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Punicalin's Inhibition of Collagen-Induced Human Platelet Activation with Attenuation of PLCγ2 Phosphorylation

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ABSTRACT

Cardiovascular diseases (CVDs) remain a leading cause of morbidity and mortality worldwide, with arterial thrombosis contributing substantially to acute cardiovascular and cerebrovascular events. Disruption of atherosclerotic plaques exposes thrombogenic components to circulating blood, triggering platelet adhesion, activation, and aggregation, which may ultimately result in arterial occlusion and ischemic complications. Platelet activation involves complex intracellular signaling pathways that promote granule secretion and amplify thrombus formation. Therefore, identifying compounds capable of suppressing excessive platelet activation remains an important strategy for preventing thrombotic disorders. Punicalin, a naturally occurring hydrolyzable ellagitannin, possesses diverse pharmacological activities, including hepatoprotective, neuroprotective, antiviral, antibacterial, and vascular endothelial protective effects. However, its effects on platelet function remain largely unexplored. Punicalin markedly inhibited human platelet aggregation induced by collagen and arachidonic acid, but had no significant effect on U46619. Furthermore, punicalin significantly reduced collagen-stimulated ATP release and P-selectin surface expression, indicating suppression of platelet granule secretion. Notably, punicalin attenuated phospholipase Cγ2 (PLCγ2) phosphorylation. Collectively, these findings demonstrate that punicalin effectively inhibits human platelet activation, particularly collagen-induced responses, in association with suppression of PLCγ2 phosphorylation and granule secretion. These results provide new insights into the pharmacological properties of punicalin on platelet activation.

Keywords

Punicalin, Collagen, PLCγ2, ATP release, P-selectin, Human platelet.

Introduction

Cardiovascular diseases (CVDs) remain a leading cause of morbidity and mortality worldwide, with atherothrombosis playing a central role in the development of acute cardiovascular and cerebrovascular events [1]. Atherosclerosis is a progressive vascular disorder involving lipid accumulation, endothelial dysfunction, and chronic inflammation, which contribute to the formation and progression of atherosclerotic plaques within arterial walls [2]. Disruption of atherosclerotic plaques through rupture or endothelial erosion exposes thrombogenic components to circulating blood, triggering platelet adhesion, activation, and subsequent thrombus formation [3]. Exposure of subendothelial collagen promotes platelet recruitment and aggregation, whereas tissue factor, particularly from ruptured plaques, activates the coagulation cascade and enhances thrombin generation. Thrombin further amplifies platelet activation and facilitates the conversion

of fibrinogen into fibrin, thereby stabilizing the growing thrombus [3,4]. The coordinated processes of platelet aggregation and fibrin deposition may eventually lead to arterial occlusion and impaired tissue perfusion, resulting in severe ischemic events such as myocardial infarction and ischemic stroke [4].

Platelets are essential components of hemostasis and thrombosis, providing a rapid response to vascular injury and contributing to thrombus development [3]. These small, anucleate blood elements originate from megakaryocytes and normally remain in a quiescent state within the circulation. However, exposure to subendothelial matrix proteins and thrombogenic agonists at sites of vascular injury rapidly triggers platelet activation and subsequent thrombus formation [3,5]. Collagen-induced platelet activation is primarily initiated through glycoprotein VI (GPVI), while integrin $\alpha_2\beta_1$ contributes to stable platelet adhesion to collagen [6]. Engagement of GPVI triggers intracellular signaling involving spleen tyrosine kinase (Syk) and phospholipase Cγ2 (PLCγ2), leading to intracellular Ca^{2+} mobilization and protein kinase C (PKC) activation. These signaling events subsequently promote cytoskeletal rearrangement, platelet shape change, and granule secretion, thereby amplifying platelet activation [7]. Following initial platelet activation, ADP released from dense granules and newly synthesized thromboxane A_2 (TXA_2) act through their respective receptors to amplify platelet responses and promote the recruitment of additional platelets [8]. These secondary agonists further enhance platelet activation and trigger inside-out signaling, inducing conformational changes in integrin αIIbβ3 that facilitate fibrinogen binding and subsequent platelet aggregation [9]. Thus, platelet adhesion, signal amplification, and integrin activation operate as interconnected processes that drive thrombus growth, providing a mechanistic basis for targeting platelet signaling in the prevention of arterial thrombosis.

