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# Curdione attenuates thrombin-induced human platelet activation: β1-tubulin as a potential therapeutic target

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ABSTRACT

*Rhizoma Curcumae*, the dry rhizomes derived from *Curcuma aromatica* Salisb., are a classical Chinese medicinal herb used to activate blood circulation, remove blood stasis and alleviate pain. Our previous study proved that curdione, a sesquiterpene compound isolated from the essential oil of *Curcuma aromatica* Salisb. can inhibit platelet activation suggesting its significant anticoagulant and antithrombotic effects. However, the underlying mechanism of curdione mediated anti-platelet effect has not been fully elucidated. Platelet proteins extracted from washed human platelets, including normal group (treated with normal saline), thrombin group and curdione group were digested and analysed by nano ESI-LC-MS/MS. UniProt database and SIEVE software were employed to identify and reveal the differentially expressed proteins. Furthermore, possible mechanisms involved were explored by Ingenuity Pathway Analysis (IPA) Software and validated by western blot experiments. Twenty-two differentially expressed proteins between the normal and thrombin group were identified. Compared with the thrombin group, the curdione treatment was significantly down-regulated only 2 proteins (Talin1 and β1-tubulin). Bioinformatics analysis showed that Talin1 and β1-tubulin could be involved in the integrin signal pathway. The results of western blot analysis were consistent with that of the proteomics data. Vinculin, identified in IPA database was involved in the formation of cell cytoskeletal. The down-regulation of β1-tubulin facilitated the decrease in vinculin/Talin1. Curdione regulated the expression of vinculin and Talin1 by β1-tubulin affecting the integrin signalling pathway and eventually inhibiting platelet activation. The β1-tubulin may be a potential target of curdione, which attenuates thrombin-induced human platelet activation.



## 1. Introduction

Platelets are small biologically active fragments derived from bone marrow megakaryocytes. Platelets are not only helpful for haemostasis and thrombus but also crucial to abnormal bleeding and thrombotic diseases. Pathological thrombus occurs under conditions of excessively activate platelets and is considered to be an important pathological basis of cardiovascular disease [1]. Many of the non-haemostatic functions of platelets may result from their capacity to store a number of biologically active molecules in the intracellular granules. These molecules such as α-granules, dense granules and lysosomes can be released into the circulation or translocated to the platelet surface during platelet activation [2]. Platelet cytoskeletal reorganization and granule secretion induced by endothelial cell injury eventually contributes to blocked arteries. Platelet activation generally occurs through inside-out signal transduction, which is launched by receptor-ligand combinations and action on G protein-coupled receptors [3]. Morphologic changes induced by cytoskeletal reorganization, protein synthesis and granule secretion result from signalling cascades triggered by these stimuli, which led to affinity variation between platelet receptors, fibrinogen and GPIIb/IIIa.

The rhizome of genus *Curcuma* (*Rhizoma Curcumae*, Chinese name: Ezhu) is a classical, traditional Chinese medicine (TCM) that has been used as a remedy for cardiovascular diseases [4], menstrual disorders, and hepatitis for more than a thousand years [5,6]. The genus *Curcuma* belongs to family Zingiberaceae, and the plants of this genus mainly distribute in India, Malaysia and China. The Chinese Pharmacopoeia recorded that *Rhizoma curcumae* should be the dry rhizomes derived from *Curcuma kwangsiensis* S.G. Lee & C.F. Liang, *Curcuma zedoaria* (Christm.) Roscoe and *Curcuma aromatica* Salisb [7]. According to the records of *Lei Gong's Moxibustion Theory* and Li Shizhen's *Compendium of Materia Medica* in ancient time, the described functions of *Rhizoma curcumae* were to remove blood stasis, improve blood circulation, monitor the menstrual cycle and relieve pain. This suggested that *Rhizoma curcumae* might possess anti-platelet activation function.

