

Effect of 6'-acetylpaeoniflorin on dinitrochlorobenzene-induced allergic contact dermatitis in BALB/c mice

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Abstract Allergic contact dermatitis (ACD) is a classical experimental model of allergic inflammatory skin disease, which is a delayed-type hypersensitivity reaction that is mediated by hapten-specific T lymphocytes. The goal of this study was to investigate the pharmacokinetic profiles of 6'-acetylpaeoniflorin (6-AP) and the effect of 6-AP on the ACD model. 6-AP was synthesized from paeoniflorin (Pae) via acetylation, and the structure was confirmed. There were statistically significant differences in the pharmacokinetic parameters including $t_{1/2\alpha}$, $t_{1/2\beta}$, AUC, MRT and $C_{\max}$ among the animals that were orally administered Pae and 6-AP. An ACD model was induced using immunization with dinitrochlorobenzene (DNCB) in BALB/c mice. The mice were orally administered 6-AP (35, 70 and 140 mg/kg/d), Pae (70 mg/kg/d) and prednisone (Pre, 5 mg/kg/d) from day 1 to day 7 after immunization. The results indicated that the topical application of DNCB to the skin provoked obvious inflammatory responses. 6-AP significantly inhibited ear swelling and decreased inflammatory cell infiltration and epidermal

keratinization. Additionally, 6-AP observably alleviated the hyperplasia of red pulp and germinal center appearance, decreased the spleen index and inhibited splenocyte proliferation in the ACD model compared to that of Pae. Furthermore, the study indicated that 6-AP could increase the IL-10 level, while simultaneously reducing the IL-17 level in splenocytes. In summary, these results suggest that 6-AP has a significantly higher anti-inflammatory effect than Pae and that 6-AP might be a candidate for the treatment of allergic skin diseases.

Keywords 6'-Acetylpaeoniflorin (6-AP) · Paeoniflorin (Pae) · Allergic contact dermatitis (ACD) · Anti-inflammatory activity · Pharmacokinetics

Introduction

Allergic contact dermatitis (ACD) is a highly prevalent and potentially chronic disease in industrialized countries. Knowledge of the pathophysiology of human ACD has been obtained mainly from studies using murine models of contact hypersensitivity (CHS) to a hapten (i.e., an allergen, drug or metal) [1, 2]. ACD is mediated by hapten-specific T lymphocytes, which are activated in an immune organ during the sensitization process [3]. A previous study found that excessive levels of IL-17 and insufficient levels of IL-10 were observed in the serum of ACD patients [4]. IL-17 facilitates the development of delayed-type hypersensitivity and antibody responses, and increases inflammatory cytokine production in immune disease [5]. By contrast, IL-10 alleviates inflammatory reactions by inhibiting the function of APCs and thus may promote immune tolerance and reduce IL-17 levels [6]. In this context, it is widely recognized that imbalances in the

Electronic supplementary material The online version of this article (doi:10.1007/s12026-016-8788-8) contains supplementary material, which is available to authorized users.

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levels of inflammatory/anti-inflammatory cytokines are important in driving the pathological development of ACD.

Compared with current therapies for ACD, glucocorticosteroids, non-steroid anti-inflammatory drugs and immunomodulators are often associated with severe adverse effects; thus, better treatment with respect to efficacy and side effects are needed. Treatments using natural compounds are expected to prevent the onset of inflammatory diseases and ameliorate allergic symptoms [7].

Total glucosides of paeony (TGP) is the active compound extracted from the dried roots of *Paeonia lactiflora* Pall, and in 1998, TGP was approved by the China Food and Drug Administration (CFDA) as a novel treatment for rheumatoid arthritis (RA) [8]. TGP and TGP capsules, which were developed by the Institute of Clinical Pharmacology of Anhui Medical University, have been used to inhibit inflammation and regulate the immune response of clinical arthritis patients for approximately 20 years [9, 10]. The effect of TGP for the treatment of inflammatory disease without evident toxicity or side effects has been demonstrated in a multicenter clinical trial [11, 12]. However, the slow effects of TCP seriously limit its clinical application [13].

Paeoniflorin (Pae; Fig. 1a) is one of the principal bioactive components of TGP and is a water-soluble monoterpene glucoside [14]. As an immune modulator and an anti-inflammation constituent, Pae has been demonstrated to be effective in the treatment of inflammatory and autoimmune animal models with RA, hepatic fibrosis, psoriasis, Sjögren's syndrome and systemic lupus erythematosus [15, 16]. Pae decreased thymocyte proliferation and regulated the balanced secretion of pro-/anti-inflammatory cytokines in the serum, thymocyte and splenocyte culture supernatants in the ACD model [17]. However, previous studies found that Pae was absorbed poorly in the gastrointestinal tract by oral administration, leading to a very poor bioavailability (3–4 %), which may be the main reason for the slow onset of action of TGP [18, 19]. The

hydrophilic structure, efflux function of P-glycoprotein (P-gp) and enzymolysis may account for the low bioavailability of Pae [20]. Therefore, enhancing the lipophilicity may be an effective way to improve the oral bioavailability of Pae [21]. A recent study has revealed that compared to Pae, Caco-2 cells can easily absorb benzoylpaeoniflorin, which is an esterified derivative of Pae [22]. Additionally, acetylation enhances lipophilicity and improves absorption and cell permeability. Moreover, acylated derivatives have more beneficial effects than the parent compounds [23, 24]. Therefore, these results strongly suggest that the lipophilicity of Pae may be improved by acetylation.