Punicalin ($C_{34}H_{22}O_{22}$; MW:782.5)

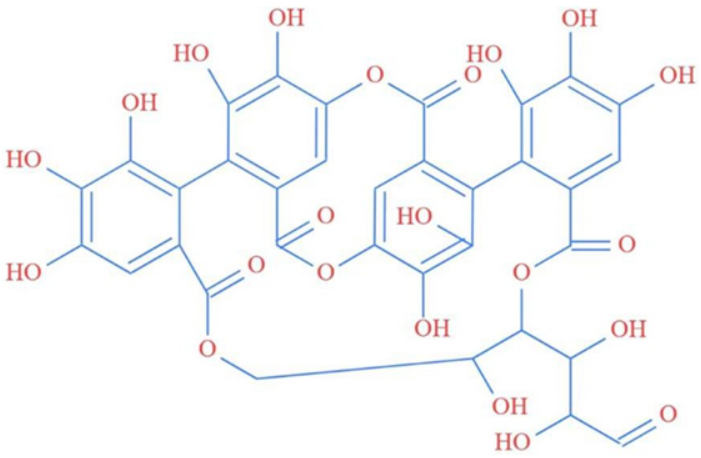


Figure 1: Chemical structures of punicalin ($C_{34}H_{22}O_{22}$).

Punicalin (Figure 1) is a naturally occurring hydrolyzable ellagitannin found in the peels of pomegranate (*Punica granatum L.*) and the leaves of *Terminalia catappa L.* [10,11]. Previous studies have demonstrated its diverse pharmacological activities, including attenuation of acetaminophen-induced liver injury in rats and improvement of cognitive performance and synaptic function in mice fed a high-fat diet [11,12]. Furthermore, punicalin has been reported to exhibit antiviral and antibacterial activities [13,14]. More recently, punicalin was shown to alleviate endothelial cell pyroptosis and improve vascular endothelial function under hyperglycemic and hyperlipidemic conditions [15]. Given the close association between endothelial dysfunction and platelet activation during arterial thrombosis [3], these findings suggest that punicalin may also influence platelet function. Therefore, the present study investigated the effects of punicalin on platelet activation and explored the underlying signaling mechanisms.

Methods

Chemicals and Reagents

Punicalin (purity >98%) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Aprotinin, arachidonic acid (AA), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), leupeptin, luciferin–luciferase, phenylmethylsulfonyl fluoride, sodium fluoride, sodium orthovanadate and sodium pyrophosphate were supplied by Sigma-Aldrich (St. Louis, MO, USA). The polyclonal antibody against phosphorylated PLCγ2 was obtained from Cell Signaling Technology (Beverly, MA, USA), whereas the monoclonal antibody against α-tubulin was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated anti-human CD62P (P-selectin) monoclonal antibody was purchased from BioLegend (San Diego, CA, USA). Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG secondary antibodies were obtained from Abcam (Cambridge, UK).

Human Platelet Preparation and Assessment of Platelet Aggregation and ATP Release

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Taipei Medical University (Approval No. TMU-JIRB-N202412004). Written informed consent was obtained from all healthy volunteers who had abstained from medications or dietary supplements affecting platelet function for at least 14 days. Blood was collected using acid-citrate-dextrose (9:1, v/v) and centrifuged to obtain platelet-rich plasma (PRP). EDTA (2 mM) and heparin (6.4 IU/mL) were added before washing. Washed platelets were suspended in Tyrode's buffer containing BSA (3.5 mg/mL) and Ca^{2+} (1 mM). Platelets were preincubated with punicalin (20–80 μM) or 0.1% DMSO for 3 min before stimulation with collagen (2 μg/mL), AA (60 μM), or U46619 (1 μM). Platelet aggregation was measured using a Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). ATP release was assessed by adding 20 μL of luciferin–luciferase reagent 1 min before stimulation, followed by luminescence measurement using a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan).

Assessment of Platelet P-Selectin Surface Expression by Flow Cytometry Washed human platelets (3.6×10^8 cells/mL) were preincubated with 0.1% DMSO (vehicle control) or punicalin (40

and 80 μM) in the presence of FITC-conjugated anti-P-selectin monoclonal antibody (2 μg/mL) for 3 min, followed by collagen stimulation (2 μg/mL) for 15 min. P-selectin expression was assessed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), with 50,000 events acquired per sample.

