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Research Article

A Naphthalenic Derivative ND-1 Inhibits Thrombus Formation by Interfering the Binding of Fibrinogen to Integrin αIIbβ3

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Integrin αIIbβ3 plays a crucial role in the process of platelet aggregation. Three integrin αIIbβ3 antagonists (abciximab, eptifibatide, and tirofiban) have been approved by FDA for clinical use. Unfortunately, they all showed severe side effects such as thrombocytopenia and bleeding risk. Thus, researches on the development of more effective and safer antiplatelet agents are needed. In this manuscript we reported a novel naphthalenic derivative compound ND-1 with potent antithrombotic effect and lower bleeding risk. ND-1 inhibited ADP-, collagen-, thrombin-, and U46619-induced platelet aggregation with IC_{50} values of 1.29, 14.46, 12.84, and 40.24 μM, respectively. Mechanism studies indicated that ND-1 inhibited the binding of fibrinogen to integrin αIIbβ3 in a dose-dependent manner with an IC_{50} value of 3.12 μM. ND-1 inhibited P-selectin expression induced by ADP, collagen, thrombin, and U46619 on the surface of platelets. Additionally, this compound reduced platelets spreading to the immobilized fibrinogen. In vivo, ND-1 potently decreased thrombus formation in an arteriovenous shunt thrombosis model in rats and slightly prolonged bleeding time in a tail cutting model in mice. Taken together, our results reveal that ND-1 is a novel antagonist of αIIbβ3 with strong antithrombotic effect and lower bleeding risk.

1. Introduction

Cardiovascular diseases (CVDs), including acute coronary syndrome, myocardial infarction, deep-vein thrombosis, and pulmonary embolism, are the leading cause of morbidity and mortality worldwide [1]. The proximal cause of CVDs is intravascular thrombus formation [2, 3]. In the process of thrombosis, platelets play an important role as they can adhere to the exposed subendothelial matrix and then change shape and release ADP, thromboxane A2, thrombin, and epinephrine, which recruit additional platelets from the blood flow to the injury sites and form plugs [4, 5]. Accordingly, antiplatelet agents have been widely developed as an important tool for preventing thrombotic events [6].

Integrin αIIbβ3 is a crucial molecule in the process of platelet aggregation. It has extracellular region for ligands binding and intracytoplasmic tail mediating intracellular signal transduction. Both inside-out and outside-in signaling in platelet involve this molecule [7, 8]. So the activation of integrin αIIbβ3 was recognized as the final common pathway of platelet aggregation [9, 10]. Three integrin αIIbβ3 antagonists (abciximab, eptifibatide, and tirofiban) have been approved by FDA for clinical use. Unfortunately, they all showed severe side effects such as thrombocytopenia and bleeding risk [11–14]. Thus, studies on the development of more effective and safer antiplatelet agents are needed.

In the search for new antiplatelet agents, we screened more than 500 synthetic compounds and natural products and found that ND-1 (Figure 1(a)), a compound derived from naphthalene, had potent antiplatelet aggregation effect. The aim of this study was to investigate the effect of ND-1 on thrombus formation, as well as the underlying mechanisms.

