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## Original Article

# Polysaccharide-containing fraction from *Artemisia argyi* inhibits tumor cell-induced platelet aggregation by blocking interaction of podoplanin with C-type lectin-like receptor 2



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## ARTICLE INFO

## Article history:

Received 22 March 2019

Received in revised form  
11 July 2019

Accepted 13 August 2019

Available online 26 September 2019

## Keywords:

Tumor cell-induced platelet  
aggregation

Podoplanin

C-type lectin-like receptor 2

## ABSTRACT

Tumor cell-induced platelet aggregation (TCIPA) is a mechanism that involves the protection of tumor cells in the circulation and the promotion of tumor cell invasion and metastases. The C-type lectin-like receptor 2 (CLEC-2) that binds podoplanin (PDPN) is on the platelet surface and facilitates the TCIPA. Selective blockage of the PDPN-mediated platelet-tumor cell interaction is thereby a plausible strategy for inhibiting metastases. In a search for antagonists of PDPN- and tumor cell-induced platelet aggregation, traditional Chinese medicines were screened and it was found that the water extract of *Artemisia argyi* leaves selectively inhibited the PDPN-induced platelet aggregation. Bioactivity-guided fractionation analysis was performed for defining a polysaccharide-containing fraction (AAWAP) characterized by inhibition of PDPN activity and tumor cell-induced platelet aggregation. The pharmacological effects of AAWAP on PDPN-activated CLEC-2 signaling were determined by using Western blot and alpha screening analyses. AAWAP was non-toxic to the cells and platelets and it suppressed PDPN- and tumor cell-induced

**Abbreviations:** 2CP, N-(2-benzamidoethyl)-2-hydroxy-5-nitrobenzamide; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; CLEC-2, C-type lectin-like receptor 2; Co-HP, cobalt hematoporphyrin; DVT, deep vein thrombosis; LDH, lactose dehydrogenase; PDPN, podoplanin; PMP, 1-phenyl-3-methyl-5-pyrazolone; TCIPA, tumor cell-induced platelet aggregation; TCM, traditional Chinese medicine.

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<https://doi.org/10.1016/j.jfda.2019.08.002>

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Polysaccharides  
*Artemisia argyi*

platelet aggregation by irreversibly blocking the interaction between PDPN and CLEC-2 in a dose-dependent manner. These findings indicate that AAWAP is an antagonist of the PDPN-CLEC-2 interaction. This action by AAWAP may result in the prevention of tumor cell metastases, and if so, could become an effective pharmacological agent in treating cancer patients.

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## 1. Introduction

Cancer-associated thrombosis commonly occurs in patients with lung, breast, head and neck, and colorectal cancer, and is a marker of a poor prognosis for cancer patients [1]. Tumor cell-induced platelet aggregation (TCIPA) protects tumor cells by forming a platelet “shield” that allows tumor cells to tolerate attacks by immune cells and promotes tumor cell invasion and metastasis [2–5]. Therefore, inhibition of the TCIPA should be considered as a potential therapeutic strategy in the treatment of cancer.

Podoplanin (PDPN) is a type-I transmembrane sialoglycoprotein that is expressed in various cancer cell types and plays a critical role in facilitating tumor invasion and metastasis [4,5]. PDPN expression is highly associated with cancer-related mortality [6]. PDPN affects cellular behavior by interacting with a number of cellular proteins including C-type lectin-like receptor 2 (CLEC-2), galectin 8, and CD44 [6]. Of these PDPN-interacting proteins, CLEC-2 is a platelet surface receptor that binds PDPN and facilitates TCIPA [4,7]. Antibodies or synthetic agents targeting PDPN or CLEC-2 inhibit tumor cell growth, migration, invasion, metastasis, and tumor cell-induced thrombus formation [6–9]. Because the suppression of PDPN/CLEC-2-induced platelet aggregation does not affect normal physiological hemostasis [4], selective blockage of PDPN-CLEC-2 binding or its downstream signaling represents an apparently safe anti-metastasis strategy.

Traditional Chinese medicine (TCM) and botanical products are important sources of healthy foods and therapeutic agents [10]. Thirteen water extracts of TCMs were prepared and their inhibitory effects on PDPN-induced platelet aggregation were evaluated as possible antagonists of TCIPA. Of these, the water extracts (100 µg/mL) of leaves of *Artemisia argyi* (AAW), spikes of *Prunella vulgaris* (PVW), seeds of *Cassia obtusifolia* (COW), rhizomes of *Davallia mariesii* (DMW), and flowers of *Inula japonica* (IJW) showed inhibitory effects on PDPN-induced platelet aggregation. AAW was the only extract that selectively inhibited PDPN- but not other agonists-induced platelet aggregation (Fig. S1) and was chosen as the candidate TCMs to develop and identify new antagonists against TCIPA.