Confocal Laser Scanning Microscopy Analysis

Resting or collagen (2 μg/mL)-stimulated platelets were adhered to poly-L-lysine-coated coverslips and fixed with 4% (v/v) paraformaldehyde for 1 h. The samples were permeabilized with 0.1% Triton X-100 and blocked with 5% BSA in phosphate-buffered saline (PBS) for 1 h, followed by incubation with primary antibodies against the target proteins for 24 h. After washing with PBS, platelets were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG secondary antibodies for 1 h. Fluorescence images were captured using a confocal laser scanning microscope (Leica TCS SP5, Mannheim, Germany) equipped with a 100× oil-immersion objective.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM), with n indicating the number of independent experiments using platelets from different donors. Differences among groups were analyzed by one-way ANOVA followed by the Tukey post hoc test. Statistical significance was defined as $p < 0.05$. All analyses were conducted using SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA).

Results

Inhibitory Effects of Punicalin on Agonist-Induced Human Platelet Aggregation The inhibitory effects of punicalin on human platelet aggregation induced by various agonists were investigated. As illustrated in Figure 2, punicalin (20–80 μM) suppressed collagen (2 μg/mL)-induced platelet aggregation in a concentration-dependent manner, with nearly complete inhibition observed at 80 μM. At this concentration, punicalin also inhibited platelet aggregation stimulated by AA (60 μM). However, no significant inhibitory effect was observed against U46619 (1 μM)-induced aggregation, even at 80 μM. The half-maximal inhibitory concentration (IC_{50}) of punicalin against collagen-induced platelet aggregation was approximately 40 μM. The vehicle control (0.1% DMSO) did not significantly alter platelet aggregation (data not shown). Given its pronounced inhibitory effect on collagen-induced aggregation, collagen was selected as the agonist for subsequent experiments.

Inhibitory Effects of Punicalin on Collagen-Induced ATP Release in Human Platelets

Platelet granule secretion was evaluated by measuring ATP release which serve as indicators of dense-granule and contribute to platelet activation and aggregation [16]. As shown in Figure 3A, punicalin significantly reduced ATP release in collagen-stimulated platelets at both 40 and 80 μM, demonstrating a concentration-dependent inhibitory effect.

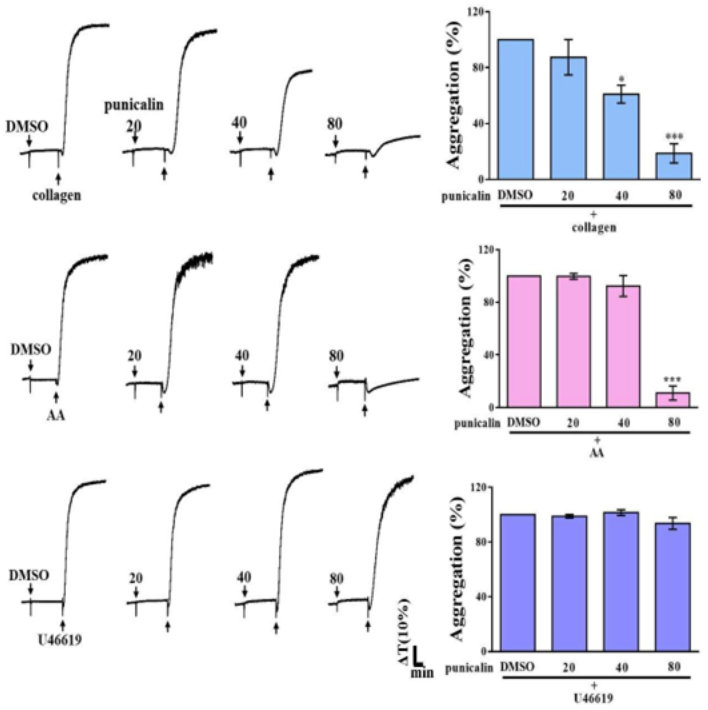


Figure 2: Effects of punicalin on platelet aggregation induced by different agonists. Washed human platelets were preincubated with 0.1% DMSO (vehicle control) or punicalin (20–80 μM), followed by stimulation with collagen (2 μg/mL), arachidonic acid (AA, 60 μM) or U46619 (1 μM). The corresponding bar graphs (right panels) illustrate the quantitative effects of punicalin on agonist-induced platelet aggregation (%). Data are presented as mean ± SEM (n = 4). *$p < 0.05$ and ***$p < 0.001$ compared with the vehicle control group.