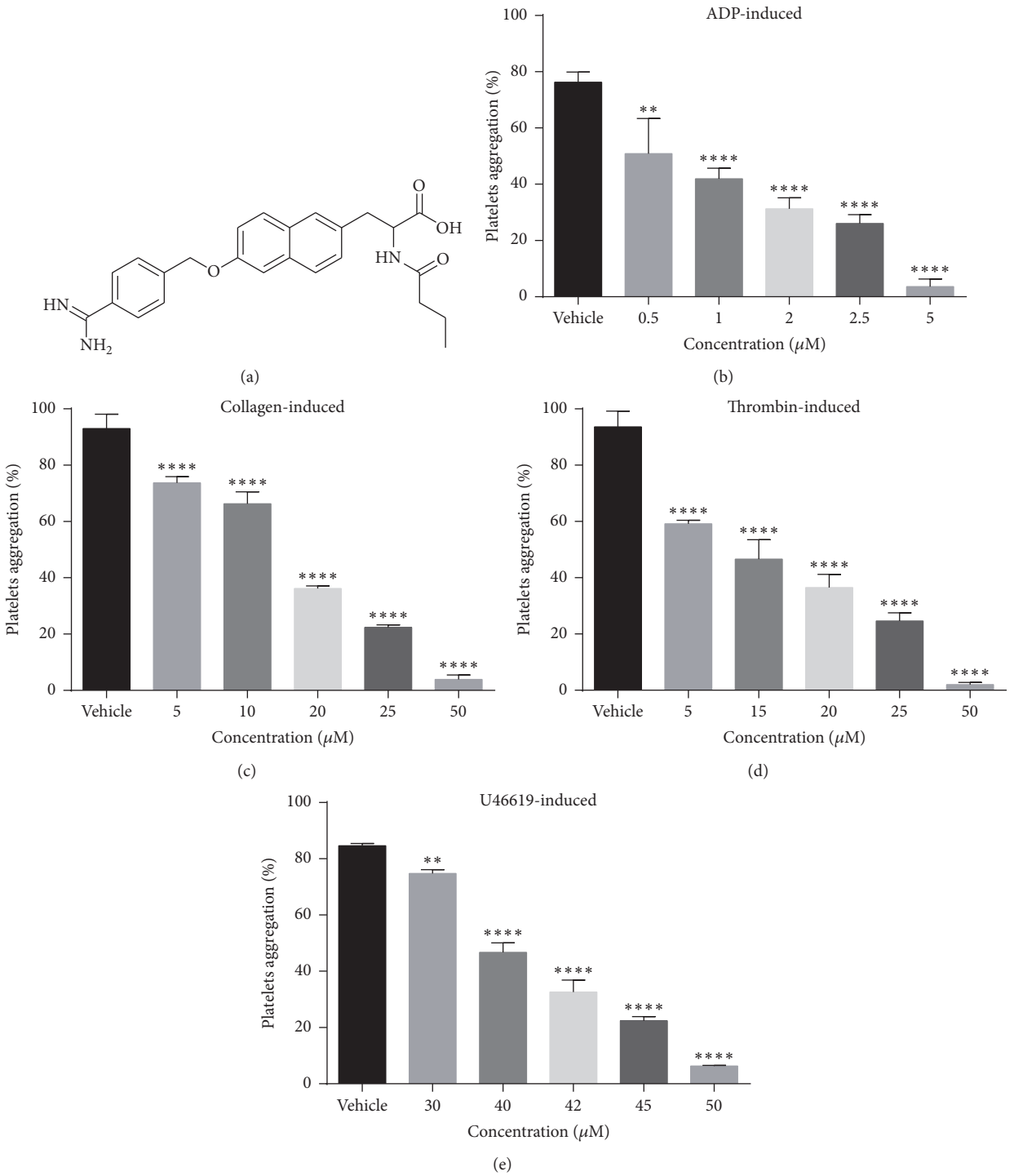

Figure 1: Chemical structure of ND-1 and effect on platelet aggregation. (a) Chemical structure of ND-1. ((b) to (e)) Effect of ND-1 on platelet aggregation. Platelet-rich plasma was preincubated with indicated concentrations of ND-1 or vehicle for 5 min at 37°C. Aggregation was initiated by the addition of 20 μM ADP (b), 1 μg/mL collagen (c), 0.25 U/mL thrombin (d), or 2 μM U46619 (e). Data are presented as mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{****}P < 0.0001$ compared with vehicle.

2. Materials and Methods

2.1. Materials. ND-1 (2-butyramido-3-(6-((4-carbamimidoylbenzyl)oxy)naphthalen-2-yl)propanoic acid) was synthesized by Zhiyu Li (China Pharmaceutical University, China) and was dissolved in DMSO as a stock solution, stored at −20°C. Aspirin, ADP, thrombin, U46619, human fibrinogen, apyrase, prostaglandin E1 (PGE1), FITC-conjugated phalloidin, and anti-mouse IgG-conjugated alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO,

USA). Collagen was from Hyphen BioMed (Neuville sur Oise, France). Purified human platelet integrin αIIbβ3 and the mouse anti-human integrin β3 antibody were from Enzyme Research Laboratories (South bend, IN, USA) and Millipore (Temecula, CA, USA), respectively. PE-conjugated anti-human CD62P, FITC-conjugated anti-human CD42a, REA Control (S)-PE, and REA Control (S)-FITC were from Miltenyi Biotec (Koln, North Rhine-Westphalia, Germany).

