

Dehydroandrographolide inhibits IgE-mediated anaphylactic reactions via calcium signaling pathway

Delu Che^a, Yajing Hou^a, Yingnan Zeng^a, Chaomei Li^a, Yongjing Zhang^a, Di Wei^a, Shiling Hu^a, Rui Liu^a, Hongli An^b, Yajun Wang^{c,*}, Tao Zhang^{a,*}

^a College of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China

^b Center for Translational Medicine, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, China

^c Department of Physical Examination, First Affiliated Hospital of Xi'an Jiao Tong University, Xi'an 710061, China

ARTICLE INFO

Keywords:

Dehydroandrographolide
IgE-mediated anaphylactic reactions
Mast cells
Degranulation
P-PLC γ inhibitor

ABSTRACT

The classical mast cells degranulation pathway is mediated by Fc ϵ RI aggregation and varies in strength among subjects. Dehydroandrographolide (DA) is one of principal components of *Andrographis paniculata* (Burm.f.) Nees (family: Acanthaceae) and considered the main contributors of its therapeutic properties, such as anti-tumor. In this study, inhibition of IgE-mediated anaphylactic reactions and anti-inflammatory potential of DA were investigated. The anti-anaphylactic activity of DA was investigated using skin swelling and extravasation assays *in vivo* and mast cell degranulation assay *in vitro*. The release of cytokines was measured using ELISA kits. Human Phospho-Kinase Array kit and western blotting were used to explore the related molecular signaling pathways. DA inhibited IgE-mediated mast cell activation, including degranulation and release of cytokines *in vitro*. Moreover, DA reduced the degree of swelling and Evans blue exudation of mice paw in a dose-dependent manner by inhibiting mast cell degranulation. DA obviously reduced the concentrations of histamine, TNF- α , MCP-1, IL-8, IL-13, and IL-4 in mice serum and inhibited IgE-mediated anaphylactic reactions as a potential P-PLC γ inhibitor. Our study reveals that DA can inhibit allergic responses *in vivo* and *in vitro*, and it may be regarded as a novel P-PLC γ inhibitor for preventing mast cell-immediate and delayed allergic diseases.

1. Introduction

Mast cells (MCs) which are filled with secretory granules containing a vast array of molecules endowed with multiple biological activities (Espinosa and Valitutti, 2018), are primary effectors in allergic reactions (Alvarez-Perea et al., 2017). Recent studies reveal that mast cells play an essential role in promoting IgE/Ag-mediated anaphylaxis. Mast cells (MCs) express high-affinity receptors (Fc ϵ RI) of immunoglobulin E (IgE) on the cell surface. MCs are activated by the crosslinking of antigen-specific IgE receptors (Fc ϵ RI), resulting in mast cell degranulation and cytokine release, classically to induce an anaphylactic reaction called "Type I hypersensitivity" (Wang et al., 2017). Type I allergic reaction is a severe, life-threatening adverse reaction, which affects both children and adults. This allergic reaction is generally visible within a few minutes after the exposure of an antigen and induces MCs to release histamine, leukotrienes, 5-hydroxytryptamine, prostaglandins, and many other mediators to cause microvascular dilatation of the mucosal airway, increased vascular permeability, tissue swelling,

and inflammation in the mucosal tissue (Bernstein, 2013; Gurish and Austen, 2012; Palm et al., 2012). A number of immune diseases, such as allergic asthma, atopic dermatitis, and allergic rhinitis are induced by type I hypersensitivity (Bossi et al., 2011; Chang, 2000; Umetsu and DeKruyff, 2004).

Currently, Histamine 1 receptor antagonist are considered as the first-line therapy for type I hypersensitivity and other IgE-related diseases (Kalinier, 2009). However, some research reported histamine 1 receptor antagonist were not very effective in > 50% patients, especially in the treatment of chronic allergic diseases, such as chronic urticarial (Confino-Cohen et al., 2012). Many kinds of nature products are used as anti-inflammatory and anti-allergy drugs in the world, but the mechanism of their effect are not clear. Nature products are a valuable resources and need further study.

Dehydroandrographolide (DA) is one of principal components of *Andrographis paniculata* (Burm.f.) Nees (family: Acanthaceae) and considered the main contributor of its therapeutic properties (Li et al., 2007a, b). Various pharmacological properties of DA are anti-cancer

* Correspondence to: T. Zhang, School of Pharmacy, Xi'an Jiaotong University, Yanta Westroad, Xi'an 710061, China.

** Correspondence to: Y. Wang, Department of Physical Examination, First Affiliated Hospital of Xi'an Jiao Tong University, Xi'an 710061, China
E-mail addresses: 13709199383@163.com (Y. Wang), taozhang@mail.xjtu.edu.cn (T. Zhang).

(Chassagne et al., 2018), anti-HIV, anti-influenza virus (Woo et al., 2008), and cardioprotection (Valdiani et al., 2013). Recently, anti-tumor properties of DA have been reported more thorough, but the mechanism is also unclear (Hsieh et al., 2017; Basak et al., 1999; Hsieh et al., 2015). However, the underlying effect of inhibition of IgE-dependent allergic reactions and the mechanism has few reports. The aim of the present study was to determine the *in vitro* and *in vivo* effects of DA on inhibition of IgE-dependent allergic reaction.

2. Materials and methods

2.1. Drugs and reagents

DA (purity $\geq 98\%$) (JOT-10143) was supplied by Chengdu Pufei De Biotech Co. Ltd. (Chengdu, Sichuan, China). Anti-DNP-specific IgE, DNP-BSA, albumin from chicken egg white and FITC-avidin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3, AM ester, and pluronic F-127 were procured from Biotium (California, USA). Tyrode's solution buffer was prepared fresh on the day of use (6.954 g/L NaCl, 0.353 g/L KCl, 0.282 g/L CaCl_2 , 0.143 g/L MgSO_4 , 0.162 g/L KH_2PO_4 , 2.383 g/L HEPES, 0.991 g/L glucose, and 1 g/L BSA, pH 7). The *p*-nitrophenyl *N*-acetyl- β -D-glucosamide and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared using ultrapure water produced by MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Animals

Adult male C57BL/6 mice (18–22 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Animals were housed in individual cages in a large colony room, with free access to water, and fed a standard dry chow twice a day. The breeding environment was 20–25 °C, with a relative humidity of 40% on a 12 h light/dark cycle. The mice were randomly divided into the following groups (6 mice per group): blank, albumin, and the experimental group. All experiments involving equal treatments of animals were conducted by experimenters blind to the experimental conditions.

