_3$-induced mesentery vessel injury. (A) Doppler images showing blood perfusion within the indicated observation time points. (B) Representative dynamic blood perfusion curves. (C) Statistical analysis of vessel occlusion time. (D) Bar graph of the mouse tail bleeding test showing the first bleeding duration and cumulative bleeding time. (E) The effects of Ada (20 μM) on activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) determined by a coagulation analyzer. All data were obtained from adult mouse or healthy donor serum. $^{**}p < 0.01$ and $^{***}p<0.001$ *vs.* control.

Adapalene inhibits thrombin-induced signaling transduction

To elucidate the mechanism by which adapalene counteracts thrombin-induced platelet activation, we delved into the modulation of the MAPK pathway. Our findings revealed that adapalene effectively inhibited the activation of Extracellular Signal-Regulated Kinase (ERK) at a concentration of 20 μM, as illustrated in Figure 5A and 5B.

The PI3K/Akt signaling pathway has been extensively involved in the process of platelet activation and has an important role in the inside-out signaling of integrins triggered by Immunoreceptor Tyrosine-Based Activation Motif (ITAM)-coupled receptors and G protein-coupled receptors [30]. We detected the phosphorylation levels of the PI3K regulatory subunits p85 (Y485) and p55 (Y199) and Akt to assess whether adapalene participates in the inside-out signal transduction of platelet PI3K/Akt. Compared with the solvent control group, adapalene significantly reduced thrombin-induced phosphorylation of PI3K and Akt (Figure 5C, D).

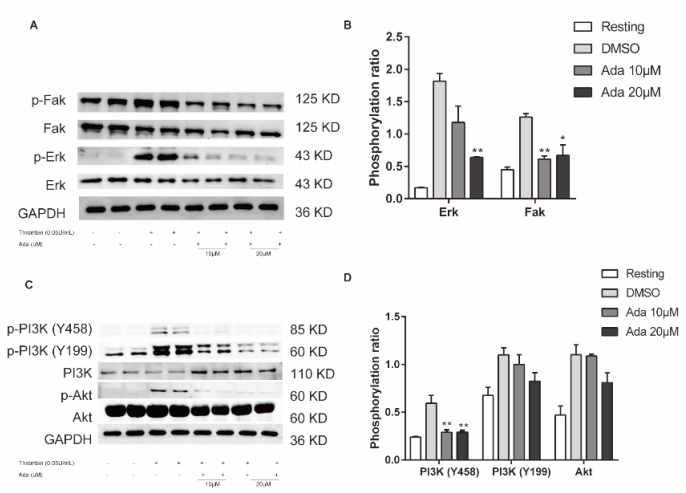


Figure 5: The influences of adapalene on the thrombin-induced platelet signaling pathway.

After preincubation with adapalene (10, 20 μM) or solvent for 10 min, washed mouse platelets were stimulated with thrombin (0.05 U/mL) for 5 min, and platelets were further harvested for western blotting. (A&B) Phosphorylation levels of Fak and Erk and quantification. (C&D) Phosphorylation alteration of PI3K and Akt and statistical analysis. Bar graphs summarize data from three

independent experiments, and the data are expressed as the mean ± SEM (n >3). $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* control.

Several direct ADA targets have been identified in other cellular systems, such as nuclear retinoic acid receptors (RARA, RARB, and RARG isotypes) in dermal fibroblasts [31] and perilipin 1 in hamster sebocytes [32]. Herein, we tested the expression of known targets of ADA in platelets. Interestingly, we found protein expression of RARB, RARG and perilipin 1 in platelets, while their expression was not affected by ADA treatment (Figure S3A). In addition, a previous study indicated that RARA is also expressed in platelets [33].

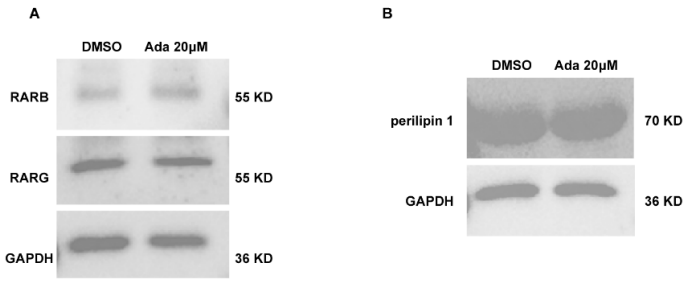


Figure S3: Expression of known targets of ADA in platelets.

