GARCINOL, A NOVEL ANTITHROMBOTIC AGENT: INHIBITION OF PLATELET ACTIVATION AND INTEGRIN $\alpha_{IIB}\beta_3$-MEDIATED SIGNALING PATHWAYS

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Abstract - Platelets hold a central role in both normal hemostasis and pathological arterial thrombosis, activated by adhesive proteins or soluble agonists that induce signal transduction through their respective receptors. This activation leads to common signaling events, triggering platelet shape changes, inside-out signaling, and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$, initiating outside-in signaling, platelet granule secretion, and aggregation. Extracted from the dried fruit rind of *Garcinia indica*, garcinol, a polyisoprenylated benzophenone, exhibits diverse bioactivities. Despite limited research on its impact on platelet activation, this study reveals garcinol's inhibition of aggregation induced by collagen, thrombin, arachidonic acid, and U46619.Garcinol directly inhibited integrin $\alpha_{IIb}\beta_3$ activation by interfering with FITC–PAC-1 binding stimulated by collagen. In addition, garcinol affected integrin $\alpha_{IIb}\beta_3$-mediated outside–in signaling. In conclusion, this study identified that garcinol, a novel antithrombotic agent, acts as a naturally occurring integrin $\alpha_{IIb}\beta_3$ inhibitor.

Keywords - Antithrombosis, Garcinol, Integrin $\alpha_{IIb}\beta_3$, Human Platelet

I. INTRODUCTION

Platelets, devoid of a nucleus, wield pivotal roles in various physiological and pathological processes, including hemostasis, thrombosis, atherosclerosis, inflammation, and tumor metastasis [1]. Their primary physiological function involves forming hemostatic thrombi to prevent blood loss and uphold vascular integrity. Pathological arterial thrombosis, a major contributor to cardiovascular and cerebrovascular diseases like cardiac arrest and ischemic stroke, poses a significant global health concern. Platelet adhesion and aggregation, triggered by vascular subendothelial connective tissue injury, contribute to the formation of platelet plugs and hemostasis. Despite their primary role in preventing blood loss, platelets can also give rise to pathogenic thrombi, leading to vascular thromboembolic diseases. While platelets typically remain inactive in circulation, damage to blood vessels prompts their adhesion, release of biologically active components, and aggregation [2]. Collagen further enhances platelet adhesion and activation through specific receptors, initiating the release of ADP and thromboxane A_2. Activated platelets recruit circulating platelets by binding fibrinogen to integrin $\alpha_{IIb}\beta_3$, formed through a calcium-dependent association between α_{IIb} and β_3. In their resting state, integrin $\alpha_{IIb}\beta_3$ exhibits low activation, impeding interaction with ligands. Agonist-activated platelets induce a conformational change in integrin $\alpha_{IIb}\beta_3$, enabling binding to ligands and initiating platelet aggregation [3]. Garcinia indica, commonly known as kokum and belonging to the Clusiaceae family, is a tropical evergreen tree native to specific regions in India. This tree is frequently utilized for treating gastric disorders and skin irritation [4]. In 1981, garcinol, a polyisoprenylated benzophenone, was first isolated from the dried fruit rind of G. indica [5]. Beyond its traditional uses, garcinol has garnered attention for its diverse health benefits. It has demonstrated anticancer properties [4,6], exerting effects on various diseases such as cardiovascular diseases (CVDs), diabetes, liver injury, and neurodegenerative disorders [4,6,7]. The anticancer impact of garcinol involves the stimulation of apoptosis, downregulation of Akt, NF-κB, and STAT3 expression, and suppression of caspase 3 activation [7-10]. Additionally, garcinol has been found to protect against neurodegeneration by inhibiting apoptosis. Overall, this natural compound exhibits promising therapeutic potential across a range of health conditions [11]. Although garcinol possesses various pharmacological properties, to the best of our knowledge, few studies have investigated its effect on platelet activation. Cao et al. [12] reported that garcinol (33 μM) strongly reduced platelet aggregation stimulated by collagen-related peptides and moderate activity in stimulation with thrombin in murine platelets. Thus, this study systemically investigated the effects of garcinol on platelet activation in humans. Our results indicate that garcinol exhibits antithrombotic activity by interfering with integrin $\alpha_{IIb}\beta_3$. Therefore, garcinol can be serve as a novel antiplatelet agent.