2.3. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for using mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animals were operated under chloral hydrate anesthesia.

2.4. Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). Cells were maintained in StemPro™-34 medium supplemented with 1% StemPro™ nutrient supplement, 1:100 penicillin-streptomycin, 2 mM-glutamine, and 100 ng/mL human stem cell factor in 5% CO_2 at 37 °C. The culture medium was replaced per week and cells maintained at a density of 2×10^6 cells/mL.

2.5. Cytotoxicity assay

Cell viability was determined using Abbkine-Cell Counting Kit assays (California, USA). LAD2 cells were seeded into 96-well plates at a density of 5×10^3 cells per well, and pre-incubate the plate for 24 h in a humidified incubator at 37 °C, 5% CO_2 . Then the 96-well plate was centrifuged at $200 \times g$ for 5 min at 4 °C and medium was removed. Each well were treated with 100 μL of DA at different concentrations (0, 6.125, 12.5, 25, 50, 100, 200 μM) prepared by medium and incubated

for 24 h in a humidified incubator at 37 °C, 5% CO_2 . Next, 10 μL of Cell Counting Kit solution was added to each well followed by incubation for 2 h. Further, the relative cell viability was assessed by detection of absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA). Survival rate of LAD2 cells was calculated as:

$$[(\text{OD}_{\text{Treated}} - \text{OD}_{\text{Blank}})/(\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}})] \times 100\%.$$

2.6. The β -hexosaminidase release assay

LAD2 cells were seeded into 96-well plates and incubated with 2 $\mu\text{g}/\text{mL}$ of DNP-IgE for at 37 °C and 5% CO_2 for 24 h. The culture medium was then replaced with 100 μL per well of tyrode's solution containing different concentrations of DA (12.5, 25, 50 μM), and incubated for 20 min at 37 °C in 5% CO_2 . Blank and negative controls were treated with tyrode's solution alone under the same incubation conditions. The plates were centrifuged at $200 \times g$ for 5 min at 4 °C and buffer removed. 100 μL of tyrode's solution containing 4 $\mu\text{g}/\text{mL}$ DNP-BSA was added to the DA-treated cells and negative control, 100 μL of tyrode's solution was added to blank control. Cells were lysed with 0.1% Triton X-100 in tyrode's solution to analyze the total β -hexosaminidase content. The β -hexosaminidase released into the supernatants and in cell lysates was quantified by hydrolysis of *p*-nitrophenyl *N*-acetyl- β -D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was developed by adding stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11). The absorbance was measured at 405 nm using a microplate spectrophotometer and the percentage of β -hexosaminidase release was calculated as follows: absorbance of culture supernatant at 405 nm $\times$ 100/absorbance of total cell lysate supernatant at 405 nm.

IC_{50} value was calculated by the inhibition ratio of β -hexosaminidase release which caused by different concentration of DA.

2.7. Histamine release assay

1×10^6 LAD2 cells per well were incubated with 2 $\mu\text{g}/\text{mL}$ of DNP-IgE for at 37 °C and 5% CO_2 for 24 h in a 96-well plate. Following incubation, the culture medium was removed and 100 μL indicated concentrations (12.5, 25, 50 μM) DA prepared by tyrode's solution were added, only tyrode's solution were added in blank and negative control. Cells were incubated for 20 min at 37 °C with 5% CO_2 . The 96-well plate was centrifuged at $200 \times g$ for 5 min at 4 °C and the buffer was removed. 100 μL tyrode's solution containing 4 $\mu\text{g}/\text{mL}$ DNP-BSA was added in DA group and negative control and 100 μL tyrode's solution was added in the blank control and incubated for 40 min. Histamine release assay was carried out using LC-ESI-MS/MS method and Histamine (HA) and formic acid (mass spectrometry grade) were purchased from Sigma, Histamine $\cdot$ 2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA), HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Mass spectrometry grade formic acid was from Sigma. In the applied LC-ESI-MS/MS method, an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used. Histamine was evaluated on the system with an HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 μm , Agela Technologies, Tianjin, China), and an isocratic elution with acetonitrile–water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 mL/min.

2.8. Chemokine release assay

1×10^6 LAD2 cells per well were incubated with 2 $\mu\text{g}/\text{mL}$ of DNP-IgE for at 37 °C and 5% CO_2 for 24 h in a 96-well plate. After the culture medium was removed, 100 μL of different concentrations of DA (12.5, 25, 50 μM), prepared in tyrode's solution and containing 4 $\mu\text{g}/\text{mL}$ DNP-BSA, was added to each well and incubated for 6 h at 37 °C. Human

TNF- α , MCP-1, and IL-8 ELISA Kits were purchased from ExCell Bio CO., LTD. (Beijing, China) and used to measure the levels of TNF- α , MCP-1 and IL-8 levels in supernatants according to provided instructions.

2.9. Hind paw swelling and extravasation in IgE-induced mice

Adult male mice (8–10 weeks) were injected intravenously with OVA (50 μ g/mL). After one week, the mice were treated with different doses of DA (0.25, 0.5, 1 mg/kg) by intragastric administration. 1 h later, the mice were anaesthetized with an intraperitoneal injection of 80 mg/kg 1% pelltobarbitalum natricum. Five minutes after the induction of anesthesia, each mouse was injected intravenously (i.v.) with 0.2 ml of 0.4% Evans blue in saline. A vernier caliper was used to measure the thickness of the paw before any injection and as needed during the experiments. 20 μ g/mL OVA in saline was injected into the left paw and saline only into the right paw as a blank control. After 15 min, paw thickness was measured again and recorded. Mice were then killed by CO₂, and a photo of each paw was taken. Paw tissues were collected, dried for 24 h at 50 °C, and weighed. Evans blue was extracted by a 12 h incubation in 500 μ L acetone-saline (7:3) at 37 °C. Tissues were cut into pieces, placed for 10 min in an ultrasonic machine, centrifuged for 20 min at 3000 rpm. The supernatant was equally distributed in 200 μ L aliquots into 96 well cell culture plates, and the OD was read at 620 nm using a spectrophotometer. For studies using drug substances, mice were gavaged with saline as a blank control.

