Introduction

Platelets play a crucial role in haemostasis and several pathophysiological processes. Collagen is a main initiator for platelet activation and aggregation. Given that Wnt signalling negatively regulates platelet function, and IWR-1 (a small molecule inhibitor for Wnt signalling) has the potential of inhibiting collagen synthesis, it is essential to investigate whether IWR-1 regulates collagen-induced platelet activation and protects against thrombogenesis. In the present study we found that IWR-1 pretreatment effectively suppressed collagen-induced platelet aggregation in a dose-dependent manner. In addition, IWR-1 also resulted in a decrease of P-selectin and phosphatidylserine surface exposure using fluorescence-activated cell sorting analysis. In vitro studies further revealed that IWR-1 had a negative effect on integrin α2β1 activation and platelet spreading. More importantly, the results from in vivo studies showed that IWR-1 exhibited a robust bleeding diathesis in the tail-bleeding assay and a prolonged occlusion time in the FeCl3-induced carotid injury model. Taken together, current results demonstrate that IWR-1 could effectively block collagen-induced platelet activity in vitro and in vivo, and suggest its candidacy as a new antiplatelet agent.

Keywords
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► collagen
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Abstract
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Note: Yanqing Wu and Ling Wang have made equally important and outstanding contributions to this paper and thus share senior authorship.

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Introduction
Platelets are the key element in terms of haemostasis with the basic function of binding to damaged blood vessels, aggregating to establish thrombi, and subsequently preventing excessive bleeding. The initiation of platelet activation is driven by stimulating products binding to platelet and relative prothrombotic factors. There are several pathways responsible for platelet activation, in which collagen receptors such as glycoprotein VI (GPVI)/Fc receptor γ-chain complex play a crucial role in mediating the process. During the platelet activation, vascular damage induces collagen to be exposed to circulating platelets followed by adhesion of platelets using collagen as a substrate. Previous study has demonstrated that the adhesion between platelets and collagen is mediated by receptor integrin α2β1, which allows the subsequent interaction between platelets and GPVI.

The prevailing evidence considers Wnt signalling pathway as one of the fundamental mechanisms associated with various
biological processes such as cell proliferation, differentiation, and migration. Previous studies also demonstrated the regulatory role of Wnt signalling in stem cell development and adult tissue homeostasis. The associated regulators in the Wnt signalling pathway are reported to significantly contribute to vascular development and endothelial cell specification. In addition, Wnt signalling pathway plays a key role in vascular inflammation, development of atherogenesis, and platelet formation. Previous studies have revealed the regulatory role of Wnt signalling pathway in platelet function in which Wnt3a is reported to modulate platelet adhesion and repress platelet activation to collagen. However, the precise mechanism of Wnt regulating the platelet formation or activation still remains unclear. A small molecule tankyrase inhibitor, IWR-1, is reported to be involved in Wnt/β-catenin signalling suppression in cancer stem-like cells. Meanwhile, recent study showed that IWR-1 is able to suppress the production of collagen in dermal fibroblasts, suggesting the correlation between the Wnt signalling pathway and collagen through inhibition from IWR-1. Given that IWR-1 can act on suppressing collagen production via regulating the Wnt signalling pathway, it is rational to propose the regulatory action of IWR-1 on platelet activation via the Wnt signalling pathway in a collagen-dependent way.

In the present study, we aimed to address the regulation on platelet activation in favour of the presence of IWR-1 to protect against thrombogenesis. The finding of the study might provide a novel insight in developing potent antiplatelet agents.

Materials and Methods

Human Subjects
The blood samples were collected from human subjects with a written consent. The blood collection was approved by the Second Affiliated Hospital of Nanchang University.

Animals
Commercially validated C57BL/6J mice (aged 8–10 weeks) were purchased from Biowit Technologies (Shenzhen, China). The mice were housed under a 12-hour light and 12-hour dark cycle with enough supplies (food and water) at 24°C. All the experiments subjected on mice were conducted under the regulation of relevant laws and guidelines, and with approval of the Ethical Committee of the Second Affiliated Hospital of Nanchang University.