*A. argyi* and its congeners are widely distributed and their leaves have been used as traditional medicine, food ingredients, and herbal teas in China, Japan, Korea, Taiwan, and Russia [11,12]. The genus *Artemisia* plants have been used for the treatment of menstruation-related symptoms and gastric disease [11]. *A. argyi* elicits anti-cancer, anti-inflammatory,

anti-obesity, anti-Trypanosome, and antioxidant activity [12–15]. The therapeutic effects of *Artemisia* plants are mainly mediated by a variety of flavonoids [16], terpenoids [17,18], and caffeoylquinic acids [19].

In this study, the water extract of *A. argyi* leaves was fractionated via a bioassay-directed fractionation method to identify the fractions that elicited anti-PDPN-induced platelet aggregation activity. The cellular toxicity and the putative mechanisms of action for the active constituents were also addressed.

## 2. Materials and methods

### 2.1. Materials and reagents

Recombinant human PDPN and His-tagged CLEC-1B were purchased from Sino Biological Inc. (Beijing, China). The recombinant human PDPN-Fc chimera was purchased from R&D Systems Inc. (Minneapolis, MN). Thrombin, apyrase, prostacyclin I<sub>2</sub> (PGI<sub>2</sub>), heparin, bovine serum albumin (BSA), mannose, rhamnose, galaturonic acid, glucose, galactose, xylose, arabinose, glucosamine hydrochloride, fucose, acetic acid, 3-phenylphenol, sodium tetraborate, and phosphate-buffered saline (PBS) were purchased from Sigma–Aldrich (St Louis, MO). Collagen and U46619 were purchased from Chrono-Log Co. (Havertown, PA). Anti-phospho-specific antibodies for PLCγ2 (Y1217), SLP-76 (Y145), Syk (Y525/526), Akt1 (S473), PDK1 (S241), PKD/PKCμ (S744/748), cPLA<sub>2</sub> (S505), p38 (Y180/182), Erk1/2 (Y202/204), and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA). The anti-β-actin antibody was purchased from Novus Biological (Mill Valley, CA). The lactate dehydrogenase (LDH) activity assay kit was purchased from Promega Inc. (Madison, WI). The Bio-Rad protein assay dye reagent concentrate (#5000006) was purchased from Bio-Rad (Hercules, CA). Dextran T standards were purchased from Pharmacosmos (Holbaek, Denmark).

### 2.2. Genomic identification of the leaves of *Artemisia argyi*

Dried leaves of *A. argyi* were purchased from a Chinese medicine store (Huang-De-An, New Taipei City, Taiwan). ITS sequence analysis was performed to confirm that the dried leaves belonged to the species of *A. argyi* (see Supplementary

information). Sequence similarity was determined and analyzed by alignment with the genome sequence in the NCBI genome database (accession #GU724269).

### 2.3. Preparation of the bioactive polysaccharide-containing fraction (AAWAP)

The bioactive crude polysaccharide fraction (8.0 g) of the dried leaves from *A. argyi* (120 g) was prepared as previously described [20]. The crude polysaccharide fraction (8.0 g) was dissolved into ddH<sub>2</sub>O (1:20, w/v), then sequentially fractionated by 100 and 300 kDa MWCO Vivaspin 20 (Little Chalfont, GE Healthcare) to yield three sub-fractions of AAWPU (>100 kDa, 2.2 g), AAWPD (<100 kDa, 4.4 g), and AAWPUU (>300 kDa, 1.4 g). AAWPUU (658.6 mg) was dissolved in ddH<sub>2</sub>O (50 mg/mL) and centrifuged at 8000 *g* for 10 min to remove insoluble material. Acetic acid (99.8%) was added to the supernatant until the pH reached 3.0. The subsequent precipitate (AAWAP, 261.2 mg) was collected by centrifuging at 8000 *g* for 10 min. The average molecular weight of AAWAP was 4255 kDa. The carbohydrate, uronic acid and protein contents in AAWAP was 15.25%, 8.33%, and 23.61%, respectively [20]. PDPN-induced platelet aggregation assays were then performed to determine the bioactivity of each fraction (Fig. S2).

### 2.4. Monosaccharide composition of AAWAP

AAWAP (10 mg) was hydrolyzed, and then the hydrolysis product or monosaccharide standards (mannose, rhamnose, galacturonic acid, glucose, galactose, xylose, arabinose, glucosamine hydrochloride, and fucose) were dissolved in ddH<sub>2</sub>O, labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP), and analyzed by HPLC as previously described [20,21].

### 2.5. Human blood collection and preparation of washed human platelets

All experimental protocols and procedures were approved by the Institutional Review Board of Chang Gung Memorial Hospital (Linkou, Taiwan, Republic of China) with the approval ID 201800448A3 and 102–4838C. Healthy volunteers had not taken any drugs that might influence hematological function within the last two weeks of blood collection and had no history of hematological diseases.