2.10. Skin avidin stain assay

Adult male mice (8–10 weeks) were injected intravenously with OVA (50 μ g/mL). After one week, the mice were deal with different doses of DA (0, 0.25, 0.5, 1 mg/kg) by intragastric administration. 1 h later, the mice were anaesthetized with an intraperitoneal injection of 80 mg/kg 1% pelltobarbitalum natricum. Anaesthetized mice were injected with 5 μ L 20 μ g/mL of OVA in the left paw; saline was used as negative control and. Fifteen minutes later, the injection site of the skin in paw was excised, washed with PBS and fixed with 4% formaldehyde for 48 h and subjected to stain. After dried at 37 °C for 30 min and pre-incubated in blocking solution (10% normal goat serum (v/v), 0.2% Triton X-100 (v/v) in PBS, pH 7.4) for 2 h at 25 °C, followed by incubation with 1/500 FITC-avidin for 45 min. Sections were washed 3 times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL, U.S.A) was added. Images were taken immediately using a confocal scanning laser microscope (Nikon, Tokyo, Japan).

2.11. Analysis of mouse serum

Adult male mice (8–10 weeks) were injected intravenously with OVA (50 μ g/mL). After one week, different doses of DA (0.25, 0.5, 1 mg/kg) were dosed by intragastric administration and the same volume of saline was gavage for both negative control and blank group. 1 h later, mice were challenged by tail vein injection with 50 μ g/mL OVA in saline through the tail vein in treated groups and control group and only saline was injected in the blank group. After 8 h, blood was collected from retroorbital plexus. The blood was collect and centrifuged to get serum. Histamine release was analyzed by LC-ESI-MS/MS method. Mouse TNF- α , MCP-1, IL-4 and IL-13 ELISA Kits were purchased from BIOTECH CO., LTD. (Beijing, China) and used to measure the levels of TNF- α , MCP-1, IL-4 and IL-13 levels in supernatants according to provided instructions.

2.12. Phosphorylated kinase analysis

1×10^7 LAD2 cells were incubated with 2 μ g/mL of DNP-IgE for at 37 °C and 5% CO₂ for 12 h in culture dishes. Total proteins from the untreated and DA-treated LAD2 cells with 4 μ g/mL DNP-BSA (6 h) were

analyzed by Human Phospho-Kinase Array kit (R&D Systems China Co., Ltd. Shanghai, China), and all the steps were finished according to provided instructions.

2.13. Western blot analysis

1×10^7 LAD2 cells were incubated with 2 μ g/mL of DNP-IgE for at 37 °C and 5% CO₂ for 12 h in culture dishes. Total proteins from the untreated and DA-treated LAD2 cells with 4 μ g/mL DNP-BSA (6 h) were extracted in ice-cold condition using RIPA lysis buffer containing 10% protease inhibitor and a phosphatase inhibitor cocktail (Roche Diagnostics). After 30 min, insoluble protein lysate was removed by centrifuging the samples at 13,500 g for 10 min at 4 °C. The protein concentration was determined using a BCA Protein Quantification kit according to manufacturer's instructions. The protein in the cell lysates was denatured by boiling the samples for 5 min with a 5 $\times$ loading sample buffer from Thermo Fisher Scientific, Inc. (MA, USA), and equal amounts of protein were separated on a 10% gel using SDS-PAGE (Shaanxi Pioneer Biotech Co., Ltd.). The separated proteins were transferred onto polyvinylidene fluoride membranes from Hangzhou Microna Membrane Technology Co., Ltd. (Hangzhou, China), and blocked by constant stirring with 5% non-fat milk in Tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd.) at room temperature for 2 h. The membranes were then incubated overnight at 4 °C with the following primary antibodies: anti-Lyn (1:1000, #2796, Cell Signaling Technology [CST]), anti-phosphorylated-Lyn (1:1000, #2731, CST), anti-Fyn (1:1000, #4023, CST), anti-phosphorylated-Fyn (1:1000, Phospho-Src Family (Tyr 461) #6943, CST), anti-Btk (1:1000, #8547, CST), anti-phosphorylated-Btk (P-Btk, Tyr223) (1:1000, #87141, CST), anti-PLC γ 1 (1:1000, #5690, CST), anti-phosphorylated-PLC γ 1 (P-PLC γ 1, Ser1248) (1:1000, #8713, CST), or anti-GAPDH (1:2000, #2118, CST). The membranes were then washed thrice with TBST at every 10 min interval and then incubated with secondary antibodies (at a dilution of 1:20,000 in TBST) for 1 h at 37 °C. The membranes were again washed three times with TBST for 10 min and developed using an enhanced chemiluminescence (ECL) kit. A Lane 1DTM transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to capture the images of the developed blots, and Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

2.14. Statistical analysis

The group data are expressed as the mean $\pm$ S.E.M. Independent sample variance analysis was used to determine the significance of statistical comparisons using SPSS software. Differences were considered significant at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

3. Results

3.1. DA inhibited IgE-mediated degranulation in LAD2 cells

It is important to evaluate the cytotoxic effect of DA on LAD2 cells, since the inhibitory effect of DA did not work as affecting mast cell proliferation. The effect of DA on mast cell viability was examined by a Cell Counting kit assay. DA showed low cytotoxicity on LAD2 cells at a concentration of 100 μ M with a cell viability rate higher than 90%, but showed higher cytotoxicity at 200 μ M (Fig. 1A). So the work concentration was set as 12.5, 25, 50 μ M. However, it was unexpected that DA did not induce β -hexosaminidase and histamine release in these cells compared with blank group ($p > .05$) (Fig. 1B and C).