After preincubation with adapalene (20 μM) or solvent for 10 min, platelets were further harvested for western blotting. (A) Expression levels of RARB and RARG. (B) Expression levels of perilipin 1.

Discussion

Thrombotic diseases contribute significantly to elevated rates of morbidity and mortality, posing a substantial burden on public health. Anti-platelet therapies represent the cornerstone of thrombotic disease management. However, the existing antiplatelet treatments are constrained by certain limitations, underscoring the pressing need for enhanced antithrombotic agents. Adapalene, a third-generation retinoic acid compound sanctioned by the U.S. Food and Drug Administration for acne treatment, has emerged as a promising candidate. Its notable safety profile distinguishes it from other retinoids, prompting the exploration of its diverse pharmacological properties. Adapalene exhibits multifaceted effects including anti-acne, anti-inflammatory, anti-proliferative, and immunomodulatory activities. Notably, ongoing investigations suggest that adapalene could serve as a valuable therapeutic avenue for multiple malignancies. The mechanism of retinoids revolves around their specific binding to retinoid receptors, with retinoid A receptor-targeting retinoids influencing cell differentiation and proliferation [34]. Based on this mechanism, adapalene has been successfully used to treat acne, psoriasis and photoaging. Adapalene binds selectively to Retinoid Acid Receptors (RAR) but not the cytoplasmic binding protein of retinoic acid; adapalene has high affinity for both RAR-γ receptors in the epidermis and RAR-β receptors in dermal fibroblasts, thereby activating genes responsible for cell differentiation. In a study of hamster sebum cells, experimental evidence indicated that adapalene had an inhibitory effect on sebum accumulation [32], as it can inhibit the transcription of diacylglycerol acyltransferase 1 (triacylglycerol synthase) and perilipin 1 (lipid droplet associated protein). Although adapalene is increasingly used off-label to treat various diseases, its mechanism of action remains largely unclear. The current knowledge indicates that adapalene mainly works as an antagonist targeting RARs or modulating cell cycle progression in nucleated cells. In the present study, our data enriched our understanding of the actions of adapalene in the enucleated cellular system, which not only extended the pharmacological role of adapalene as an antiplatelet reagent but also disclosed the underlying mechanism by interrupting platelet activation signaling transduction. Our findings provide evidence for a novel therapeutic potency of adapalene toward thrombotic diseases.

In the current investigation, we conducted a comprehensive analysis of the antithrombotic and antiplatelet properties of adapalene, both *in vitro* and *in vivo*, while also elucidating the underlying molecular mechanisms. Adapalene demonstrated a dose-dependent inhibition of thrombin- and ADP-induced murine platelet aggregation, along with the suppression of platelet α-granule formation, dense granule release, and αIIbβ3 activation. Additionally, it exhibited a decrease in platelet spreading on immobilized fibrinogen. These findings collectively suggest that adapalene effectively mitigated platelet activation and outside-in signaling mediated by Integrin αIIbβ3. Mechanistically, adapalene was found to markedly impede the phosphorylation of key downstream signaling molecules, such as SRC-Syk-PLCγ2 and PI3K-Akt. Moreover, it hindered αIIbβ3-mediated downstream signaling pathways, including FAK activation. Concordantly, adapalene greatly decreased thrombosis formation and vessel occlusion times in mouse mesentery vessels injured by $FeCl_3$. Moreover, a slight but not significant increase in tail bleeding duration time was observed in adapalene-treated mice, suggesting less hemorrhagic risk (Figure 6).

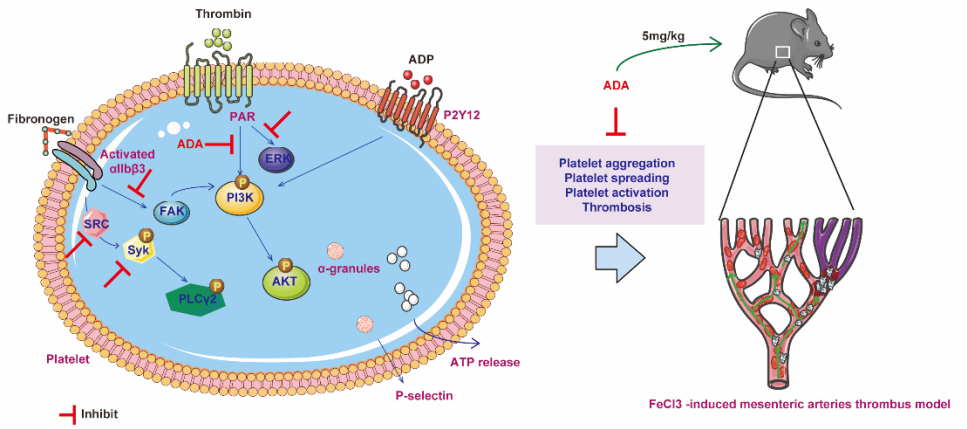


Figure 6: Schematic diagram of the antithrombosis mechanism of adapalene.