The washed platelets were prepared as previously described with some modifications [22]. Briefly, platelet-rich plasma (PRP) was obtained by centrifugation of the whole blood premixed with 3.2% trisodium citrate solution (9:1) at 220 *g* for 15 min. PRP was centrifuged at 980 *g* for 8 min to pellet the platelets. After washing the platelets twice using Tyrode's buffer (137 mM NaCl, 12 mM NaHCO<sub>3</sub>, 5.5 mM glucose, 5 mM HEPES, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.3) containing 0.6 μM PGI<sub>2</sub>, 8 units/mL heparin, and 0.02 units/mL apyrase, the platelets were re-suspended in Tyrode's buffer and then diluted to 3 × 10<sup>5</sup> platelets/mL for the thrombin, collagen, or U46619-induced platelet aggregation assays, or 3 × 10<sup>8</sup> platelets/mL for the PDPN- and tumor cell-induced platelet aggregation assays.

### 2.6. Platelet aggregation and TCIPA

Washed platelets were pre-incubated with PBS or sample solutions (2.5 μL) for 3 min (4 min for TCIPA assay) at 37 °C with constant stirring (900 rpm) prior to the addition of the platelet agonists: collagen (2 μg/mL), U46619 (2 μM), thrombin (0.1 U/mL), or tumor cells (1.5 × 10<sup>6</sup> cells). For the PDPN-induced platelet aggregation assays, washed platelets were pre-incubated with PBS or sample solutions (2 μL) for 3 min at 37 °C with constant stirring (1000 rpm) in the presence of the anti-human CD32 (FcγRII) blocking solution (1 μL). Subsequently, PDPN (5 μg/mL) was added to trigger platelet aggregation [22,23]. These assays were performed by using a platelet aggregometer (Chrono-Log Corp., Havertown, PA). The time to reach 50% maximal aggregation was recorded and defined as the aggregation time.

### 2.7. Lactose dehydrogenase (LDH) cytotoxicity assays

Cytotoxicity toward platelets and tumor cells was evaluated using a commercial LDH activity assay kit according to the manufacturer's instruction. Briefly, washed platelets (1 × 10<sup>9</sup>/mL) were pre-incubated for 1 min at 37 °C with constant stirring (900 rpm). Then 2 μL of PBS, AAWAP (10–100 μg/mL) or a 1% Triton X-100 solution (positive control) was added to the reaction mixtures and incubated for an additional 15 min. The supernatants were collected after centrifugation at 3000 *g* for 5 min and 10,000 *g* for 5 min. Tumor cells (1 × 10<sup>5</sup> cells/well) were cultured in a 12-well plate and treated with AAWAP (100 μg/mL) for 0–24 h. The supernatants of the cell cultures were collected as described above for the LDH cytotoxicity assay.

The LDH cytotoxicity assay was performed by mixing 50 μL of the cell or platelet suspension with 50 μL of the reagent mix (diaphorase, tetrazolium INT, NAD<sup>+</sup>, and lactate) in an optical 96-well flat microplate. The reaction was incubated at room temperature for 30 min and then quenched by adding 50 μL of the stop solution. The LDH activity of the samples was determined by measuring the absorbance at 492 nm using an ELISA plate reader (Thermo Labsystems, Franklin, MA). The supernatant from platelets that were lysed with 0.1% Triton X-100 was used as a control for total LDH activity [22,24].

### 2.8. Western blot analysis

Platelets were mixed with 125 μL of 5X lysis buffer (300 mM NaCl, 100 mM Tris-HCl, pH 7.5, 5 mM EGTA, 5 mM PMSF, 5 mM Na<sub>3</sub>VO<sub>4</sub>, 5% Triton X-100, 20 μg/mL leupeptin, and 20 μg/mL aprotinin) and kept on ice for 2 h. Subsequently, 156 μL of the sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 50 mM DTT) was added to the platelet lysates and the mixture was heated to 95 °C for 10 min. The lysates were kept at –80 °C until use.

The extracted proteins were fractionated using a 10% polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Pall Corp., Ann Arbor, MI). After blocking with 5% non-fat dry milk, membranes were incubated with appropriate primary antibody at 4 °C overnight. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), and then incubated with a HRP-conjugated

secondary antibody prepared in 5% BSA for 1 h at room temperature. After washing with TBS-T, the proteins were detected by using an enhanced chemiluminescence detection kit (Millipore Corp., Bedford, MA). The relative protein expression was normalized to  $\beta$ -actin expression [22].

### 2.9. Alpha screen

AAWAP (2.44–156.25  $\mu\text{g}/\text{mL}$ ) or PBS (10  $\mu\text{L}$ ) was incubated with 5  $\mu\text{L}$  of His-tagged human CLEC-2 protein (330 nM) in a 96-well plate for 15 min at 37 °C. Subsequently, 5  $\mu\text{L}$  of PDPN-Fc protein (2.75 nM) was added to the reaction and the solution was mixed by shaking for 1 h at 37 °C. Protein A acceptor beads (20  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{L}$ ) and nickel chelate donor beads (20  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{L}$ ) (Perkin Elmer, Waltham, MA) were then added to the mixture. After incubation for 1 h at room temperature, binding intensity was determined using an EnVision plate reader (Perkin Elmer, Waltham, MA) [25].