Therefore, we measured the release of β -hexosaminidase and histamine, the pre-stored mediators, to determine whether DA treatment could inhibit IgE-mediated degranulation of MCs. Further research confirmed that after incubation of DNP-IgE for 12 h, DNP-BSA induced degranulation of LAD2 cells, the release rate of β -hexosaminidase was

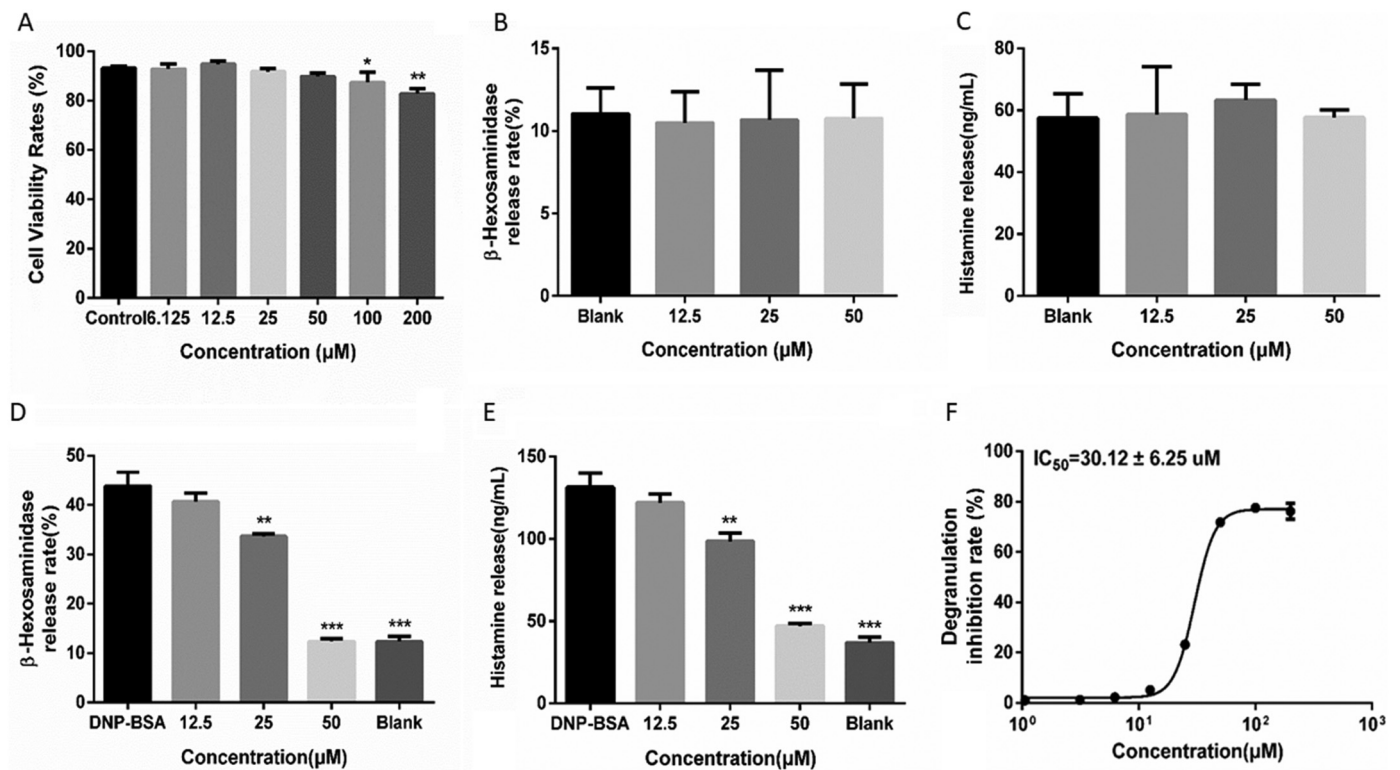


Fig. 1. DA inhibited IgE-mediated degranulation in LAD2 cells.

(A) DA showed no cytotoxic effect on LAD2 cells. (B–C) DA failed to induce LAD2 cell degranulation and release of β-hexosaminidase and histamine. Tyrode's solution was used as blank control. (D–E) DA inhibited DNP-BSA-induced degranulation in DNP-IgE-treated LAD2 cells, reducing the release of β-hexosaminidase and histamine (2 μg/mL DNP-BSA was used as an agonist and introduced in DNP-IgE-treated LAD2 cells, and was also used as a negative control). Tyrode's solution was used as blank control. Different concentrations of DA (12.5, 25, and 50 μM) were used to inhibit the degranulation caused by DNP-BSA. (F) The IC₅₀ value of DA was calculated by inhibition of β-hexosaminidase release in DA-treated LAD2 cells (experiments were repeated thrice, and data are represented as mean ± S.E.M. and analyzed using two-tailed unpaired Student's *t*-test. Differences were considered significant at * *p* < .05, ** *p* < .01, and *** *p* < .005).

43.68 ± 3.72% and the histamine release was 132.87 ± 10.21 ng/mL. Whereas treatment with DA dose-dependently inhibited the release of β-hexosaminidase and histamine in these cells, at 50 μM, DA showed a highest inhibition effect, the degree of degranulation was about for one third compared with the control group (Fig. 1D and E). The IC₅₀ value of DA, calculated by inhibition of β-hexosaminidase release in DA-treated LAD2 cells, was 30.12 ± 6.25 μM (Fig. 1F). These results are consistent with the observations that DA treatment inhibited IgE-mediated degranulation of MCs.

3.2. DA reduced IgE-mediated cytokine release in LAD2 cells

A number of chemokines are released in IgE-mediated anaphylactic reaction, which can be harmful to patients and may induce delayed allergic reactions. We analyzed chemokine release via ELISA analysis, and found that DA reduced IgE-mediated cytokine release *in vitro*. DA had the obvious effect in reduction of MCP-1, IL-8, and IL-13 levels (Fig. 2B–D) with a dose-dependent manner. It also reduced the release of TNF-α and IL-4, but the effect was not significant (*p* < .05) (Fig. 2A and E). DA showed a significant inhibition effect on MCP-1, IL-8 and IL-13 released by LAD2 cells. MCP-1, IL-8 and IL-13 release in control group were 71.14 ± 3.28, 67.48 ± 5.16 and 39.86 ± 1.53 pg/mL, respectively. However, at a dose of 50 μM DA, the release of MCP-1, IL-8 and IL-13 were 38.73 ± 2.54 (*p* < .01), 23.41 ± 0.86 (*p* < .001) and 17.22 ± 1.04 (*p* < .001) pg/mL, respectively (Fig. 2B–D).

