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Original Research Article

Picroside I inhibits asthma phenotypes by regulating T-bet/GATA-3 ratio and Th1/Th2 balance in a murine model of asthma

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Abstract

Purpose: *To examine the anti-asthmatic activity of picroside I in murine asthma model, and to elucidate the mechanism(s) involved.*
Methods: *The study involved systematic sensitization of acclimatized BALB/c mice with ovalbumin (OVA), and subsequent exposure to aerosol allergens. The effect of picroside I on associated IgE formation was determined. All assays were performed using standard protocols. Protein expression was assessed using western blotting.*
Results: *Picroside I inhibited allergic airway inflammation, AHR, and the production of OVA-associated IgE and Th2 cytokines. Moreover, it altered the T-bet/GATA3 ratio by suppressing the phosphorylation of STAT6 in a dose-dependent manner.*
Conclusion: *These results indicate that the anti-asthmatic effect of picroside I occurs via a mechanism involving inhibition of Th2 cytokines by suppression of the expressions of pSTAT6 and GATA-3, and upregulation of the expression of T-bet. Thus, picroside I is a promising agent for the management of asthma.*

Keywords: *Picroside, Asthma, Allergic response, IgE, GATA-3, pSTAT6*

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INTRODUCTION

Asthma is considered one of the main chronic inflammatory airway diseases. It impacts over three hundred million people the world over, and is expected to affect another hundred million by 2025 [1]. The frequency of allergic asthma has elevated considerably in the recent past, thereby posing a serious health problem [2]. Allergic asthma is primarily caused by a number of allergens which include, but are not limited to house dust, foods, and drugs. The major symptoms of asthma are wheezing, breathlessness and cough [3]. These symptoms

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result from bronchoconstriction and thickening of bronchial mucosa due to inflammation of the eosinophilic airway, remodelling of the airways, and production of larger-than-normal amounts of mucus. Indeed, allergic asthma is allied with inflammation of the airways and disproportionate production of mucus [4].

In allergic asthma, the inflammation of the airways is regulated by a multifaceted mechanism [4,5]. During an asthmatic attack, the allergens processed by antigen-presenting cells trigger the initiation of Th2 cells and release of various cytokines. These cytokines intensify the allergic response by enhancing inflammatory cell infiltration into the airways, and by initiating disproportionate formation of mucus [6].

The differentiation of Th2 and regulation of the transcription of Th2 cytokines involves GATA-3. In this process, the initiation of signal transducer and transcription-6 (STAT6) activator via interleukin-4 signal transduction causes dimerization and STAT6 phosphorylation to pSTAT6 which, in the nucleus, triggers the expression of GATA-binding protein-3 (GATA3), leading to Th2 cell differentiation [5].

Picrorhiza kurroa is a highly imperilled, medicinal herb found only in the North Western Alpine Himalayas (altitude, 2800 – 4800 m) [7]. It is the source of picroside 1. Studies have shown that picrosides exhibit diverse medicinal effects which include, but are not limited to hepatoprotective, anti-inflammatory, and anti-carcinogenic effects [5,6]. In the present investigation, the impact of picroside I on inflammation of airway in asthma model was evaluated.

EXPERIMENTAL

Animals

Five-week old BALB/c female mice were procured from Shantou University Medical College, and were kept under controlled conditions. The animals were given standard pellet diet *ad libitum*. Prior to commencement of the study, the mice were acclimatised to laboratory conditions for seven days. The study was approved by the animal ethics committee of Luoding People's Hospital, Luoding, (Approval no. C7A547LH/2017) and all the procedures were carried out as per standard international guidelines [8].

Animal grouping and treatment

The mice were randomly grouped into 6 different groups. Mice in group 1 (sham, normal control) received phosphate-buffered saline (PBS, vehicle) only. Group 2 mice were OVA control (OVA-sensitized and OVA-challenged (OVA/OVA + vehicle); while mice in group 3 were OVA-sensitized, OVA-challenged, and given 0.7 mg/kg dexamethasone (OVA/OVA/DEXA). Groups 4 - 6 mice OVA-sensitized, OVA-challenged, and given picroside 1 at concentrations of 0.2, 2.0 and 20 mg/kg (OVA/OVA/picroside 1-treated). Dexamethasone were given orally, once daily from day 20 to day 32, with PBS as vehicle. The procedure used for inducing allergic asthma is summarised in Figure 1.