### 2.10. Statistical analysis

All experiments were repeated at least three times and the results were expressed as the mean  $\pm$  S.E.M. Data were analyzed by a Student's t-test or a one-way ANOVA as appropriate using GraphPad Prism 4 (San Diego, CA, USA).  $P < 0.05$  indicates statistical significance.

## 3. Results

### 3.1. AAWAP selectively inhibited PDPN-induced platelet aggregation

Using a bioactivity-guided fractionation protocol, a crude polysaccharide fraction of AAWPU (5  $\mu\text{g}/\text{mL}$ ) elicited inhibitory activity against PDPN-induced platelet aggregation. This crude polysaccharide fraction was subdivided by molecular weight and the sub-fraction AAWPUU (>300 kDa) was found to be more potent than the crude polysaccharide fraction. After acetic acid precipitation, the precipitate fraction (AAWAP) showed the most potent inhibitory effect on PDPN-induced platelet aggregation in a dose-dependent manner (Figs. 1A, D, and S2). This fraction was selected for further analysis in the following studies.

Analysis of AAWAP showed that its contents included carbohydrates (15.25%), uronic acid (8.33%), and proteins (23.61%). AAWAP may belong to a group of sulfated polysaccharides that have a low content of carbohydrates, uronic acid, and protein [26,27]. The monosaccharide molar ratio of AAWAP was glucose (2.42), galactose (1.00), arabinose (0.79), mannose (0.34), and galaturonic acid (0.25, respectively (Fig. S3).

### 3.2. AAWAP inhibited tumor cell-induced platelet aggregation

Two PDPN-expressing human osteosarcoma cell lines, HOS and MG63, were used to test the effects of AAWAP on TCIPA. AAWAP inhibited TCIPA in both cell lines in a dose-dependent manner (Fig. 1B, C). The time to reach 50% platelet aggregation

for AAWAP-treated (10  $\mu\text{g}/\text{mL}$ ) HOS and MG63 cells ( $944.6 \pm 53.1$  and  $1626.0 \pm 111.0$  s) was significantly longer than that for the control ( $633.7 \pm 26.7$  and  $723.0 \pm 41.3$  s), respectively (Fig. 1E, F). The LDH assay revealed that AAWAP (100  $\mu\text{g}/\text{mL}$ ) was not cytotoxic to tumor cells. LDH release occurred only in platelets pre-treated with high concentrations of AAWAP (>50  $\mu\text{g}/\text{mL}$ ) (Fig. 2). At concentrations lower than 50  $\mu\text{g}/\text{mL}$ , cytotoxicity is not a factor related to the inhibitory effects of AAWAP on PDPN- and tumor cell-induced platelet aggregation.

### 3.3. AAWAP suppressed PDPN-induced CLEC-2 signaling

An analysis of the effect of AAWAP on PDPN-activated signaling proteins downstream of CLEC-2 was compared to the effect of 2CP, an antagonist of PDPN-CLEC-2 binding, on PDPN-induced platelet aggregation (Fig. 3A) [22]. Phosphorylation of Syk (Y525/526), PLC $\gamma$ 2 (Y1217), Akt1 (S473), p38 (Y180/Y182), cPLA2 (S505), PKC $\mu$  (S748), and Erk (Y202/204) was increased in the PDPN-treated group. Both AAWAP and 2CP significantly suppressed PDPN-induced dose-dependent phosphorylation of these proteins (Fig. 3B). The phosphorylation of PDK1 (S241) that was decreased by PDPN was restored by AAWAP and 2CP (Fig. 3B). These results indicate that AAWAP, similar to 2CP, inhibits PDPN-induced platelet aggregation through modulation of CLEC-2-mediated signaling.

