



Sulfuretin alleviates atopic dermatitis-like symptoms in mice via suppressing Th2 cell activity

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Abstract

Atopic dermatitis (AD) is a chronic skin inflammatory disease characterized by uncontrolled Th2 cells response to environmental allergens. Long-term topical application of corticosteroids for treating AD may induce severe side effects. Sulfuretin is a major flavonoid found in *Rhus verniciflua* and carries antioxidant and anti-inflammatory properties. Its therapeutic effect on AD has not been characterized. We first studied the cytotoxic and regulatory effects of sulfuretin on differentiated Th2 cells. Next, we evaluated therapeutic effect of sulfuretin on AD-like damages caused by 2,4-dinitrochlorobenzene (DNCB) in a mouse model. Serum IgE level, overall symptomatic score, and cytokine accumulation at the lesions were measured. Lastly, we investigated the regulatory mechanism of sulfuretin on GATA3 pathway in primary mouse CD4⁺ cells. Study on in vitro differentiated Th2 cells showed sulfuretin inhibited IL4 production in dose-dependent manner without any cytotoxicity. In vivo study showed 10 μ M sulfuretin alleviated the AD symptoms including skin lesion severity, scratching incidence, IgE serum level, and proinflammatory cytokine accumulation at local skin lesion site. Mechanistic study suggested sulfuretin attenuated Th2 cytokine production by suppressing GATA3 expression. Our results demonstrate that sulfuretin could be used as therapeutic application for treating AD.

Keywords Sulfuretin · Atopic dermatitis · Th2 cell · GATA3 · DNCB

Introduction

Atopic dermatitis (AD) is a common pruritic skin inflammatory disease primarily due to cutaneous hyperreactivity to environmental triggers. AD commonly occurs during early childhood, but is also found in adulthood. In children, the lifetime prevalence of AD is 10~20% as compared with 1~3% in adults. And its prevalence is on the rise all over the world, particularly in the industrialized societies. Although it is not a life-threatening disease, AD is still considered as a major skin disease that causes significant burdens upon patients' life quality, work productivity, and health outcomes [1–3].

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The interplay among skin barrier dysfunction, environmental allergic triggers, and allergic inflammation is at the critical stage in the AD pathogenesis. Dysfunction in skin barrier, as primary defect, results in itchy dry skin that leads to scratching. Frequent scratching inflicts mechanical damage to skin barrier that eventually leads to allergic sensitization to environmental antigens and allergic skin inflammation. And the secondary defect is the immune response activated by the exposure to immunologic triggers such as food and inhalant allergens. AD-associated immune response usually involves increased Th2 cells in acute skin lesion. As effector cells, their systemic response is the key cellular step during pathogenesis of AD. This is evidenced by upregulation of Th2 cytokines such as IL13 and IL4, elevated number of eosinophils, and increased serum IgE level in most AD patients or AD-like animal models [4–6]. Transgenic mice overexpressing IL4 specifically in their skin tissue develop inflammatory itchy skin lesions similar to AD [7].

Mechanistic understanding of AD pathogenesis has made great progress in recent decades, but the effective solutions to cure AD are still not available [8]. The aim of AD treatment is relieving symptoms and manage disease with multi-step approaches in long term. Currently, topical corticosteroid

application is still the gold standard of anti-inflammatory treatment of AD. However, chronic use of topical corticosteroids was associated with many adverse effects, such as growth retardation, acne, atrophy, red burning skin, and cataracts [9, 10]. The future challenge will be the development of more effective and safer treatments on AD. And those anti-inflammatory molecules from natural products have become attractive candidates.

Rhus verniciflua stokes (Anacardiaceae) is commonly used in Asian medicines to treat cancer and oxidative damage. *Rhus verniciflua* extract has been reported to exert multiple biological functions including anti-inflammatory effects, antioxidant activity, anti-mutagenic activity, and apoptotic effects on human cancer cells [11]. Sulfuretin is the major flavonoid component found in *Rhus verniciflua*. Recent animal studies have reported that the administration of sulfuretin alone can attenuate allergic airway inflammation [12], ameliorate the severity of experimental arthritis, and reduce lymphedema volume in mouse model [13]. In skin study, sulfuretin has been suggested as key active that exert the protective function of *Rhus verniciflua* stokes on human dermal fibroblasts and keratinocytes against oxidative damage [14]. Given the antioxidant and anti-inflammatory properties of sulfuretin, we hypothesized that sulfuretin may give a beneficial effect on skin inflammation disorders like AD.

