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A tryptophan derivative TD-26 attenuates thrombus formation by inhibiting both PI3K/Akt signaling and binding of fibrinogen to integrin αIIbβ3

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ABSTRACT

The incidence and mortality of thrombotic disorders are rapidly increasing worldwide. The existing antithrombotic drugs, however, are associated with side effects, especially bleeding complications. Therefore, there remains a need for the development of more effective and safer antithrombotic agents. In this study, we discovered a new synthetic tryptophan derivative TD-26, producing potent inhibitory effect on platelet aggregation while without causing obvious bleeding risk. It has been shown that TD-26 inhibited platelet aggregation induced by ADP, thrombin, U46619 and collagen *in vitro* and suppressed the platelet aggregation induced by ADP *ex vivo*. Mechanism studies indicated that TD-26 inhibited platelet adhesion to fibrinogen-coated surfaces, blocked the binding of fibrinogen to integrin αIIbβ3 and reduced Akt^{Ser473} phosphorylation in platelet phosphatidylinositol 3-kinase (PI3K) signaling. Furthermore, TD-26 exhibited potent antithrombotic activity *in vivo*. In animal models, it decreased death of mice with acute pulmonary thrombosis by 90% and attenuated thrombosis weight by 60.3%, both at a dose of 3 mg/kg. Additionally, TD-26 did not obviously prolong bleeding time in mice. Taken together, our results reveal that TD-26 is a novel antithrombotic compound exhibiting both integrin αIIbβ3 inhibition and PI3K signaling blockage, with a low bleeding risk.

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1. Introduction

Thrombotic disorders, including myocardial infarction, acute coronary syndrome, and pulmonary embolism, are a major cause of morbidity and mortality throughout the world [1]. Platelets play an important role in the development of these diseases [2]. Platelets circulate in blood and play significant roles in hemostasis, angiogenesis, inflammation and metastasis [3–5]. Once vessel injury occurs, platelets rapidly adhere to the exposed subendothelial matrix and then change shape, spread on fibrinogen, and release/produce ADP, thromboxane A_2, thrombin and epinephrine, which recruit additional platelets from the flowing blood to the injury site [6,7]. In this process, the major platelet receptor integrin αIIbβ3 is activated and bind plasma fibrinogen and von Willebrand factor, leading to the formation of platelet plug and thrombosis [8,9]. Accordingly, antiplatelet treatment has been established as an important approach to treat thrombotic diseases. Many kinds of antiplatelet drugs have been approved by FDA for use in clinical practice [10]. The cyclooxygenase inhibitor aspirin [11], the ADP receptor antagonist clopidogrel [10,12] and the integrin αIIbβ3 antagonist tirofiban are widely used to prevent and treat thrombosis. However, they have side effects, including increased bleeding risk, neutropenia and thrombocytopenia [13–15]. Consequently, there remains a need for the development of more effective and safer antiplatelet agents.

In our ongoing efforts to discover new antiplatelet agents, more than 500 synthetic compounds and natural products were screened for their ability to inhibit platelet aggregation. Among those, 2- (4-bromophenylsulfonamido) -3- (5- (4-(piperidin-4-yl) butoxy) -1H-indol-3-yl) propanoic acid (TD-26), a synthetic tryptophan derivative, was found to produce inhibitory effect on platelet aggregation induced by various agonists. There are few reports on platelet inhibitory effects of tryptophan derivatives. In this study, we

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investigated the effect of TD-26 on platelet aggregation, as well as the actions of TD-26 on both integrin αIIbβ3 receptor and intracellular signaling pathway. *In vivo* antithrombotic effect of TD-26 was also investigated using mouse acute pulmonary thrombosis and rat arterio-venous shunt models.

2. Materials and methods

2.1. Reagents

TD-26 was synthesized by Dr. Zhiyu Li (China Pharmaceutical University, China). ADP, thrombin, U46619, human fibrinogen, prostaglandin E1 (PGE1), anti-mouse IgG-conjugated alkaline phosphatase and disodium 4-nitrophenyl phosphate substrate were all 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 (CD61) antibody were from Enzyme Research Laboratories (South bend, IN, USA) and Millipore (Temecula, CA, USA), respectively. The PI3Kβ inhibitor, TGX-221 was from Cayman Chemical (Ann Arbor, MI, USA). Anti-Akt, anti-phospho-Akt, anti-p38, anti-phospho-p38, anti-Src, and anti-phospho-Src antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Animals and human blood samples

New Zealand rabbits (male, 2.0–2.5 kg), Sprague–Dawley (SD) rats (180–220 g), and Institute of Cancer Research (ICR) mice (18–22 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. Human blood was obtained from healthy donors in accordance with the Declaration of Helsinki and the permission from the Ethical Committee of China Pharmaceutical University.