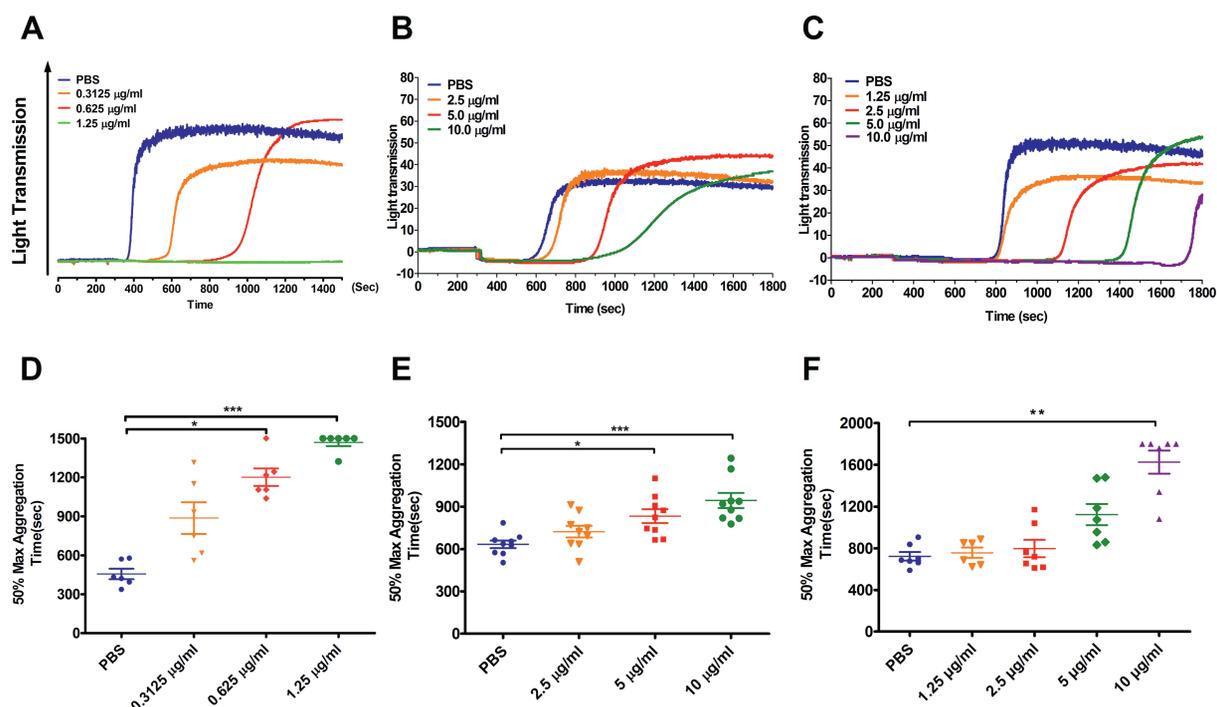
### 3.4. AAWAP irreversibly blocked the interaction between PDPN and CLEC-2

An alpha screen-based competition assay was performed to determine whether or not AAWAP interfered with PDPN binding to CLEC-2. The PDPN-CLEC-2 interaction was attenuated by AAWAP in a concentration-dependent manner with an  $\text{IC}_{50}$  of 26.53  $\mu\text{g}/\text{mL}$  (Fig. 4A). AAWAP (25  $\mu\text{g}/\text{mL}$ ) suppressed the maximum value and changed the slope of the PDPN-CLEC-2 response curve, indicating that AAWAP may irreversibly block the interaction between PDPN and CLEC-2 (Fig. 4B).

## 4. Discussion

Polysaccharides are major bioactive constituents of natural products with diverse bioactivities, including immunomodulatory, anti-diabetic, anti-inflammatory, and antitumor activities [20,28]. In particular, injection of the polysaccharide of *Astragalus membranaceus* (PG2®) was approved for treatment of cancer-related fatigue in Taiwan [29]. Polysaccharides such as lentinan, schizophyllan, and krestin have been used as immunomodulatory agents in several countries [30]. Polysaccharides thereby represent a source of healthy foods and therapeutic agents.

Several polysaccharides have been isolated from *Artemisia* sp. with potent immunomodulatory effects [21,30]. Among them, ASKP-1, a novel polysaccharide of *Artemisia sphaerocephala* composed of mannose, glucose, and galactose with an average molecular weight of 908 kDa elicits macrophage-activating effects through modulating MAPK, PI3K/Akt and NF- $\kappa$ B signaling [22]. FAAP-02 is an octomeric polysaccharide



**Fig. 1** – AAWAP inhibited PDPN- and tumor cell-induced platelet aggregation. (A and D) Washed platelets were treated with PBS or different concentrations of AAWAP (0.3125  $\mu\text{g/ml}$ : orange; 0.625  $\mu\text{g/ml}$ : red; 1.25  $\mu\text{g/ml}$ : green) followed by stimulation with PDPN. Representative traces of platelet aggregation and the time to reach 50% of the maximal aggregation was defined as the aggregation time. The value is set to 1500 s when no platelet aggregation was observed. (B and E) AAWAP (1.25  $\mu\text{g/ml}$ : orange; 2.5  $\mu\text{g/ml}$ : red; 5.0  $\mu\text{g/ml}$ : green; 10.0  $\mu\text{g/ml}$ : purple) attenuated HOS cell-induced platelet aggregation in a dose-dependent manner. (C and F) AAWAP (2.5  $\mu\text{g/ml}$ : orange; 5.0  $\mu\text{g/ml}$ : red; 10.0  $\mu\text{g/ml}$ : green) attenuated MG-63-induced platelet aggregation in a dose-dependent manner. The value is set to 1800 s when no platelet aggregation was observed. Data represent the mean  $\pm$  S.E.M of six independent experiments. \*P < 0.05 and \*\*\*P < 0.001 when compared to the PBS-group.

from *A. argyi* exhibiting antitumor activity via immunostimulatory effects [30]. In the present study, a polysaccharide-containing fraction (AAWAP) with an average molecular weight of 4255 kDa inhibited PDPN- and tumor cell-induced platelet aggregation through direct and irreversible inhibition of the PDPN and CLEC-2 interaction. To the best of our understanding, this is the first natural product reported to act as an antagonist of PDPN-CLEC-2 binding. TCMs may be excellent sources for screening more novel agents that inhibit the PDPN-CLEC-2 interaction.