3.3. DA inhibited IgE-mediated anaphylaxis by inhibiting mast cell degranulation

In order to investigate the anti-anaphylactic effect of DA, a mouse

model of ovalbumin (OVA)-induced skin inflammation, which was previously shown to be IgE-dependent, was used. The mice were injected intravenously with OVA. A week later, the mice were dosed with DA (0.25, 0.5, and 1 mg/kg) by intragastric administration. After OVA was injected into the left paw, we analyzed the degree of swelling and Evans blue exudation of mice paw, and saline was injected into the right paw as control. The results showed that DA reduced the degree of swelling and Evans blue exudation of mice paw with a dose-dependent manner. 1 mg/kg DA showed a highest inhibition effect in this study, the degree of swelling and Evans blue exudation were about for one fourth compared with the control group. As the dose of DA increased, the otherness of treatment-paw and saline-paw was reduced. (Fig. 3A). Subsequently, we analyzed the related paw skin sections for degranulated MCs by avidin staining and found that DA antagonized MCs *in vivo* to cause their degranulation (Fig. 3B). The inhibiting effect was seen as a decrease in hemangiectasis caused by reducing histamine release.

In addition, the effects of DA on IgE-MC-dependent reactions were evaluated by intravenously administering OVA to C57BL/6 mice. DA obviously reduced the concentration of histamine, and cytokine, such as TNF-α, MCP-1, IL-13, and IL-4 in mice serum (Fig. 4). The results were quite the same with the data *in vitro* (Fig. 3). Both the results confirmed that DA suppressed IgE-mediated systemic anaphylaxis reactions *in vivo*.

3.4. DA inhibited IgE-mediated anaphylaxis by downregulating phosphorylation level of PLC-γ1 in the calcium signaling pathway

Upregulation phosphorylation level of many key protein in FcεRI receptor downstream signaling pathways are relative to mast cell

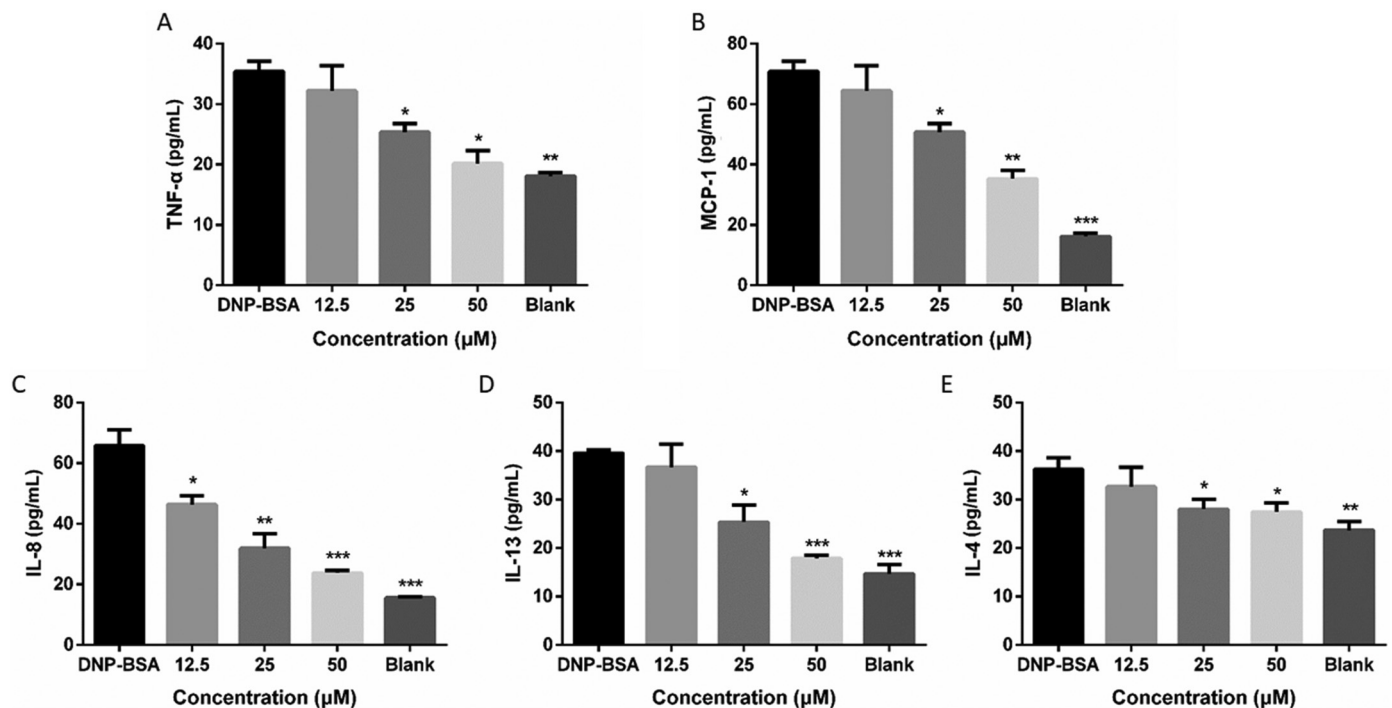


Fig. 2. DA reduced IgE-mediated cytokine release in LAD2 cells.

DNP-BSA (2 μg/mL) was used as an agonist and introduced in DNP-IgE-treated LAD2 cells and was also used as a negative control. Tyrode's solution was used as control. Different concentrations of DA (12.5, 25, and 50 μM) were used to inhibit cytokine release caused by DNP-BSA. Experiments were repeated thrice, and data are represented as mean $\pm$ S.E.M. and analyzed using two-tailed unpaired Student's t-test. Differences were considered significant at * $p < .05$, ** $p < .01$, and *** $p < .005$. (A-E) DA reduced TNF- α , MCP-1, IL-8, IL-13, and IL-4 release in DNP-IgE-treated LAD2 cells acted upon by DNP-BSA.