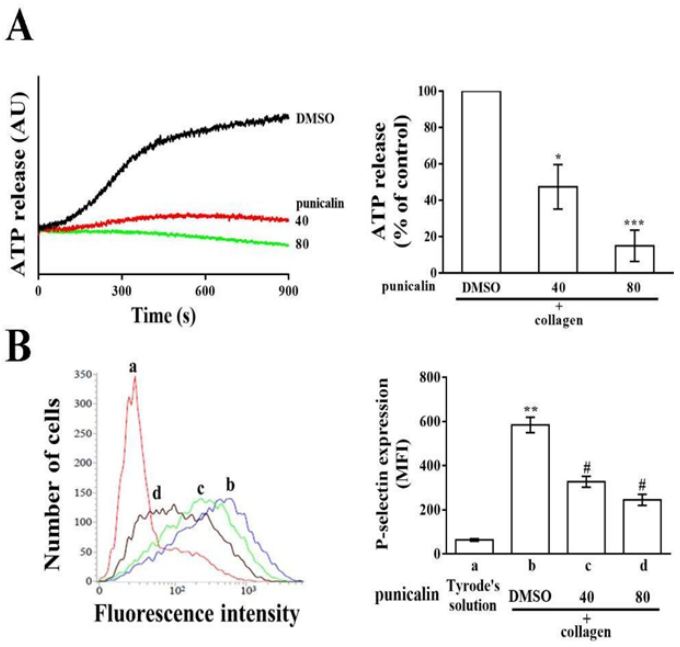


Figure 3: Effects of punicalin on ATP release and P-selectin surface expression in collagen-stimulated platelets. Washed human platelets were preincubated with 0.1% DMSO (vehicle control) or punicalin (40 and 80 μM), followed by collagen (2 μg/mL) stimulation. (A) ATP release was

expressed in arbitrary units (AU). (B) P-selectin surface expression was determined by flow cytometry and expressed as mean fluorescence intensity (MFI). The experimental groups included (a) Tyrode's solution (resting control), (b) 0.1% DMSO + collagen, (c) punicalin (40 μM) + collagen, and (d) punicalin (80 μM) + collagen. Data are presented as mean ± SEM (n = 4). *p < 0.05 and ***p < 0.001 versus the vehicle control group in (A); **p < 0.01 versus the resting control and #p < 0.05 versus the vehicle control group in (B).

Suppression of Collagen-Stimulated P-Selectin Surface Expression by Punicalin P-selectin is primarily stored in the α-granule membranes of resting platelets and becomes exposed on the platelet surface following activation-induced granule secretion [17]. As shown in Figure 3B, resting platelets (Tyrode's solution) exhibited minimal P-selectin surface expression, whereas collagen (2 μg/mL) stimulation markedly increased its expression. However, pretreatment with punicalin (40 and 80 μM) significantly attenuated collagen-induced P-selectin expression.

Punicalin Attenuates PLCγ2 Phosphorylation in Collagen-Stimulated Human Platelets

PLCγ2 is a key signaling protein in collagen-induced platelet activation that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [18]. IP_3 promotes Ca^{2+} release from the dense tubular system (DTS), followed by store-operated Ca^{2+} entry [19]. In Figure 4, confocal microscopy revealed that collagen markedly increased phosphorylated PLCγ2 fluorescence (green) compared with resting platelets, whereas punicalin (80 μM) significantly attenuated this response. In contrast, α-tubulin fluorescence (red) remained unchanged among the groups. These findings suggest that the antiplatelet activity of punicalin is associated with suppression of PLCγ2 phosphorylation.

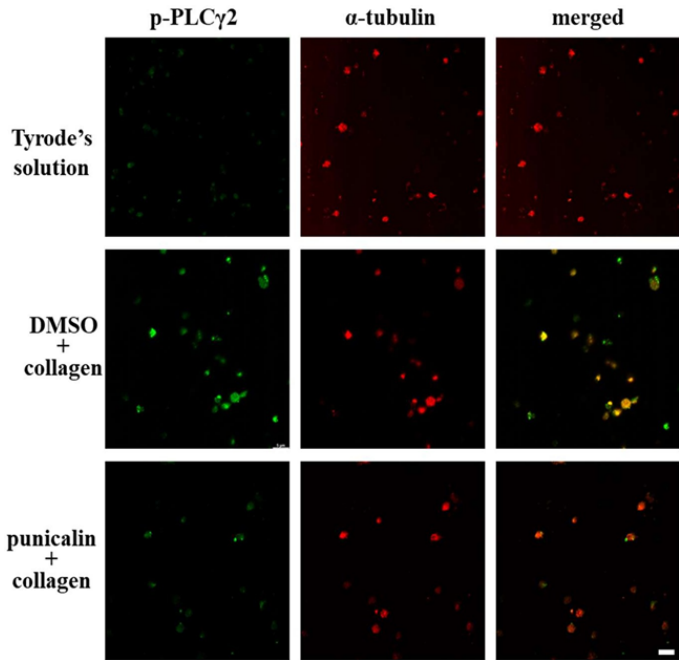


Figure 4: Effects of punicalin on collagen-induced phospholipase Cγ2 (PLCγ2) phosphorylation in human platelets. Washed human platelets were preincubated with 0.1% DMSO (vehicle control) or punicalin (40 μM), followed by collagen (2 μg/mL) stimulation. Phosphorylated PLCγ2 (green) and α-tubulin (red) were visualized using Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG, respectively. Scale bar = 5 μm. Images are representative of four independent experiments.