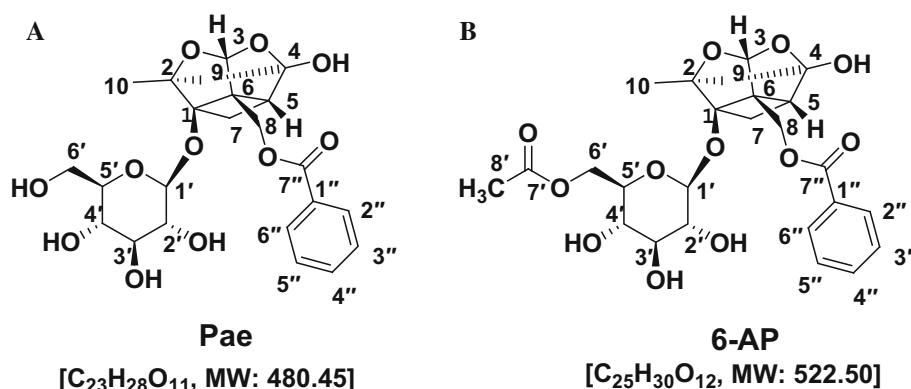
In the present study, a novel chemical structure 6'-acetylpaeoniflorin (6-AP) was synthesized via acetylation, which may show enhanced lipophilicity. Thus, the aim of this study was to compare the differences in the pharmacokinetics process of 6-AP and Pae in rats after oral administration and subsequently to compare the anti-inflammatory and immunomodulatory activities of 6-AP and Pae in BALB/c mice with ACD.

Materials and methods

Animals

Five-week-old male BALB/c mice (18–20 g) and Sprague–Dawley (SD) rats (male, 180–220 g) were purchased from the Animal Department of Anhui Medical University (Hefei, Anhui Province, China) and were quarantined for 1 week. Animals were healthy and housed under standard laboratory conditions at a constant temperature of $24 \pm 1^\circ\text{C}$. Water and a standard diet were available ad libitum. The lighting duration in the breeding room was 12 h (7:00 a.m.–7:00 p.m.). The Ethical Committee on Animal Research at the Institute of Clinical Pharmacology of Anhui Medical University approved the experiments in this study.

Fig. 1 The chemical structure of Pae (a) and 6-AP (b)



Drugs and reagents

Pae (white powder, purity > 95.0 %) was provided by the Institute of Clinical Pharmacology, Anhui Medical University (Fig. 1a). 6-AP (white powder, purity > 99.0 %) was synthesized from Pae via acetylation, and the target compound was confirmed by $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and mass spectrometry (Fig. 1b). Pre was purchased from Xian Ju Pharmaceutical Co. Ltd. (Hangzhou, China). Before administration, 6-AP, Pae and Pre were suspended in physiological saline separately. Methanol was of chromatography grade (Merck & Co., Darmstadt, Germany). Dinitrochlorobenzene (DNCB) was purchased from the Shanghai Chemical Agent Co. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-10 and IL-17 were products of the Shanghai Huiyin Biological Technology Co. Ltd. (Shanghai, China). Dulbecco's minimum essential medium (DMEM) was purchased from Gibco Co. (CA, USA). Concanavalin A (ConA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All of the other chemicals used were of reagent grade or better.

Synthesis of 6-AP

A mixture of Pae (3.0002 g, 6.25 mmol), 4-dimethylaminopyridine (DMAP, 25 mg), pyridine (15 mL) and CHCl_3 (20 mL) was stirred at room temperature for 0.5 h. Then, acetic anhydride (0.6375 g, 6.25 mmol) was added dropwise to a stirred solution and stirred for 6 h. The progress of the reaction was monitored using thin-layer chromatography (TLC; dichloromethane: methanol = 8:1, V/V). After solvent recovery, chromatography on silica gel (dichloromethane: methanol = 30:1, V/V) produced a white solid of 1.3627 g, yield of 41.77 %.

Pharmacokinetics of 6-AP and Pae

Drug administration

Rats were randomly divided into six groups for the oral administration of 6-AP (25, 50 and 100 mg/kg) and Pae (25, 50 and 100 mg/kg). Rats were fasted for 12 h with free access to water prior to the experiment.

Plasma sample preparation

Before and at different time points after oral administration (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0 and 12 h), approximately 0.2 mL of blood samples was collected in heparinized tubes from anesthetized rats by diethyl ether. Blood samples were centrifuged (4000 rpm, 15 min and

4 °C), and plasma was separated and kept frozen at −20 °C. Plasma (0.1 mL) was added to 0.9 mL of methanol, vortex-mixed for 2 min and centrifuged at 15,000 rpm for 15 min at 4 °C. Finally, the supernatant was transferred to a clean test tube and evaporated to dryness under a flow of nitrogen gas. The residue was reconstituted in 50 μL of methanol, vortex-mixed for 1 min and centrifuged for 15 min (15,000 rpm, 4 °C), and 30 μL of solution was injected for HPLC measurement. In addition, these procedures were also applied for the determination of the calibration curve, recovery and precision test.

HPLC analysis

An Agilent 1200 series HPLC system (Agilent Technologies, CA, USA) was used in the analysis. An Agilent HC-C18 column (4.6 mm $\times$ 250 mm, 5 μm) was also used. Detection was performed at a wavelength of 230 nm under a constant temperature of 30 °C. The mobile phase for the chromatographic separation consisted of methanol and distilled water (48:52, v/v). The flow rate was set at 0.8 mL/min.

Calibration curve, recovery, precision and stability tests

A calibration curve was constructed based on the analysis of blank plasma spiked with various levels of Pae (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 $\mu\text{g/mL}$) and 6-AP (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 20 $\mu\text{g/mL}$). The concentrations of Pae and 6-AP in samples were calculated from the peak area using a linear regression equation obtained from the calibration curve.

Three concentrations of Pae in blank plasma were prepared. For the recovery tests, the peak area of Pae in blank plasma was recorded and compared with that of the standard in the mobile phase to provide recovery values. For the precision tests, intraday variance was determined by assaying each sample for quartic on the same day, and inter-day variance was also performed over four consecutive days. For stability test, the plasma samples of Pae were stored at −20 °C and then assayed at day 0, day 7 and day 14. In addition, these processes were also applied to 6-AP.

Effect of 6-AP on allergic contact dermatitis

Induction and evaluation of allergic contact dermatitis in BALB/c mice

ACD was induced in BALB/c mice according to a published method [25]. DNCB was dissolved in acetone/olive oil (AOO ratio of 4:1). To induce sensitization, mice were sensitized with 100 μL of a 0.5 % DNCB solution applied

to the shaved abdomen (2 cm × 2 cm). Five days later, mice were challenged by painting both surfaces of the right ear with 20 µL of 0.5 % DNCB and rechallenged after 1 day, which resulted in an extensive disease presentation. The left ear of mice was painted with an identical volume of AOO as the control. Then, 24 h after the second challenge, small round pieces from the right and left ears were cut using a sharp clamp (diameter 0.8 cm), and the weight of the pieces was determined using an electronic balance. Ear swelling was evaluated by measuring the difference in the weight between the right and left ears.