To test this hypothesis, we firstly evaluated the effect of sulfuretin on in vitro differentiated Th2 cells. We then employed a DNCB-induced mouse AD model to evaluate the therapeutic benefit of sulfuretin in vivo. Scratching behavior, dermatitis scores, IgE serum level, and proinflammatory cytokine accumulation were used to assess the improvement in AD-like mouse model. At last, we also studied the regulatory mechanism of sulfuretin on primary mouse CD4⁺ cells, which provided a mechanistic explanation on the therapeutic effect of sulfuretin on DNCB-caused AD-like skin lesions.

Methods

Reagents

Sulfuretin (HPLC, $\geq 99\%$, #7569.1) was purchased from Carl Roth. DNCB (#237329) was obtained from Sigma. GATA3 (D13C9) and GAPDH (14C10) antibodies were purchased from Cell Signaling, USA. siRNA against GATA3 and its scramble control were from Dharmacon.

TUNEL assay

Sulfuretin cytotoxicity was determined using the TUNEL assay (#ab66108, Abcam). Briefly, cells were seeded overnight and then changed with new medium at different concentrations of Sulfuretin once per 2 days for 1 week. Thereafter, the

cells were harvested and fixed using paraformaldehyde. The apoptotic cells were labeled with APO-BrdUTM assay kit (#23210, Thermofisher, Waltham, MA, USA) and measured by flow cytometry.

Functional characterization of in vitro differentiated Th2 cells

Human primary CD4⁺ T cells were purchased from Lonza. The cells were first cultured for 1 week. Then, they were differentiated into Th2 CD4⁺ cells in vitro with CellXVivo Th2 Differentiation kit (#CDK019, R&D Systems, Minneapolis, MN, USA). The differentiated Th2 cells were then incubated with various concentrations of sulfuretin. After incubation for 48 h, the cell culture supernatant was harvested and cytokine IL4 secretion were determined by enzyme-linked immunosorbent assay (#EH3IL4, ELISA) kit from Thermo Fisher.

Mouse strain

All experimental studies involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Wuxi No.2 People's Hospital. The 8 to 10-week-old BALB/c mice were obtained from the Animal House and maintained at room temperature of 22 ± 2 °C and at $50 \pm 5\%$ humidity throughout the experiment. Animal treatment and maintenance procedure followed the guidelines developed by the ethics committee for animal welfare.

DNCB-induced atopic dermatitis model

DNCB was purchased from Sigma-Aldrich (USA) and was used to induce atopic dermatitis-like symptoms [6, 15, 16]. The dorsal hair of the mice was first removed 2 days before DNCB challenge. Then, shaved skin on the dorsal part of the mice was challenged with 250 μ l of 1% DNCB dissolved in acetone:olive oil (4:1) for sensitization (counted as day 1). The repeated application was performed on the same lesion site once every 3 days with 0.2% DNCB. The mice were sacrificed on day 18. The mice were randomly divided into four groups ($n = 8$ on each group): (1) vehicle treated; (2) DNCB treated; (3) 10 μ M sulfuretin treated; and (4) DNCB + 10 μ M sulfuretin treated.

Assessment of scratching behavior in AD-like mice model

Scratching behavior was monitored at the end of all treatments. The mice were allowed to acclimatize in cages for 20 min; the number of scratching incidences was recorded for the next 10 min. We repeated the counting for six times (1 h in total). The scratching scores were defined as average

times every 10 min. Scratching behavior was measured as hind paw movements. Scratching of the ears, nose, and dorsal skin with hind paws was measured, while licking of the dorsal skin and belly for grooming was not counted. All assessments were done in a blinded manner.

Determination of serum IgE level by ELISA

Whole blood was harvested from the subjects in each treatment group at the end of the study (day 18). The serum was collected by centrifugation at 10,000 rpm for 30 min and kept at minus 80 °C. The serum level of IgE was determined with IgE ELISA kit (#ab157718, Abcam, USA) following the instruction of manufacturer.

Scoring evaluation of AD-like skin lesions

The severity of the AD-like lesions in the dorsal skin of DNCB-induced AD mice was assessed at the end of the study (day 18). The severity of four symptoms (excoriation/erosion, edema, erythema/hemorrhage, and scarring/dryness) was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of these individual scores was used to determine dermatitis score following the method of Matsuda et al. [17].