2.2. Animals. Institute of Cancer Research (ICR) mice (male, 18–22 g) and Sprague-Dawley rats (male, 180–220 g) were purchased from Nanjing Qinglongshan Animal Center (Nanjing, Jiangsu, China). All experiments were carried out in accordance with the guidelines and the regulations of the Ethical Committee of China Pharmaceutical University. The protocols were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University.

2.3. Human Blood Samples and Washed Platelet Preparation. Human platelet-rich plasma (PRP) was obtained from Jiangsu Province Blood Center. Washed platelets were prepared as described previously [15]. Briefly, PRP containing 5 mM EDTA, 2 U/mL apyrase, and 0.1 μg/mL PGE1 was centrifuged at 1100 ×g for 10 min, and then pellets were resuspended in Tyrode buffer and the concentration of platelets was adjusted to approximately 3×10^7 platelets/mL.

2.4. Platelet Aggregation Assay. Platelet aggregation was measured as previously described using a four-channel aggregometer (LBY-NJ4, Pulisheng Science Instrument Company, Beijing, China) [9, 16]. Briefly, PRP was centrifuged at 1580 ×g for 10 min to obtain platelet-poor plasma (PPP). PRP was preincubated with or without ND-1 for 5 min at 37°C, and aggregation was induced by the addition of ADP (20 μM), collagen (1 μg/mL), thrombin (0.25 U/mL), or U46619 (2 μM). The maximum platelet aggregation rate was determined within 5 min by continuously stirring.

2.5. Fibrinogen/Integrin αIIbβ3 ELISA. Similar to the previous method [17, 18], the 96-well microplate (Corning Incorporated, Corning, NY, USA) was coated with human fibrinogen (10 μg/mL) at 4°C overnight. After washing with TACTS (20 mM Tris, 0.15 M NaCl, 2 mM $CaCl_2$, and 0.05% Tween 20, pH 7.5), wells were blocked with 1% BSA in TACTS for 1 h at 37°C and then washed with TACTS three times. Purified human platelet integrin αIIbβ3 receptor (20 μg/mL) and ND-1 or vehicle were added and incubated for 2 h at 37°C. Wells were washed and then mouse anti-human integrin β3 antibody (1 : 2000) was added. After incubation for 1 h at 37°C, wells were washed and anti-mouse IgG-conjugated alkaline phosphatase (1 : 2000) was added and incubated for 1 h at 37°C. Disodium 4-nitrophenyl phosphate substrate was added and incubated for 30 min, and the reaction was stopped by adding 3 M NaOH. The OD values of samples were estimated at 405 nm.

2.6. P-Selectin Expression on Platelets. The expression of P-selectin on the surface of platelets was studied using a flow cytometric method as described previously with some modifications [19, 20]. Briefly, PRP was incubated with or without compound ND-1 for 10 min at 37°C. The platelets were then separately stimulated with ADP (20 μM), collagen (1 μg/mL), thrombin (0.25 U/mL), and U46619 (2 μM) at 37°C within 10 min, and then PE-conjugated anti-human CD62P and FITC-conjugated anti-human CD42a were added and incubated for 10 min at 4°C in the dark. After that platelets were fixed with 1% paraformaldehyde at 4°C and analyzed with a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). For each sample, 20000 platelets were analyzed.

2.7. Platelet Spreading on Immobilized Fibrinogen. Platelet spreading was measured as previously described [21, 22]. Glass coverslips were coated with 40 μg/mL fibrinogen in 0.1 M $NaHCO_3$ (pH 8.3) at 4°C overnight. Washed platelets incubated with ND-1 (50, 25, and 10 μM) or vehicle for 5 min at 37°C were allowed to spread on the fibrinogen-coated surfaces for 60 min at 37°C. After three washes, the platelets were fixed with 4% paraformaldehyde and permeabilized with 0.2% triton X-100 solution and stained with FITC-conjugated phalloidin. Adherent platelets were viewed by an upright fluorescent microscope AXIO ScopeA1 (ZEISS Group, Jena, Germany). Three images were chosen randomly per experiment. The spreading area of individual platelet was assessed using ImageJ software (National Institutes of Health, Bethesda, MD).

