

Ginsenoside Rk3 Inhibits Human Platelet Activation via cAMP Elevation and PI3K/MAPK Pathway Suppression

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ABSTRACT

Previous studies have established the aggregation-inhibitory properties of synthetic ginsenoside derivatives, such as G-Rp (1, 3, and 4), as well as natural ginsenosides isolated from *Panax ginseng*, including 20(S)-Rg3, Rg6, F4, and Ro. However, the specific inhibitory mechanisms of G-Rk3, another bioactive compound derived from *Panax ginseng*, require further detailed investigation. This research aimed to evaluate the effects of G-Rk3 on agonist-induced human platelet aggregation. We assessed the suppression of platelet signaling components, including integrin α IIb β 3-mediated fibrinogen binding via flow cytometry, along with the mobilization of intracellular calcium, the release of dense granules, and the secretion of thromboxane B2. Furthermore, the modulation of phosphorylation within the PI3K/MAPK pathway was examined, alongside observations of thrombin-stimulated clot retraction in platelet-rich plasma. Treatment with G-Rk3 markedly elevated cyclic adenosine monophosphate (cAMP) levels, resulting in the substantial phosphorylation of the cAMP-dependent kinase targets vasodilator-stimulated phosphoprotein (VASP) and inositol 1,4,5-trisphosphate receptor (IP3R). G-Rk3 administration suppressed Ca²⁺ release from the dense tubular system and diminished overall platelet reactivity by deactivating the integrin α IIb/ β 3, which subsequently decreased fibrinogen binding. Additionally, G-Rk3 attenuated the phosphorylation of the MAPK and PI3K/Akt pathways, leading to a decline in both intracellular granule secretion and TXA2 generation. Ultimately, G-Rk3 successfully prevented platelet aggregation and subsequent fibrin-dependent thrombus formation. These findings indicate that G-Rk3 holds significant potential as a valuable prophylactic or therapeutic agent in the management of cardiovascular disorders driven by aberrant platelet aggregation and thrombogenesis.

Keywords: G-Rk3, Platelet aggregation, Cyclic nucleotide, Intracellular Ca²⁺, Granule secretion

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Introduction

Cardiovascular disease (CVD) remains the primary cause of global mortality among all medical conditions. In the United States specifically, coronary heart disease accounts for one in seven fatalities, while heart failure is responsible for one in nine deaths [1].

Following vascular damage, effective hemostasis depends on appropriate platelet activity to facilitate blood clotting and prevent hemorrhage. Conversely, aberrant platelet activation and subsequent aggregation can generate pathological thrombi, which contribute to prevalent cardiovascular conditions, including atherosclerosis, coronary artery disease, heart failure, and stroke [2].

Pharmacological management of thrombotic complications typically operates by suppressing platelet function. While contemporary CVD therapies and preventative measures utilize this mechanism, they are frequently associated with significant adverse effects, underscoring the necessity for superior therapeutic alternatives [3].

Utilizing naturally occurring bioactive molecules has emerged as a promising approach for managing thrombosis and platelet aggregation-related pathologies [4]. Specifically, elements of the traditional Mediterranean diet, alongside various medicinal plants, have demonstrated significant antiplatelet and cardioprotective efficacy in the context of CVD prevention [5-7].

Under healthy physiological conditions, vascular endothelial cells release prostaglandin I₂ and nitric oxide into the bloodstream, triggering the synthesis of cAMP and cGMP within platelets. Elevated cAMP levels stimulate protein kinase A (PKA), whereas increased cGMP stimulates protein kinase G (PKG). These two kinases subsequently phosphorylate vasodilator-stimulated phosphoprotein (VASP) and the inositol 1,4,5-trisphosphate receptor (IP3R) [8]. The phosphorylation of IP3R blocks the mobilization of Ca²⁺ from the dense tubular system into the cytoplasm [9]. Furthermore, VASP, serving as a critical target for both PKA and PKG, plays a key role in modulating integrin α Ib/ β 3 and actin filament dynamics [10, 11].

A crucial phase of aggregation involves the release of ATP- and serotonin-rich platelet granules, a process closely linked to the phosphorylation of mitogen-activated protein kinases (MAPKs) and the PI3K/Akt pathway [12]. The MAPK family, which includes p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), governs intracellular signaling networks vital for hemostasis and thrombus generation [13]. These kinases are expressed in human platelets and serve as essential signaling mediators that promote the secretion of platelet granules [14-18]. Furthermore, MAPK signaling induces the phosphorylation of membrane-bound cPLA₂, which amplifies TXA₂ synthesis and thereby exacerbates platelet activation and clustering [19, 20]. Similarly, the PI3K/Akt cascade modulates dense granule secretion, general platelet regulation, and aggregation [21].

Ginsenosides derived from *Panax ginseng* mediate a wide array of biological responses. The primary constituents of red and white ginseng include ginsenosides Rb1, Rc, Rd, Re, and Rg1, while the minor constituents encompass ginsenosides Rg5, Rk1, Rg6, and F4 [22, 23]. Prior research indicates that G-Rk3 exerts anti-carcinogenic properties in lung cancer models, suppresses apoptotic and inflammatory responses during hepatic injury, and mitigates nephrotoxicity induced by cisplatin [24-26]. Indeed, corroborating studies have confirmed G-Rk3's capacity to combat lung malignancies, restrict liver tissue inflammation and apoptosis, and defend against cisplatin-associated renal impairment [24-26]. Nevertheless, because there is a scarcity of research exploring the specific antiplatelet mechanisms of G-Rk3, the present study sought to investigate how G-Rk3 modulates the aggregation of human platelets.