Real-time PCR analysis of proinflammatory cytokines in skin lesion

Total RNA in skin tissue harvested from the AD lesions was extracted using Trizol® reagent following instruction manual. One microgram of RNA was reversely transcribed into cDNA using Maxima™ H Minus cDNA Synthesis Master Mix with dsDNase. Quantitative real-time PCR was carried out using QuantiTect SYBR Green PCR kit from Qiagen. Data were normalized against the expression level of gapdh gene. Specific primer sequences of TNF α , IL6, and GAPDH were listed here:

TNF α
 5'-TACTGAACCTTCGGGGTGATTGGTCC-3'
 5'-CAGCCTTGTCCTTGAAGAGAACC-3'
 IL6
 5'-CCGGAGAGGAGACTTCACAG-3'
 5'-GGAAATTGGGGTAGGAAGGA-3'
 GAPDH
 5'-CACCACCCTGTTGCTGTAGCC-3'
 5'-ACCACAGTCCATGCCATCAC-3'

Western blotting analysis

Th2 cells were collected by centrifuge and the lysate from the cell pellets was harvested with lysis buffer (125 mM Tris-HCl,

pH 6.8, 10% glycerol (v/v), 1% SDS) at 4 °C. Protein concentration was measured using the Bradford protein assay kit (Bio-Rad Laboratories, USA). Forty micrograms of cell lysate was mixed with SDS sample buffer for 5-min incubation at 75 °C and resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel were transferred onto a PVDF membrane. After blocking with 10% milk in TBST at RT for 1 h, the membrane was then probed with primary antibodies against GATA3 and GAPDH proteins at 4 °C for overnight.

Functional characterization of ex vivo differentiated primary Th2 cells

Mouse naïve CD4⁺ T cells were purified from spleen using the EasySep system (STEMCELL technologies) and cultured in RPMI 1640 medium (Gibco®, USA), supplemented with 10% FCS. After 4-day of culturing, dead cells were removed by centrifuging over Lympholyte M (Cedarlane). The remaining live Th2 cells were differentiated ex vivo into Th2 CD4⁺ cells using CellXVivo Mouse Th2 Cell Differentiation kit (#CDK002, R&D Systems, USA). At day six of differentiation, cells were then activated with anti-Mouse CD28 and anti-Mouse CD3 (from the kit). The activated Th2 CD4⁺ cells were then incubated with various concentrations of sulfuretin as indicated in the study. After 24-h incubation, the cell culture supernatant was harvested and cytokines IL5 and IL4 secretion were determined by ELISA.

Statistical analysis

All data from in vitro experiments were showed as mean $\pm$ S.D. and analyzed by two-tailed Student's *t* tests. The data from in vivo studies with more than two experimental groups were analyzed by one-way ANOVA with multiple comparisons among the groups. Only difference between the groups with *p* < 0.05 was considered statistically significant.

Results

Sulfuretin shows no impact on cell viability

Sulfuretin is a major polyphenol found in the heartwood of *Rhus verniciflua* (Fig. 1a). It has been applied to reduce oxidative stress or inhibit proinflammatory response in different inflammatory disease models. We sought to understand its therapeutic potential on AD. In AD model, Th2 cells play central part in mediating immune response that drives the manifestation of AD disease. We hypothesized sulfuretin could repress Th2 cell activity. Firstly, we evaluate the cytotoxic effect of sulfuretin on T cells. Human peripheral blood CD4⁺ T cells were cultured and incubated with different doses

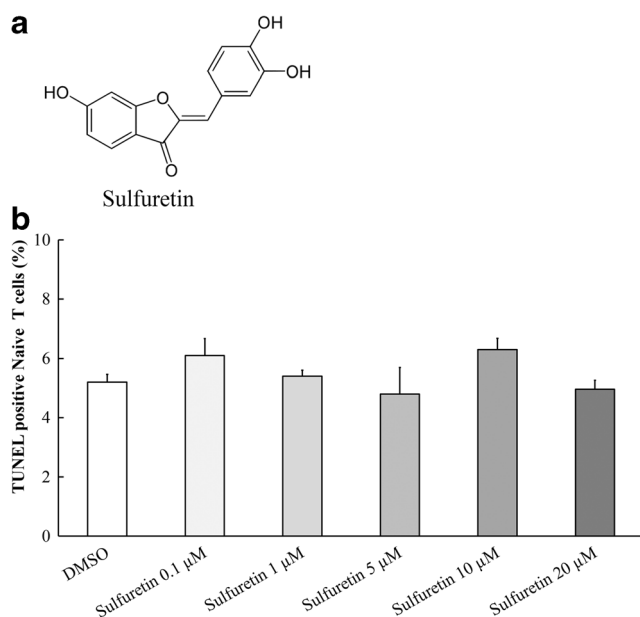


Fig. 1 Effect of sulfuretin on in vitro differentiated Th2 cell viability. **a** Chemical structure of sulfuretin. **b** In vitro differentiated Th2 cells were treated with different concentrations of sulfuretin for 7 days. The cell viability was assessed by the percentage of apoptotic cells as measured by TUNEL assay. The data are presented as the mean $\pm$ SD ($n = 6$, for each condition)

of sulfuretin from 0.1 to 20 μ M for 1 week. TUNEL assay was then used to assess the percentages of dead cells. As shown (Fig. 1b), with DMSO treatment only around 5% cells were labeled as apoptotic. As expected, sulfuretin even at the highest concentration showed no cytotoxicity towards T cells in culture in comparison with the control.