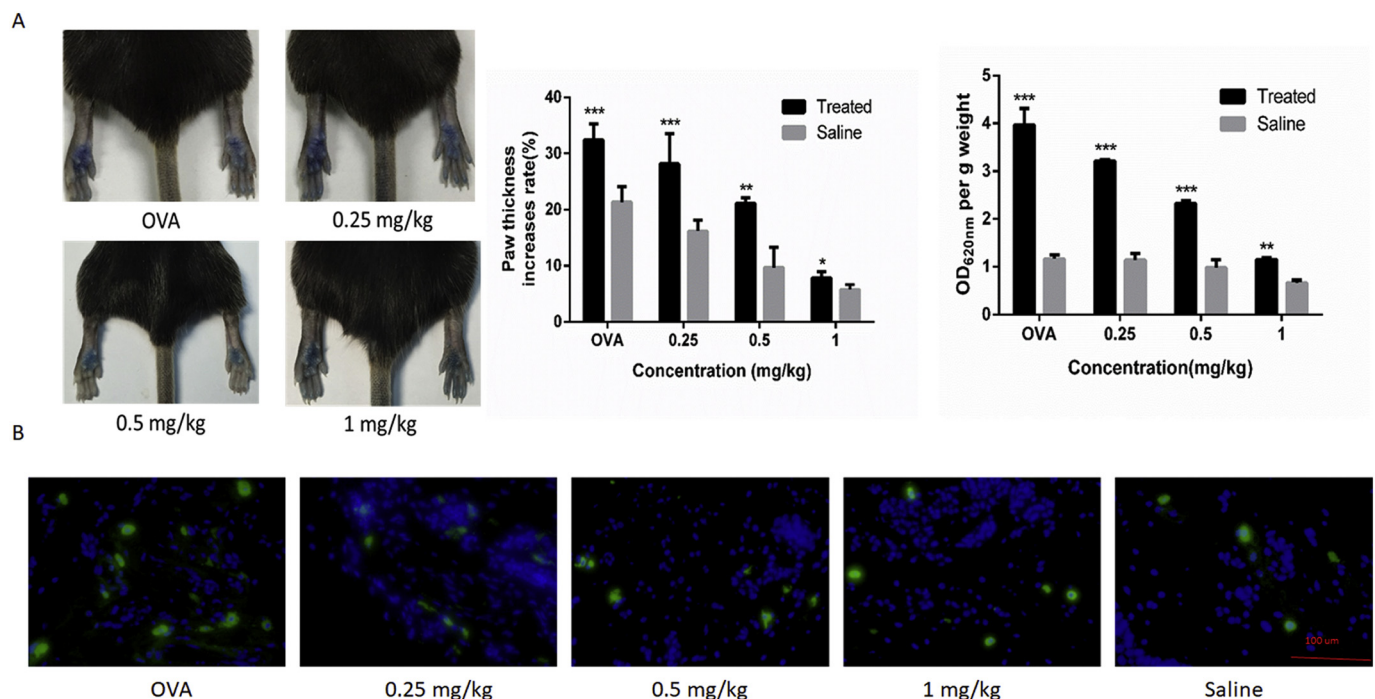


Fig. 3. DA inhibited IgE-mediated local anaphylaxis by inhibiting MC degranulation.

OVA (50 μg/mL) was used to treat C57BL/6 mice ($n = 6$ per group; experiments were repeated 3 times. The data are presented as the mean $\pm$ S.E.M. Two-tailed unpaired Student's t-test was used for analysis). Treatment group was intragastrically administered with different concentrations of DA (0, 0.25, 0.5, and 1 mg/kg). OVA (10 μg/mL) was injected into the left paw, while saline was injected into the right one as control. (A) DA reduced the degree of swelling and Evans blue exudation induced by OVA from hind paws of the mice. (B) DA inhibited skin mast cell degranulation analyzed by avidin staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