Sensitization, airway OVA challenge and treatments

For sensitization of the mice, 40 µg of OVA plus 2.6 mg of $Al(OH)_3$ in PBS (200 µL) were given intraperitoneally on days 0 and 7. The mice were thereafter administered 5 % OVA in PBS from days 21 to 32. Dexamethasone and picroside were given once per day from day 19th to 25th. Some mice were sacrificed on 24th day, and broncho alveolar lavage was carried out to assess lung eosinophilia.

Measurement of airway hyper-responsiveness (AHR) and collection of BALF

The measurement of AHR was carried out as

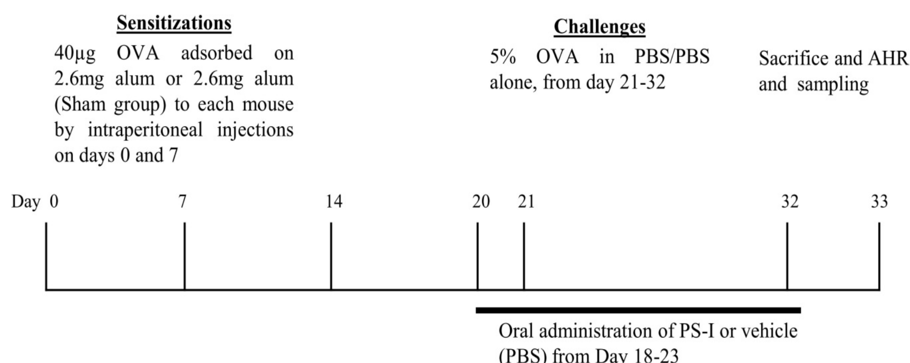

Figure 1: Procedure used for triggering allergic asthma in the mice

described previously [9]. On day 33, the mice were sacrificed under ether anaesthesia, and their BALF portions were harvested for differential cell counting and estimation of cytokine levels. The whole procedure was carried out as described earlier [9].

Evaluation of cytokines and OVA-specific Immunoglobulin E (IgE)

Serum cytokine (IL-5, IL-4 IL-13 and IFN- γ) were estimated using ELISA (R & D Systems) kits in line with the manufacturer's guidelines. The assay of OVA-specific IgE, was done using a microtiter plate as described previously in literature [9,10].

Western blotting analysis for protein expression

The lungs were homogenized in buffer and their protein concentrations were determined with Bradford method. Protein expression was measured using western blotting as described previously [2].

Histological examination

After the collection of BALF from the lungs, the left lung was carefully isolated and subjected to fixation in neutral buffered formalin (10 %) for 24 h. The specimens were subjected to dehydration and subsequently paraffin-embedded. Thereafter, 5-μm sections of the fixed and embedded tissues were subjected to H & E staining. Histological analysis was carried out as described previously [8].

Statistical analysis

Data are shown as mean ± SEM. Statistical analysis was done using Students *t*-test with GraphPad prism 7 software. Values of $p < 0.05$ were considered as indicator of significant difference.

RESULTS

Picroside I reduced AHR in experimental asthma model

The airway resistance developed by treatment with methacholine at concentrations ranging from 0 to 16 mg/ml was considerably enhanced in the OVA group. Assessment of the picroside I on AHR revealed no significant differences in the baseline airway resistance among the six groups. In contrast, there was significant decreases in airway resistance in the control, DEXA and picroside I (2 and 20 mg/kg)-treated groups (Figure 2). However, the decline in airway resistance in the 0.2 mg/kg was not significant.

Picroside I influenced the release of Th1 and Th2 cytokines

The Th2 cytokines IL-4 (Figure 3A), IL-5 (Figure 3B) and IL-13 (Figure 3C) were measured in mice sera. Mice administered picroside I at concentrations of 0.2, 2 and 20 mg/kg exhibited no remarkable changes in these cytokines relative to control group. However, picroside I enhanced the secretion of IFN-γ (Figure 3D), a Th1 cytokine, dose-dependently, indicative of its impact on T cell differentiation.