Treatment

The mice were randomly divided into seven groups ($n = 8$). Five groups of mice were treated with DNCB and orally administered 6-AP (35, 70 and 140 mg/kg/d), Pae (70 mg/kg/d) and Pre (5 mg/kg/d) from day 1 to day 7 after immunization. Normal group and ACD model group were given an identical volume of physiological saline.

Histopathology

The histopathological examination was carried out according to a published method [26]. Briefly, the punched piece of the right ear and spleen tissue were fixed with 10 % formaldehyde and embedded in paraffin, and then, thin sections were prepared. After the skin and spleen section were stained with hematoxylin and eosin, board-certified pathologist that was unaware of the treatment of the samples conducted a microscopic examination. The pathological scores for the ear samples were assessed using a previously published method [25].

Assay of spleen indices

We used the method previously reported by Zhang et al. [27]. On day 7 after immunization, the mice were weighed and then killed by cervical dislocation. The spleen was removed and weighed. The spleen indexes were defined as spleen wet weight versus body weight.

Splenocytes proliferation assay

Splenocytes proliferation assay was performed as previously described [25]. After the spleen weight was measured, the spleen was minced aseptically. The splenocytes were collected and suspended in DMEM (containing 5 % (v/v) calf serum) at a concentration of 5×10^6 cells/mL. The cell suspension (100 µL) and ConA (100 µL with a final concentration of 5 mg/L) were simultaneously seeded into a 96-well flat-bottom plate. Triplicate wells were designed. The cultures were incubated at 37 °C in an

atmosphere of 5 % CO₂ for 48 h. Four hours before the end of the incubation period, 10 µL of MTT (5 g/L) was added to each well. The absorbance was measured using an ELx808 ELISA Microwell Reader (BioTek Co., USA) at 490 nm. The results are described as the average of triplicate measurements of the absorbance.

Analysis of cytokine production

Cytokine production was analyzed referred to the previous procedure [28]. After the spleen weights were measured, each spleen was minced aseptically under sterile conditions. The splenocytes were then collected and suspended in DMEM (containing 5 % (v/v) calf serum) at a concentration of 5×10^6 cells/mL. The suspensions (500 µL) and ConA (500 µL with a final concentration of 5 mg/L) were simultaneously seeded into a 24-well culture plate. The cultures were incubated at 37 °C in an atmosphere of 5 % CO₂ for 48 h. Following the incubation, the plate was centrifuged at 2000 rpm (4 °C) for 15 min. The supernatant was collected and reserved at −20 °C. The levels of IL-10 and IL-17 in the supernatant of the splenocytes were measured using ELISA, according to the manufacturer's instructions.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD). The concentration–time data were subsequently processed using drug and statistics for Windows version 2.0 (Chinese Pharmacology Society). The pharmacokinetic parameters, including $t_{1/2}$, MRT, AUC, $C_{\max}$ and $T_{\max}$, were calculated based on moment methods. Significant differences between means were evaluated using one-way ANOVA, Dunnett's test and Student's t test (SPSS Software Products, USA). A difference was considered significant at $p < 0.05$. Spearman's coefficient was used to investigate the correlation between the severity of the inflammatory responses and the cytokine levels. A value of $p < 0.05$ was used to indicate statistically significant differences.

Results

Structure elucidation of 6-AP

The chemical shifts of 6-AP were quasi-identical with those of Pae, as shown in Table 1, with the exception of the signals for 6'-H, which moved dramatically downfield by 0.25 and 0.28 ppm (from 4.95 or 4.90 ppm in Pae to 5.20 or 5.18 ppm in 6-AP), and 6'-C, which moved dramatically downfield by 2.3 ppm (from 61.1 ppm in Pae to 63.4 ppm

Table 1 ^{13}C NMR and ^1H NMR spectral data of Pae and 6-AP (400 MHz, DMSO)

No.	Pae		6-AP	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	87.4		87.5	
2	84.9		84.7	
3	43.5	1.66 (1H, d, 12.0 Hz) 2.06 (1H, d, 12.4 Hz)	43.5	1.65 (1H, d, 14.4 Hz) 2.39 (1H, d, 6.8 Hz)
4	104.6		104.5	
5	42.2	3.01–2.97 (1H, m)	42.2	3.06–3.02 (1H, m)
6	70.1		70.1	
7	21.9	1.82 (1H, d, 10.8 Hz) 2.39 (1H, d, 10.4 Hz)	21.6	1.71 (1H, d, 10.8 Hz) 2.46 (1H, d, 6.8 Hz)
8	60.4	4.68 (1H, d, 5.2 Hz); 4.62 (1H, br d, 12.0 Hz)	60.3	4.68 (1H, d, 5.2 Hz) 4.61 (1H, br d, 12.4 Hz)
9	99.9	5.32 (1H, s)	99.9	5.34 (1H, s)
10	19.0	1.24 (3H, s)	18.8	1.20 (3H, s)
1'	98.5	5.09 (1H, d, 4.8 Hz)	98.4	5.07 (1H, d, 4.8 Hz)
2'	73.3	3.66–3.62 (1H, m)	73.3	4.02–3.99 (1H, m)
3'	76.8	4.39–4.37 (1H, m)	76.5	4.42–4.40 (1H, m)
4'	69.9	4.39–4.37 (1H, m)	69.7	4.28–4.25 (1H, m)
5'	76.8	3.10–3.04 (1H, m)	73.3	3.14–3.11 (1H, m)
6'	61.1	4.95 (1H, d, 5.2 Hz) 4.90 (1H, d, 5.2 Hz)	63.4	5.20 (1H, d, 5.2 Hz); 5.18 (1H, d, 4.8 Hz)
7'			170.0	
8'			20.6	2.00 (3H, s)
1''	129.6		129.6	
2''	129.2	8.00 (1H, d, 7.2 Hz)	129.2	8.00 (1H, d, 7.2 Hz)
3''	128.7	7.57 (1H, t, 7.6 Hz)	128.7	7.57 (1H, t, 7.6 Hz)
4''	133.4	7.72–7.66 (1H, m)	133.4	7.70–7.66 (1H, m)
5''	128.7	7.57 (1H, t, 7.6 Hz)	128.7	7.57 (1H, t, 7.6 Hz)
6''	129.2	8.00 (1H, d, 7.2 Hz)	129.2	8.00 (1H, d, 7.2 Hz)
7''	165.5		165.7	

in 6-AP). In addition, the chemical shifts of 7'-C (170.0 ppm), 8'-C (20.6 ppm) and 8'-H (2.00 ppm) are identified as 6-AP.