2.8. Arteriovenous Shunt Model in Rats. The arteriovenous shunt thrombosis in rat was measured with a modified method [23]. Male Sprague-Dawley rats were randomly divided into five groups, six for each group. ND-1 (1, 3, and 7 mg/kg), aspirin (50 mg/kg), or vehicle was injected through caudal vein. After 15 minutes, rats were anesthetized with intraperitoneal 10% chloral hydrate (3 mL/kg). The right jugular vein and the left carotid artery were isolated, and two 4 cm polyethylene 50 (PE50) catheters were inserted separately. The two PE50 catheters were linked by a 12 cm PE60 catheter containing 10 cm long silk thread soaked with saline to induce thrombosis. After 20 min of blood circulation, the silk thread carrying thrombus was pulled out. The weights of the thrombus were measured after the thread was dried for 30 min at 60°C.

2.9. Bleeding Time Assay. Bleeding time in mice was measured according to previous method [24]. Male ICR mice were randomly divided into five groups, ten for each group. ND-1 (2, 6, and 14 mg/kg), aspirin (50 mg/kg), or vehicle was injected through caudal vein. After 15 min, mice were anesthetized with intraperitoneal 5% chloral hydrate (6 mL/kg), followed by a cut of 3 mm from the tail tip. The tail was immediately immersed into 12 mL saline at 37°C and the accumulated bleeding time was recorded within 20 min.

2.10. Statistical Analysis. Statistical analysis was performed using GraphPad Prism Statistical Software (version 6.0, San Diego, CA). The data are presented as means ± SD or means

± SEM. Differences between the sample-treated group and vehicle were analyzed by one-way analysis of variance assay (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Effect of ND-1 on Platelet Aggregation. To investigate the effect of ND-1 on platelet aggregation, we used human PRP induced by a panel of agonists as an in vitro model. As shown in Figures 1(b)–1(e), ND-1 significantly inhibited ADP-, collagen-, thrombin-, and U46619-induced platelet aggregation in a dose-dependent manner, and IC_{50} values were 1.29 μM (95% CI, 0.55–99 μM), 14.46 μM (95% CI, 13.02–16.05 μM), 12.84 μM (95% CI, 10.86–15.18 μM), and 40.24 μM (95% CI, 39.54–40.95 μM), respectively. ND-1 exhibited stronger inhibition to ADP-induced platelet aggregation than other three inducers, and the inhibition rate was 95.29 ± 3.58% at a dose of 5 μM.

3.2. Effect of ND-1 on the Binding of Fibrinogen to Integrin αIIbβ3. To verify the inhibition of ND-1 for the binding of fibrinogen to integrin αIIbβ3, fibrinogen/integrin αIIbβ3 solid-phase ELISA was performed. As shown in Figure 2(a), ND-1 strongly inhibited the binding of fibrinogen to purified integrin αIIbβ3 in a dose-dependent manner with an IC_{50} value of 3.12 μM (95% CI, 2.75–3.56 μM).

3.3. Effect of ND-1 on Platelet P-Selectin Expression. P-selectin is a major marker protein of platelet activation, which appears on the surface of platelets during platelet activation. To test whether ND-1 affected platelet activation, the level of P-selectin expression was assessed by flow cytometry. As shown in Figures 2(b)–2(e), ND-1 at 50 μM inhibited U46619-, ADP-, collagen-, and thrombin-induced P-selectin expression by 96.55%, 86.61%, 75.53%, and 41.16%, respectively.

3.4. Effect of ND-1 on Platelet Spreading. Platelet spreading on immobilized fibrinogen is dependent on cytoskeletal reorganization driven by integrin αIIbβ3 outside-in signaling. To investigate the role of ND-1 in the outside-in signaling, the platelets' spreading on immobilized fibrinogen was assessed. The results in Figures 3(a) and 3(b) showed that ND-1 significantly inhibited platelets' spreading in a dose-dependent manner with inhibition rates of 66,06 ± 5.45%, 50.64 ± 4.48%, and 34.55 ± 4.92% at concentrations of 50, 25, and 10 μM, respectively. Therefore, ND-1 is involved in the regulation of integrin αIIbβ3 outside-in signaling.

