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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 3×10^8/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 (3×10^8/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

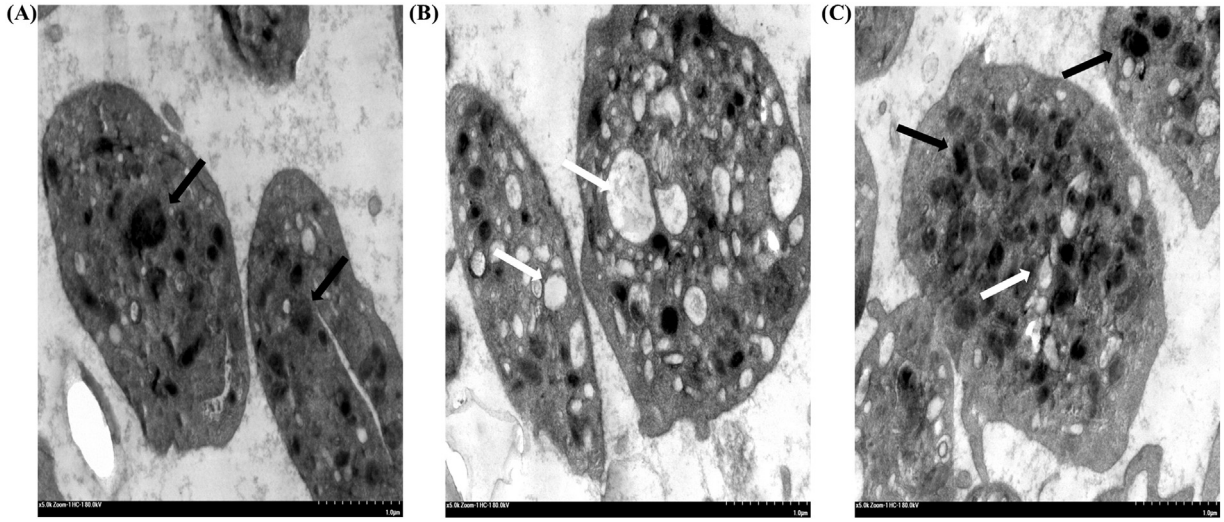

Fig. 1. Transmission electron microscopy analysis the granules secretion of human washed platelet. (A) The resting platelets; (B) platelets were stimulated with thrombin (0.3 U); (C) after pre-incubation with 100 μM curdione and stimulation with thrombin (0.3 U). The white narrows were empty vesicles that have been secreted α-granules, and the black narrows point to α-granules ($n = 5$).

(Thermo Fisher Scientific Inc.). The protein lists were generated with set *p*-value 0.05 for the identified proteins.

2.6. Pathway analysis

To understand the pathways modulated by thrombin and curdione, a list of differentially expressed proteins (<0.6 or >1.8-fold) was compiled. These proteins were categorized in accordance with their Gene Ontology (GO) descriptions using information from the PANTHER (Protein ANalysis THrough Evolutionary Relationships, http://pantherdb.org) classification systems. The proteins were projected on the basis of their molecular functions, biological processes and protein classes. The canonical pathways, disease/function pathways and protein-protein interactions were analysed using Ingenuity Pathway Analysis Software (IPA trial version, Ingenuity Systems, http://www.ingenuity.com) systems. The predicted protein-protein interaction networks and canonical pathways were generated using inputs of gene identifiers, fold-changes and *p*-values among the three groups.

2.7. Western blot analysis

Differentially expressed proteins identified in LC-MS/MS analysis and label-free quantification software SIEVE were selected for further validation by immunoblot analysis. After pre-treatment, the washed human platelet (3×10^8 platelets/ml) suspension was divided into different groups. Successively, platelet suspension was accumulated, washed with PBS and centrifuged. The protein concentration was determined using a BCA Protein Assay Kit (Beyotime, China) and the proteins were then resuspended in 5× SDS-PAGE loading buffer and heated at 95 °C for 10 min with agitation. For immunoblot analysis, a total of 10 μg protein per sample was subjected to run with 12% separating and 5% stacking SDS-PAGE gels. Proteins were transferred from the gels onto nitrocellulose membrane by electroblotting. The membranes were saturated by incubation with non-fat dry milk (5% w/v) for 2 h at room temperature and then incubated with an anti-β1-tubulin antibody (1:1000), an anti-Talin-1 antibody (1:500) and an anti-vinculin antibody (1:5000) overnight

Table 1
Identified proteins with differential expression in thrombin group compared with normal group, showing >1.8 fold up-regulation and <0.66 down-regulation with statistical significance. **A** represented the thrombin group, **B** represented the normal group.