Platelet Preparation
The platelets were prepared as described previously. Blood was collected into a tube containing ACQ solution (100 mM dextrose, 75 mM trisodium citrate, and 35 mM citric acid) and centrifuged at 237 × g for 10 minutes at room temperature. The platelets were then recovered from platelet-rich plasma and centrifuged at 483 × g for 10 minutes at room temperature. The platelets were washed with piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES) buffer (20 mM PIPES, 137 mM NaCl, pH 6.5) and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Platelets counts were analysed using an automated haematology analyser (Drew Scientific, Miami, Florida, United States).

Platelet Aggregation
The platelet-rich plasma was incubated with dimethyl sulfoxide (DMSO) control or 5 or 30 µM IWR-1 at 37°C for 3 minutes followed by activation with 2.0 µg/mL collagen type I. The aggregation of platelet was analysed using model 700 aggregometry systems (BD Biosciences, United States).

Platelet Adhesion and Spreading
The platelet spreading and adhesion was assessed with immunofluorescence microscopy as previously described. Briefly, the platelets were washed and incubated in the presence of IWR-1 at different concentrations (5 and 30 µM) separately for 5 minutes. The resting platelets were then activated with collagen for 10 minutes at 37°C and applied on a fibrinogen-coated coverslip for half an hour. Then, the platelets were washed again and fixed, permeabilized, and stained with tetramethylrhodamine (TRITC)-conjugated phalloidin. The coverslip was analysed from three consecutive fields using a microscope (Leica, Wetzlar, Germany).

Flow Cytometry
Flow cytometry was conducted to analyse the level of P-selectin, annexin-V, and integrin α2β1 as described previously. Briefly, the washed human platelets were incubated in the presence of IWR-1 for 5 minutes and activated with collagen (5 µM) for 10 minutes. Platelets were then incubated with fluorescein isothiocyanate (FITC) conjugated anti-P-selectin (304903; BioLegend, California, United States) to measure phosphatidylserine exposure, or antibody against integrin α2β1 (ab30483; Abcam, California, United States) and subsequently with appropriate secondary antibodies (ab150117, Abcam) to measure integrin α2β1 activation. The fluorescence intensities were analysed with a FACScan (Cell Signaling, United States). Experiments were repeated three times.

In Vivo Tail-Bleeding Assay
Mice were injected with 200 µL control (DMSO) or 2 mg/kg (body weight) IWR-1 dissolved in 200 µL DMSO, followed by the tail-bleeding assay after 1 hour, as previously described. The tail was cut into a length of 5 mm from the tip after the anaesthetization. The transected tail was kept in saline at constant temperature of 37°C. The time of bleeding until cessation was recorded.

In Vivo FeCl3-Induced Carotid Injury Model
Mice were injected intravenously with DMSO or IWR-1 (2 mg/kg) and a FeCl3-induced carotid injury assay was performed as previously described with some modifications. Briefly, mice at 21 to 32 weeks were anaesthetized with isoﬂurane (4% during induction of anaesthesia; 2–2.5% during maintenance of anaesthesia), and then the left carotid artery was exposed and observed using a dissecting microscope (Leica). The vascular blood flow of the exposed artery was measured by a microvascular probe after using a saturated (6% FeCl3) filter paper to injure the artery. The formation times of stable, occlusive thrombi were recorded.
Experiments were terminated at 3 minutes postocclusion or 30 minutes postinjury, whichever occurred first.

Statistical Analysis
All data are presented as mean ± standard deviation from at least three separate experiments. The differences between groups were analysed using unpaired Student’s t-test or one-way analysis of variance (ANOVA), followed by the Scheffé test (SPSS software version 18; IBM Corp., New York, United States). Differences were deemed statistically significant at $p < 0.05$.

Results
IWR-1 Inhibited Collagen-Induced Platelet Aggregation in a Dose-dependent Manner
Collagen, the main component of vascular subendothelial matrix, is the most potent vascular wall component that initiates platelet activation and aggregation.\textsuperscript{21,22} It is also a strong inducer for platelet adhesion. A previous study showed that IWR-1 (a specific inhibitor of the Wnt signalling pathway) inhibits the production and secretion of collagen from the fibroblasts.\textsuperscript{13} Additionally, given that Wnt signalling negatively regulates platelet function,\textsuperscript{11} it is possible that IWR-1 inhibits collagen-induced platelet activation and aggregation, and thus protects against thrombogenesis. To assess the regulatory act of IWR-1 on collagen-induced platelet aggregation, light transmittance aggregometry was performed. Human platelets were incubated in the presence 5- and 30-µM IWR-1 for 5 minutes followed by activation with collagen. As shown in \textsuperscript{→}Fig. 1A, B, platelets pretreated with 5-µM IWR-1 exhibited significant reduction in collagen-induced platelet aggregation, when compared with the control groups. 30 µM of IWR-1 displayed further inhibition on platelet aggregation, thus this inhibition was in a dose-dependent manner (\textsuperscript{→}Fig. 1A, B). IWR-1 still exerted an inhibitory effect on the platelet aggregation when a 10 µg/mL collagen concentration was used (\textsuperscript{→}Fig. 1C, D). But adenine diphosphate-mediated platelet aggregation was not affected by IWR-1 (\textsuperscript{→}Supplementary Fig. S1A). IWR-1 treatment did not cause platelet lysis (data not shown). These results