Validation of HPLC methodology

Specificity

The specificity was evaluated by comparing the chromatograms of blank plasma, blank plasma spiked with Pae, blank plasma spiked with 6-AP and plasma samples obtained from rats after oral administration of a 6-AP solution. The retention times of Pae and 6-AP were approximately 5.75 and 8.07 min, respectively. As presented in Fig. 1S (Supplementary Material), no significant

endogenous peaks interfering with Pae and 6-AP were obtained in the blank plasma.

Standard curve, recovery, precision and stability

The calibration curves of Pae and 6-AP in rat biological samples were linear, over the range from 0.05 to 20 $\mu\text{g/mL}$ (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 20 $\mu\text{g/mL}$). With the least-squares method, two regression equations were obtained (Fig. 2S in Supplementary Material). The recoveries of Pae at each QC level (0.1, 1 and 10 $\mu\text{g/mL}$) were 98.20 ± 3.13 , 95.62 ± 2.26 and 98.16 ± 1.90 %, and the recoveries of 6-AP at each QC level (0.1, 1 and 10 $\mu\text{g/mL}$) were 95.56 ± 2.65 , 96.77 ± 2.30 and 98.51 ± 2.14 % (Tab. 1S in Supplementary Material). The variations of

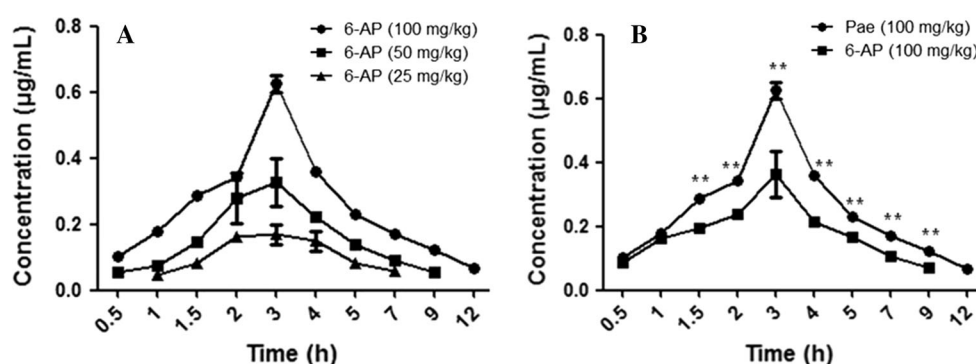


Fig. 2 Mean plasma concentration–time profiles of 6-AP and Pae. Data are presented as the mean $\pm$ SD * $p < 0.05$; ** $p < 0.01$, compared to concentrations of Pae; **a** mean plasma concentration of 6-AP ($n = 5$); **b** comparison of mean concentrations between Pae and 6-AP ($n = 5$)

intra-daily and inter-daily were all lower than 5 %, meeting the requirements of quantitative analysis (Tab. 2S, Tab. 3S in Supplementary Material). Pae and 6-AP are both stable in plasma under refrigeration (Tab. 4S in Supplementary Material). Consequently, these results indicate that the analytical method is suitable for the present study.

Plasma concentration profile of Pae and 6-AP

For oral administration, Fig. 2 presents the curves of 6-AP (Fig. 2a). The plasma concentrations of Pae in orally administered groups (25 and 50 mg/kg) were undetectable. Compared with the Pae group (100 mg/kg), the plasma levels of 6-AP were observably improved from 1.5 to 9 h (Fig. 2b, $p < 0.05$; $p < 0.01$).

Pharmacokinetic study

The pharmacokinetic parameters of Pae and 6-AP summarized in Table 2 show that there were statistically significant differences in parameters including the T_{max} , C_{max} , AUC, MRT and $t_{1/2}$. Compared with parameters of Pae,

$t_{1/2\alpha}$, $t_{1/2\beta}$, AUC, MRT (0– t) and C_{max} of 6-AP increased dramatically ($p < 0.05$; $p < 0.01$).

Effect of 6-AP on allergic contact dermatitis

6-AP inhibited ear swelling in mice treated with DNCB

Compared with normal mice, the ear of mice treated with DNCB provoked observable swelling. Administration of 6-AP (70 and 140 mg/kg/d), Pae (70 mg/kg/d) and Pre (5 mg/kg/d) significantly inhibited DNCB-induced ear swelling in mice ($p < 0.05$ and $p < 0.01$, respectively; Fig. 3). In addition, 6-AP showed significant results better than Pae at 70 mg/kg/d in reducing skin inflammation ($p < 0.05$).