2CP, a 5-nitrobenzoate derivative, inhibits PDPN- and tumor cell-induced platelet aggregation through direct binding to CLEC-2 with a dissociation constant of 33.2  $\mu\text{M}$ , thereby inhibits PDPN- and tumor cell-induced platelet aggregation at concentration of 12.1 and 20  $\mu\text{M}$  [22]. This inhibition is also observed with protoporphyrin IX (H2-PP). H2-PP was modified at its hematoporphyrin moiety, and a cobalt-conjugating derivative (cobalt hematoporphyrin, Co-HP) was synthesized. At concentrations between 1.53  $\mu\text{M}$  and 3.05  $\mu\text{M}$ , Co-HP selectively inhibits platelet aggregation induced by PDPN-expressing CHO cells and rhodocytin [4]. AAWAP inhibited PDPN- and tumor cell-induced platelet aggregation at the concentrations of 0.3–2.3 nM (1.25–10.0  $\mu\text{g/ml}$ ) and was more potent than other known inhibitors of the PDPN-CLEC-2

interaction. In addition, it is a firstly inhibitors of the PDPN-CLEC-2 interaction from natural source.

PDPN and CLEC-2 may be involved in tumor cells metastasis and are recognized as critical regulators of many diseases such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and deep vein thrombosis (DVT) [4,31–34]. The interaction between platelets and immune cells regulates the pathogenic mechanisms of ARDS and ALI. CLEC-2 expressed on platelets interacts with PDPN expressed on alveolar macrophages. The interaction inhibits neutrophil recruitment and improves lung function in animal models of ARDS/ALI [31,32]. DVT and its major complication, pulmonary embolism is associated with high mortality. Platelet CLEC-2 and PDPN expressed on the pathological venous wall may be responsible for thrombus formation and DVT [4,33,34]. Treating DVT with Co-HP and platelet-specific deficiency in CLEC-2 significantly decreases thrombus formation and protects against DVT [4,33]. The inhibitory effect of AAWAP on PDPN binding may be effective in preventing metastases and protection against DVT.

In conclusion, AAWAP, a non-cytotoxic polysaccharide-containing fraction, was prepared from water extracts of *A. argyi* leaves using a bioactivity-guided fractionation protocol. AAWAP suppressed PDPN- and tumor cell-induced platelet

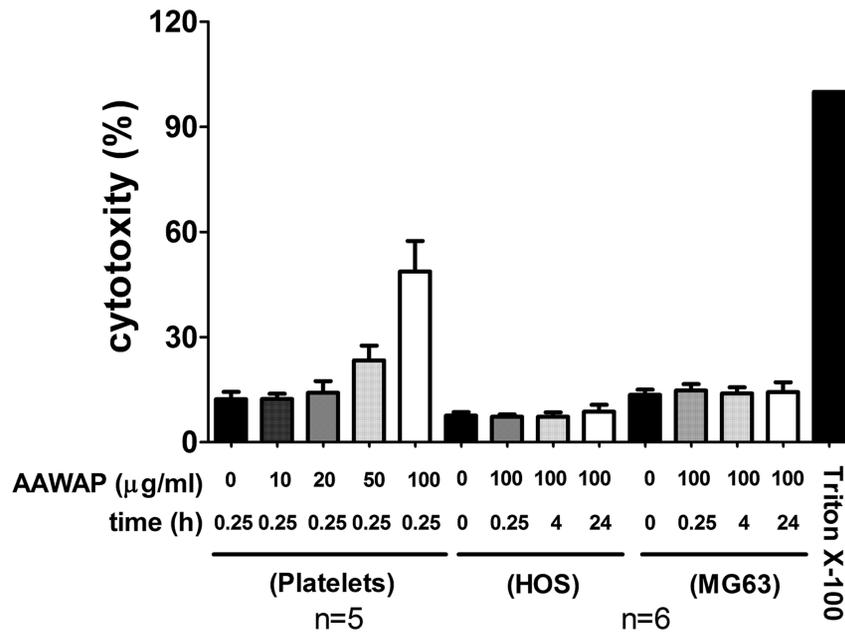


Fig. 2 – The effect of AAWAP on LDH release in human platelets and osteosarcoma cells. Platelets and osteosarcoma cells (HOS and MG63) were incubated with the indicated concentrations of AAWAP at 37 °C. The cell-free supernatants were collected for measurement of LDH activity. The supernatant from platelets lysed by 0.1% Triton X-100 was used as a control for total LDH activity (100%). The released LDH activity was used as an indicator of cytotoxicity. Data represent the mean ± S.E.M of five to six independent experiments.