Materials and Methods

Materials

Antibodies and western blotting lysis buffers were obtained from Cell Signaling (Beverly, Massachusetts, USA). Ginsenoside Rk3 (G-Rk3) was procured from the Ambo Institute (Daejeon, South Korea). Platelet agonists, including collagen, U46619, and thrombin, were purchased from Chrono-Log Corporation (Havertown, Pennsylvania, USA). Fura 2-AM and Alexa Fluor 488-conjugated fibrinogen were supplied by Invitrogen Molecular Probes (Eugene, Oregon, USA). Enzyme immunoassay (EIA) kits for quantifying TXB₂, serotonin, ATP, and cyclic nucleotides (cAMP/cGMP) were sourced from Cayman Chemical Co. (Ann Arbor, Michigan, USA). Polyvinylidene difluoride (PVDF) membranes and enhanced chemiluminescence reagents were acquired from Thermo Fisher Scientific (Gangnam-gu, Seoul, South Korea). All additional unlisted chemicals and reagents were obtained from Sigma Chemical Corporation (St. Louis, Missouri, USA).

Preparation of human washed platelets

Human platelets were prepared following an established and validated methodology [27]. Platelet-rich plasma (PRP) was obtained from the Korean Red Cross Blood Center (Suwon, South Korea). To isolate the platelets, the PRP was centrifuged at 1,300 × g for 10 minutes. The collected platelets were subsequently washed twice using a buffer containing 2.7 mM KCl, 138 mM NaCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 1 mM Na₂EDTA, and 5.5 mM glucose, adjusted to a pH of 6.9. Following the washing steps, the platelets were resuspended in a buffer composed of 2.7 mM KCl, 138 mM NaCl, 12 mM NaHCO₃, 0.49 mM MgCl₂, 0.36 mM NaH₂PO₄, 5.5 mM glucose, and 0.25% gelatin at a pH of 7.4. This procedure yielded a final platelet concentration of 108 cells/mL. All preparation steps were conducted at a constant temperature of 25°C to prevent spontaneous platelet

aggregation. The utilization of human biological samples was formally approved by the Institutional Review Board at Namseoul University, South Korea (approval number 1041479-HR-202110-002).

Measurement of platelet aggregation

Suspended platelets (108 cells/mL) were incubated with varying concentrations of G-Rk3 for 3 minutes at 37°C. Subsequently, 2 mM CaCl₂ was added to the suspension, followed by the introduction of collagen to induce stimulation for a duration of 5 minutes. Aggregation was monitored using a Chrono-Log aggregometer (Havertown, Pennsylvania, USA) with a continuous stirring speed of 1,000 rpm. The rate of aggregation was determined based on the increase in light transmittance. A baseline reference value of 0% (permeability) was established using the suspension buffer. G-Rk3 was dissolved in dimethyl sulfoxide (DMSO) at a consistent final concentration of 0.1% across all experimental assays.

Measurement of cytotoxicity

Cytotoxicity was evaluated by detecting the release of cytoplasmic lactate dehydrogenase (LDH). Platelet suspensions (108 cells/mL) were treated with varying doses of G-Rk3 and incubated at room temperature for 2 hours. Following this incubation period, the samples were centrifuged at 12,000 × g for 2 minutes. The resulting supernatant was then analyzed using an LDH EIA kit and a Synergy HT multi-mode reader (BioTek Instruments, Winooski, Vermont, USA).

Measurement of cyclic nucleotide (cAMP and cGMP) production

Platelet suspensions at a concentration of 108 cells/mL were incubated with varying doses of G-Rk3 for 3 minutes at 37°C. After this incubation, 2 mM CaCl₂ and collagen were introduced to trigger stimulation for 5 minutes. The reaction was subsequently halted by adding 1 M HCl. Levels of cyclic nucleotides (cAMP and cGMP) were quantified using respective EIA kits and analyzed with a Synergy HT multi-mode reader (BioTek Instruments, Winooski, Vermont, USA).

Measurement of intracellular Ca²⁺ mobilization

PRP was incubated with 5 μM of Fura 2-AM at 37°C for 60 minutes. After preparing the platelet suspension according to the previously outlined protocol, the washed platelets were supplemented with 2 mM CaCl₂ and incubated for 3 minutes at 37°C. Collagen was then introduced to stimulate the cells for 5 minutes. Fura 2 fluorescence was recorded utilizing an SFM 25 spectrophotometer (BioTek Instruments, Winooski, Vermont, USA). Excitation wavelengths alternated between 340 nm and 380 nm every 0.5 seconds, while the emission wavelength was recorded at 510nm. The concentration of mobilized intracellular Ca²⁺ was determined utilizing the established Grynkiewicz equation [28].

Measurement of fibrinogen binding

Suspended human platelets (108 cells/mL) were treated with 2 mM CaCl₂ and combined with 30 μg/mL of Alexa Fluor 488-conjugated human fibrinogen. Collagen was then added to stimulate the platelets for 5 minutes. The reaction was halted by introducing phosphate-buffered saline (PBS, pH 7.4) containing 0.5% paraformaldehyde. To ensure experimental precision, all procedures were conducted in the dark. Fibrinogen binding was quantified using a flow cytometer (FACS) and analyzed with Cell-Quest software (both supplied by BD Biosciences, San Jose, California, USA).

Measurement of TXB₂ production

Platelet suspensions at a concentration of 108 cells/mL were pre-incubated with various concentrations of G-Rk3 for 3 minutes at 37°C. Stimulation was then induced for 5 minutes through the addition of 2 mM CaCl₂ and collagen. Because TXB₂ serves as a stable metabolic byproduct of TXA₂, TXB₂ production levels were quantified utilizing a specific TXB₂ EIA kit and measured with a Synergy HT multi-mode reader (BioTek Instruments, Winooski, Vermont, USA).

Measurement of ATP and serotonin release

Platelet suspensions (108 cells/mL) were incubated with various concentrations of G-Rk3 for 3 minutes at 37°C. Subsequently, 2 mM CaCl₂ and collagen were added to induce stimulation for a period of 5 minutes. The reaction

was then halted by introducing 2 mM ice-cold EDTA. Following this, the mixture was centrifuged to isolate the released serotonin and ATP within the supernatant. The respective EIA kits (serotonin and ATP) along with a Synergy HT multi-mode reader (BioTek Instruments, Winooski, Vermont, USA) were utilized to quantify the levels of serotonin and ATP secretion.