Sulfuretin suppresses Th2 cell activation in vitro

Next, we proceeded to study the effect of sulfuretin on in vitro differentiated Th2 cells. Th2 cells are first differentiated from naïve CD4⁺ T cells as promoted by Th2 polarized cytokines such as IL4. Abnormal Th2 cell activity is a hallmark of allergic inflammation. IL4, as a classic Th2 cytokine, triggers a number of events that drive Th2-mediated adaptive immune response. The secretion level of IL4 therefore can be used as indicative of Th2 functional activity. We firstly differentiated naïve T cells into Th2 polarized cells in vitro. The differentiated Th2 cells were then incubated with various concentrations of sulfuretin for 48 h. The activities of Th2 cells in different condition were assessed by the secretion level of Th2 signature cytokine IL4. As shown (Fig. 2), differentiated Th2 cells produced a significant higher level of IL4 than naïve T cells. Low concentration of sulfuretin (0.1 μ M and 1 μ M) showed no significant effect on IL4 secretion from Th2 cells. But sulfuretin in higher concentration (from 5 to 20 μ M) reduced markedly on the IL4 production in differentiated Th2 cells. The repressing effect exhibited dose-dependent manner.

Ten micromolar and 20 μ M treatments reduced IL4 secretion to nearly 50%. This in vitro result suggested sulfuretin had suppressing effect on Th2 cell activation.

Sulfuretin treatment exerts beneficial effects on AD mouse model

Next, we moved on to examine the in vivo effect of sulfuretin on AD model. DNCB-induced skin lesion on murine model is widely used to study the pathological mechanism of AD or evaluate the therapeutic candidates for AD [6, 18]. In our experimental design, BALB/c mice were divided randomly into four groups ($n = 8$ in each group): vehicle, DNCB, sulfuretin, and DNCB + sulfuretin. Vehicle group was the control group that received no challenge. DNCB group was challenged with DNCB on dorsal skin to induce AD-like lesions. DNCB + sulfuretin group received sulfuretin treatment after DNCB-mediated skin lesions were established. Similar to vehicle group, sulfuretin group received no DNCB challenge but only sulfuretin to assess any unwanted effect on healthy skin. Since in vitro study showed 10 μ M and 20 μ M produced similar reduction effect, we chose 10 μ M as in vivo dose. The overall disease manifestation was assessed by evaluating the scratching behavior and measuring dermatitis scores in AD mouse model. AD-like lesion causes itching at the wound site. We firstly measured the scratching frequency in different treatment groups. As shown (Fig. 3a), within the 60-min observation time frame, DNCB alone group exhibited about 12 times of scratching per 10 min as compared with control group with only about two times of spontaneous scratching. Sulfuretin group was comparable to vehicle control, which suggested this compound caused no irritation. Intriguingly, DNCB plus sulfuretin treatment group showed markedly decreased scratching incidence to about five times per 10 min. The severity of the dermatitis in AD mouse model was also scored by four symptoms (edema, erythema/hemorrhage, scarring/dryness, and excoriation/erosion). As shown in Fig. 3b, mice from control group showed a baseline score of 1. DNCB-challenged mice exhibited severe dermatitis symptom with average lesion score of 9. In line with the improvement in scratching behavior, sulfuretin treatment in DNCB-mediated lesions significantly reduced the dermatitis score to about 4, with more than 50% decrease. While sulfuretin treatment alone did not affect the baseline. Here, we used DMSO as solvent of sulfuretin. To exclude the solvent effect, we also repeated the study to compare the treatment effect of sulfuretin with DMSO on AD model. As shown (Supplementary Figure S1A&1B), DMSO alone had no benefits in AD-like symptoms in the mouse model. Taken together, sulfuretin treatment markedly reduced the overall scratching frequency as well as severity scores of skin lesions. These results indicated that sulfuretin exerted beneficial effect on AD-like lesions on in vivo model.