3.5. Antithrombotic Activity of ND-1 In Vivo. To examine the antithrombotic activity of ND-1, the arteriovenous shunt thrombosis model was used. ND-1 inhibited the formation of thrombus in a dose-dependent manner as shown in Figure 4(a). ND-1 at 7 mg/kg inhibited thrombus formation by 31.84 ± 6.76% ($n = 6$), whereas aspirin at 50 mg/kg inhibited thrombus formation by 26.32 ± 9.5% ($n = 6$), indicating that this compound at a low dose showed similar effect to that of aspirin at a high does.

3.6. Bleeding Risk of ND-1 In Vivo. To assess the bleeding risk incurred by ND-1, we measured the bleeding time of ND-1-treated mice by a tail cutting assay at ND-1 doses of 2, 6, and 14 mg/kg. As shown in Figure 4(b), ND-1 at 2 and 6 mg/kg did not significantly prolong bleeding time, compared with vehicle group. But when the dose of ND-1 increased to 14 mg/kg, which represented two times the high dose used in the arteriovenous shunt thrombosis model, it significantly prolonged bleeding time (13.67 ± 2.67 min), while the positive control aspirin prolonged bleeding time more (17.18 ± 1.47 min).

4. Discussion

In this study, we found that ND-1 inhibited platelet aggregation via blocking the binding of fibrinogen to integrin αIIbβ3. In vivo, ND-1 potently decreased thrombus formation in a rat arteriovenous shunt thrombosis model and slightly prolonged bleeding times in mice, suggesting that ND-1 is a lead compound for development of cardiovascular drug.

The diverse platelet activation signaling pathways stimulated by various agonists converge into common signaling events, which induce the inside-out signaling transduction, leading to activation of integrin αIIbβ3, which is a final effector for platelet aggregation [25]. Our studies showed that ND-1 inhibited platelet aggregation induced by various agonists (ADP, collagen, thrombin, and U46619), suggesting a possible inhibition of integrin αIIbβ3. Fibrinogen/integrin αIIbβ3 ELISA was conducted and the result showed that ND-1 inhibited the binding of fibrinogen with αIIbβ3. The activation of platelets leads to degranulation, which results in exposure of platelet granule membranes and their associated antigens. The CD62P (P-selectin), one of α-granule membrane antigens, is highly expressed on the activated platelets surface [26]. The present studies showed that ND-1 inhibited platelet activation (indicated by P-selectin expression) induced by ADP, collagen, thrombin, and U46619. These agonists have different receptors on the platelet surface and activate platelets via different pathways [27]. So, we speculated that ND-1 can act on integrin αIIbβ3, the "final common pathway" of platelet activation. This result further confirms our conclusion that ND-1 affects the binding of fibrinogen to integrin αIIbβ3.

Integrin αIIbβ3, a dual-direction effector, not only mediates inside-out signaling but also affects outside-in pathway. Ligands such as fibrinogen bind to integrin αIIbβ3 and mediate outside-in signaling transduction, resulting in platelet spreading. ND-1 reduced platelet spreading on immobilized fibrinogen, demonstrating its inhibition on integrin αIIbβ3-mediated outside-in signaling.

Three agents targeting αIIbβ3 have been approved for human use in the USA, starting with abciximab, a monoclonal antibody fragment, followed by eptifibatide, a cyclic peptide, and tirofiban, a peptidomimetic molecule [28]. These drugs have shown efficacy in the adjunctive therapy to ischemic complications of percutaneous coronary interventions in a large number of randomized studies. However, they are involved in an increased bleeding risk