Western immunoblot analysis

A 1x lysis buffer was introduced to terminate the reaction. The protein concentration of the resulting platelet lysates was determined employing a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Subsequently, 20 µg of the extracted protein was separated via 4-20% SDS-PAGE and transferred onto a PVDF membrane. The membranes were then incubated with primary antibodies at a 1:1000 dilution, followed by treatment with secondary antibodies at a 1:2000 dilution. Protein bands were visualized utilizing an ECL reagent supplied by Thermo Fisher Scientific (Gangnam-gu, Seoul, South Korea).

Assessment of platelet-mediated fibrin clot formation

First, 2 mM CaCl₂ was introduced to 500 µL of PRP, followed by the addition of 0.05 U/mL of thrombin to induce stimulation. This procedure was conducted in a polyethylene tube to prevent surface adhesion. The samples were incubated at 37°C for 15 minutes. The resulting fibrin clots were captured using a digital camera, and the total coagulation area was subsequently quantified using ImageJ software (v. 1.46, National Institutes of Health, USA).

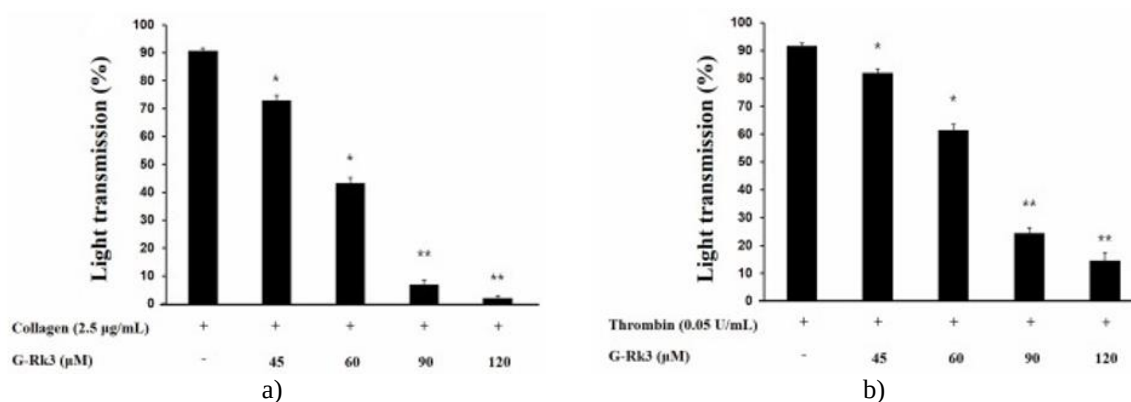
Statistical analysis

All experimental findings are expressed as the mean ± standard deviation (SD). Statistical significance was established at $P < 0.05$, determined via Student's t-test or analysis of variance (ANOVA). When ANOVA indicated significant variations among group means, Scheffe's post hoc test was applied for further evaluation. All statistical computations were executed using SPSS version 21.0.0.0 (SPSS, Chicago, IL, USA), with a p-value of < 0.05 denoting statistical significance.

Results and Discussion

Effects of G-Rk3 on human platelet aggregation and cytotoxicity

Human platelet aggregation was successfully triggered by collagen (2.5 µg/mL), U46619 (200 nM), and thrombin (0.05 U/mL) (**Figures 1a-1c**). The resulting aggregation rates reached 90.8% for collagen, 91% for U46619, and 90.3% for thrombin. Upon treatment with G-Rk3, the most pronounced suppression of aggregation occurred in the collagen-stimulated platelets. The degree of inhibition exhibited a dose-dependent relationship with G-Rk3 concentrations (19.0% at 45 µM, 44.0% at 60 µM, 81.2% at 90 µM, and 96.5% at 120 µM) (**Figure 1a**). Furthermore, G-Rk3 exhibited no evidence of cytotoxicity (**Figure 1d**). Collectively, these findings indicate that G-Rk3 potently suppresses platelet aggregation while avoiding detrimental cytotoxic effects.



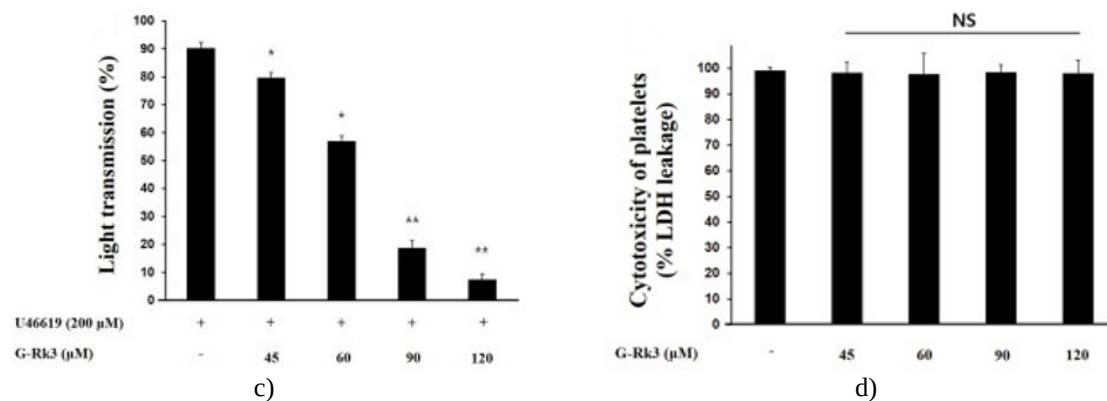
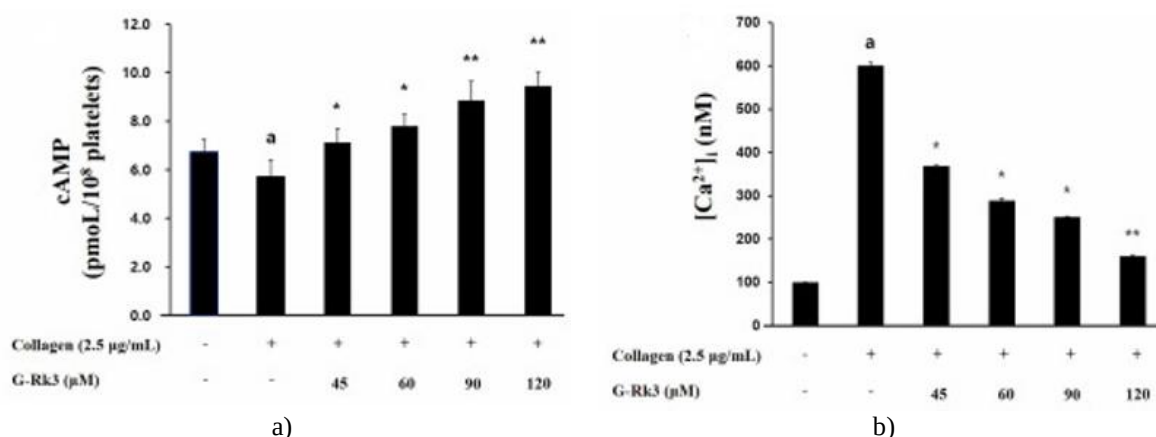