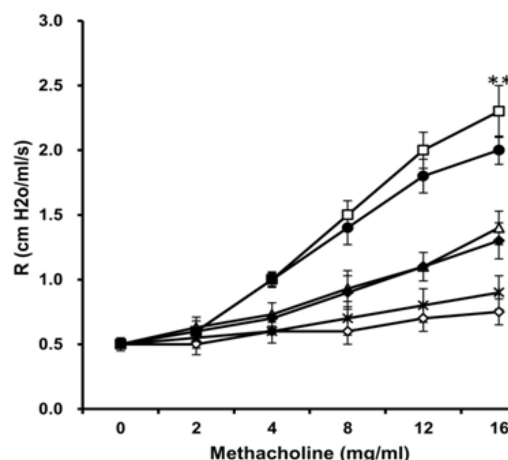

Figure 2: Estimation of airway hyper-responsiveness (AHR). Picroside I treatment caused reductions of airway hyper-responsiveness in mice. Data are shown as mean ± SEM; $^*p < 0.05$; $^{**}p < 0.01$, compared with sham/PBS group. (◊ = Control, □ = OVA control, Δ = DEXA (0.7 mg/kg), ● = PS-1 (0.2 mg/kg), ♦ = PS-1 (2 mg/kg), ■ = PS-1 (20 mg/kg)

Picroside I reduced OVA-associated IgE levels

Picroside 1 administration (0.2, 2 and 20 mg/kg) did not resulted in any marked alterations in the OVA-associated IgE levels, when compared to the control group (Figure 4).

Picroside I attenuated airway inflammation

Mice BALF was analysed for inflammatory cells. Only a few inflammatory cells were observed in the control group. In contrast, there were marked increases in the population of inflammatory cells in mice sensitized and challenged with OVA. The impact of picroside I on allergen-triggered inflammatory cell penetration was determined in mice administered 3 varied concentrations of picroside I. As depicted in Table 1, picroside I at

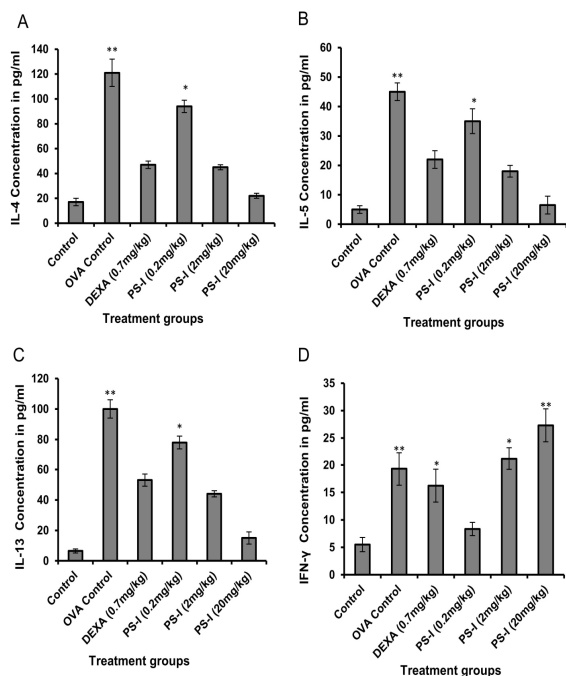

Figure 3: Effect of picroside I on Th1 and Th2 release: IL-4 **(A)**, IL-5 **(B)** IL-13 **(C),** and IFN-γ (**D**) IL-4, IL-5, IL-13 and IFN-γ levels in BAL fluid. Data are shown as mean ± SEM; *$p < 0.05$, **$p < 0.01$, vs sham/PBS

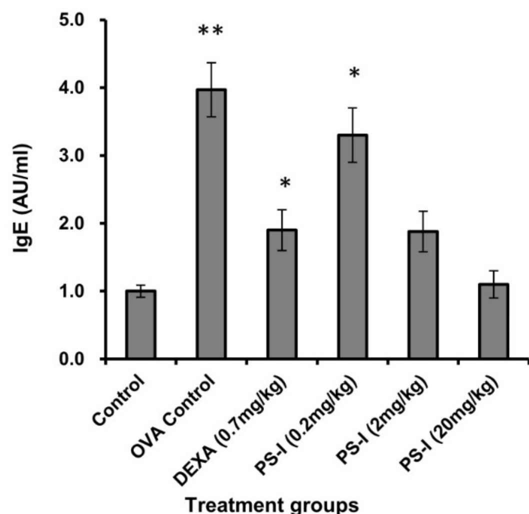

Figure 4: Effect of picroside I on the release of OVA-specific IgE, as evaluated by ELISA and presented as arbitrary units. Data are shown as mean ± SEM; *$p < 0.05$, compared with sham/PBS