II. PLATELET PREPARATION AND AGGREGATION

After obtaining approval from the Institutional Review Board of Taipei Medical University (TMU-JIRB-N201812024) in accordance with the Declaration of Helsinki guidelines, human platelet suspensions were prepared as described previously

[13]. Blood samples were collected from healthy human volunteers aged between 20 to 35 years who had not taken any medication for the 14 days prior to collection. The collected blood was mixed with an acid-citrate-dextrose solution (9:1, v/v) and centrifuged at 120 × *g* for 10 min. The resulting supernatant was supplemented with EDTA (2 mM) and heparin (6.4 U/mL) for 5 min before undergoing another centrifugation at 500 × *g* for 10 min. The platelet pellet obtained was resuspended in 5 mL of Tyrode's solution and kept at 37°C for 10 min. Following another spin at 500 × *g* for 10 min, the washing procedure was repeated. The washed platelets were then suspended in Tyrode's solution containing BSA (3.5 mg/mL). Platelet numbers were counted using a Coulter counter (Beckman Coulter, Miami, FL, USA). The concentration of washed platelet suspensions is approximately 3.6×10^8 –1.2×10^9 cells/mL for following studies. The final Ca^{2+} concentration in the Tyrode's solution was 1 mM.

III. DETECTION INTEGRIN $\alpha_{IIb}\beta_3$ ACTIVATION AND IMMUNOBLOTTING

Integrin $\alpha_{IIb}\beta_3$ activation were detected using fluorophore-labeled antibodies. Washed platelets (3.6×10^8 cells/mL) were preincubated with garcinol (20 and 40 μM) and the FITC-conjugated PAC-1 mAb (2 μg/mL) for 3 min and subsequently stimulated by collagen (1 μg/mL) for another 5 min.Fluorescein-labeled platelets in the suspensions were detected using a flow cytometer equipped with BD FACSuite™ software (FACScan system, Becton Dickinson, San Jose, CA, USA). Data were collected from 10,000 platelets per experimental group, and platelets were identified on the basis of their characteristic forward and orthogonal light-scattering profiles.

For other experiments,washed platelets (3.6×10^8 cells/mL) were preincubated with garcinol (20 and 40 μM) or solvent control (0.1% DMSO) for 3 min, and collagen was subsequently added to trigger activation. In another study, dishes (6-cm diameter) were precoated with fibrinogen (100 μg/mL) overnight and then blocked with 1% BSA, followed by the addition of washed platelets preincubated with garcinol (20 and 40 μM) or solvent control (0.1% DMSO); the platelets were allowed to spread on the dishes for 60 min. The platelets were resuspended in 200 μL of lysis buffer, and supernatants containing 80 μg of protein were separated through 12% sodium dodecyl sulfate gel electrophoresis. The Bradford protein assay (Bio-Rad, Hercules, CA, USA) was performed to quantitate protein concentrations. Targeted proteins were detected using their respective primary antibodies. The optical density of protein bands was measured using a video densitometer and the Bio-ProfilBiolight software program (version V2000.01; VilberLourmat, Marne-la-Vallée, France). Relative protein expression was calculated after normalization to the expression of the total protein of interest.

IV. GARCINOL'S EFFECT ON INHIBITING HUMAN PLATELET AGGREGATION

This study investigated mechanisms underlying the inhibitory effect of garcinol on platelet activation in humans. As presented in Fig. 1, 10–40 μM garcinol exhibited the highest inhibitory activity against collagen (1 μg/mL)-stimulated platelet aggregation. At higher concentrations of 40–80 μM, garcinol exhibited moderate activity against platelet aggregation triggered by thrombin (0.02 U/mL), AA (60 μM), and U46619 (1 μM), a prostaglandin endoperoxide.

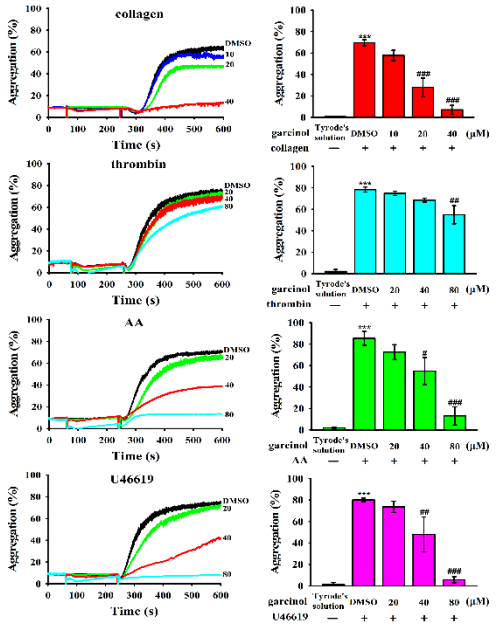


Figure 1: Inhibition of agonists-induced platelet aggregation by garcinol.