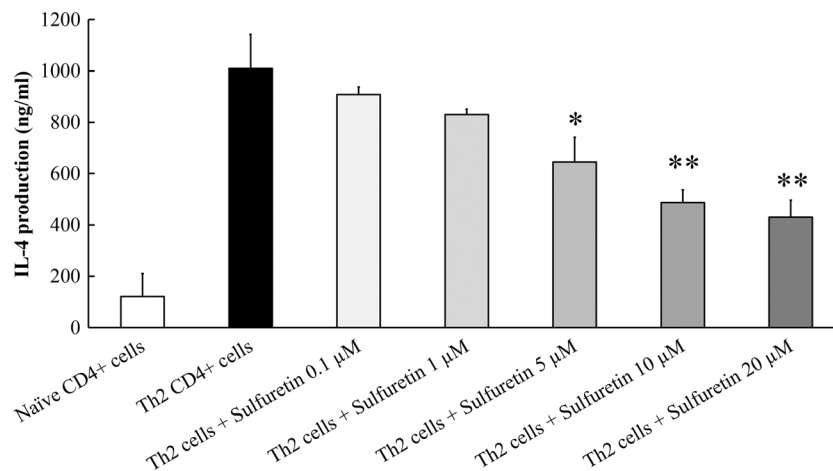


Fig. 2 Effect of sulfuretin on IL4 production in differentiated Th2 cells. Naïve CD4⁺ cells were differentiated into Th2 cells in vitro. The differentiated Th2 cells were treated with different concentrations of sulfuretin for 2 days. The cell supernatant was collected and cytokine

IL4 level was determined by ELISA. The data are presented as the mean $\pm$ SD ($n=6$, for each condition). * $p < 0.05$, significantly different from Th2 cell control group without treatment; ** $p < 0.01$, significantly different from Th2 cell control group without treatment

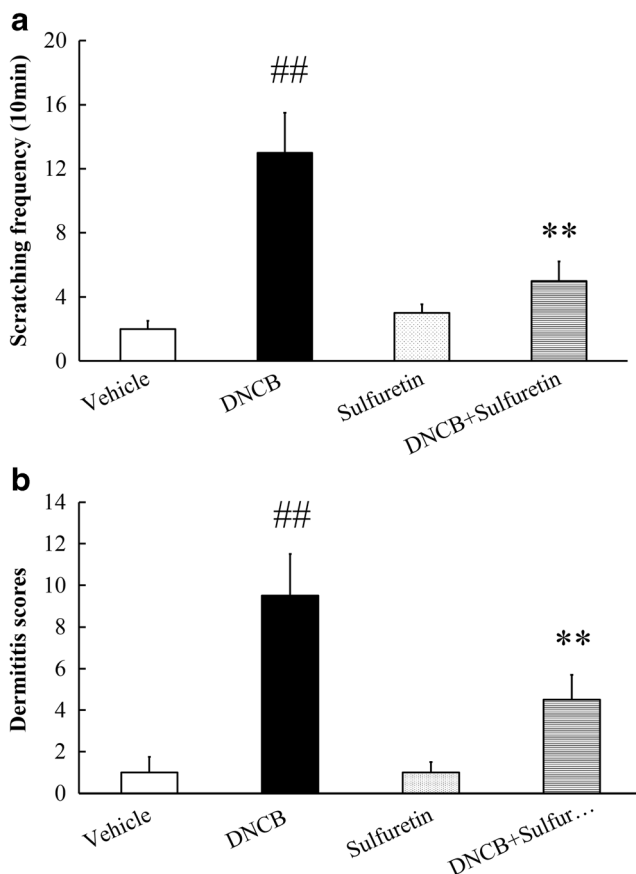


Fig. 3 Effect of sulfuretin on DNCB-induced AD-like symptoms in mice. **a** The scratching behavior was assessed on the last day of the study (day 18). Briefly, the number of scratching behaviors was counted for 10 min. Measurement was repeated for six times (60 min in total). **b** The severity of clinical symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scarring/dryness) of the AD-like skin lesions was evaluated macroscopically as the dermatitis scores. The data are presented as the mean $\pm$ SD ($n=8$, for each experimental group). ### $p < 0.01$, significantly different from control group; * $p < 0.05$, ** $p < 0.01$, significantly different from DNCB-treated group