0.2, 2 and 20 mg/kg inhibited allergen-triggered inflammatory cell infiltration. Nonetheless, in the group given 0.2mg/kg, the infiltration of inflammatory cells was profound, when compared to other picroside I-treated groups. The anti-inflammatory effect of picroside I was evident from the histological examination of H & E stained sections of lung (Figure 4A). A remarkable increase in inflammatory cells in the airway was found in OVA-adminstrated mice, when compared with PBS-treated control mice. However, there were remarkable reductions in infiltration of inflammatory cells in mice administered different concentrations of picroside I.

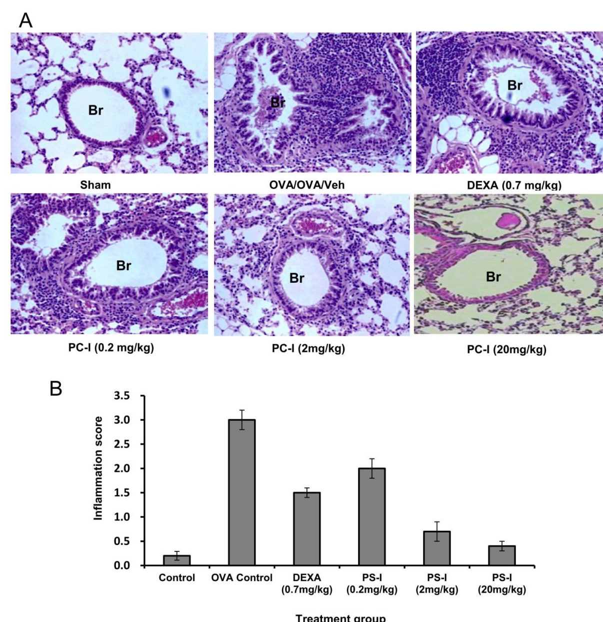

Figure 5: (A) Picroside I administration considerably decreased airway infiltration of inflammatory cells. **(B)** Quantitative determination of inflammation using inflammation score

Table 1: Effect of picroside I on total cell count and differential cell count

TCC (x 10^4/ml)		Differential count (%) Macro	Mono	Eosino	Neutro
SHAM/PBS/VEH	3.1±1.4	37.5±4.4	43.1±4.2	1±0.12	2±0.3
OVA/OVA/VEH	55.2±4.3**	10.6±3.1*	11.4±2.3*	57.1±3.2**	23.2±3.4*
PS-I-0.2mg/kg	37.2±9.4*	15.2±3.2*	18.5±3.5	28.5±4.1*	15.2±2.2
PS-I -2mg/kg	12.3±2.2	24.5±4.5	22.1±4.3	8.4±3.1	10.4±1.4
PS-I -20mg/kg	8.5±3.6	40.1±4.6	40.2±4.3	3.2±0.2	2.6±0.4
OVA/OVA/DEXA	10.4±2	24.4±5.2	37.2±2.4	10.4±5	10.3±2.2

Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, compared with sham/PBS

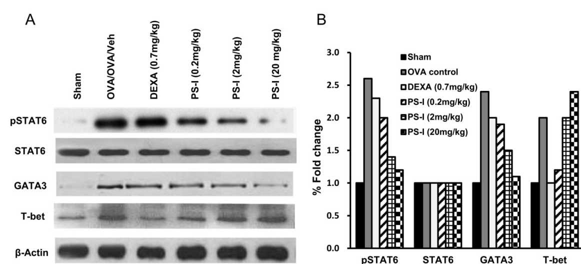

Figure 6: **(A)** Effect of picroside 1 on pSTAT6, GATA3 and T-bet expressions, as determined using western blot; **(B)** densitometric analysis of pSTAT6, STAT6, GATA3 and T-bet expressions