S·NO	ProteinID	Gene name	Description	MW [kDa]	Peptides	Hits	Score	%Coverage	P value_AvsB	Fold change
1	Q9Y490	TLN1	Talin_1	269.6	7	30	692.35	62.10	9.90E-20	2.71
2	Q9H4B7	TUBB1	Tubulin beta_1 chain	50.3	3	10	84.26	67.41	9.90E-20	3.32
3	P27105	STOM	Isoform 2 of Erythrocyte band 7 integral membrane protein	31.7	1	2	40.57	42.71	1.22E-02	3.16
4	P18206-2	VCL	Isoform 3 of Vinculin	116.6	1	2	197.03	44.56	4.54E-02	3.09
5	P12814-2	ACTN1	Alpha_actinin_1	102.6	3	6	124.21	37.66	1.18E-03	2.93
6	P08514	ITGA2B	Integrin alpha_IIb	113.3	2	4	96.49	27.82	5.04E-04	2.91
7	P07737	PFN1	Profilin 1_ isoform CRA_b	15.0	2	7	79.40	35.71	1.42E-02	2.24
8	P07195	LDHB	L_lactate dehydrogenase B chain	36.6	1	3	27.55	16.77	1.81E-03	2.08
9	Q01518	CAP1	Isoform 2 of Adenylyl cyclase_associated protein 1	51.9	1	2	21.80	13.26	6.69E-03	0.63
10	Q86UX7-2	FERMT3	Fermitin family homolog 3	75.4	2	11	127.45	47.81	1.64E-03	0.57
11	P02768	ALB	Serum albumin	69.3	1	4	23.97	20.20	7.78E-10	0.52
12	Q9NY65	TUBA8	Tubulin alpha_8 chain	50.1	1	4	29.53	29.18	2.39E-04	0.51
13	Q60FE5	FLNA	Filamin_A	278.1	2	11	557.18	44.16	3.71E-08	0.41
14	P05106	ITGB3	Integrin beta	87.0	1	1	79.36	20.94	1.10E-03	0.40
15	P06733	ENO1	Isoform MBP_1 of Alpha_enolase	47.1	1	4	41.22	31.57	7.89E-04	0.37
16	J3KTJ1	MYL12A	Myosin regulatory light chain 12A	13.0	1	4	26.59	57.89	3.44E-06	0.33
17	P35579	MYH9	Myosin_9	226.4	5	18	116.31	18.72	9.90E-20	0.28
18	P02679-2	FGG	Fibrinogen gamma chain	49.5	1	2	31.93	23.57	1.33E-04	0.08
19	P07996	THBS1	Isoform 2 of Thrombospondin_1	129.3	4	10	50.54	15.21	2.32E-12	0.06
20	P02675	FGB	Fibrinogen beta chain	55.9	1	2	20.51	10.18	1.52E-02	0.04
21	P02042	HBD	Hemoglobin subunit delta	16.0	1	2	128.23	63.27	3.03E-03	0.00
22	P68871	HBB	Hemoglobin subunit beta	16.0	4	24	174.95	88.44	2.75E-06	0.00

Table 2
Identified proteins with differential expression in curdione group compared with thrombin group, showing >1.8 fold up-regulation and <0.66 down-regulation with statistical significance. **A** represented the curdione group, **B** represented the thrombin group.

S·NO.	ProteinID	Gene name	Description	MW [kDa]	Peptides	Hits	Score	Coverage%	P value_AvsB	Fold change
1	Q9Y490	TLN1	Talin_1	269.6	1	4	746.80	61.35	3.14E-05	0.45
2	Q9H4B7	TUBB1	Tubulin beta_1 chain	50.3	1	4	79.31	58.76	6.20E-04	0.54

at 4 °C. Membranes were then incubated with the secondary antibody for 2 h at room temperature. GAPDH (1:5000) was selected as the control. Protein bands were detected using the enhanced chemiluminescence detection kit (Thermo Scientific, USA) and the intensity of bands were quantified using GraphPad Prism 5 software (GraphPad Software Inc.).

2.8. Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis was performed using SPSS 16.0 software. Differences between groups were evaluated by ANOVA followed by Student-Newman-Keul's test. Statistical significance was considered as $P < 0.05$ and $P < 0.01$.