Adapalene can inhibit platelet activation and platelet aggregation by inhibiting the phosphorylation of the SRC-Syk-PLCγ2, PI3K-AKT and ERK signaling pathways, thus alleviating FeCl3-induced mesentery vessel injury.

Platelet activation commences by the binding of surface glycoprotein receptors to their respective ligands, including ADP receptors, thrombin receptors, TXA2 and other G protein-coupled and tyrosine kinase receptors. The ligands bind to the receptors to trigger platelet inside-out signaling. *Ex vivo* platelet aggregation assays can reflect the effect of antiplatelet agents. Here, we show that adapalene can significantly inhibit the aggregation induced by thrombin and ADP, indicating that it may act on the common signaling pathway of platelet activation caused by multiple receptors. However, we adopted plasma-free washed platelets for analyses, and further studies with platelet-rich plasma are needed to determine whether plasma interferes with the action of the drug. Platelets undergo a process of activation that involves substantial morphological alterations. In our investigation, we observed that adapalene treatment did not have any influence on platelet parameters, including platelet count, Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), and Plateletcrit (PCT). However, we did find that adapalene remarkably hindered the morphological extension of platelets on Fibronectin (FN). Consequently, additional studies are warranted to gain a more comprehensive understanding of the impact of adapalene treatment on platelet shape changes, as outlined in this review [35].

Platelets contain three distinct classes of granules: α-granules, dense granules and lysosomes. α-granules are rich sources of various proteins, such as P-selectin, vWF, chemokines, cytokines and growth factors, which are packaged in platelets by megakaryocytes and are required for appropriate physiological function in platelets. Dense granules contain small molecules essential for hemostasis, e.g., ADP, ATP, 5-hydroxytryptamine, polyphosphate, glutamic acid, histamine and calcium [36,37]. Importantly, platelet activation is accompanied by the release of the aforementioned substances, which may potentially enhance the activation process. Typically, the release of granules can be assessed by quantifying the levels of Adenosine Triphosphate (ATP) and P-selectin. According to the findings from flow cytometry analysis, it was evident that adapalene significantly suppressed the expression of P-selectin and the release of ATP. Additionally, a noticeable downregulation of Lamp1 was observed in response to adapalene treatment. Based on these observations, it can be inferred that adapalene not only inhibits the release of alpha-granules but also exerts inhibitory effects on the release of dense granules and lysosomal granules. Further investigations are required to elucidate the precise mechanisms underlying these effects.

Integrin αIIbβ3 activation is the eventual step of platelet activation [38], and it is also the link transmitting outside-in and inside-out signaling. Activated αIIbβ3 promotes Src activation *via* autophosphorylation, and Src phosphorylation subsequently remodels its downstream cytoskeletal proteins. Platelets often undergo a transformation characterized by the extension of filopodia and lamellipodia, followed by complete spreading, which marks the initial phase of platelet activation through the "outside-in" pathway. Subsequently, the platelet cytoskeleton contracts, pulling fibrinogen from the plasma and forming stable thrombi, representing a more advanced stage. In the present study, we employed flow cytometry to investigate the effects of adapalene on platelet function. Our results demonstrate a significant reduction in the binding of fibrinogen to αIIbβ3 integrin in the presence of adapalene, indicating a substantial suppression of αIIbβ3 integrin activation by adapalene. Moreover, our findings also reveal a noticeable inhibition of platelet spreading, strongly implying that adapalene hinders outside-in signaling mediated by platelet integrin αIIbβ3.