Picroside I modulated pSTAT6, GATA-3 and T-bet expressions in lung tissue

The expressions of pSTAT6, GATA-3 and T-bet in lung tissue homogenates were determined in control, OVA control, DEXA-administrated, and picroside I-treated mice. There were no expressions of pSTAT6, GATA-3 and T-bet in control mice. However, the expressions of pSTAT6 and GATA-3 were highly upregulated in OVA control mice. In picroside I-treated group, the expressions of pSTAT6 and GATA-3 were downregulated, indicating an inhibitory effect on Th2 cell differentiation (Figure 6). The picroside 1-induced decreases were concentration-dependent, but there was no detectable expression in the 20 mg/kg picroside administrated group. The dose-dependent modulation of T-bet expression by picroside I further confirmed that the decrease in Th2 cytokine release was due to modulation of GATA3 by picroside I.

DISCUSSION

The spotlight in the development of drugs for asthma currently is either on improving the efficiency of available drugs or searching for novel compounds that can target the Th2-specific transcription factors [11]. The latter is based on the central and principal function of Th2 cytokines in escalating inflammation of airway and the transcription factor genes that control their production. However, this approach requires inhibition of the monoclonal antibodies and Th2-cell transcription factors [3,12].

The transcription factor family GATA which includes 6 members, harbours a prevalent DNA binding domain that is evolutionarily preserved across vertebrates. With respect to the immune system, GATA3 is the imperative among all members of GATA family [13,14-16]. This transcription factor is regarded as the main controller of Th2 cell differentiation. It is a downstream gene expressed after IL-4-induced STAT6 phosphorylation [17,18]. Coordinated control of Th2 cytokines is vital for an allergic response such as asthma. The cytokines have a major function in AHR advancement, IgE generation, eosinophills of airway, and high mucus secretion, all of which are the main features of allergic asthma [12,19]. Furthermore, T-bet transcription factor along with GATA-3 maintains a suitable Th1/Th2 cell ratio in the body under normal physiologic conditions. An imbalance in this ratio may result in a disease condition. The two transcription factors control the expression of each other. An increase in the expression of one subsequently suppresses the expression of the other. Therefore, T-bet/GATA-3 ratio is used to investigate immune equilibrium in the Th1/Th2 responses in asthma [9]. Enhanced expression of T-bet transcription factor shifts the equilibrium to Th1, and causes increased release of Th1 cytokines [3,20].

In the current investigation, standard OVA-triggered murine model of asthma was used to study the anti-asthmatic activity of picroside I. It was observed that picroside I abridged the employment of inflammatory cells to the microenvironment of the lungs. In this model, picroside I caused decrease in the methacholine-triggered AHR in OVA-immunized asthmatic mice. In addition, it caused reduction in the release of Th2 cytokines by Th2 cells and production of OVA-specific IgE in a concentration-dependent fashion. Histology results showed absence of inflammation in the lung sections around the bronchioles in the control group. However, in the OVA control group, maximum inflammation was observed. Picroside I reduced the inflammation significantly at its higher dose. It also downregulated pSTAT6 and GATA3 expressions. Reduction in the expression of these two transcription factors is related to the reduction in the release of cytokines, IgE production and airway eosinophilia or airway inflammation. In addition, picroside I treatment dose-dependently increased the serum levels of IFN-γ, a Th1 cytokine, and the expression of T-bet in the lung dose dependently.

T-bet expression varied indirectly with that of GATA3 expression, i.e., for a decrease in GATA3 expression, there was a corresponding increase in T-bet expression. Therefore, it can be postulated that picroside I attenuates asthma features in a mouse model of asthma by altering T-bet/GATA-3 ratio which reflects alterations in the Th1/Th2 balance. Furthermore, picroside I suppressed STAT6 phosphorylation which resulted in decreased GATA3 expression and

increased expression of T-bet at increasing doses of picroside I.

CONCLUSION

The findings of this study show that picroside I suppresses asthma phenotypes by altering T-bet/GATA-3 ratio, thereby altering Th1/Th2 equilibrium in a murine model of asthma. Therefore, picroside I is a potential therapeutic agent for the management of asthma via amelioration of allergic responses.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Sen Zhang, Yuemei Lu, Jinhong Zheng, Hua Qin, Xiaoling Qiu performed all the experiments. Haile Huang, Guangzhou Mo, Songrong Liang, Yaowei Zhang carried out literature survey and compiled the data.

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