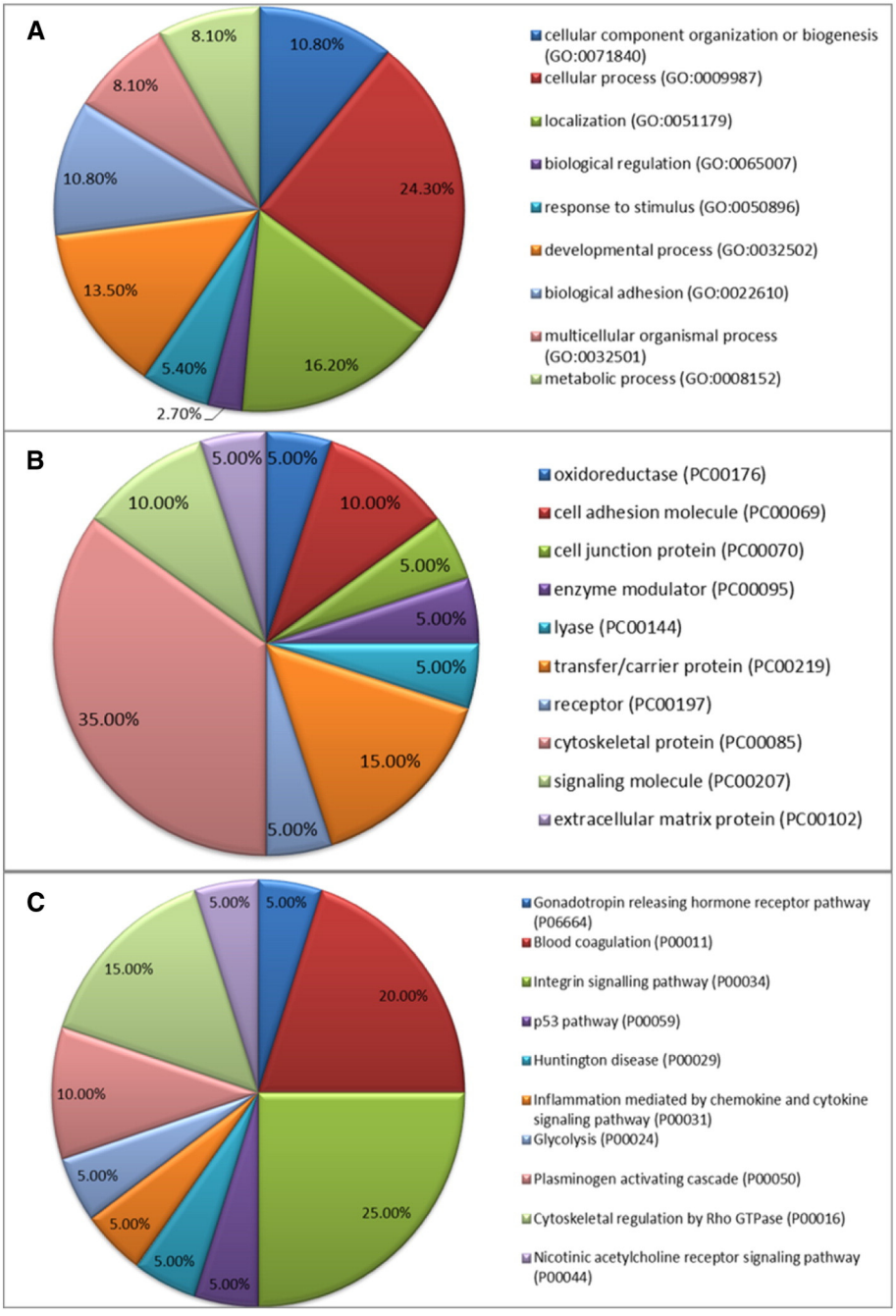

Fig. 2. Gene ontology analysis of proteome changes. Differentially expressed proteins between the normal group and thrombin group were systematized analysis by PANTHER classification system. (A) the biological processes; (B) the protein classes; (C) the pathway analysis.

3. Results and discussion

3.1. Inhibition effect of curdione on α-granule secretion

In this study, the inhibition effect of curdione on activated human platelets was conducted using electron microscopy before proteome analysis. Platelets include three types of granules: α-granules, dense granules and lysosomes. The α-granule contains P-selectin, growth factors, coagulation factors and chemokines. We used 100 μM of curdione to pretreat the cells, and performed transmission electron microscopy to observe the α-granule secretion to investigate the morphological changes in α-granule after thrombin induction. In the normal group, most of the particle contents were stored in the platelets (Fig. 1A). This was significantly different in the context of 0.3 U of thrombin (Fig. 1B) where most of the contents in α-granules were secreted following platelet activation, leaving empty vesicles and pseudopodia extensions. The 100 μM curdione seemed to inhibit thrombin-induced α-granule secretion (Fig. 1C) completely. Determination on platelets ultrastructure and secretion indicated that curdione almost inhibited the content of released particles induced by thrombin.

3.2. Identification of differential proteins by LC-MS/MS

Platelet proteins, pre-treated with or without curdione and successively stimulated by thrombin, were investigated by proteomics approach using a nano ESI-LC-MS/MS system. Three parallel experiments were performed on a LTQ XL Orbitrap mass spectrometer (ThermoFisher Science, USA) for analysing the trypsin digestion peptides. BSA was used as the control to verify the effect of enzymatic hydrolysis. Our results indicated that the BSA score was >600, which proved that the enzyme hydrolysis effect was better (data not shown).

Based on MS analysis, we used 4 injections of each sample in our experimental protocol. After the mass spectrometry analysis, Proteome Discoverer software (v.1.2, ThermoFisher Science) was used for the identification of proteins. The filter criteria of protein identification was set with the confidence level of the peptide run up to medium reliability and above it, which made the FDR value < 0.05. In total, 249, 256, and 288 proteins were identified in the normal group, the thrombin group and the curdione group, respectively (Supplementary Tables 1 to 3). The output data were searched against the UniProt human proteome database with decoy using the Sequest HT search engine and further analysed with the SIEVE software (v 1.3, ThermoFisher Science, USA) to expose the proteins with altered expression. The inclusion criteria were P value < 0.05, ratio < 0.6 or >1.8, and scored >20 points. Twenty-two different proteins were found between the normal and the thrombin group, of which 8 were up-regulated and 14 were down-regulated (Table 1). Some of the differentially expressed proteins were also found in porcine platelet proteome [18] corroborating our results and verifying the accuracy of our proteomics data and suggested that these proteins are involved in the activation of platelets.

It is noteworthy that compared to the thrombin group, only two proteins (Talin1 and β1-tubulin), also found in Table 1, were shown significantly down-regulated in the curdione treatment (Table 2) indicating that the two proteins were associated with the process of platelet activation and aggregation. Relevant information on protein function was retrieved from the UniProt and Gene Ontology databases and appropriated references therein.