Sulfuretin treatment suppresses immune response in AD mouse model

Despite of the high heterogeneity of AD, the elevated serum IgE level is consistently observed in this pathogenesis of AD. The severity of AD also correlates with serum IgE level. To have an overall assessment on serum IgE, we next measured IgE levels in the different experimental groups. The whole blood was harvested at the end of the protocol and the serum IgE levels were determined by ELISA. As shown in Fig. 4a, control group showed the baseline level of IgE in serum about 10 ng/ml. In AD-like group challenged with DNCB, IgE serum level increased readily to above 20 ng/ml, more than twofold increase than in the control group. Interestingly, sulfuretin treatment markedly decreased IgE serum level nearly to baseline, suggesting a systematic improvement in AD-like symptom. To be noted, sulfuretin alone did not change the baseline level of IgE in serum. Next, we proceeded to measure the accumulation of proinflammatory cytokines at the AD-like lesions. Permeability barrier abrogation is one of the most important events in the pathogenesis of AD. The dysfunctional skin barrier during AD induces the secretion of proinflammatory cytokines including TNF α and IL6 in the damaged cutaneous tissue. As shown in Fig. 4b, c, DNCB-challenged group showed significantly elevated levels of TNF α and IL6 at the skin lesions to about eight and four times of the levels in vehicle group. Sulfuretin alone group showed similar levels of TNF α and IL6 as control group. DNCB + sulfuretin treatment showed markedly decreased levels of TNF α (Fig. 4b) and IL6 (Fig. 4c) at about 50% of the amount observed in DNCB group. Taken together, the downregulation of serum IgE and the attenuation of proinflammatory cytokine production in DNCB + sulfuretin treatment group

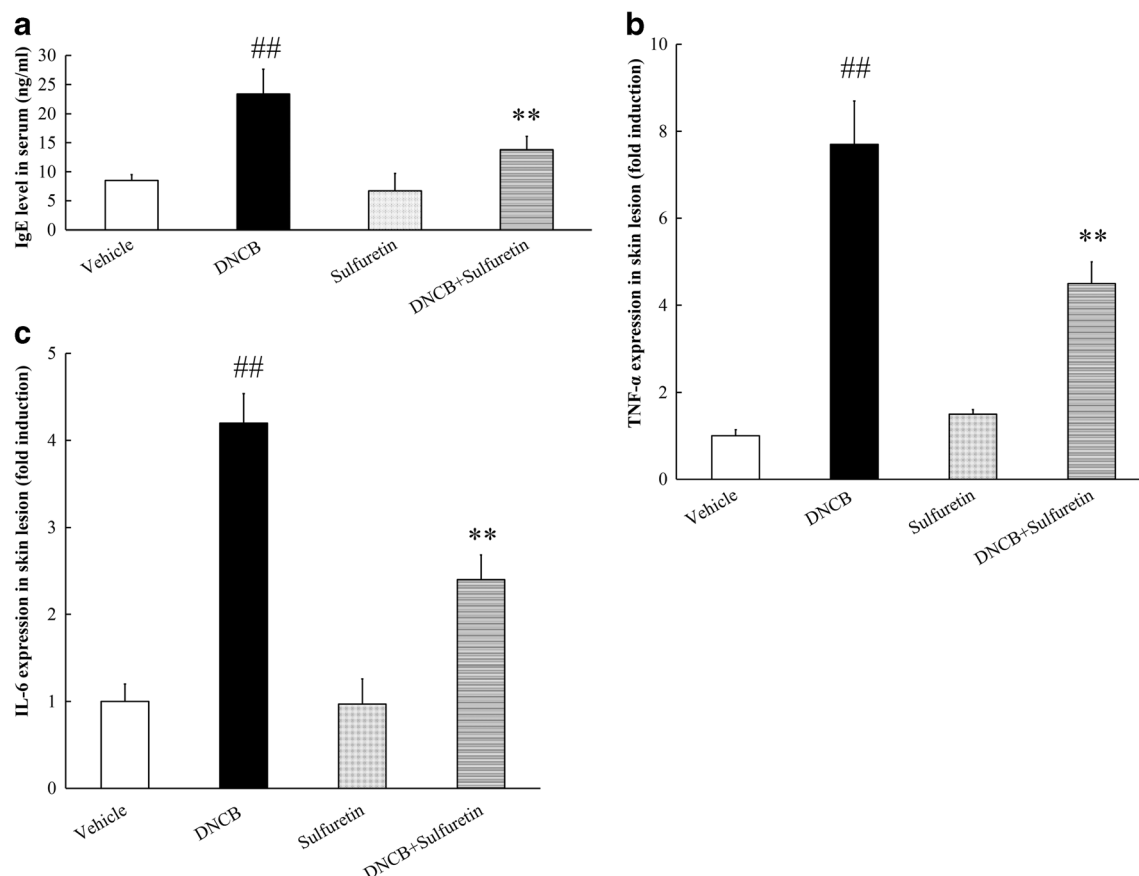


Fig. 4 Effects of sulfuretin on IgE serum level and the proinflammatory cytokine accumulation at the AD lesions of DNCB-treated mice. **a** Sample blood was collected on the last day of the study (day 18). The level of serum IgE was measured with ELISA. **b** The dorsal skin was collected on the last day of the study (day 18). Total RNA was extracted

from skin tissue with TRIzol reagent. The proinflammatory cytokines TNF α and IL6 were determined by real-time PCR. ^{##} $p < 0.01$, significantly different from control group ($n = 8$, for each experimental group); ^{*} $p < 0.05$, ^{**} $p < 0.01$, significantly different from DNCB-treated group

were consistent with previous scratching behavior and dermatitis score assessment, which suggested sulfuretin carried a suppressing effect on immune response elicited by DNCB in AD animal model.