Figure 1. Influence of G-Rk3 on agonist-stimulated aggregation of human platelets. (a) Impact of G-Rk3 on platelet aggregation triggered by collagen. (b) Impact of G-Rk3 on platelet aggregation triggered by thrombin. (c) Impact of G-Rk3 on platelet aggregation triggered by U46619. (d) Evaluation of G-Rk3 cytotoxicity in human platelets. Data are presented as the mean $\pm$ SD ($n = 4$). * $P < 0.05$, ** $P < 0.001$ relative to the collagen-stimulated platelets.

Effects of G-Rk3 on cyclic nucleotide generation, intracellular Ca^{2+} mobilization, and IP3R phosphorylation

Because the cyclic nucleotides cAMP and cGMP are known to downregulate platelet activation, we examined the capacity of G-Rk3 to elevate these signaling molecules (cAMP/cGMP). A dose-dependent elevation in cAMP generation was noted following G-Rk3 treatment. Conversely, no significant alterations in cGMP levels were detected (data not shown). These findings indicate that G-Rk3 suppresses platelet activation primarily by robustly enhancing cAMP synthesis, rather than cGMP, in collagen-stimulated platelets.

Given that intracellular Ca^{2+} mobilization is a crucial event for proper platelet activation, we evaluated the impact of G-Rk3 on calcium dynamics. Following collagen exposure, intracellular calcium concentrations surged from 100.2 ± 0.5 nM to 600.5 ± 7.5 nM. Upon administering various concentrations of G-Rk3 (45~120 μM), a dose-dependent reduction in calcium levels was documented (**Figure 2b**). Because the IP3R protein plays a key role in regulating intracellular calcium stores, the influence of G-Rk3 on IP3R phosphorylation was also assessed. Similarly, treatment with escalating doses of G-Rk3 (45~120 μM) demonstrated a positive dose-response relationship with IP3R phosphorylation, with all concentrations exceeding 45 μM yielding statistically significant effects. Collectively, these data suggest that G-Rk3 decreases intracellular calcium concentrations by promoting the phosphorylation of IP3R.



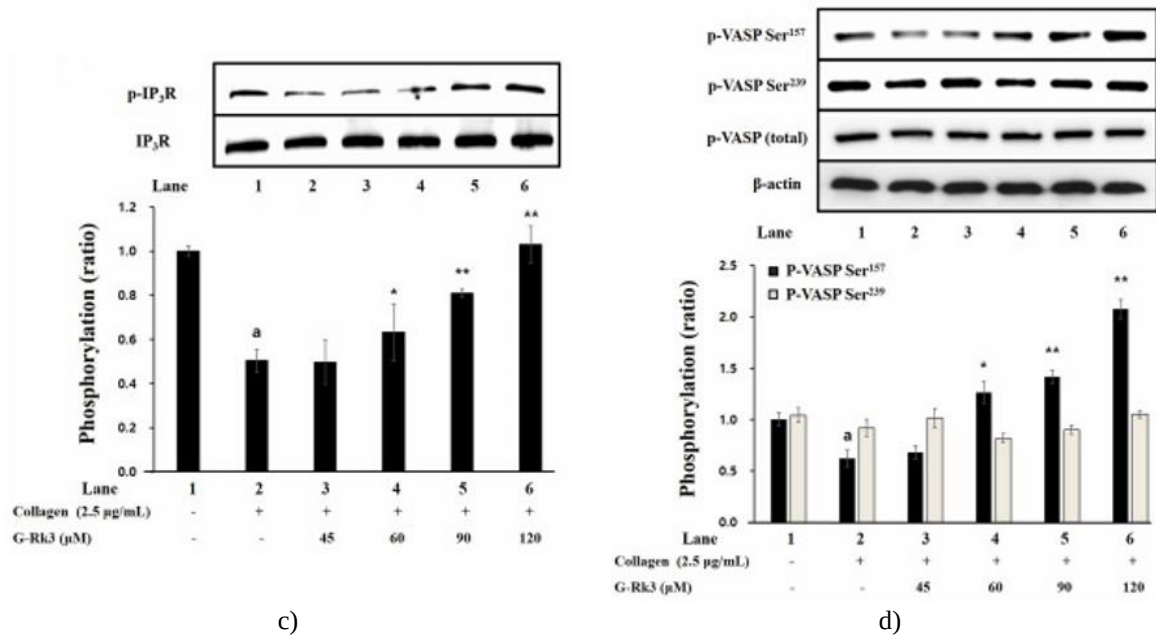


Figure 2. Impact of G-Rk3 on cAMP generation, intracellular Ca²⁺ mobilization, and the phosphorylation of associated proteins. (a) Influence of G-Rk3 on cAMP synthesis. (b) Influence of G-Rk3 on the mobilization of intracellular Ca²⁺. (c) Influence of G-Rk3 on the phosphorylation of IP3R. (d) Influence of G-Rk3 on the phosphorylation of VASP. Data are expressed as the mean $\pm$ SD (n = 4). aP < 0.05 relative to unstimulated platelets; *P < 0.05, **P < 0.001 relative to collagen-stimulated platelets.