Discussion

Punicalin, a naturally occurring hydrolyzable ellagitannin found in pomegranate and *Terminalia catappa L.*, has attracted attention for its hepatoprotective, neuroprotective, and vascular endothelial protective properties [11,12,15]. However, its pharmacological effects on platelet function remain poorly understood. In the present study, punicalin exhibited concentration-dependent inhibition of collagen-induced platelet aggregation. At high concentration, punicalin also inhibited AA-induced aggregation, whereas U46619-induced aggregation remained unaffected. Moreover, punicalin significantly reduced ATP release, P-selectin surface expression, and PLCγ2 phosphorylation in collagen-stimulated platelets, providing further evidence of its inhibitory effects on platelet activation.

Platelet activation involves coordinated intracellular signaling events that promote granule secretion and amplify aggregation. Dense granules contain ADP, ATP, and Ca^{2+}, whereas α-granules store various proteins, including P-selectin, fibrinogen, and platelet-derived growth factor. Upon activation, ADP released from dense granules reinforces platelet recruitment and aggregation, while P-selectin translocates from α-granule membranes to the platelet surface [16,17]. Accordingly, ATP release and P-selectin surface expression serve as important indicators of platelet secretion. In this study, punicalin significantly suppressed collagen-induced ATP release. Similarly, both concentrations markedly reduced P-selectin surface expression. These findings indicate that punicalin attenuates both dense-granule and α-granule secretion, thereby potentially limiting the amplification of platelet activation.

Collagen-induced platelet activation primarily involves GPVI, which initiates a tyrosine kinase signaling cascade involving Syk, Bruton's tyrosine kinase (Btk), and PLCγ2 [18,20]. Activated $PLC\gamma_2$ hydrolyzes PIP_2 to generate IP_3 and DAG, leading to intracellular Ca^{2+} mobilization and PKC activation. These downstream events are essential for platelet secretion and aggregation [18-20]. In the present study, confocal fluorescence analysis demonstrated that collagen markedly enhanced PLCγ2 phosphorylation, whereas punicalin significantly attenuated this response without altering α-tubulin fluorescence. These observations suggest that suppression of PLCγ2 phosphorylation may contribute to the inhibitory effects of punicalin on collagen-induced platelet activation. However, whether punicalin directly targets PLCγ2 or interferes with its upstream signaling components requires further investigation.

The different responses to platelet agonists may provide additional insight into the possible mechanisms of punicalin. Unlike collagen, which predominantly activates GPVI-dependent PLCγ2 signaling, U46619 initiates platelet responses mainly through

G protein-coupled receptors and downstream PLCβ signaling [21,22]. AA serves as a substrate for cyclooxygenase-1 (COX-1) and thromboxane synthase, leading to TXA_2 production and subsequent platelet activation through TXA_2 receptors. In contrast, U46619 directly activates TXA_2 receptors without requiring endogenous TXA_2 synthesis [21,22]. Interestingly, punicalin inhibited AA-induced aggregation but showed no significant effect on U46619-induced responses. This distinction raises the possibility that punicalin interferes with events upstream of TXA_2 receptor activation, although its effects on AA metabolism and TXA_2 generation remain to be determined. Taken together, these findings indicate that punicalin suppresses collagen-induced platelet activation in association with reduced PLCγ2 phosphorylation and granule secretion. Further studies examining TXA_2 production, upstream kinases, and downstream Ca^{2+}/PKC signaling are warranted to clarify its molecular targets and potential antithrombotic activity.

Conclusion

Our findings demonstrate that punicalin effectively inhibits human platelet activation, particularly collagen-induced aggregation, in association with suppression of PLCγ2 phosphorylation. These inhibitory effects were accompanied by reduced ATP release and P-selectin surface expression, suggesting that interference with PLCγ2 signaling contributes to its antiplatelet activity. However, the involvement of other signaling pathways cannot be excluded.

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