It has been reported that diarylheptanoids and essential oils are the main constituents of genus *Curcuma* plants [8]. Essential oils are considered as active constituents, which possess anti-angiogenesis [4], antiplatelet [9], antimicrobial, anti-inflammatory, and anti-cancer properties [10], and have been promoted as a supplement or therapy for a

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diversity of symptoms. Curdione, isolated from the essential oils of *Curcuma aromatica* Salisb., is one of the major sesquiterpene compounds (Supplementary Fig. 1). Curdione is the main active ingredient and commonly used as a TCM quality control marker [6]. Recent case reports have highlighted the anti-inflammatory [11] and hepatoprotective activity [12] of curdione. Moreover, our previous study proved that curdione has a significant anticoagulant and antithrombotic effect [13]. Moreover, we also found that curdione resulted in the obvious attenuation of thrombin-induced platelet aggregation [14]; however, the underlying mechanism was not fully elucidated.

Platelet function depends on protein expression and dynamic changes in post-translational modifications (PTM) in the context of enucleated cells. Platelet proteomics can map the platelet proteome and characterize the protein profiles and PTM to reveal the differences in platelet function [15]. In the present study, we exploited the proteomic approach to investigate the potential targets of curdione on thrombin induced platelet activation. We found that Talin1 (TLN1) and β1-tubulin (TUBB1) were significantly down-regulated in the context of curdione treatment. We investigated the role of the TUBB1 and TLN1-modulated proteins in platelet activation and aggregation. Furthermore, we discuss the inhibitory effect of vinculin and the consequences of its combination with Talin1 on the integrin signalling pathway.

## 2. Materials and methods

### 2.1. Reagents

Curdione (>97%) was extracted from the essential oils of *Curcuma aromatica* Salisb. as previously described [14]. Thrombin, HEPES, EGTA and Triton X-100 were purchased from Sigma (St Louis, MO). For Western blot, the primary antibodies rabbit monoclonal anti-β1-tubulin and anti-vinculin were purchased from Abcam (Cambridge, UK). The primary antibodies mouse monoclonal anti-Talin1 was obtained from Boster Biological Engineering Co., LTD (Wuhan, China). The primary antibodies rabbit monoclonal anti-GAPDH was obtained from Bioworld Technology, Inc. (Nanjing, China). The horseradish peroxidase conjugated secondary antibodies were obtained from Zhongshan Biosciences (Beijing, China). ABT-751, the inhibitor of β1-tubulin, was obtained from Selleck Chemicals (Shanghai, China). The inhibitor of vinculin, Melittin, was obtained from Cool Seoul Chemical Science and technology Co., LTD (Beijing, China).

### 2.2. Human platelet preparation

Venous blood was collected from healthy volunteers, who did not use any medication 14 days before sampling and in accordance with approved guidelines from the Local Research Ethics Committee of The First Affiliated Hospital of Anhui Medical University, anticoagulated with 3.2% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 800 rpm for 15 min at 25 °C. Platelets were isolated from PRP by a 5-min centrifugation at 3000 rpm, washed in ACD buffer (45 mM trisodium citrate, 20 mM citric acid, 74.5 mM D-glucose; final concentration, pH 5.0) and wash buffer (173 mM NaCl, 4 mM KCl, 24 mM $NaH_2PO_4$, 4 mM $Na_2HPO_4$, and 0.2 mM EGTA, 5.5 mM D-glucose, pH 6.0) with 10 ng/ml of prostacyclin, washed platelets were resuspended in Tyrode's/ Hepes buffer at a final concentration of $3 \times 10^8$/ml. Platelets were not used until after the isolation of 30 min [16]. The platelet suspension was divided into three aliquots. The curdione group was pre-incubated with 100 μM curdione and then 0.3 U thrombin was added to assess the inhibitory effect of curdione on platelet aggregation. Normal saline (NS) was used as control and the thrombin group was added 0.3 U thrombin. The entire process was finished within 10 min and replicated five times. All of the three groups were centrifuged for 3 min at 3000 rpm to obtain platelet cells. After resting, platelets aliquots were disrupted with lysis buffer kit (Beyotime, China) according to the instructions, and finally stored at −80 °C until further processing.