3.3. Gene ontology (GO) and UniProt analysis of proteome changes

To investigate the mechanism of anti-platelet activate of curdione, we elucidated the biological pathways affected by the modulation of protein abundance in response to thrombin. The selected 22 differentially expressed proteins were annotated with GO terms using PANTHER to assess the biological processes involved. The distribution of proteins for biological processes, protein classes and pathways are shown in Fig. 2. The biological processes obtained from GO analysis showed that these proteins were associated with the cellular process, the biological adhesion and the developmental process (Fig. 2A). Notably, the majority of the differentially expressed proteins are cytoskeletal proteins, transfer/carrier proteins, cell adhesion molecules, signalling molecules, receptors and enzyme modulators (Fig. 2B). For pathway analysis, the result suggests that the proteins are involved in the integrin signalling pathway, blood coagulation and cytoskeletal regulation by Rho GTPase, plasminogen activating cascade and inflammation mediated by chemokine and cytokine signalling pathway (Fig. 2C). Among them, TUBB1 was involved in the cytoskeletal regulation by Rho GTPase, while TLN1 was involved in the integrin signalling

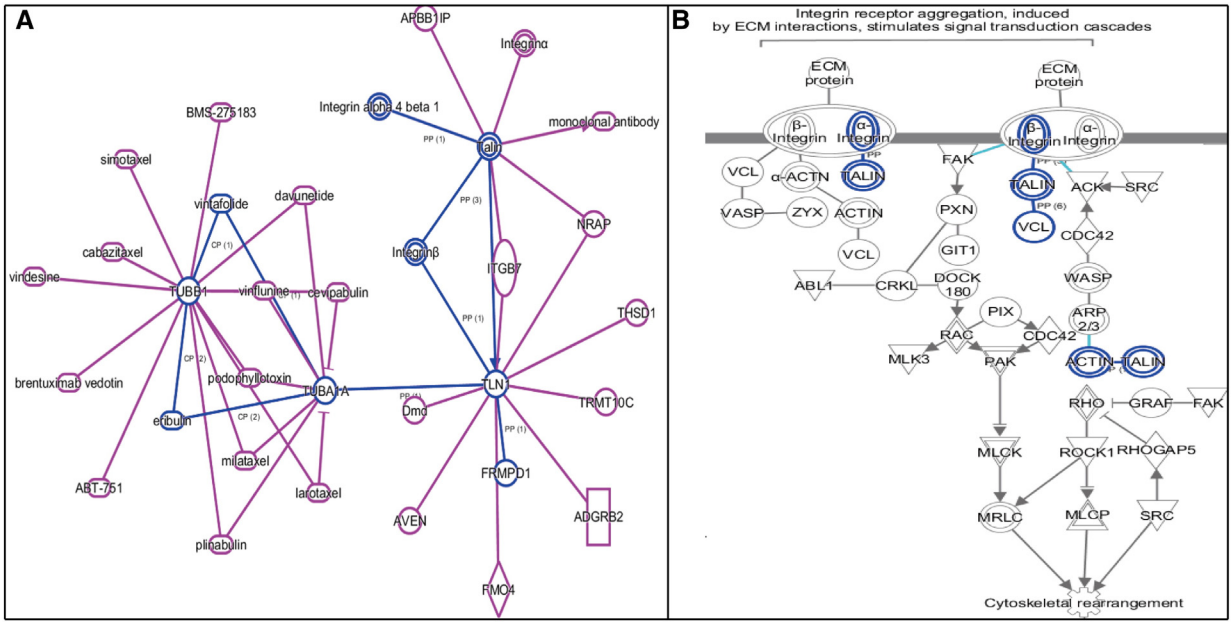

Fig. 3. Ingenuity network and pathway analysis. (A) IPA analysis of the differential proteins (β1-tubulin and Talin1) between the thrombin group and the curdione group from LC-MS/MS data; (B) ingenuity pathway analysis of the integrin signalling.

pathway. Integrin-dependent aggregation defects are reported to be correlated with myelodysplastic syndromes. The identified proteins play a critical role in the integrin αIIb/β3 signalling; and thus, platelet aggregation [19]. Given the known biological function on platelet activation, the data from gene ontology suggest that curdione affects critical cellular processes that are relevant to integrin signalling pathway.

UniProt database analysis showed that the majority of differential proteins were of the actin cytoskeleton, including TLN1, VCL, ACTIN1, PFN1, CAP1, FLNA and MYH9. The main functions of these proteins were cell adhesion and platelet activation or blood coagulation. Integrin (ITGA2B, ITGB3) was another class of differential proteins. Integrin αIIb/β3, the specific binding site to Arginine-glycine-aspartic acid (RGD) ligands [20] is a receptor for fibrinogen, fibronectin, prothrombin, plasminogen, thrombospondin and vitronectin. We observed a simultaneous down-regulation of integrinβ3, fibrinogen, and thrombospondin. The alterations in cytoplasmic conformation and aggregation of integrin clusters resulting from integrin activation occurred when combined with fibrinogen. The β3 subunit was then phosphorylated on tyrosine residues (Tyr-747 and Tyr-759) in response to thrombin-induced platelet aggregation [21] such that the expression of integrin β3 was decreased. Other differential proteins such as FERMT3 act by activating the integrin beta1–3 (ITGB1, ITGB2 and ITGB3) [22]. ENO1 is involved in gluconeogenesis and canonical glycolysis, and STOM functioned RNA polymerase binding [23].