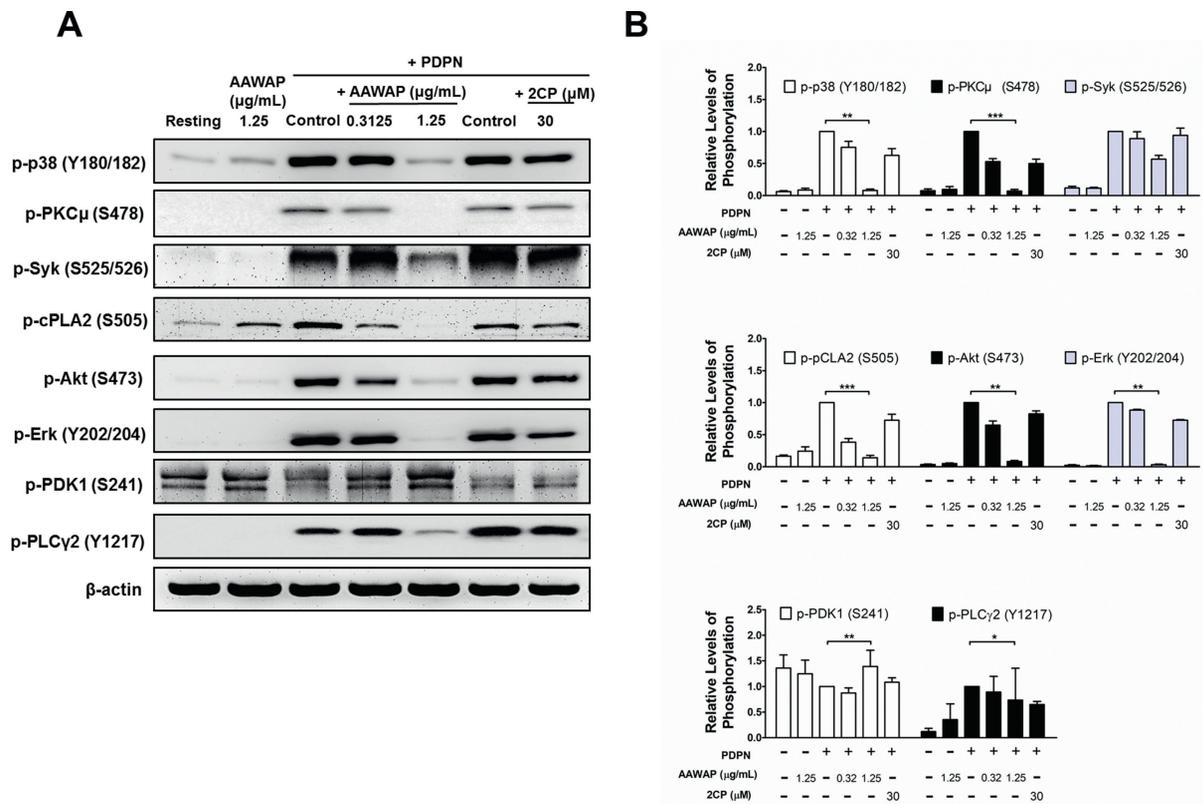
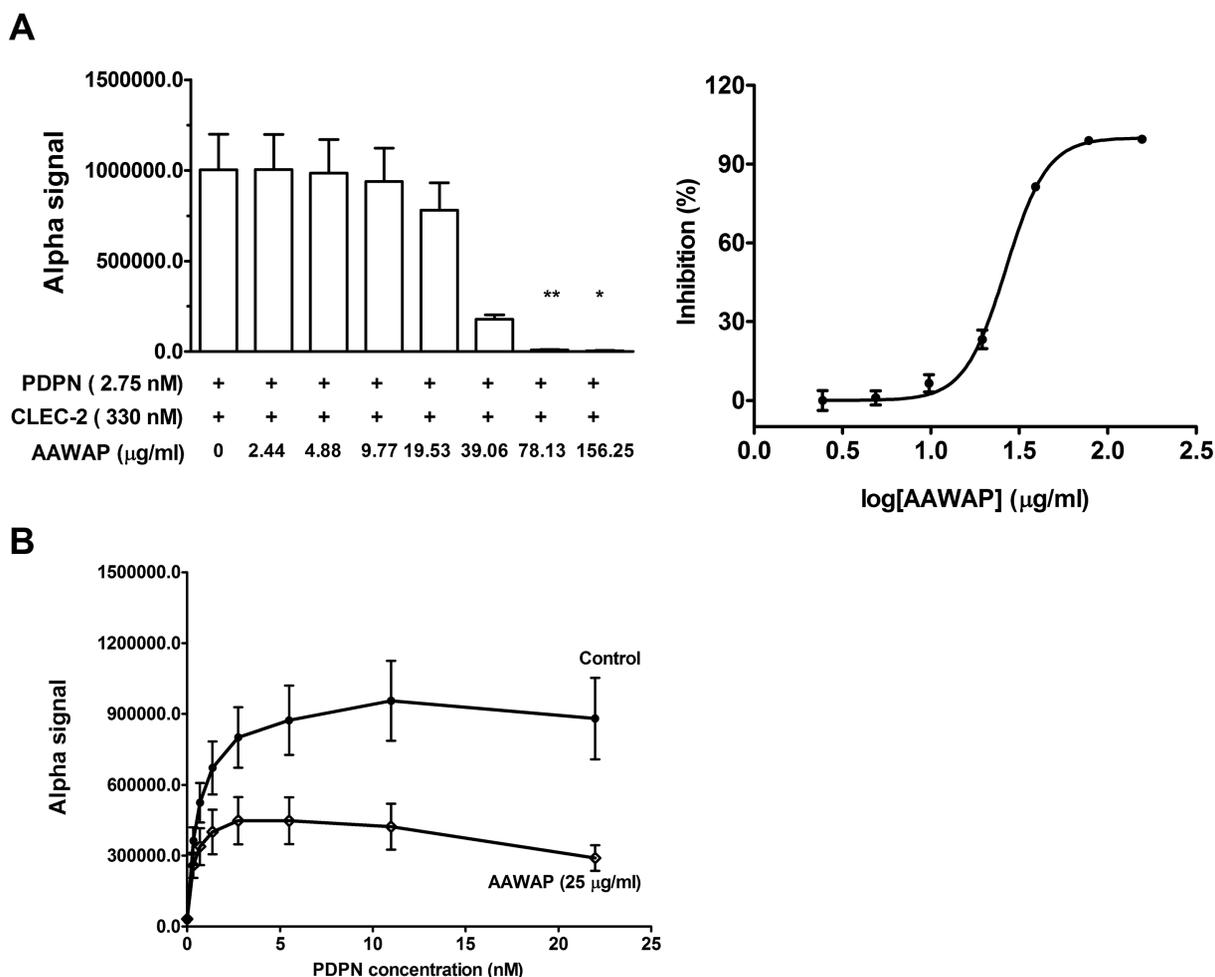