2.3. In vitro platelet aggregation assay

In vitro platelet aggregation was measured using the turbidimetric method [16,17] with a minor modification. Briefly, carotid artery blood was collected from New Zealand rabbits, anticoagulated with a 1/10 volume 3.8% sodium citrate and centrifuged for 15 min at 950 rpm to prepare platelet-rich plasma (PRP) or 10 min at 3000 rpm to obtain platelet-poor plasma (PPP). PRP was preincubated with TD-26 or vehicle (saline) at 37 °C for 5 min. Then platelet aggregation was induced by ADP (10 μM), collagen (2.5 μg/ml), thrombin (2 U/ml) or U46619 (3 μM). The maximum platelet aggregation rate was determined within 5 min with continuous stirring.

2.4. Ex vivo platelet aggregation assay

Similar to the method described previously [18], SD rats were randomly divided into 5 groups with 6 rats (males and females in half) in each group. The rats were injected intravenously with TD-26 (0.3, 1, 3 mg/kg), aspirin (50 mg/kg) or vehicle. Thirty minutes after injection, blood samples were obtained from the rats and anticoagulated with 3.8% sodium citrate. The maximum platelet aggregation rate was measured as mentioned above.

2.5. Platelet adhesion

The effect of TD-26 on platelet adhesion to fibrinogen was examined as described previously [8,19]. To prepare labeled platelets, rat PRP was centrifugated for 8 min at 2700 rpm. The platelet pellets were resuspended in HBMT (138 mM NaCl, 12 mM $NaHCO_3$, 10 mM HEPES, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 7.4) and labeled with 14 μM calcein-AM for 30 min at 22 °C in the dark. After washing and centrifugation, the labeled platelets were resuspended in HBMT containing 2 mM $CaCl_2$. The platelet count was adjusted to 5×10^8 platelets/ml using HBMT. Black-walled 96-well microplates (Corning no.437111; Denmark, NUNC) were coated with human fibrinogen (100 μg/ml) at 4 °C overnight. Wells were then washed 3 times with Tris/saline (100 mM NaCl, 50 mM Tris, pH 7.4) and blocked with HBMT containing 2% BSA for at least 1 h. After 3 washes, TD-26 or HBMT and calcein-labeled platelets were added and incubated for 2 h in the dark. Wells were washed 4 times with HBMT/$CaCl_2$. The fluorescence intensity was measured by an automated plate reader ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 515$ nm, TECAN infinite M200 PRO).

2.6. Fibrinogen/integrin αIIbβ3 ELISA

Similar to the previous method [20], the 96-well microplates (Corning Incorporated, Corning, NY, USA) were coated with human fibrinogen (10 μg/ml) at 4 °C overnight. After washing with TACTS (20 mM Tris, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, pH 7.5), wells were blocked with 1% BSA in TACTS for 1 h. Purified human platelet integrin αIIbβ3 receptor (20 μg/ml) and TD-26 were added and incubated for 2 h at 37 °C, wells were washed followed by addition of mouse anti-human integrin β3 antibody (1:2000). After 1 h incubation at 37 °C, wells were washed and anti-mouse IgG-conjugated alkaline phosphatase (1:2000) was added. Three washes were performed after 1 h incubation. Disodium 4-nitrophenyl phosphate substrate was added and incubated for about 30 min, and 3 M NaOH was added to stop the reaction. The absorbance was estimated at 405 nm.

2.7. Western blot analysis

Western blot assay was performed as described by Su and Huang et al. [21,22]. Gel-filtered human platelets were pre-incubated with of TD-26, TGX-221 or vehicle for 10 min and then stimulated by agonists for 5 min with stirring at 37 °C. The reaction was stopped by RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM Tris, 150 mM NaCl containing protease inhibitors and phosphatase inhibitors). Samples were then boiled for 5 min. The proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a poly vinylidene difluoride (PVDF) membrane (Millipore) and subjected to western blot. The membranes were incubated with corresponding antibodies at 4 °C overnight, and then secondary antibodies HRP conjugated coat anti-Rabbit (catalog no. GGHL-15P-25) was added and incubated for 2 h. Protein levels were detected by a Tanon infrared imaging system.

2.8. Acute pulmonary thrombosis model in mice

To verify the anti-thrombotic activity of TD-26 *in vivo*, a modified method [18] was used. ICR mice were randomly divided into 6 groups with 10 mice (males and females in half) in each group. Fifteen minutes after intravenous injection with TD-26 (0.3, 1, 3 mg/kg), aspirin (50 mg/kg) or vehicle, ADP (300 mg/kg) was injected to induce thrombosis. The mice were observed for 15 min to record the mortality and recovered time.

2.9. Arterio-venous shunt model in rats

To estimate thrombus formation, a previously described method [23,24] was used. SD rats were randomly divided into 5 groups with

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