6-AP ameliorated the histopathology in mice treated with DNCB

Compared with normal mice, the skin histopathology (Fig. 4B) in mice treated with DNCB revealed significant inflammatory cell infiltration and epidermal keratinization,

Table 2 Pharmacokinetics parameters of Pae and 6-AP after oral administration in rats

Pharmacokinetics parameters	Pae (100 mg/kg)	6-AP		
		25 mg/kg	50 mg/kg	100 mg/kg
$t_{1/2\alpha}$ (h)	1.163 $\pm$ 0.394	1.253 $\pm$ 0.098	1.354 $\pm$ 0.404	1.940 $\pm$ 0.034*
$t_{1/2\beta}$ (h)	1.509 $\pm$ 0.292	1.395 $\pm$ 0.124	1.581 $\pm$ 0.301	2.082 $\pm$ 0.108*
AUC (0– t ; mg/L h)	1.477 $\pm$ 0.063	0.697 $\pm$ 0.056	1.336 $\pm$ 0.022	2.622 $\pm$ 0.039**
AUC (0– ∞ ; mg/L h)	1.796 $\pm$ 0.107	0.938 $\pm$ 0.071	1.628 $\pm$ 0.054	2.974 $\pm$ 0.163**
MRT (0– t ; h)	3.993 $\pm$ 0.066	3.534 $\pm$ 0.079	3.919 $\pm$ 0.042	4.675 $\pm$ 0.043**
MRT (0– ∞ ; h)	5.673 $\pm$ 0.274	5.543 $\pm$ 0.382	5.238 $\pm$ 0.429	6.176 $\pm$ 0.639
T_{max} (h)	3.058 $\pm$ 0.421	2.682 $\pm$ 0.473	2.719 $\pm$ 0.293	3.624 $\pm$ 0.861
C_{max} (mg/L)	0.363 $\pm$ 0.023	0.179 $\pm$ 0.015	0.366 $\pm$ 0.033	0.626 $\pm$ 0.026**

Data are presented as the mean $\pm$ SD ($n = 5$); * $p < 0.05$; ** $p < 0.01$, compared to Pae group

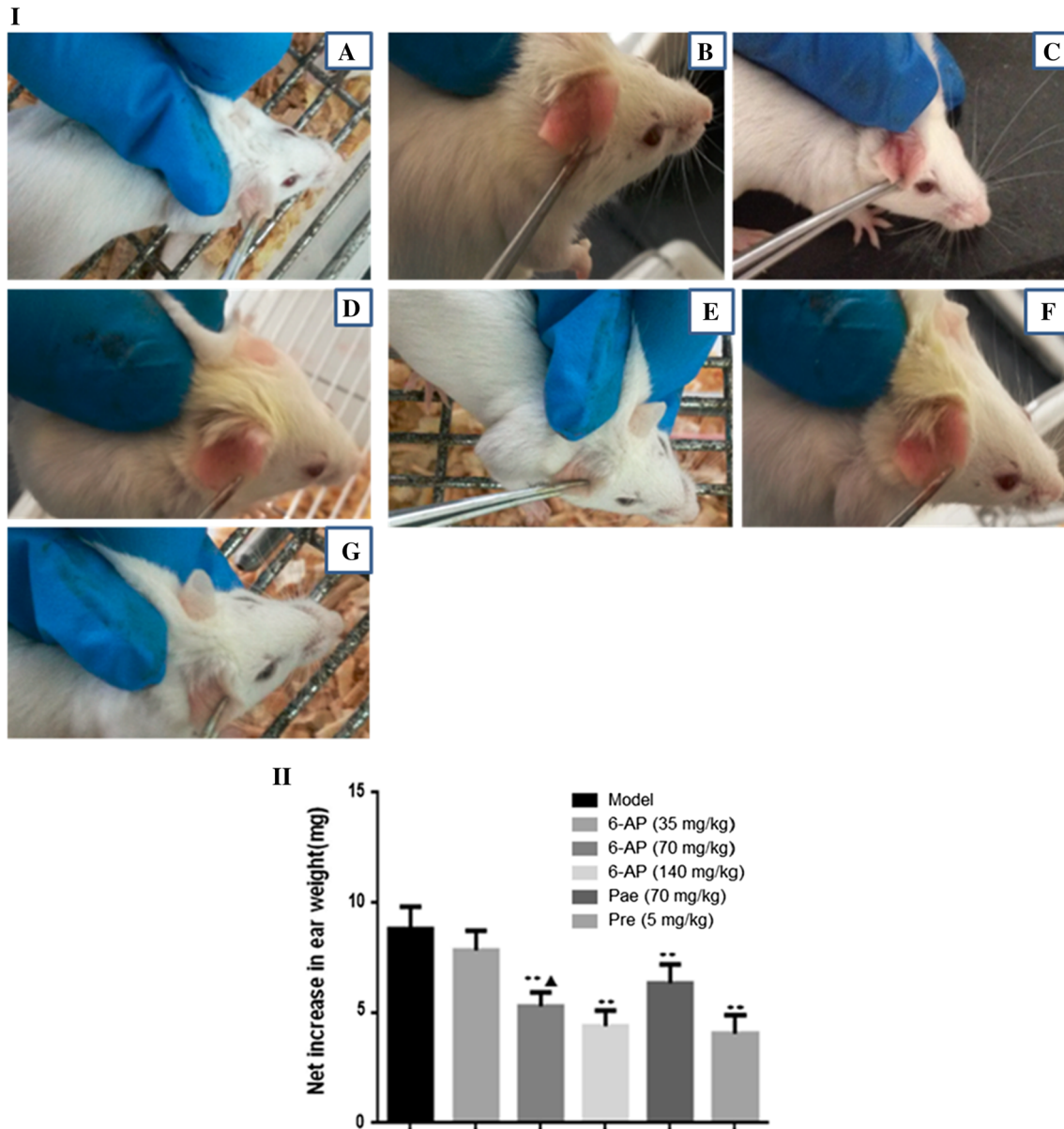


Fig. 3 Effects of 6-AP on ear swelling in mice treated with DNCB. The data are presented as the mean $\pm$ SD ($n = 8$). * $p < 0.05$ and ** $p < 0.01$ compared with the ACD model group; $\Delta p < 0.05$ compared with the Pae group. **I** Appearance of the ear treated with

DNCB showed significant swelling at day 7. (A) Normal group. (B) ACD group. (C) 6-AP (35 mg/kg/d). (D) 6-AP (70 mg/kg/d). (E) 6-AP (140 mg/kg/d). (F) Pae (70 mg/kg/d). (G) Pre (5 mg/kg/d). **II** Ear swelling in mice with ACD

and the spleen histopathology (Fig. 5B) demonstrated observable red pulp hyperplasia and germinal center (GC) appearance. After treatment with 6-AP (70 and 140 mg/kg/d), inflammatory cell infiltration and epidermal keratinization were markedly decreased (Fig. 4D, E), and the degree of spleen injury was also significantly alleviated in the hyperplasia of red pulp and GC appearance (Fig. 5D, E). In addition, 6-AP (70 and 140 mg/kg/d) and Pre (5 mg/kg/d) significantly reduced the histopathological scores of the ear and spleen tissues in mice treated with DNCB ($p < 0.05$ and $p < 0.01$, respectively; Fig. 6). Similar

results were observed after treatment with Pae (70 mg/kg/d) and Pre (5 mg/kg/d).