(A) Washed human platelets were preincubated with either a solvent control (0.1% DMSO) or various concentrations of garcinol (10-80μM) before being stimulated with different agonists to induce platelet aggregation. The bar diagrams on the right side illustrate the corresponding statistical analyses.The data are expressed as the mean ± standard error of the mean (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs 0.1% DMSO + Tyrode's solution;$^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs 0.1% DMSO + agonists group.

V. INTERFERENCE OF INTEGRIN $\alpha_{IIb}\beta_3$ OUTSIDE–IN SIGNALING BY GARCINOL

Platelet aggregation relies on the interaction between fibrinogen and integrin $\alpha_{IIb}\beta_3$; however, inactivation of integrin $\alpha_{IIb}\beta_3$ can induce the disaggregation of previously aggregated platelets [14]. To assess the potential interference of garcinol with integrin $\alpha_{IIb}\beta_3$ activation, we utilized flow cytometry to analyze the binding of FITC-PAC-1 monoclonal antibody to the activation-induced conformational epitope of integrin

$\alpha_{IIb}\beta_3$. Garcinol at concentrations of 20 and 40 μM significantly impeded PAC-1 binding to integrin $\alpha_{IIb}\beta_3$ on collagen-stimulated platelets (Fig. 2A). This suggests that the mechanism of action of garcinol may involve disrupting the interaction between fibrinogen and integrin $\alpha_{IIb}\beta_3$. Moreover, we elucidated the mechanisms through which garcinol interferes with integrin $\alpha_{IIb}\beta_3$ outside–in signaling by examining key kinases involved in outside–in signaling, including integrin β_3, Src, FAK, and Syk phosphorylation (Fig. 2B-E). These findings unequivocally demonstrate that garcinol directly targets integrin $\alpha_{IIb}\beta_3$.

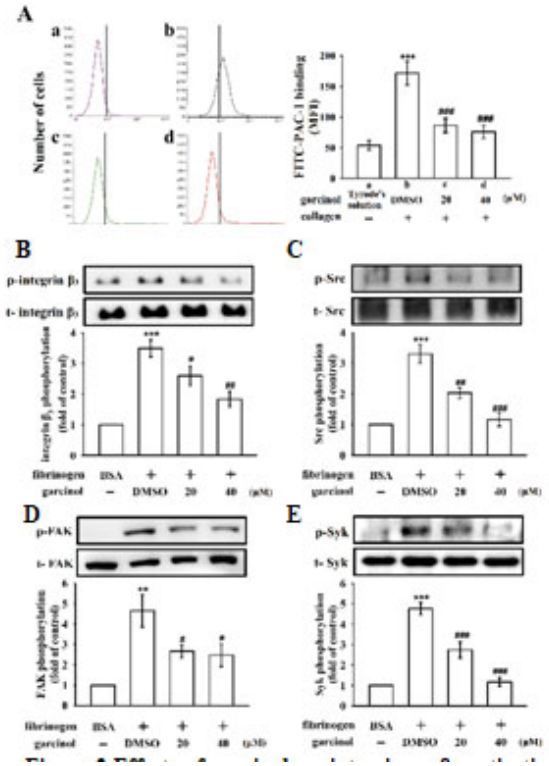


Figure 2:Effects of garcinol on integrin $\alpha_{IIb}\beta_3$ activation and integrin $\alpha_{IIb}\beta_3$-mediated cell signaling in human platelets.

(A) Resting platelets (3.6×10^8 cells/mL) (a) or platelets were preincubated with 0.1% DMSO (b) or garcinol (c, 20; d, 40 μM) and PAC-1 mAb (2 μg/mL) for 3 min and then stimulated with collagen (1 μg/mL) for another 5 min. The suspensions were assayed for fluorescein-labeled platelets on a flow cytometer.For other experiments, washed platelets were preincubated with solvent control (0.1% DMSO) or garcinol (20 and 40 μM) and subsequently activated by immobilized fibrinogen (100 μg/mL). Platelets were collected, and their subcellular extracts were analyzed to determine the levels of (B) integrin β_3, (C) Src, (D) focal adhesion kinase (FAK) and (E) Syk phosphorylation.Data are presented as the mean ± standard error of the mean (n = 4). (A)$^{***}p < 0.001$ compared with the resting (Tyrode's solution) group; $^{###}p < 0.001$ compared with the 0.1% DMSO + collagen group.(B-E) $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the resting (Tyrode's solution) group; $^{#}p < 0.05$, $^{##}p < 0.01$, and $^{###}p < 0.001$ compared with the immobilized fibrinogen + 0.1% DMSO group.

VI. CONCLUSION

This study identified that garcinol, a novel antithrombotic agent, acts as a naturally occurring integrin $\alpha_{IIb}\beta_3$ inhibitor.

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