### 2.3. Electron microscopy

The washed human platelets ($3 \times 10^8$/ml) were pre-incubated with 100 μM curdione or NS for 10 min, followed by stimulation with 0.3 U thrombin at 37 °C for 5 min, and collection at 3000 rpm for 8 min. After fixing in 2.5% glutaraldehyde at 4 °C for 4–6 h, the collected platelets were incubated in 1% osmic acid for 1 h. The platelets were dehydrated in ethanol (30% and 50% with each step lasting 15 min) and incubated in 70% Uranium saturated acetate ethanol for 6–12 h. The samples were then dehydrated in ethanol (80% and 95% with each step lasting 15 min and also dehydrated in 100% ethanol twice for 40 min). After the removal of 100% ethanol, the platelets were subsequently incubated with oxirane, a solution of oxirane and Epon (1:1) and a solution of oxirane and Epon (1:2), with each step lasting 30 min, 1 h and 2 h, respectively. The liquid was replaced with Epon812 for 2 h, and embedded in Epon in a 45 °C oven for 12 h and transferred to a 60 °C oven for 48 h. The pellets were ultramicrocut in 70 nm sections. The ultrathin sections were stained with uranyl acetate and lead citrate and observed using HT-7700 transmission electron microscopy (HITACHI, Japan) and photographed.

### 2.4. Trypsin digestion

Treated human platelet samples were added to four times of the sample volume of pre-cold acetone (−20 °C) to precipitate at −20 °C overnight, and then centrifuged at 10,000 rpm at 4 °C for 5 min. The protein pellet was then resuspended in ULB buffer (7 M urea, 1.5 M Tris-HCl pH 8.8, 2 M thiourea, 4% w/v CHAPS, 50 mM $NH_4HCO_3$), and then reduced using 100 mM DTT for 15 min at 50 °C and alkylated with 300 mM iodoacetamide for 15 min at room temperature in darkness. Alkylated proteins (150 μg/sample) were digested with mass spectrometry grade trypsin (Promega, USA) at 37 °C for 18 h. The resulting peptides obtained were desalted using Strata-X column (Phenomenex, China) and dried in a SpeedVac (Thermo Electron, USA). Dried peptides were stored at −80 °C for the LC-MS/MS analysis.

### 2.5. Nano ESI-LC-MS/MS analysis

The peptides were fully dissolved in 0.1% formic acid (v/v) and prepared for nano ESI-LC-MS/MS using C18 Zip-Tip purification. For LC-MS/MS, 2 μL of the peptides were loaded onto a reverse phase C18 column (75 μm × 100 mm), with 1.7 μm, 300 Å pore particles size using an ACCELA 600 liquid chromatography system (Thermo Electron, USA). Peptides were eluted from the column using a 100 min increasing organic gradient. Mobile phrase A was water/0.1% formic acid (v/v), while mobile phrase B was 100% acetonitrile. The gradient started at 5% mobile phrase B in 10 min, 5–40% mobile phrase B in 50 min. 40–95% mobile phrase B in 30 min. At 90 min the gradient returned to 5% to re-equilibrate the column for 10 min for the next injection. Peptides eluted from the column were analysed by data-dependent MS/MS on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc.). The instrument settings were as follows: the activation type of scan range was from 300 to 2000 *m/z*, the resolution was set to 60,000, the min signal required 500, the neutral loss in top was set to 3, the activation time was set to 30 s and dynamic exclusion duration was set to 60 s [17].

For protein acquisition, the data were analysed by Sequest HT search engine, against the UniProt human proteome database (http://www.uniprot.org/uniprot, version: November 2015) under the Proteome Discoverer 1.3 software (Thermo Fisher Scientific Inc.). Proteins were detected at a false discovery rate < 0.05. The following protein identification data were aligned by ChromAlign software. Label-free peptides eluted between 0 and 100 min were conducted by SIEVE 1.3 software