6-AP reduced splenocyte proliferation in mice treated with DNCB

As shown in Fig. 7, the spleen index of the model group was significantly greater than that of normal mice ($p < 0.01$). Treatment with 6-AP (70 and 140 mg/kg/d) significantly reduced the spleen index of mice treated with DNCB, while 6-AP (70 mg/kg/d) was significantly better

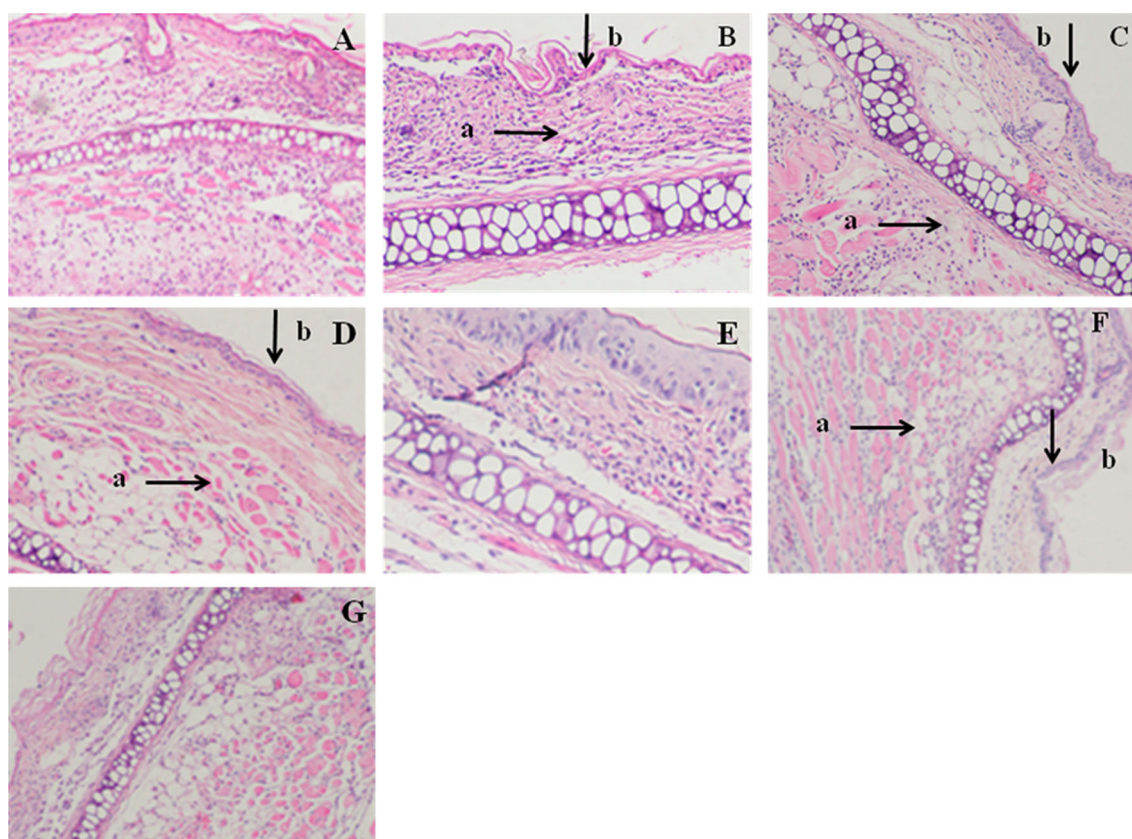


Fig. 4 Effects of 6-AP on the skin histopathology in mice treated with DNCB (original magnification $\times 200$). **A** Normal ear tissue, **B** the ACD group, in which the histopathology displayed obvious inflammatory cell infiltration (*a*) and epidermal keratinization (*b*), **C** the 6-AP group (35 mg/kg/d), in which the histopathology showed similar inflammation to those observed in the ACD model, **D** the 6-AP group (70 mg/kg/d), in which the histopathology displayed an

obvious decrease in inflammatory cell infiltration, **E** the 6-AP group (140 mg/kg/d), in which the histopathology showed obvious reductions in inflammatory cell infiltration and epidermal keratinization, **F** the Pae group (70 mg/kg/d), in which the histopathology showed similar inflammation to those observed in the ACD model, **G** the Pre group (5 mg/kg/d), in which the histopathology showed similar effects to those observed in **E**

than Pae at the same dose for the improvement of the spleen index ($p < 0.05$). Further study indicated that DNCB treatment promoted splenocyte proliferation in mice ($p < 0.05$). However, treatment with 6-AP (35, 70 and 140 mg/kg/d) significantly inhibited the abnormal proliferation of splenocyte in mice treated with DNCB ($p < 0.05$ and $p < 0.01$, respectively; Fig. 8). The similar results were observed after treatment with Pae (70 mg/kg/d) and Pre (5 mg/kg/d).

6-AP regulated IL-10 and IL-17 levels in splenocytes

As observed in Fig. 9, compared with splenocytes from the model group, the mice treated with DNCB exhibited a markedly increased level of IL-17 and a decreased level of IL-10 ($p < 0.01$). Specifically, 6-AP observably increased the IL-10 level and reduced the IL-17 level in the splenocyte culture supernatant of ACD mice. In addition, similar results were obtained after treatment with Pre and Pae. However, 6-AP was significantly better than Pae at 70 mg/

kg/d in regulating IL-10/IL-17. To further evaluate the relationship between the severity of skin inflammation and cytokine production, we calculated the correlation coefficients for the skin histopathological scores and cytokine production (Table 3). A significant negative correlation was observed between the skin histopathological scores and IL-10, while a significant positive correlation was found between the skin histopathological scores and IL-17.