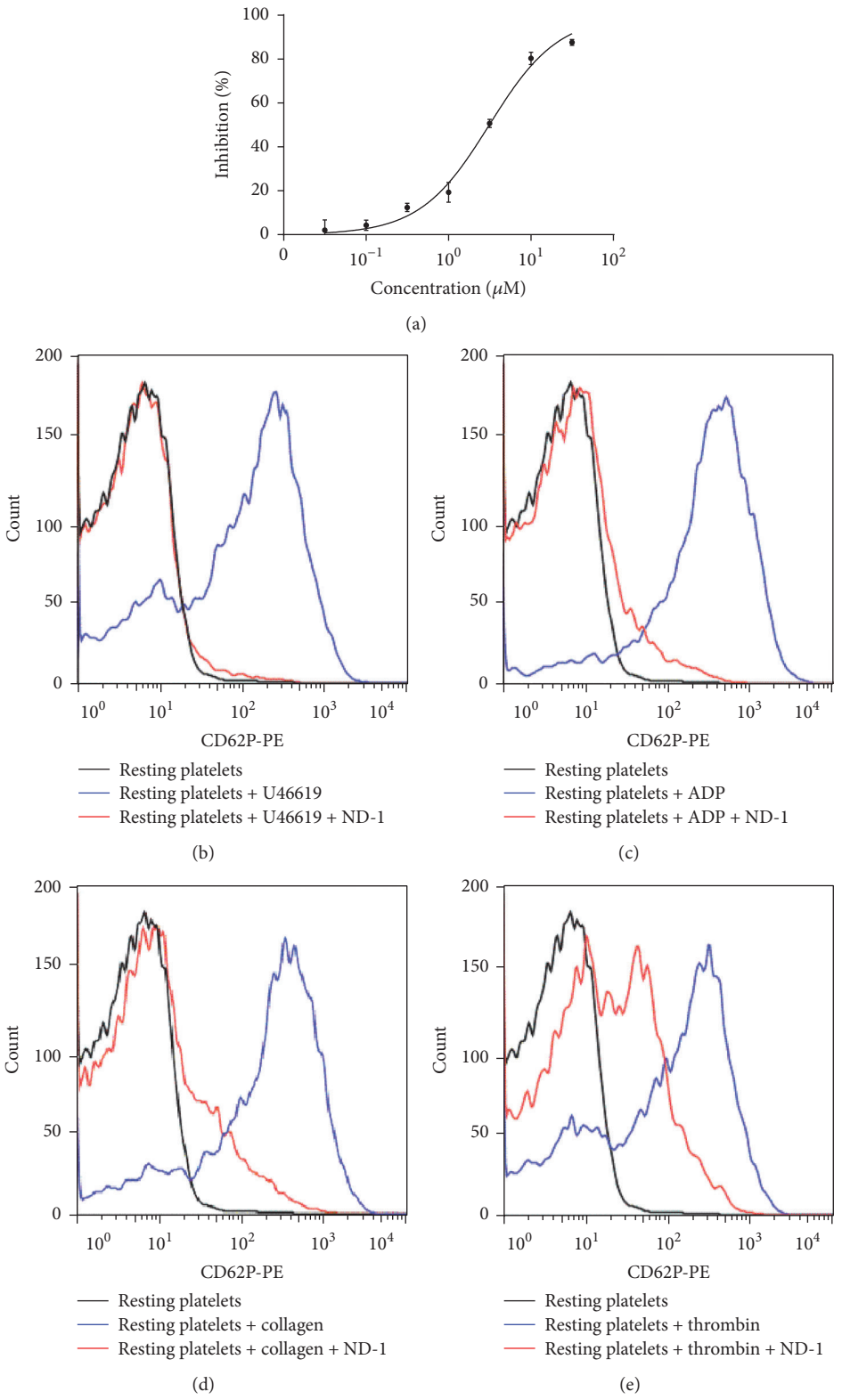

FIGURE 2: Effect of ND-1 on the binding of fibrinogen to integrin αIIbβ3 and P-selectin expression. (a) Effect of ND-1 on fibrinogen/integrin αIIbβ3 ELISA. Purified human integrin αIIbβ3 with or without ND-1 was added to wells coated with fibrinogen for 2 h at 37°C, followed by adding mouse anti-human integrin β3 antibody. The binding of fibrinogen to αIIbβ3 was determined using anti-mouse IgG-conjugated alkaline phosphatase and disodium 4-nitrophenyl substrate at 405 nm. All experiments were performed in triplicate. ((b) to (e)) Effect of ND-1 on P-selectin expression on platelets. Platelet-rich plasma was preincubated with ND-1 (50 μM) or vehicle for 10 min at 37°C. Activation was initiated by the addition of 20 μM ADP (b), 1 μg/mL collagen (c), 0.25 U/ml thrombin (d), or 2 μM U46619 (e) for 10 min at 37°C. P-selectin expression on platelet surface was detected by flow cytometry.