\textbf{Fig. 1} IWR-1 inhibited collagen-induced platelet aggregation in a dose-dependent manner. (A) Human platelet-rich plasma was incubated in the presence of IWR-1 in different concentrations (5 and 30 µM). Platelets were stimulated with DMSO control or collagen (2.0 µg/mL) and then platelet aggregation was assayed. Each experiment was performed at least three times. (B) Values of mean percentage (%) of aggregation are indicated in the figure. $^* p < 0.05$. (C) Human platelet-rich plasma was incubated in the presence of IWR-1 (30 µM). Platelets were stimulated with DMSO control or collagen (10 µg/mL) and then platelet aggregation was assayed. Each experiment was performed at least three times. (D) Values of mean percentage (%) of aggregation are indicated in the figure. $^* p < 0.05$. DMSO, dimethyl sulfoxide.
suggest that the IWR-1 has the capacity to exert ‘broad’ inhibitory effects on platelet aggregation.

**IWR-1 Inhibited Collagen-Induced P-selectin and Phosphatidylserine Surface Exposure**

Platelet secretion prior to the activation is an essential step in the activation process, thus the inhibitory effects of IWR-1 on this functional response were investigated. Before the activation with collagen, platelets pretreated with IWR-1 showed a significantly lower level of P-selectin expression, compared with the IWR-1-treated platelets (Fig. 2A). On the other hand, 30 µM of IWR-1 displayed further inhibition on the collagen-induced P-selectin secretion, showing that this inhibition was in a dose-dependent manner (Fig. 2A). These data suggest that the IWR-1 significantly diminished the secretion of P-selectin in activated platelets, and are consistent with the aggregation results. We did not observe the effect of IWR-1 on platelet ATP secretion (Supplementary Fig. S1B). The interaction between platelets and agonists is followed by another activation event, phosphatidylserine exposure. Phosphatidylserine is normally on the plasma membrane inner surface in the resting platelets, and the activation of platelets exposes the phosphatidylserine to the platelet’s outer surface. To assess the regulatory role of IWR-1 in platelet phosphatidylserine exposure, fluorescence-activated cell sorting (FACS) analysis was performed. As shown in Fig. 2B, the IWR-1-treated platelets displayed a decrease in collagen-induced phosphatidylserine exposure, compared to the control.

**IWR-1 Inhibited Collagen-Induced α2β1 Integrin Activation and Platelet Spreading**

The next investigation was addressed on whether IWR-1 affected integrin activation as a result of integrin α2β1 as the key collagen-binding receptor on the platelet membrane. Flow cytometry was performed to assess the expression of integrin α2β1. The IWR-1 was found to inhibit the levels of integrin α2β1 expression in a dose-dependent manner, in response to collagen (Fig. 3A). The activated platelet spreading occurs after its cytoskeletal remodelling, resulting in adhesions with the surrounding activated platelets, leading to further platelet aggregation. To address this process, we used phalloidin staining to observe the platelet spreading rate under the act of IWR-1. As shown in Fig. 3B, collagen treatment showed a promotion in the process of platelet spreading compared with the control, while pretreatment with IWR-1 significantly repressed collagen-induced platelet spreading. These results demonstrated that pretreatment of IWR-1 inhibited collagen-induced integrin activation and platelet spreading.