Discussion

ACD models of mammals have been successfully established by repeatedly applying small molecular haptens to the skin, which are frequently used for studying ACD pathology and searching for new drugs for ACD [2, 3]. Among these haptens, DNCB is commonly used to induce sensitization, which is mainly due to its availability and reproducibility [29]. ACD is a T cell-mediated skin reaction that is associated with an imbalance in the secretion of

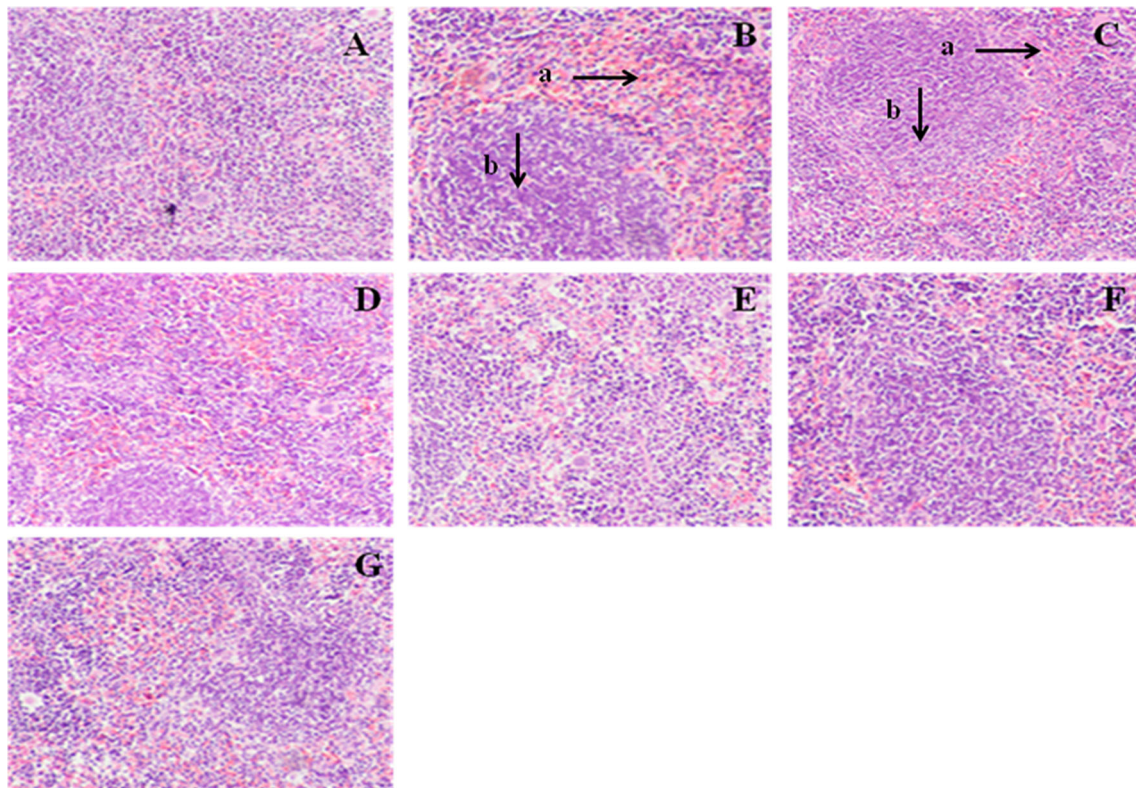
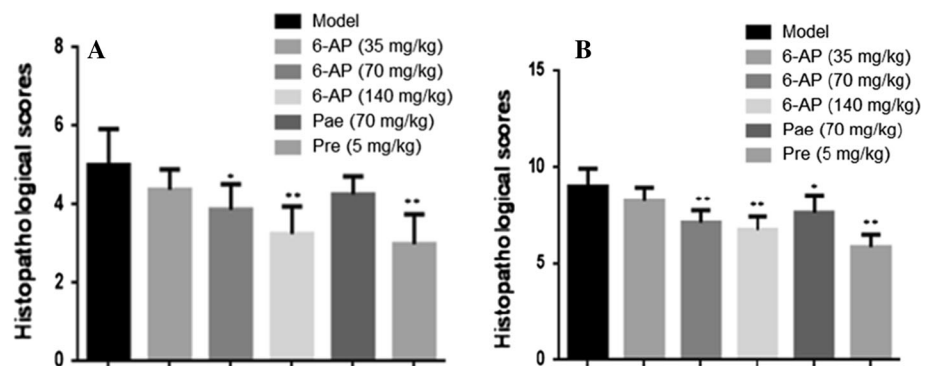


Fig. 5 Effects of 6-AP on spleen histopathology in mice treated with DNCB (original magnification $\times 200$). **A** Normal spleen tissue, **B** ACD group, in which the histopathology displayed obvious red pulp hyperplasia (*a*) and GC appearance (*b*), **C** the 6-AP group (35 mg/kg/d), in which the histopathology displayed a similar appearance to those observed in the ACD model, **D** the 6-AP group (70 mg/kg/d), in which the histopathology showed most reduction in

red pulp hyperplasia and GC appearance, **E** the 6-AP group (140 mg/kg/d), in which the histopathology showed obvious reductions in red pulp hyperplasia and GC appearance, **F** the Pae group (70 mg/kg/d), in which the histopathology showed some reduction in red pulp hyperplasia and GC appearance, **G** the Pre group (5 mg/kg/d), in which the histopathology showed similar effects to those observed in **E**

Fig. 6 Effects of 6-AP on histopathological scores of the ear (*a*) and spleen tissues (*b*) in mice treated with DNCB. The data are presented as the means $\pm$ SD ($n = 8$). $*p < 0.05$; $**p < 0.01$ compared with the ACD model group



pro- and anti-inflammatory cytokine and predominantly with a Th1 immune response that is involved in both the sensitization and elicitation phases [30, 31]. Recent studies have stressed the importance of both Th1/type1 and Th17/type17 cells for the full development of the ACD reaction in humans and CHS in mice [32]. Consistently, other studies have shown that allergen sensitization induces the development of CD8⁺ T cells that produce IFN- γ and