Effects of G-Rk3 on VASP phosphorylation and fibrinogen binding

Given that the treatment of collagen-stimulated platelets with G-Rk3 elevated cAMP levels, we evaluated the impact of G-Rk3 on the cAMP/cGMP-dependent substrate VASP. The phosphorylation of Ser157 on cAMP-dependent VASP was markedly enhanced following G-Rk3 administration, whereas the phosphorylation of Ser239 on cGMP-dependent VASP remained largely unaltered (**Figure 2d**). Quantitatively, concentrations exceeding 60 µM demonstrated statistically significant induction, confirming that the G-Rk3-mediated elevation of cAMP drives the pronounced phosphorylation of Ser157 on cAMP-dependent VASP.

Prompted by the capacity of G-Rk3 to enhance cAMP synthesis and VASP Ser157 phosphorylation, we next investigated the effects of G-Rk3 on fibrinogen binding to the integrin α IIB/ β 3. Under collagen stimulation, fibrinogen binding to α IIB/ β 3 escalated to $92.1 \pm 1.4\%$ (**Figures 3ab and 3b**). Nevertheless, G-Rk3 treatment suppressed this fibrinogen/ α IIB/ β 3 interaction in a dose-dependent manner. At the maximum evaluated concentration of 120 µM, G-Rk3 achieved a substantial inhibition rate of 93.3% (**Figures 3af and 3b**).

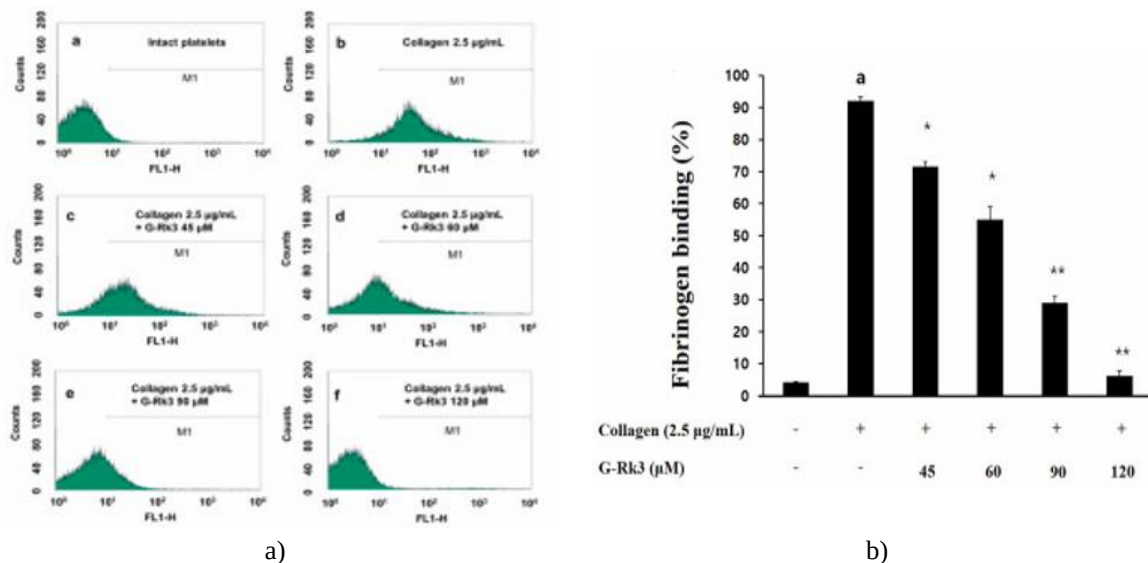


Figure 3. Modulation of fibrinogen binding by G-Rk3. (a) Flow cytometric histograms depicting fibrinogen binding. a, Unstimulated platelets (baseline); b, collagen (2.5 µg/mL); c, collagen (2.5 µg/mL) + G-Rk3 (45 µM); d, collagen (2.5 µg/mL) + G-Rk3 (60 µM); e, collagen (2.5 µg/mL) + G-Rk3 (90 µM); f, collagen (2.5 µg/mL) + G-Rk3 (120 µM). (b) Impact of G-Rk3 on the percentage of collagen-stimulated fibrinogen binding. Data represent the mean ± SD (n = 4). ap < 0.05 relative to unstimulated platelets; *p < 0.05, **p < 0.001 relative to collagen-stimulated platelets.

Influence of G-Rk3 on the phosphorylation of PI3K/Akt and MAPK

Because the PI3K/Akt signaling axis regulates the exocytosis of platelet granules, the regulatory influence of G-Rk3 on PI3K/Akt phosphorylation was assessed. Stimulation with collagen led to a robust increase in the phosphorylation of both PI3K and Akt. However, concurrent treatment with G-Rk3 significantly attenuated this collagen-induced phosphorylation (**Figure 4a**), confirming that G-Rk3 effectively blocks activation of the PI3K/Akt pathway.