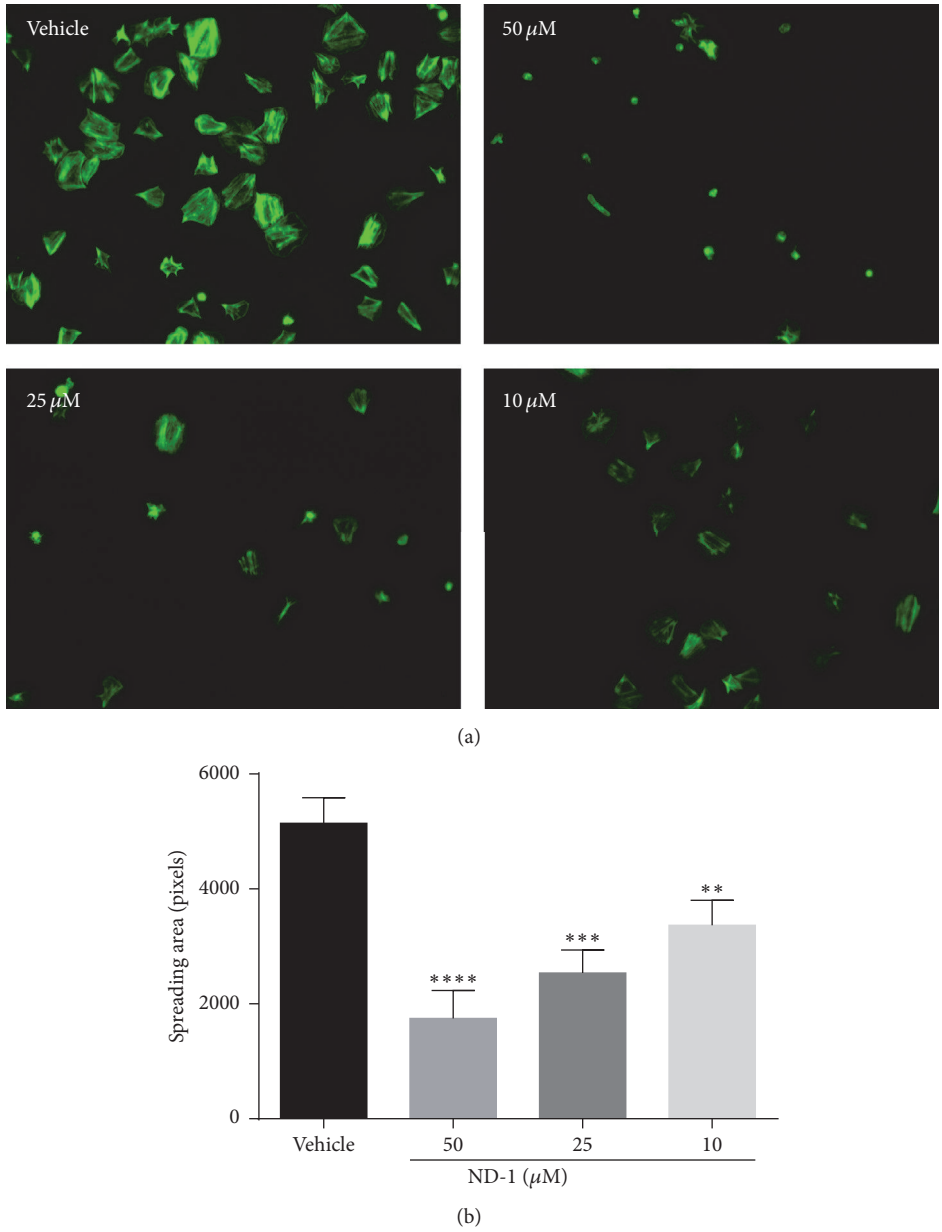

FIGURE 3: ND-1 inhibited platelet spreading on immobilized fibrinogen. (a) Washed platelets were incubated with ND-1 (50, 25, and 10 μM) or vehicle for 5 min at 37°C. Platelets were allowed to spread on fibrinogen-coated glasses for 60 min at 37°C. Then platelets were fixed, labeled, and photographed under a fluorescence microscope. Representative images from at least three independent experiments with similar results. (b) Quantification of the areas (pixel number) of 3 random fields per experiment. Data are presented as mean ± SEM ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ compared with vehicle.