Sulfuretin negatively regulates Th2 cell activity via downregulating GATA3

So far, our *in vitro* and *in vivo* results suggest sulfuretin may target Th2 cells to promote the resolution of AD-like skin lesion. To provide a mechanistic understanding on the therapeutic function of sulfuretin in AD, we sought to investigate the molecular regulation of sulfuretin on Th2 cells. GATA3 is a key transcriptional factor that determines Th2 cell lineage. It is also an essential regulator that promotes Th2 responses including the production of Th2 signature cytokines. Here, we hypothesized sulfuretin may regulate GATA3 activity in Th2 cells. Primary CD4⁺ cells harvested from mice were cultured and differentiated into Th2 lineage before being treated with different concentrations of sulfuretin (at 1 μ M, 5 μ M, 10 μ M, and 20 μ M). Intriguingly, as shown in Fig. 5a, starting

from 5 μ M, sulfuretin treatment significantly decreased GATA3 protein expression in primary Th2 cells. The suppression effect of sulfuretin on GATA3 was clearly dose dependent (Fig. 5a). This pattern was consistent with what we observed in the study on differentiated Th2 activity (Fig. 2). To confirm this trend, we also measured Th2 signature cytokines IL4 and IL5 production downstream of GATA3 activation. In agreement with western blot data, both 10 μ M and 20 μ M but not 1 μ M sulfuretin treatments readily downregulated the production of IL4 and IL5 in Th2 cells (Fig. 5b, c). Twenty micromolar sulfuretin exert the strongest inhibiting effect with more than 60% reduction on both IL4 and IL5. To a lesser extent, 5 μ M sulfuretin only marginally reduced these cytokines, which was in line with the effect on GATA3 expression (Fig. 5b, c). To further validate the role of GATA3 in sulfuretin-mediated regulation of Th2 cell activity, we knocked down GATA3 in Th2 cells and proceeded to measure its impact on Th2 cytokine production. As shown (Supplementary Figure S2A), IL4 and IL5 levels were markedly suppressed in GATA3 knocked down cells but not in control transfected with scramble siRNA. Furthermore, we

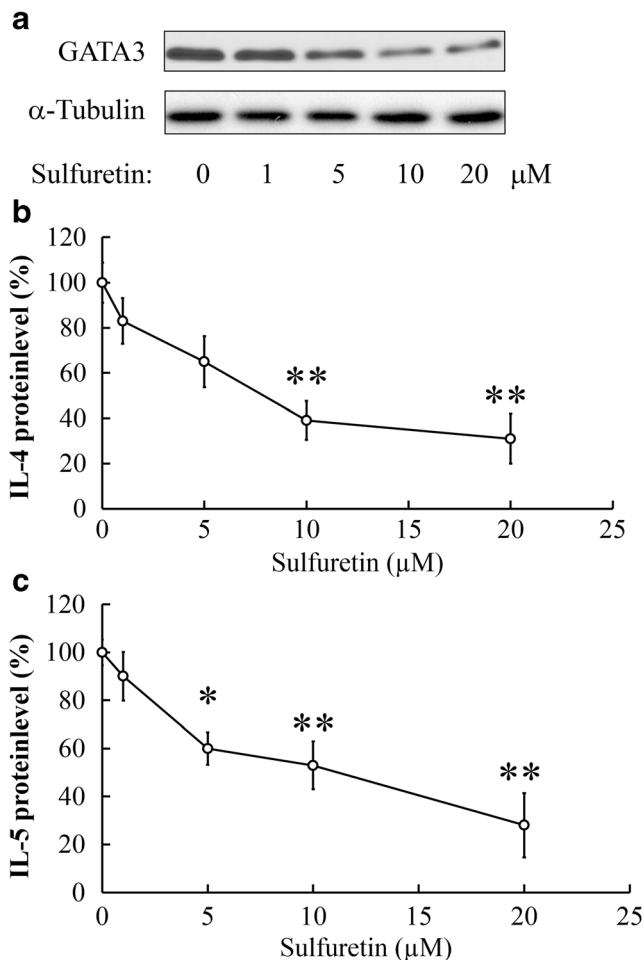


Fig. 5 Effects of sulfuretin on GATA3 pathway of primary mouse Th2 cells. **a** Primary naïve CD4⁺ cells were differentiated into Th2 cells in vitro. Then, the cells were treated with sulfuretin for the indicated concentrations for 48 h before harvesting. Cell lysate was subjected to SDS-PAGE to determine GATA3 expression. Data are representatives of at least three independent experiments. **b** IL4 and IL5 protein expression levels were also determined with ELISA. * $p < 0.05$, ** $p < 0.01$, significantly different from the control group ($n = 6$, for each condition)