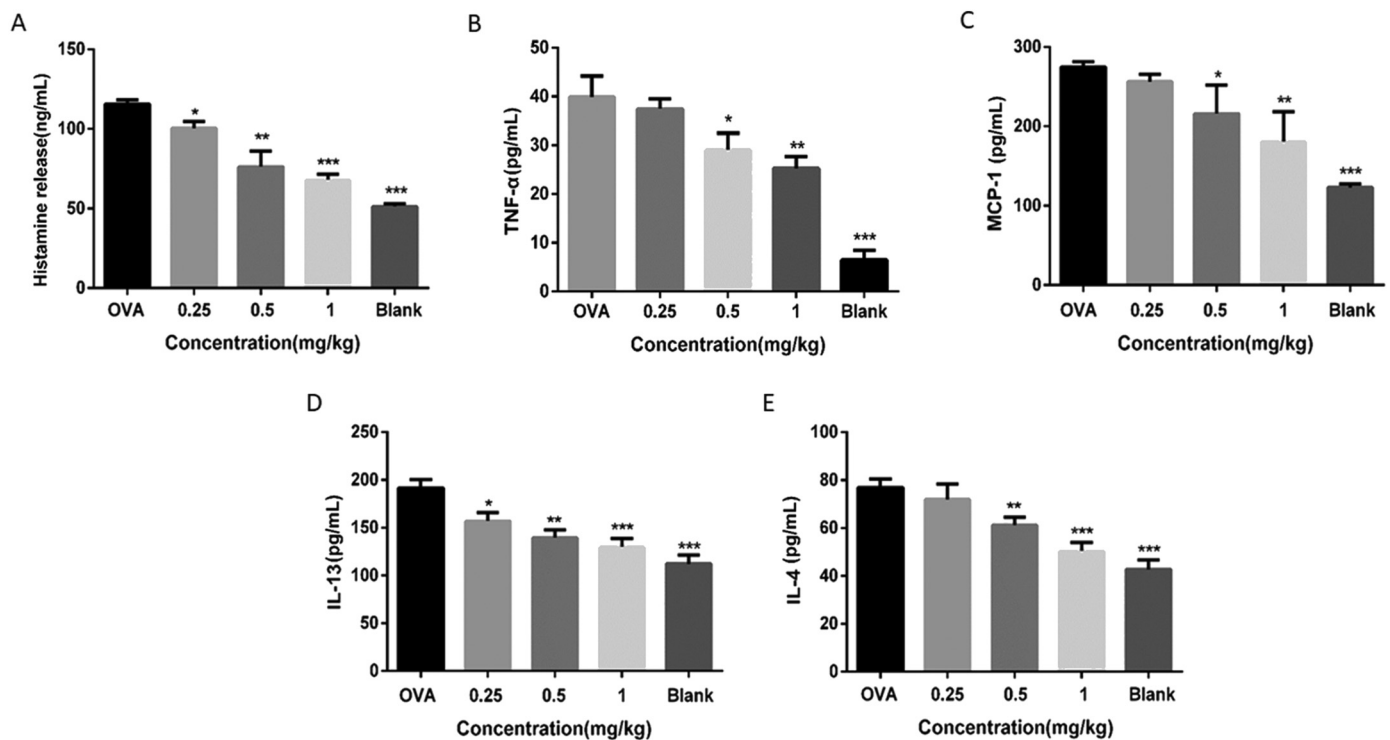


Fig. 4. DA reduced IgE-mediated histamine and cytokine release in mice serum. OVA (50 µg/mL) was used to treat C57BL/6 mice ($n = 6$ per group; experiments were repeated > 3 times. The data are presented as the mean $\pm$ S.E.M. Two-tailed unpaired Student's t-test was used for analysis). Treatment group was intragastrically administered with different concentrations of DA (0, 0.25, 0.5, and 1 mg/kg). OVA (50 µg/mL) injected into the caudal vein, while saline was used as blank control. (A-E) DA obviously reduced the concentration of histamine, TNF- α , MCP-1, IL-13, and IL-4 in mice serum.

activation and lead to type I hypersensitivity. The results of phosphorylated kinase analysis showed that DA downregulated phosphorylation level of p38 α , AKT1/2/3, and PLC- γ 1 ($p < .05$) which are important proteins in mast cell degranulation reaction and cytokine release (Fig. 5A).

The calcium signaling pathway is important for mast cell degranulation and intracellular Ca^{2+} increases is necessary to both degranulation and cytokine release. DNP-BSA increased Ca^{2+} flux and resulted in MC activation mediated by DNP-IgE. So the phosphorylation level of the key proteins in calcium signaling pathway must be up regulation. However, PLC- γ 1 is a key protein in both calcium signaling pathway and Fc ϵ RI pathway. The phosphorylation levels of PLC- γ 1 in LAD2 cells after action by DNP-BSA were decreased by DA. In addition, PLC- γ 1 is a key protein in the calcium signaling and Fc ϵ RI signaling pathways. The results of western blot showed that after treatment of LAD2 mast cells with 12.5, 25, and 50 µg/mL of DA for 1 h decreased the level of phosphorylated PLC- γ 1 mediated by IgE in a dose-dependent manner (Fig. 5E). However, The inhibition effect of DA on some of the signaling molecules located upstream of PLC- γ 1 could inhibit anaphylactic reaction in fact. So we analyzed other key protein in the calcium signaling pathways. The results of western blot showed that DA had little effect on the phosphorylation levels of Fyn, Lyn or Btk (Fig. 5B–D). This suggested that DA might inhibit MC degranulation and cytokine release via the calcium signaling pathways, acting as a P-PLC- γ 1 inhibitor to downregulate phosphorylation level of PLC- γ 1.

4. Discussion

Type I hypersensitivity is a highly efficient protective inflammatory response mediated by IgE and is induced by many kinds of diseases. Mast cell is the key effector related to inflammatory response (Hoda and Cheng, 2017). Inhibition of mast cell degranulation reaction and release cytokine is significant meaningful to cure some anaphylactic diseases, such as urticarial, atopic dermatitis and allergic asthma.

Natural products are a huge resource to find potential drugs. DA is a bicyclic diterpenoid lactone, which is extensively used in traditional Chinese medicine for a long time, and the anti-inflammatory effects of which, have a research value (Li et al., 2007a, b). It has been reported that DA might affect gene expression to reduce cytokine release and inhibit inflammation (Parichatikanon et al., 2010). Furthermore, DA enhanced innate immunity of the intestinal tract through up-regulation the expression of hBD-2 (Xiong et al., 2015). In the present study, the results suggest that DA inhibited IgE-mediated allergic reaction by decreasing MC degranulation and cytokine release (Figs. 1 and 2). In addition, the degree of swelling and Evans blue exudation from mice paw were reduced by DA. It reduced hemangiectasis by inhibiting the degranulation of skin mast cells (Fig. 3). DA inhibited the increase in Ca^{2+} concentration induced by DNP-BSA, which was mediated by PLC- γ 1 phosphorylation in MCs (Fig. 5D). Our study suggested that DA was a potential anti-inflammatory and anti-allergic compound.

Study confirmed that the incidence of allergic diseases had increased significantly and about 20% population suffered from IgE-mediated diseases. Allergic rhinitis, asthma, conjunctivitis, eczema, food allergies and drug allergies have seriously affected people's lives (Wamer et al., 2006). Mast cells are the most important initiating cells in IgE mediated tachycardia allergic inflammation in the early stages of allergic reactions. Mast cells release mediators that can cause major pathological changes in acute allergic reactions, such as increased vascular permeability, smooth muscle contraction, and excessive mucus secretion (Norman et al., 2008). Inhibiting degranulation of mast cells is an important way to cure allergic and inflammatory diseases. Nevertheless, histamine receptors antagonist are widely used in clinical to alleviate the symptom of allergic and inflammatory diseases which cannot cure the diseases. PLC- γ 1 might be a new target molecule for the treatment of allergic and inflammatory diseases.