Fig. 3 – AAWAP reversed the signal transduction of PDPN-induced platelet aggregation. (A) AAWAP and 2CP reversed PDPN-modulated phosphorylation of p38, PKCμ, Syk, cPLA2, Akt, Erk, PDK1, and PLCγ2. (B) The relative phosphorylation level of the indicated proteins were determined and quantified using ImageJ software (NIH). Data represent the mean ± S.E.M (n ≥ 3). \*\*\*P < 0.001, \*\*P < 0.01, and \*P < 0.05 when compared to the control group.



**Fig. 4** – AAWAP irreversibly blocked the interaction between PDPN and CLEC-2. (A) His-tagged CLEC-2 protein was incubated with PBS or different concentrations of AAWAP in a 96-well plate. PDPN was then added to each well. After incubating with alpha screen beads, the intensity of alpha screen fluorescence signals representing the binding intensity of PDPN-CLEC-2 was determined (left panel). AAWAP elicited dose-dependent inhibition of PDPN-CLEC-2 binding (right panel). (B) His-tagged CLEC-2 protein was incubated with AAWAP (25 µg/mL) in a 96-well plate. The indicated concentrations of PDPN were then added to each well followed by an alpha screen assay to determine the intensity of alpha screen fluorescence signal. Data represent the mean  $\pm$  S.E.M ( $n \geq 4$ ). \*\* $P < 0.01$  and \* $P < 0.05$  when compared to the control group.

aggregation by irreversibly blocking the interaction between PDPN and CLEC-2. These findings indicate that AAWAP is an antagonist of the PDPN-CLEC-2 interaction. This action by AAWAP may result in the prevention of tumor cell metastases, and if so, could become an effective pharmacological agent in treating cancer patients.

### Conflicts of interest

The authors declare no conflict of interest.

### Author contributions

Conception and design of the research: Y.L.H., C.P.T., and P.W.H.; performed experiments: Y.L.H., C.P.T., and P.W.H.; acquired and analyzed data: C.P.T., Y.L.H. and P.W.H.; drafted

and edited the manuscript: C.P.T., Y.L.H. and P.W.H.; assisted with experiments: Y.W.C, H.R.L. and Y.L.C.; data discussion and review of the manuscript; C.P. T., Y.L.H. and P.W.H.

### Acknowledgements

We acknowledge Professor Arnold Stern (New York University School of Medicine) for his editorial help, and the technique supports from Bioproduction Engineering Technology Department, Biomedical Technology and Device Research Laboratories, ITR (Hsinchu, Taiwan) for performing genomic analysis of plant material. This work was supported by the Ministry of Science and Technology grants (MOST102-2320-B-182-008-MY3 and MOST106-2320-B-182-003-MY3 to P.-W. Hsieh and MOST105-2320-B-182-029-MY3 and MOST106-2320-B-182-027-MY3 to C.-P. Tseng), and the Chang Gung

Memorial Hospital grants (CMRPD1F0561-2 and BMRPB23 to P.-W. Hsieh, and CMRPD1E0181-3, CMRPD1F0611-3, CMRPD1H0211-3 and BMRP466 to C.-P. Tseng). The funders had no role in the study design, the data collection and analysis, the decision to publish, or the preparation of the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jfda.2019.08.002>.

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