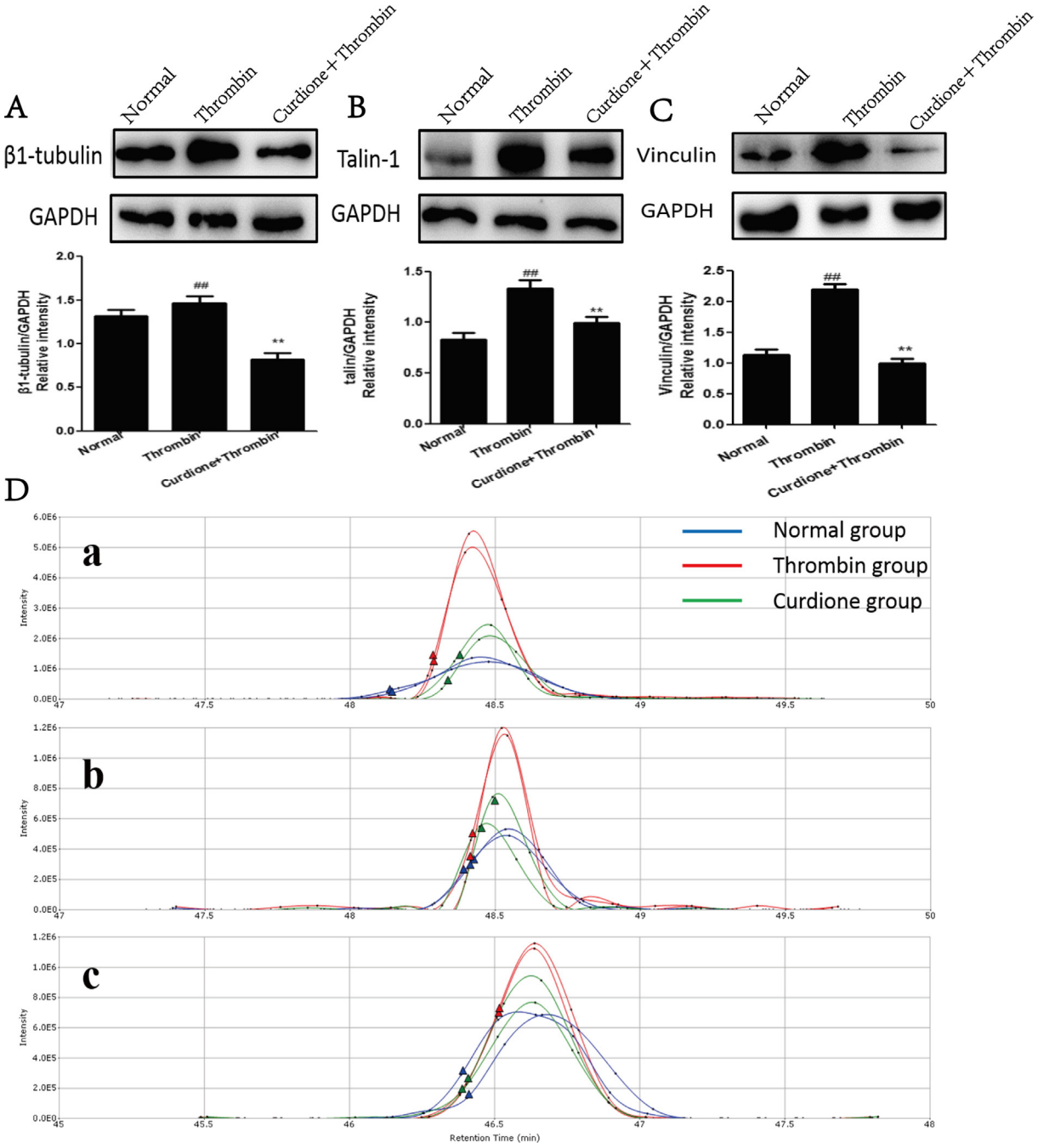

Fig. 4. Differentially expressed proteins validated by western blot. (A, B, C) β1-tubulin, Talin1 and vinculin were selected for further validation by western blot analysis, respectively. (D) A graphical representation of differences in intensity of different group proteins in the SIEVE software. (a) intensity of β1-tubulin in the three groups; (b) intensity of Talin1in the three groups; (c) intensity of vinculin in the three groups. The blue lines represent the normal group, the red lines represent the thrombin group and the green lines represent the curdione group. Data are shown as the mean ± S.D. ($n = 3$–4 per group). $^{\#\#}P < 0.01$ vs. normal group, $^{**}P < 0.01$ vs. thrombin group, $^{++}P < 0.01$ vs thrombin group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Pathway analysis of TUBB1 and TLN1

As TUBB1 and TLN1 were demonstrated to be highly expressed in the thrombin group, we performed pathway analysis of the two proteins and their associated proteins using IPA software. Canonical pathway analysis showed that TLN1 was involved in ERK, FAK and integrin signal pathway, while the TUBB1 was associated with the Epithelia Adherens Junction Signal pathway. Molecular interaction analysis indicated no direct link between TUBB1 and TLN1 (Fig. 3A). To further investigate the mechanism of inhibition effect of curdione on platelet activation, we analysed the interactions of Talin1 and other proteins in the integrin signalling pathway. As showed in Fig. 3B, the related protein connected with TLN1 was vinculin (VCL) in the integrin regulated inside-out signal transduction. It is well known that VCL is an intracellular protein with a critical role in the maintenance and regulation of cell adhesion and migration [24]. It appears that vinculin also has this characteristic in platelets.