**IWR-1 Led to Impaired Haemostasis and Thrombus Formation**

An in vivo study was performed to assess the capacity of IWR-1 to act as an antiplatelet reagent. The carotid artery thrombosis model was applied to determine whether IWR-1 (2 mg/kg into C57BL/6 mice) showed any inhibitory effects on thrombogenesis. The occlusion of the vessel was completed in 174 seconds after FeCl₃ treatment; in the meantime, it was significantly delayed in the IWR-1-treated mice at 474 seconds after the treatment (Fig. 4A). We next investigated whether IWR-1 would exert negative consequences on haemostasis by measuring the tail-bleeding time. It was found that mice injected with IWR-1 had a significantly prolonged tail-bleeding time, relative to controls (Fig. 4B). Taken together, our data confirmed that the IWR-1 exhibited antithrombotic activity and antithrombus formation.

**Discussion**

It is well acknowledged that platelets have the key influence on haemostasis, where they bind to damaged blood vessels with the formation of thrombi. However, the platelet activation can lead to relative pathological conditions due to the unfavourable aggregation of activated platelets. Hence, it has been an exciting topic to search for the potent approach to regulate those platelet activation processes at unfavourable sites. Furthermore, the platelet activation is reportedly initiated by...
collagen, and previous studies have demonstrated the inhibitory effect of IWR-1 on the production and secretion of collagen from the fibroblasts, whereas the downregulation of platelet function driven by the Wnt signalling was also reported. On the ground of the previous findings of platelet activation and associated factors, in the present study we aimed to address the capacity of IWR-1 to inhibit collagen-induced platelet aggregation. We found that the IWR-1-pretreated platelets exhibited significant reduction in collagen-induced platelet aggregation in a dose-dependent manner, indicating the inhibitory act of IWR-1 on platelet aggregation. The platelet secretion was also assayed as the IWR-1-pretreated platelets displayed a low expression level of P-selectin in a dose-dependent way. The data suggested the inhibitory role of IWR-1 in platelet activation. These results are not consistent with a previous report. Steele et al demonstrated that canonical Wnt signalling negatively regulates platelet function; thus, disruption of the Wnt frizzled 6 receptor results in a hyperactive platelet phenotype. However, current results reveal that IWR-1, an inhibitor for Wnt signalling, inhibits collagen-induced platelet activation and protects against thrombogenesis. The potential mechanism of the difference is worth further study.

The agonists that induce the platelet activation are reportedly related to the act of various receptors in which the phosphatidylinerine exposure is involved. The activation of platelets is able to expose the inner-surface phosphatidylinerine to the outer surface. To address the phosphatidylinerine exposure with the addition of IWR-1, FACS analysis was conducted...
with the result of a significant decrease of collagen-induced phosphatidylserine exposure in pretreating platelets with IWR-1. Integrin α2β1 is reported to be the major collagen-binding receptor on the platelet membrane.24 Thus, it was selected as our target to assess the capacity of IWR-1 to inhibit collagen function. The flow cytometry result displayed a significant decrease of integrin α2β1 expression in the presence of IWR-1 in a dose-dependent manner, consistent with the reduced level of collagen-induced phosphatidylserine exposure.

Activated platelets show the capacity of secreting inflammatory mediators as well as adhering to surrounding platelets.25 Interactions between platelets and neutrophils and monocytes are also identified.26 It is well acknowledged that platelet spreading plays a key role in haemostatic plug formation and thrombosis with various receptor–ligand adhesion and biochemical signals involved. In the present study, we found that pretreatment with IWR-1 on platelets repressed the spreading process while collagen itself promoted the process. This result further confirmed the inhibitory role of IWR-1 in both activation and spreading processes. Subsequent in vivo study used a carotid artery thrombosis model to measure the capacity of IWR-1 against thrombotic activity and thrombus formation. The results displayed a delayed cessation time after the injury in the presence of IWR-1, as well as in FeCl3 treatment, and the occlusion time was delayed by nearly 300 seconds. Taken together, our data confirmed the antithrombotic activity of IWR-1 in vivo.

To conclude, our study showed that IWR-1 displayed significant inhibitions on both platelet secretion and aggregation as well as platelet spreading after activation. In vivo study established the antithrombotic effect of IWR-1 in the tail-bleeding assay and FeCl3-induced carotid injury model. Future studies are suggested to focus on the pathway regulation as IWR-1 might be involved. Additionally, the effect of IWR-1 on other stimulus to platelet activation except collagen is worth further study. Our data strongly suggest IWR-1 as a potent target for treatment against thrombogenesis in clinical use.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Acknowledgments
The study was supported by projects of the National Natural Science Foundation of China (grants 81660062 and 81560145).

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