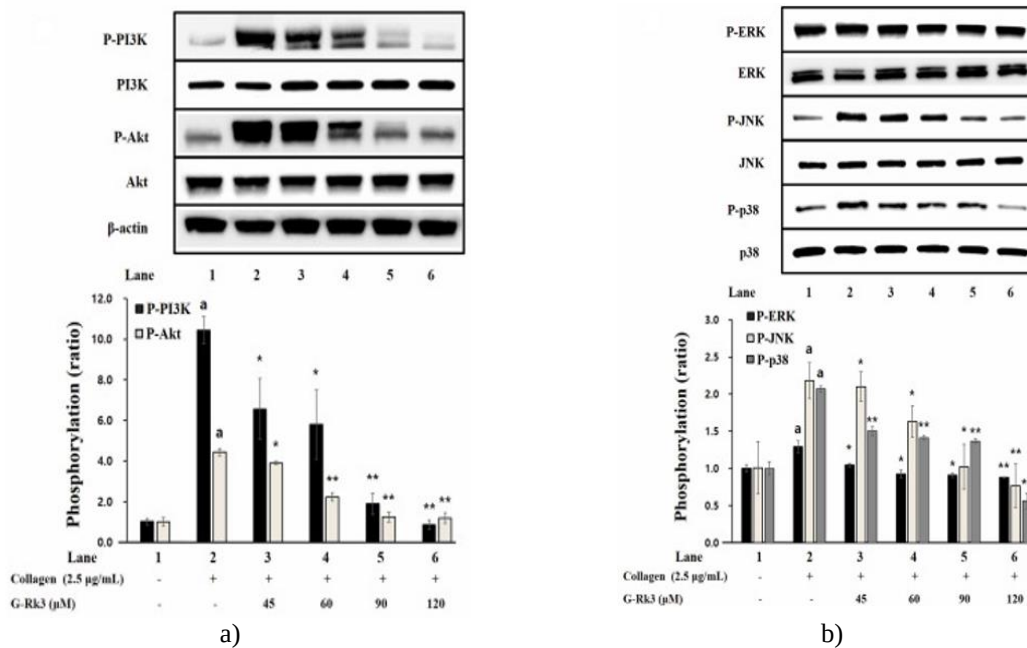


Figure 4. Effects of G-Rk3 on the phosphorylation status of PI3K/Akt and MAPK pathways. (a) Influence of G-Rk3 on PI3K/Akt phosphorylation. (b) Influence of G-Rk3 on MAPK phosphorylation. Data are expressed as the mean ± SD (n = 4). ap < 0.05 relative to unstimulated platelets; *p < 0.05, **p < 0.001 relative to collagen-stimulated platelets.

The MAPK cascades—comprising p38, ERK, and JNK—participate in platelet granule exocytosis and TXA₂ synthesis. Consequently, the effect of G-Rk3 on MAPK phosphorylation was examined. Exposure to collagen markedly upregulated the phosphorylation of all three MAPK components (p38, ERK, and JNK) relative to resting platelets. Conversely, the introduction of G-Rk3 significantly suppressed this collagen-induced phosphorylation of MAPK proteins (**Figure 4b**). These observations confirm that G-Rk3 modulates platelet aggregation via the inhibition of ERK, JNK, and p38 MAPK activation.

Actions of G-Rk3 on granule secretion, thromboxane B₂, and cPLA₂ dephosphorylation

Because the exocytosis of platelet granules (releasing ATP and serotonin) drives platelet aggregation, we examined whether G-Rk3 could modulate this secretory process. Upon stimulation with 2.5 µg/mL of collagen, ATP secretion rose sharply to 9.2 ± 0.2 µM from a baseline of 1.1 ± 0.1 µM in resting cells (**Figure 5a**). However, pre-treatment with various concentrations of G-Rk3 (45, 60, 90, and 120 µM) significantly blunted this increase. Similarly, collagen exposure (2.5 µg/mL) elevated serotonin release to 125.8 ± 6.2 ng/108 cells compared to 16.3 ± 2.1 ng/108 cells in resting platelets. G-Rk3 administration similarly countered this collagen-induced serotonin secretion in a dose-dependent manner, yielding release values of 96.5 ± 8.4 ng/108 cells at 45 µM, 76.5 ± 2.16 ng/108 cells at 60 µM, 69.0 ± 2.9 ng/108 cells at 90 µM, and 43.9 ± 7.5 ng/108 cells at 120 µM (**Figure 5b**).

Collectively, these findings demonstrate that G-Rk3 effectively curtails dense granule exocytosis by restricting ATP and serotonin release.