3.5. Western blotting analysis to verify TUBB1 and TLN1

Although curdione seems to affect several regulatory molecules, its effects on TUBB1 and TLN1 expression were predominantly visible. Therefore, western blotting analysis was employed to validate the changes of TUBB1 and TLN1. As shown in Fig. 4A to C, western blotting analysis revealed an up-regulation of TUBB1 and TLN1 in thrombin induced platelet samples, and down-regulation of these two proteins in the curdione and normal groups. In the Fig. 4D, we have re-plotted the proteomics data obtained for TUBB1 and TLN1 for comparison. In Fig. 3B, it can be observed that VCL was tightly linked to TLN1. VCL, which binds Talin and actin, is important for the full engagement of adhesion complexes with the force machinery [25,26]. In our present study, the expression of VCL in the protein level was unexpectedly consistent with that of both TUBB1 and TLN1. It has shown that TUBB1 was related to platelet adhesion [27], and in the IPA analysis and some articles reported, Talin1 is the critical actin in the integrin activation [28]. Overall, the accumulated evidence suggested that TUBB1 and TLN1, along with their correlated proteins-VCL, were associated with integrin signalling and the activation of inflammation response mechanisms. We hypothesized that β1-tubulin might regulate the expression of vinculin and Talin1, successively affect the integrin signal pathway, eventually inhibit platelet activation. These hypotheses were further verified by conducting a western blot of β1-tubulin regulation of vinculin/Talin1.

3.6. β1-tubulin could positive regulate vinculin

It has been reported that the platelets of β1-tubulin–deficient mice, generated by the targeted disruption of the β1-tubulin gene, show a disproportionately prolonged bleeding time and an attenuated platelet response to thrombin. These results – for the first time – established a role for β1-tubulin in platelet function [29]. In addition, the Q43P β1-tubulin variant resulting from a double-nucleotide substitution is probably a very ancient mutation, suggesting that it could confer an evolutionary advantage, reducing ischaemic cardiovascular risk [27]. However, β1-tubulin as a potential therapeutic target of curdione on anticoagulation remains unclear.

As β1-tubulin and vinculin appeared to have identical actions on the same processes, it is unclear whether β1-tubulin positively regulates vinculin function. To evaluate if β1-tubulin can mediate the expression of vinculin, we first investigated a dose-dependent inhibited β1-tubulin expression in washed platelets stimulated with thrombin. Washed platelets were treated with ABT-751, an inhibitor of β1-tubulin, for 1 h at 37 °C. As showed in Fig. 5A and B, ABT-751 inhibited β1-tubulin expression by nearly 2 times compared with the thrombin group at a concentration of 10 μg/ml for platelets. Moreover, ABT-751 down-regulated the expression of both vinculin and Talin1 in response to curdione treatment.

3.7. Vinculin positively regulates Talin1

Several studies reported that vinculin could bind with Talin to promote the integrin-mediated cell adhesion. Moreover, activated vinculin promotes Talin-mediated integrin activation by binding to accessible

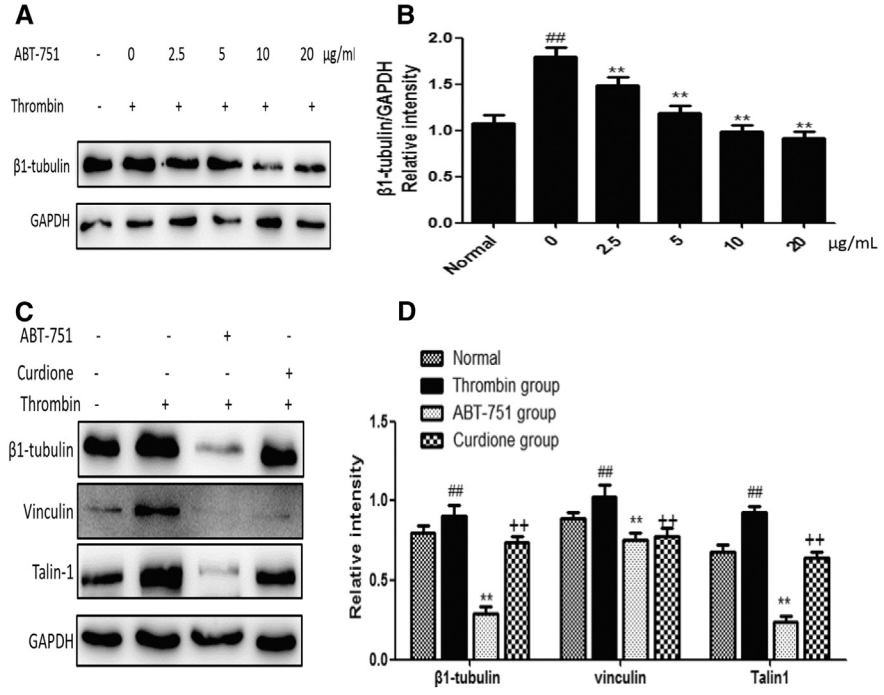

Fig. 5. Western blot analysis was used to validate the regulation of β1-tubulin to vinculin. (A) Inhibited effects of ABT-751 on the β1-tubulin. Washed human platelets were treated with 0, 2.5, 5, 10 and 20 μg/ml ABT-751 for 60 min then stimulated with 0.3 U thrombin (B) Protein expression of vinculin and Talin1 in washed platelets. The representative bands were from different gels for repeated experiments. After densitometric analysis, the values of proteins were normalized against GAPDH, respectively. Data are shown as the mean ± S.D. ($n = 3$–4 per group). $^{\#\#}P < 0.01$ vs normal group, $^{**}P < 0.01$ vs thrombin group, $^{++}P < 0.01$ vs thrombin group.