and thrombocytopenia, which restrict their use clinically [29]. Thus, numerous compounds have been designed and evaluated to overcome these drawbacks for the past several years. RUC-1 (a small molecular inhibitor of integrin αIIbβ3) only binds to the αIIb subunit but not to β3 and does not induce conformational changes of αIIbβ3. By avoiding such a change, it is anticipated that associated thrombocytopenia, induction of fibrinogen binding, and paradoxical platelet activation would be prevented [28, 30]. RUC-2 and RUC-4 were developed following RUC-1. Currently RUC-4 is under development for the prehospital therapy of ST segment elevated myocardial infarction [31, 32]. Ur-3216/2922, binding to αIIb D224 and not engaging the MIDAS metal ion, is a high affinity αIIbβ3 antagonist without ligand-induced binding site (LIBS) expression. In vivo UR-3216 showed antithrombotic effect without bleeding at effective doses [33]. We found that ND-1 potently inhibited thrombus formation in a dose-dependent manner in an arteriovenous shunt thrombosis model in rat and slightly prolonged bleeding time in a tail cutting model in mice.

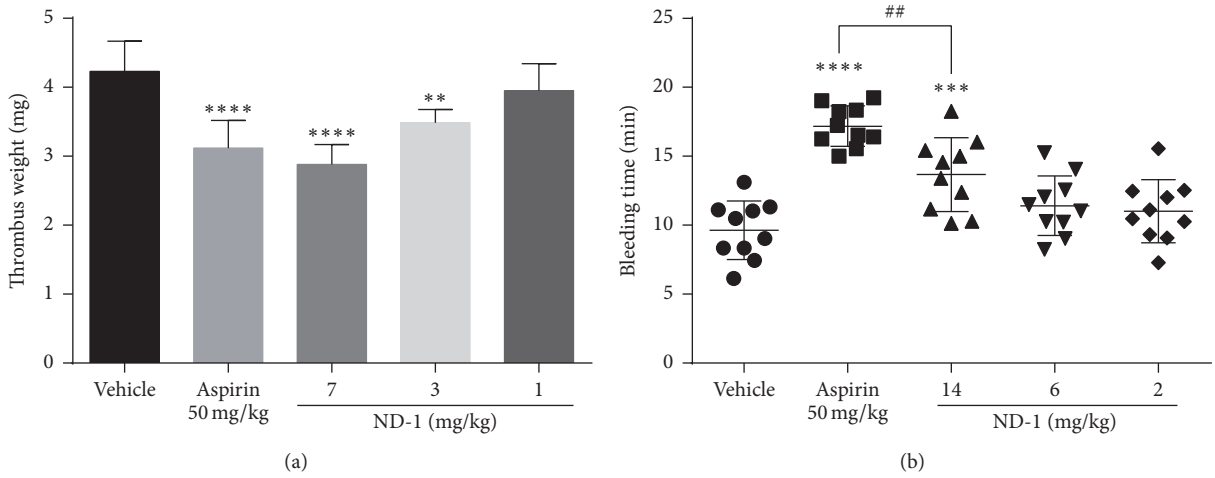

Figure 4: Effect of ND-1 on thrombus formation in rats and bleeding time in mice. (a) Effect of ND-1 on arteriovenous shunt thrombosis in rats. Animals were separated into five groups and ND-1 (1, 3, and 7 mg/kg), aspirin (50 mg/kg), or vehicle was injected through caudal vein. Fifteen minutes later, the rats were under thrombogenic challenge. Data are presented as mean ± SD ($n = 6$). (b) Effect of ND-1 on bleeding time in mice. Animals were separated into five groups and ND-1 (2, 6, and 14 mg/kg), aspirin (50 mg/kg), or vehicle was injected through caudal vein. The accumulated bleeding time was recorded within 20 min. Data are presented as mean ± SD ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus vehicle; $^{\#\#}P < 0.01$ versus aspirin-treated group.

In conclusion, ND-1 inhibited platelet aggregation via interfering the binding of fibrinogen to integrin αIIbβ3 and significantly attenuated the thrombus formation with lower bleeding risk. Thus, ND-1 can be a lead compound for antithrombotic agents development.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Xue Ding and Tong-dan Liu contributed equally to this work.

Acknowledgments

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