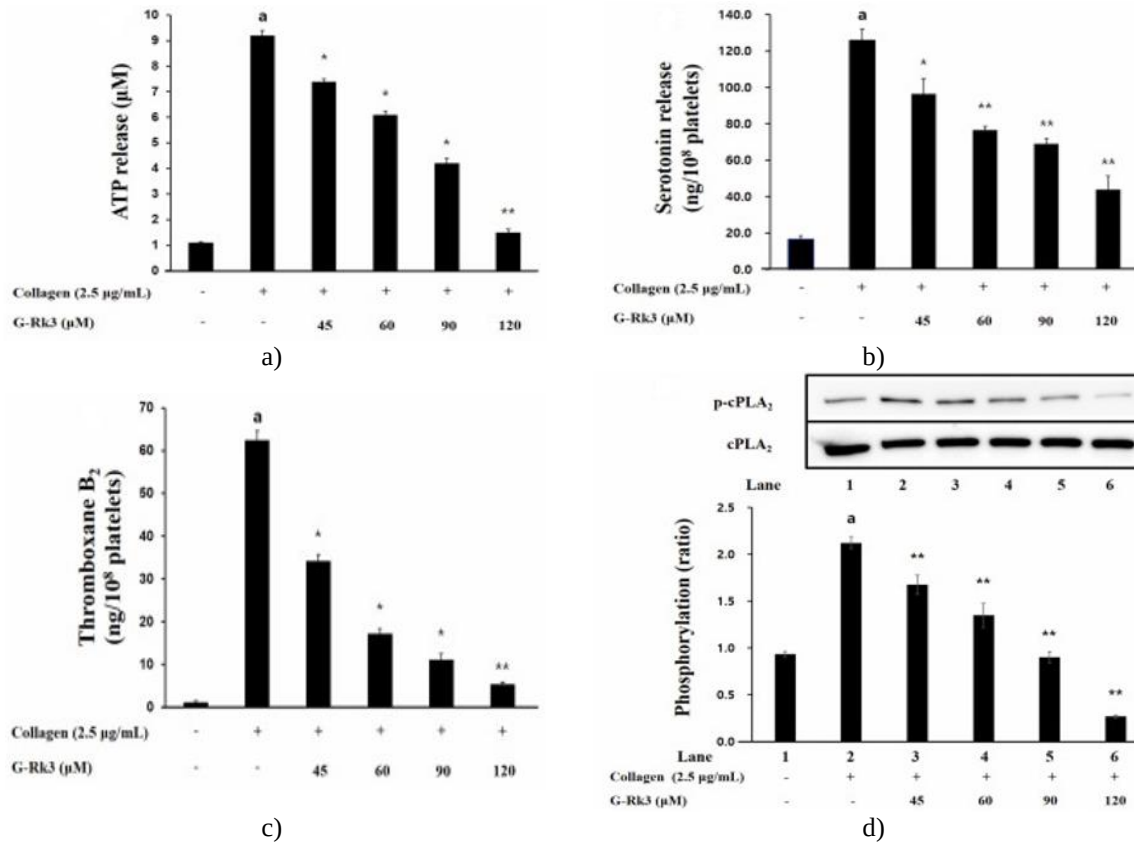


Figure 5. Regulatory effects of G-Rk3 on granule exocytosis, TXA₂ synthesis, and cPLA₂ phosphorylation. (a) Influence of G-Rk3 on ATP release. (b) Influence of G-Rk3 on serotonin release. (c) Influence of G-Rk3 on TXA₂ production. (d) Influence of G-Rk3 on cPLA₂ phosphorylation. Results are expressed as the mean $\pm$ SD (n = 4). aP < 0.05 relative to unstimulated platelets; *P < 0.05, **P < 0.001 relative to collagen-stimulated platelets.

TXA₂ functions as a potent autacoid that amplifies platelet aggregation; hence, the ability of G-Rk3 to modulate TXA₂ levels was evaluated. TXA₂ generation (quantified via its stable metabolite TXB₂) rose substantially from 1.2 ± 0.3 ng/10⁸ platelets in resting cells to 62.5 ± 2.1 ng/10⁸ platelets following 2.5 μg/mL collagen stimulation (**Figure 5c**). Upon G-Rk3 administration, this collagen-stimulated surge in TXA₂ was markedly reduced, falling to 5.4 ± 0.5 ng/10⁸ platelets. Notably, a dose of 120 μM achieved a striking 98% level of suppression (**Figure 5d**).

Cytosolic phospholipase A₂ (cPLA₂) mediates the liberation of arachidonic acid from the cell membrane into the cytosol; thus, we evaluated the impact of G-Rk3 on cPLA₂ phosphorylation. Elevated intracellular calcium levels stimulate cPLA₂ activation and drive its translocation to the membrane, with optimal enzymatic activity achieved upon phosphorylation at the Ser505 residue. While collagen stimulation induced robust cPLA₂ phosphorylation, the presence of G-Rk3 significantly inhibited this modification in a dose-dependent manner.

Influence of G-Rk3 on platelet-mediated fibrin clot formation

Fibrin clot formation is initiated by platelet agonists that trigger cellular activation and aggregation, ultimately driving outside-in signaling cascades. Consequently, we investigated whether G-Rk3 could modulate thrombin-induced fibrin clot formation. Fibrin clot retraction proceeded efficiently upon thrombin exposure (**Figure 6a**); however, treatment with escalating doses of G-Rk3 significantly inhibited this process. The rate of clot inhibition was directly proportional to the G-Rk3 concentration, showing values of 17.7% at 60 μM, 54.7% at 90 μM, and 97.1% at 120 μM (**Figure 6b**). These findings demonstrate that G-Rk3 strongly impairs fibrin clot formation and thrombogenesis.

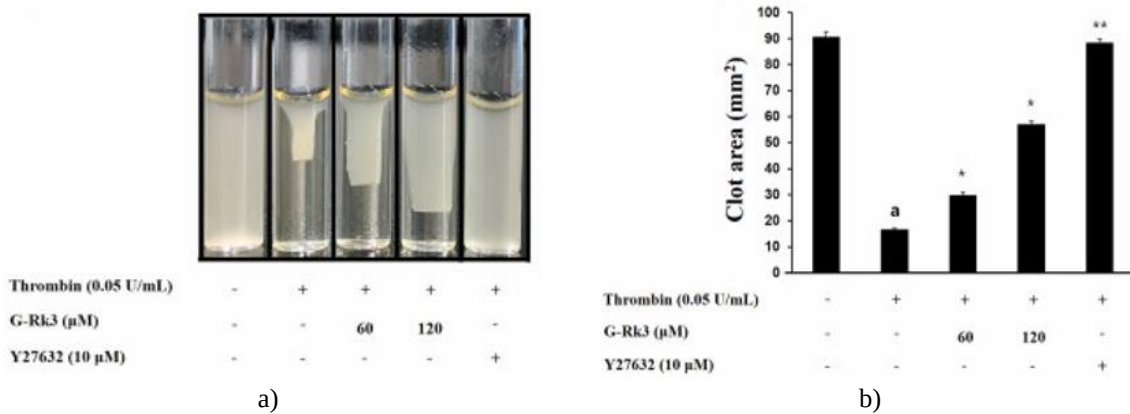


Figure 6. Suppressive effects of G-Rk3 on platelet-driven fibrin clot formation. (a) Visual representation of thrombin-induced fibrin clot retraction under G-Rk3 treatment. (b) Quantitative evaluation of G-Rk3 on the surface area of thrombin-retracted fibrin clots. Results are presented as the mean $\pm$ SD (n = 4). $a_p < 0.05$ relative to unstimulated platelets; $*p < 0.05$, $**p < 0.001$ relative to thrombin-stimulated platelets.

On the platelet membrane, the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C- γ 2 (PLC- γ 2) yields two distinct molecules: diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP₃). Subsequently, IP₃ triggers the efflux of calcium (Ca²⁺) from the dense tubular system into the cytosolic space [29]. This influx of intracellular calcium facilitates the phosphorylation of pleckstrin and myosin light chain, which are critical steps in platelet aggregation [30]. Conversely, elevated levels of the intracellular cyclic nucleotides cAMP and cGMP lead to a reduction in calcium concentrations. These two cyclic nucleotides stimulate cyclic-dependent protein kinases—specifically, protein kinase A (PKA) and protein kinase G (PKG)—which act to suppress platelet aggregation [31].