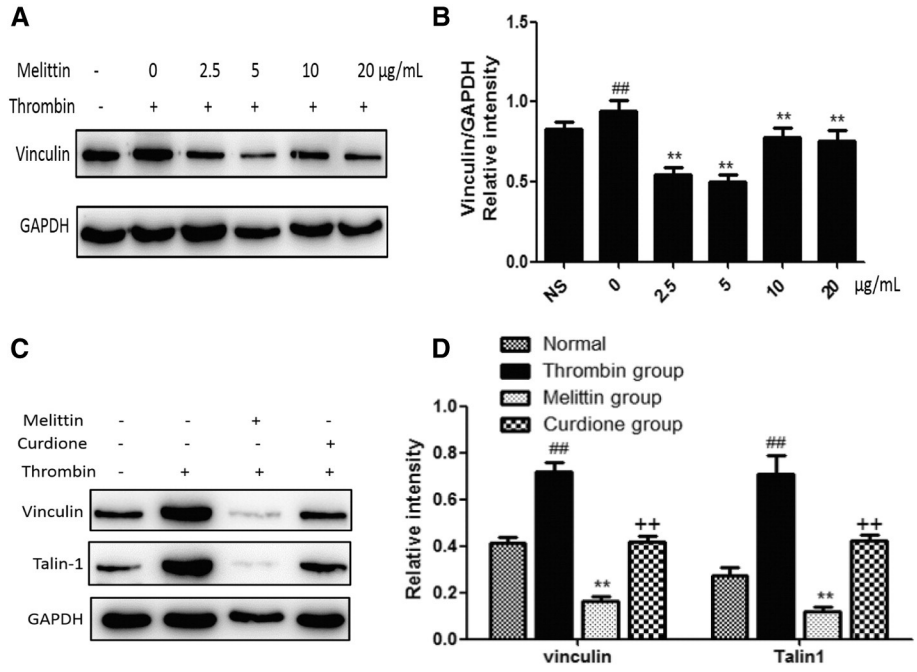

Fig. 6. Western blot analysis was used to validate the regulation of vinculin to Talin 1. (A) The inhibited effect of melittin on the vinculin. Washed human platelets were treated with 0, 2.5, 5, 10 and 20 μg/ml melittin for 60 min then stimulated with 0.3 U thrombin (B) protein expression of Talin1 in washed platelets. The representative bands were from different gels for repeated experiments. After densitometric analysis, the values of proteins were normalized against GAPDH, respectively. Data are shown as the mean ± S.D. ($n = 3$–4 per group). $^{\#\#}P < 0.01$ vs normal group, $^{**}P < 0.01$ vs thrombin group, $^{++}P < 0.01$ vs thrombin group.

vinculin binding sites in Talin rod; and thus, displacing the Talin rod from the β3 subunit [30]. Talin is indeed essential for integrin activation, cell adhesion and spreading and is at the core of adhesion complex assembly, the regulation of adhesion strength and engagement with actin.

To evaluate the regulation effect of vinculin on the expression of Talin1, we investigated a dose-dependent inhibition of vinculin expression in washed platelets stimulated with thrombin. Washed platelets treated with melittin, an inhibitor of vinculin [31], significantly down-regulated vinculin expression compared with the thrombin group at 5 μg/ml for platelets. As shown in Fig. 6A and B, melittin down-regulated the expression of Talin1 similar to that observed with curdione treatment.

β1-tubulin in human platelets is not simply a structural cell frame [32]; however, its novel function had not been explored. In this study, we determined the role of β1-tubulin in platelet activation using thrombin and the specific β1-tubulin inhibitor ABT-751. We found that thrombin increased platelet activation by stimulating the production of integrin activation and increasing the expression levels of proteins recruited by integrin. The signalling pathway from the αIIb/β3