Recent discoveries suggest that PI3K/AKT can regulate platelet activation and thrombosis. PI3K can be categorized into types I, II, and III. Among them, type I PI3Ks (PI3Kα, PI3Kβ, PI3K8, and PI3Ky) are ubiquitously expressed in human and mouse platelets and are most strongly implicated in platelets. We found that adapalene significantly reduced the phosphorylation levels of PI3K in platelets after thrombin stimulation, indicating that adapalene can affect the "inside-out" signal transduction of platelets by regulating the PI3K/AKT pathway. In the process of platelet spreading, adapalene significantly decreased the phosphorylation levels of Src and PLCγ2, indicating that adapalene can inhibit the "outside-in" signal transduction of integrin αIIbβ3 through the Src-PLCγ2 signaling pathway. MAPK is also a common pathway of agonist-stimulated platelet activation, which results in increased granule secretion and shape changes [39]. Here, adapalene significantly attenuated the activation of ERK, JNK and p38MAPK. According to previous studies, adapalene is involved in cell cycle arrest by regulating cell Cycle-Independent Kinase (CDK). For example, adapalene inhibits the proliferation of melanoma cells by blocking S phase and subsequently inducing apoptosis caused by DNA damage [40]. Adapalene can also inhibit the proliferation of prostate cancer cells by inhibiting the level of S-phase CDK2 and inducing apoptosis by upregulating the Bax/Bcl-2 ratio [19]. The above evidence suggests that adapalene functions primarily through retinoic acid receptors (RARα, β, γ) and mitosis. In our study, we demonstrated for the first time that adapalene actively affects PI3K/AKT, MAPK and SRC-Syk-PLCγ2 signaling transduction in a nucleated cell platelet, revealing a completely new mechanism to dissect adapalene-mediated antiplatelet actions. In addition, platelets indeed express known ADA receptors. However, it is still not clear which receptor is involved in or whether there are new therapeutic targets mediating the antiplatelet effects of ADA. However, it is difficult to attribute ADA-mediated antiplatelet effects to those RARs as a lack of direct data supporting the functional roles of those RAR in regulating platelet activities by genetic KO models, although the interactions of ADA with platelet RARs are theoretically possible. These matters all need to be further clarified in the future. FeCl3-induced mesenteric vessel injury is the most frequently employed thrombosis model. $FeCl_3$ predominantly causes oxidative stress and generates free radicals, which leads to lipid peroxidation, endothelial cell destruction and the formation of occlusive thrombi. Here, we report that adapalene can significantly prolong vessel occlusion time, indicating its ability to protect against *in vivo* thrombosis. Given common defects for antiplatelet reagents, toxic side effects and hemorrhage risk are nonnegligible issues. In the current study, we demonstrated experimentally that adapalene could neither cause apparent toxicity to platelets with a broad dose range nor prolong mouse tail bleeding times. Furthermore, we also evaluated the effect of adapalene on coagulation function, and no overt changes were observed between adapalene- and solvent-treated plasma.

Conclusion

From the perspective of "conventional drugs in new use", our data reveal, for the first time, the inhibitory effects of adapalene on platelet functions. Adapalene showed few toxic effects on platelet viability over the large concentration range tested. Adapalene significantly suppressed thrombin- or ADP-triggered human and mouse platelet aggregation and extocytosis of active substances stored in α- and β-granules and lysosomes. Adapalene inhibited integrin αIIbβIII activation-mediated "inside-out" signaling by inactivating agonist-stimulated PI3K/AKT signaling transduction. Adapalene also impaired platelet "outside-in" signaling by restricting the SRC/Syk/PLCγ2 pathway. Moreover, we demonstrated that adapalene greatly attenuated $FeCl_3$-induced thrombus formation in mouse mesenteric vessels without obvious bleeding risk. Therefore, we identified a novel pharmacological role of adapalene in inhibiting platelet function and thrombosis formation, suggesting its prospect to be developed as an innovative antiplatelet drug for thrombotic diseases.

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Informed Consent

Approval for this study was obtained from the Ethics Committee of Xiamen Cardiovascular Hospital of Xiamen University. The ethics statement encompasses both the animal and patient experiments. Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Xiamen University (Permit No. XMULAC20200150). We guarantee that we have considered all animal welfare during the experiment. And, our study was designed, approved and monitored by animal welfare committee of Xiamen Cardiovascular Hospital of Xiamen University.

Consent for Publication: Not applicable.

Conflict of Interest: The authors declare that they have no competing interests.

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Data Availability: All data generated or analyzed during this study are included in this published article.

Data and material availability: The authors declare that all data supporting the findings of this study are available from the authors and are included within the paper.

Author Contributions: Y.L. and L.Z. were responsible for planning the study and designing the experiments; L.Z. conducted the experiments; L.Z. wrote the manuscript; C.C. contributed to the project design and discussion concept; Y.L. provided funding and gave final approval of the manuscript.

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