IL-17 which are both required for eliciting an immune response in the ACD model [33]. During CHS responses, the neutralization of IL-17 using antibodies markedly reduces inflammatory cell infiltration, as is observed in mice in which the IL-17 receptors are knocked out [34]. As expected, these results are consistent with the positive correlations between IL-17 production and the inflammation scores that were obtained in the present study

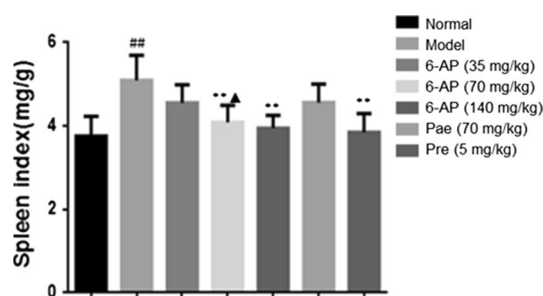


Fig. 7 Effects of 6-AP on the spleen index in mice treated with DNCB. The data are presented as the means $\pm$ SD ($n = 8$). ## $p < 0.01$ compared with the normal group; *** $p < 0.01$ compared with the ACD model group; ▲ $p < 0.05$ compared with Pae group

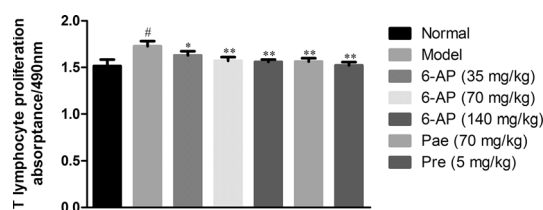
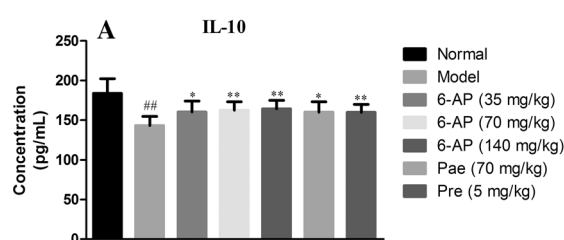


Fig. 8 Effects of 6-AP on splenocytes proliferation in mice treated with DNCB. The data are presented as the means $\pm$ SD ($n = 8$). # $p < 0.05$ compared with the normal group; *** $p < 0.01$ compared with the ACD model group

(Table 3). Furthermore, many types of immunocytes secrete IL-10, which is an anti-inflammatory cytokine. During the elicitation phase of ACD, IL-10 may exert its anti-inflammatory effects by inhibiting APC migration and reducing the production of pro-inflammatory cytokines,



such as tumor necrosis factor (TNF) and IL-1 [35]. Therefore, IL-10-deficient mice show enhanced inflammatory responses to haptens, and exogenous rIL-10 significantly reduces the skin inflammation induced by haptens [36].

In the present study, the plasma concentration profiles showed that 6-AP and Pae had statistically significant differences in plasma levels. Further study discovered that the oral administration of 6-AP resulted in higher values of $t_{1/2\alpha}$, $t_{1/2\beta}$, AUC, MRT and C_{max} in comparison with those of Pae. Therefore, the present study suggests that acetylation significantly changed the pharmacokinetic process of Pae. It is generally believed that the bioactivity of a drug is closely associated with its pharmacokinetic process. Thus in the present study, treatment with 6-AP (35, 70 and 140 mg/kg/d) significantly inhibited ear swelling in the ear tissue similar to the effects observed after oral administration of Pre (5 mg/kg/d), and 6-AP (70 mg/kg/d) showed significantly higher inhibitory effect than Pae on skin inflammation. It was further observed that 6-AP can inhibit splenocytes proliferation and restore the balanced secretion of pro-inflammatory cytokines and anti-inflammatory cytokines.

In conclusion, our results showed that the inhibitory effect of 6-AP on ACD was significantly higher than Pae, which might be attributed to its regulation of the imbalanced secretion of pro-inflammatory and anti-inflammatory cytokines. Our results also suggest that 6-AP might be an alternative or complementary therapeutic option for ACD. More in-depth studies are necessary to clarify the molecular mechanisms of action of 6-AP.

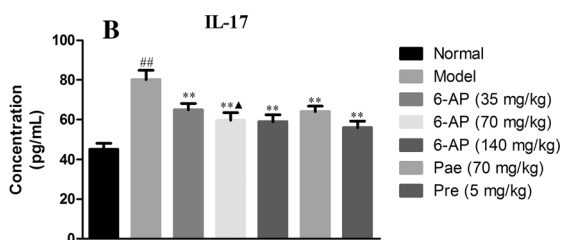


Fig. 9 Effects of 6-AP on cytokine production in the splenocyte culture supernatant of mice treated with DNCB. **a** IL-10 level in the splenocyte culture supernatant, **b** IL-17 level in the splenocyte culture supernatant. The data are presented as the means $\pm$ SD ($n = 4$).

$p < 0.01$ compared with the normal group; * $p < 0.05$ and *** $p < 0.01$ compared with the ACD model group; ▲ $p < 0.05$ compared with Pae group

Table 3 Correlations between cytokines levels and the skin histopathological scores

	Cytokines	Regression equation	Correlation coefficient	p value
Splenocyte	IL-10	$Y = -0.057X + 12.71$	-0.600	0.00004**
	IL-17	$Y = 0.117X - 3.86$	0.802	0.00010**

Correlation analysis between cytokine levels and the skin histopathological scores included the normal group, the model group and the three 6-AP groups

** Correlation is significant at the 0.01 level

Supplementary Material

The results of specificity, calibration curve, recovery, precision and stability of Pae and 6-AP are available as Supporting Information.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (Nos. 81330081, 81302845, 81473223), the Training programme of Academic and technical leaders in universities of Anhui province (No. 34), the Anhui Province Nature Science Foundation in University (No. KJ2013A158) and the Grants for Scientific Research of BSKY (No. XJ201211) from Anhui Medical University.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical standard All of the applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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