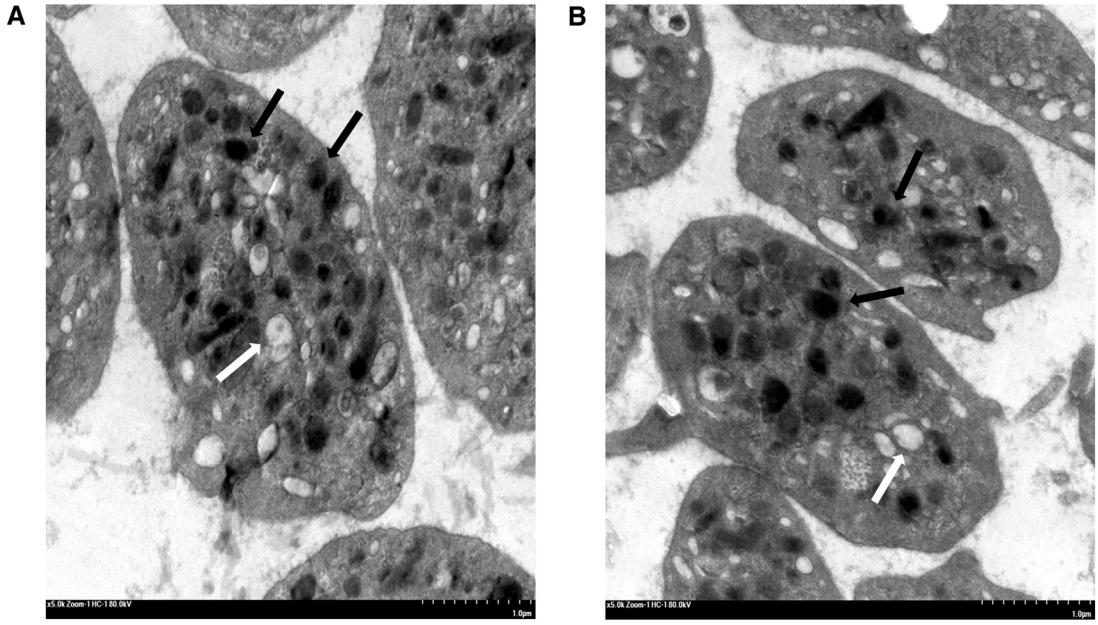

Fig. 7. Two inhibitors' effect on the granules secretion analysed by transmission electron microscopy. (A) After pre-incubation with ABT-751 (10 μM) and stimulation with thrombin (0.3 U) (B) After pre-incubation with mellitin (5 μg/ml) and stimulation with thrombin (0.3 U). The white narrows were empty vesicles that have been secreted α-granules, and the black narrows point to α-granules ($n = 5$).

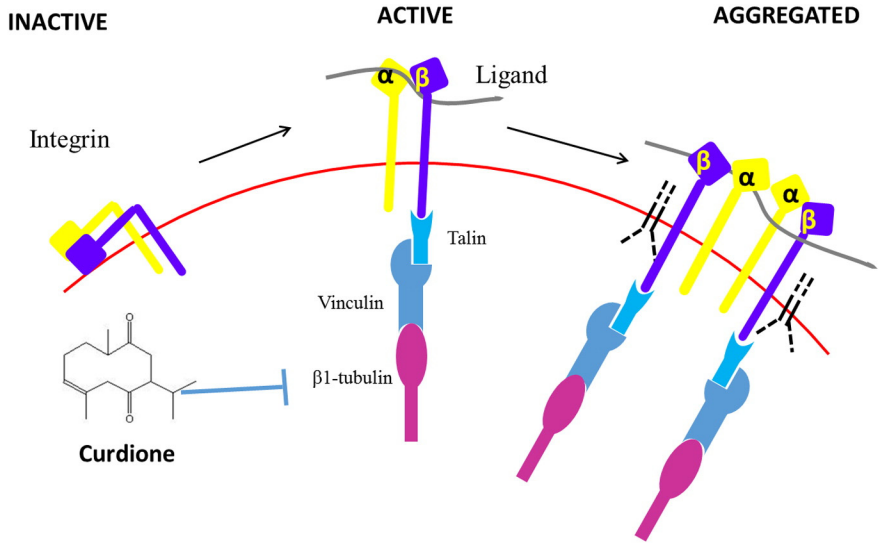

Fig. 8. Schematic overview of curdione attenuates thrombin-induced human platelet activation integrin signalling pathway. Vinculin is a talin-binding protein that promotes integrin-mediated cell adhesion, activated vinculin promotes talin-mediated integrin activation. It has been seen that the down-regulation of β1-tubulin lead to the decrease of vinculin/Talin1. The curdione works by decreasing the levels of β1-tubulin and eventually suppressed the expression of Talin1 to inhibit platelet activation and aggregation.

platelet membrane receptor to the cytoplasmic region is still unknown. Talin-1 shows lower expression levels in the curdione group and binds to the β3-subunit of integrin αIIb/β3, which was referred to as the common final step necessary for integrin activation. David A. Calderwood reported that an integrin-binding site is localized within the N-terminal Talin head domain. The binding of the Talin head domain to integrin b tails can directly enhance the combination of GPIIb/IIIa and fibrinogen [33]. Figs. 5 and 6 showed that the inhibition of TUBB1 suppressed the expressions of VCL and TLN1. Moreover, the expression of TLN1 was suppressed when VCL was inhibited.

3.8. ABT-751 and melittin attenuated the α-granule secretion

We further verified the inhibitory effect of ABT-751 and melittin on human platelets. We employed electron microscopy to analyse the α-granule secretion with 10 μg/ml ABT-751 and 5 μg/ml melittin by incubating the platelets before 0.3 U thrombin induction. In accordance with previous results, ABT-751 and melittin can inhibit the release of α-granules (Fig. 7A and B). The platelet shape remained unchanged when incubated with ABT-751 indicating that none of the platelets were activated. Therefore, we suggest that β1-tubulin may be a potential target for platelet activation. Inhibitor studies showed that ABT-751 and melittin have an effect to platelet activation and three proteins were down-regulated simultaneously when the platelets were incubated with 100 μM curdione. This suggested that curdione regulated thrombin-induced human platelet activation through the integrin signalling pathway (Fig. 8).

4. Conclusions

In our present study, β1-tubulin was found to be a potential target for the inhibitory effect of curdione on the signal transduction of platelet activation. This may provide a new direction for the research and development of anti-platelet drugs. Moreover, the results of β1-tubulin or vinculin suppression suggested that curdione works by decreasing the levels of β1-tubulin and eventually suppresses the expression of Talin1, thereby inhibiting platelet activation and aggregation.

The authors declare that they have no conflicts of interest.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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