Moreover, DA also downregulating phosphorylation level of p53, MSK1/2, p70s6 kinase, RSK1/2/3, eNOS, STAT3, p27, WNK1 and PYK2 (Fig. 5A–C). These reminded that DA might have more physiological

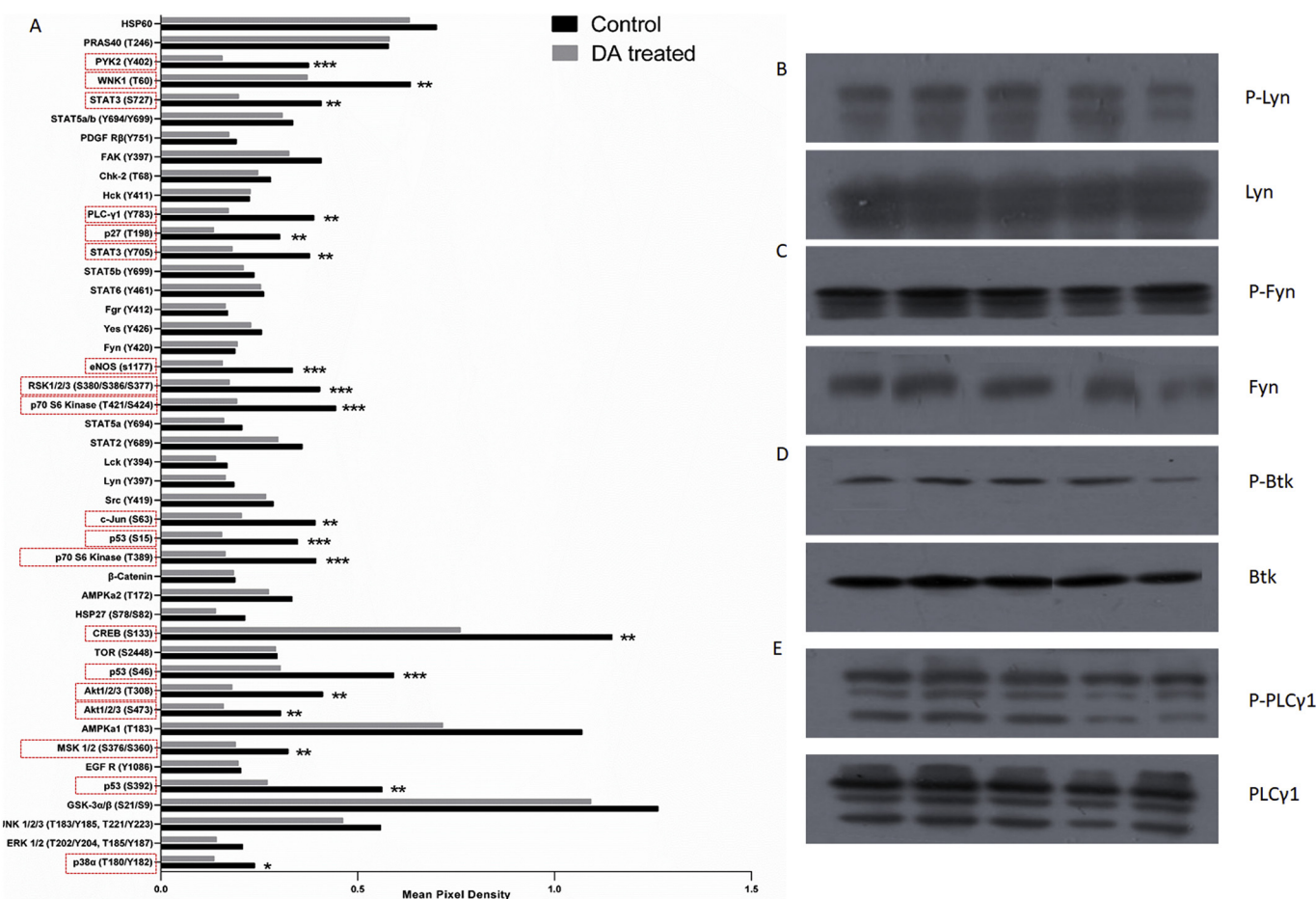


Fig. 5. DA inhibited PLCγ phosphorylation in LAD2 cells.

Experiments were repeated thrice. The data are presented as the mean $\pm$ S.E.M. Two-tailed unpaired Student's t-test was used for analysis. Treatment group was administered with 50 μ M of DA and 4 μ g DNP-BSA, and 4 μ g/mL DNP-BSA was administered in the control group. (A) Kinase phosphorylation analysis in LAD2 cells. (B-D) DA had little on the phosphorylation level of Lyn, Fyn and Btk. (E) DA decreased the level of phosphorylated PLCγ mediated by IgE in a dose-dependent manner.

activity need further study. IL-33 upregulates phosphorylation level of MSK1/2 and aggravated bronchial asthma, inhibiting MSK1/2 phosphorylation may be a new target for cure bronchial asthma (Saglan et al., 2013).

PLC-γ1 is expressed widely in a number of somatic cells (Liao et al., 2002) and is a key protein in the calcium signaling pathway and has many other physiological effects (Yu et al., 2017; Yamada et al., 2016). In type I hypersensitivity and pseudo-allergic reactions, phosphorylation of PLC-γ1 increases intracellular Ca^{2+} concentration (Liu et al., 2017; Che et al., 2018), induces mast cell degranulation, and release of cytokines. Hence, our results suggest that DA could inhibit both type I hypersensitivity and pseudo-allergic reactions. In addition, PLC-γ1 has an important effect on the development, spread, and transfer of tumors (Kochhar and Iyer, 1996; Xie et al., 2010). PLC-γ1 inhibitors can promote apoptosis of tumor cells (Wang et al., 2001), spread, and transfer of tumors (Weiger et al., 2002).

What is more, upregulation phosphorylation level of many kinase can promotes the proliferation of tumor cells, such as upregulation WNK1 could promote the proliferation of CCA cells (Wang et al., 2007). However, upregulation mTOR and p70s6 kinase could increase fibroblast proliferation and induce fibrotic diseases (Fingar and Blenis, 2004). RSK1/2/3 and STAT3 are closely related to the proliferation of tumor cells. DA has significant inhibition to phosphorylation level of these protein, it is suggest that DA is a nature product of great development value using in treatment of both cancer and immune diseases.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Number: 81872837, 81230079 and 81872837).

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