The present investigation revealed that G-Rk3 substantially upregulated the synthesis of cAMP and effectively prevented the release of intracellular calcium ([Ca²⁺]_i). The resultant elevation in cAMP triggers PKA activation, prompting the phosphorylation of multiple downstream targets, including the inositol 1,4,5-trisphosphate receptor (IP₃R) [8]. As illustrated in **Figure 3b**, IP₃R phosphorylation escalated in a dose-dependent manner following G-Rk3 administration. Consequently, the stimulation of the cAMP/PKA/IP₃R signaling axis and the concurrent decline in cytosolic calcium are directly attributable to the G-Rk3 treatment.

Furthermore, the G-Rk3-mediated rise in cAMP promoted the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) through the activation of PKA. VASP represents an essential substrate for both PKA and PKG, playing a pivotal role in modulating platelet function by governing adhesion and secretion processes. The phosphorylation of this protein suppresses platelet aggregation by obstructing the activation of integrin α IIb/ β 3 [32, 33]. Based on our findings, treating platelets with G-Rk3 successfully prevented the association between fibrinogen and α IIb/ β 3. This inhibitory action is likely driven by the capacity of G-Rk3 to activate the cAMP/PKA/VASP signaling cascade, culminating in VASP Ser157 phosphorylation and the subsequent blockade of α IIb/ β 3 and fibrinogen binding.

Additional research is required to elucidate the precise mechanisms by which G-Rk3 elevates cAMP levels. One potential avenue to explore is the reliance of cyclic nucleotides (cAMP and cGMP) on the dynamic balance between activating cyclases (adenylyl and guanylyl) and phosphodiesterase (PDE) [34]. Inhibiting PDE during the platelet aggregation process allows cyclic nucleotides to accumulate; accordingly, PDE inhibitors have proven efficacious in the clinical management of thrombosis [35]. Numerous widely used PDE inhibitors, including cilostazol, dipyridamole, and triflusal, are administered clinically to enhance the generation of cyclic nucleotides [36, 37]. Consequently, there is strong reason to hypothesize that G-Rk3 may yield comparable therapeutic benefits.

Moreover, the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways are crucial phosphoproteins that regulate primary platelet functions, including secretion, aggregation, and activation [20, 21]. As noted by Mei-Chi *et al.*, the phosphorylation of specific MAPKs, such as p38, is instrumental in generating TXA₂. This process triggers the liberation of arachidonic acid (the precursor to TXA₂) and drives platelet aggregation via the phosphorylation of cyclic PLA₂ [38]. The synthesis of TXA₂ functions as a robust autacoid that promotes

subsequent platelet activation and aggregation, serving as a vital indicator for assessing compounds designed to inhibit platelet reactivity [38].

In this investigation, G-Rk3 exerted a notable, concentration-dependent suppression of collagen-induced platelet aggregation. Furthermore, G-Rk3 dose-dependently diminished the phosphorylation of cyclic PLA₂, profoundly blunted the collagen-stimulated synthesis of TXA₂, and strongly restricted the secretion of ATP and serotonin, which are key indicators of intracellular granule release. Additionally, G-Rk3 significantly impeded the phosphorylation of the MAPK and PI3K/Akt pathways. By suppressing the activation of these critical phosphoproteins (PI3K/Akt and MAPK), G-Rk3 attenuates TXA₂ generation and restrains platelet aggregation alongside the release of intracellular granules containing serotonin and ATP.

Beyond these intracellular pathways, augmented signaling via integrin α IIb/ β 3 and the release of granular contents structurally modify the platelet cytoskeleton. This cytoskeletal rearrangement alters platelet aggregation and drives thrombus formation, which represents the primary mechanism for repairing vascular injury. During this phase, activated platelets aggregate, and a clot begins to develop through the cross-linking of fibrin and platelets. Following the initial formation of a fibrin clot, a contraction process takes place over 30 to 60 minutes, ultimately producing a stable clot plug. The contractile stabilization of these fibrin clots relies heavily on the interaction between fibrin-one and α IIb/ β 3, meaning that inhibiting α IIb/ β 3 activity can decelerate or completely halt thrombogenesis [39].

Thrombin is capable of stimulating coagulation and activating platelet α IIb/ β 3, which enhances the binding capacity of fibrinogen to α IIb/ β 3 and leads to clot formation. In the current study, the antiplatelet properties of G-Rk3 effectively inhibited thrombin-provoked fibrin coagulation in a concentration-dependent manner. This outcome highlights the potential of G-Rk3 as a potent antiplatelet compound capable of retarding or preventing thrombus generation. Prior investigations into ginseng have documented that specific minor ginsenosides, such as F4 and Rg6, as well as synthetic derivatives including 20(S)-Rg3, Ro, G-Rp1, G-Rp3, and G-Rp4, similarly exert antiplatelet properties [22, 23].

Additionally, we previously explored the antiplatelet mechanism of Rk1, a compound structurally analogous to Rk3. That research demonstrated that Rk1 impacts Ca²⁺ mobilization by increasing cAMP levels, thereby preventing platelet-driven thrombosis via the downregulation of granular secretion and integrin α IIb/ β 3 activation [40]. The current study verifies that Rk3 exerts comparable antiplatelet actions through a mechanism closely mirroring that of Rk1.

Conclusion

In conclusion, G-Rk3 elevates cAMP concentrations within human platelets, stimulates the phosphorylation of VASP and IP3R, and substantially suppresses both Ca²⁺ recruitment and the cytosolic activation of integrin α IIb/ β 3. Furthermore, G-Rk3 demonstrates potent antiplatelet efficacy by attenuating granule release and the synthesis of TXA₂, achieved through the targeted regulation of key signal transduction phosphoproteins, notably PI3K/Akt and MAPK. Ultimately, G-Rk3 serves as a formidable inhibitor of thrombin-induced fibrin clot formation.

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Ethics Statement: None

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