treated GATA3 knocked down cells with sulfuretin. Interestingly, we observed the suppressing effect of sulfuretin on IL4 and IL5 production was abolished in knocked down cells as compared with control line (Supplementary Figure S2B&C), which strongly suggested sulfuretin effect mediated through GATA3 in Th2 cells. Overall, our results revealed sulfuretin a beneficial role in attenuating Th2 cell activity through downregulating GATA3 pathway in AD-like model.

Discussion

AD is among the most common chronic inflammatory skin disorders that often leads to allergic symptoms. The pathogenesis of AD involves dysfunctional skin barrier and

subsequent abnormal type 2 immune response that is provoked by the environmental allergens. Th2 cells are the main regulator for this pathogenic immune response in AD patients. Recent studies have made a significant progress to understand the molecular and cellular mechanism underlying AD process [2, 19]. However, we are still in lack of effective solution to cure AD. In our study, we have proposed an innovative therapy of using sulfuretin, a flavonoid compound found in *Rhus verniciflua*, to treat AD. Since Th2 cells are the master regulator that drives AD, we have firstly shown sulfuretin can downregulate Th2 cell function in vitro. And the effective concentrations we used in this study did not affect the vitality of Th2 cells. Next, we proceeded to in vivo AD model. We used DNCB to induce AD-like lesion on mice. And interestingly, we have demonstrated a topical application of sulfuretin can alleviate AD-like symptom in AD mouse model. We have also shown the improvement in cellular and molecular levels including the downregulation of serum IgE and proinflammatory cytokines at the skin lesion sites. In the last part of our study, we revealed an uncharacterized molecular function of sulfuretin in antagonizing Th2 cell functional activity via GATA3 pathway, which may contribute to its beneficial impact in treating AD-like symptom in mouse model.

So far, there have been many studies reporting therapeutic function of sulfuretin in treating multiple inflammation-associated disorders such as allergic airway inflammation [12], arthritis [20], and amyloid- β -induced neurotoxicity [21]. Although generally sulfuretin is considered as anti-inflammatory agent, it can target different pathways in different tissues. In lung inflammation, a single application of sulfuretin can reduce the recruitment of airway inflammatory cell and suppress the levels of a number of cytokines in bronchoalveolar fluid. The protective effect of sulfuretin is through inhibiting NF- κ B signaling pathway [12]. Similar to its role in controlling lung airway inflammation, sulfuretin has been demonstrated to suppress collagen-induced arthritis in mouse model. In arthritis mouse model, sulfuretin treatment can markedly reduce joint destruction and synovial inflammation. These physiological protective functions of sulfuretin are also mediated through targeting NF- κ B signaling pathway [20]. In comparison, in the study on evaluating sulfuretin in protecting against amyloid- β -induced neurotoxicity, the results indicated sulfuretin protects neuron cells from neurotoxicity through activating PI3/Akt and Nrf2/HO-1 signaling pathways, suggesting its therapeutic potential for managing Alzheimer's disease [21]. Interestingly, our investigation is the first study to report the therapeutic potential of sulfuretin in AD skin disorder. In this model, sulfuretin has been shown to suppress Th2 cell function via controlling GATA3 expression. In this study, we have identified an uncharacterized role of sulfuretin in the adaptive immune system. GATA3 is the key transcriptional factor promoting Th2 cells differentiation and also plays an essential role in activating Th2 through inducing Th2

cytokine production [22, 23]. More work needs to be done to elucidate regulatory mechanism of sulfuretin on GATA3. It will be also interesting to validate the therapeutic role of targeting GATA3 signaling with siRNA experiment.

Currently, the gold standard of successful management of AD is anti-inflammatory treatment. The topical application of corticosteroids is the mainstay of anti-inflammatory treatment. This approach mediates through the binding of corticosteroid with glucocorticoid receptor (GCR) in target cell. The activated GCR will inhibit many transcription factors such as AP-1 and NF- κ B that regulate a panel of proinflammatory cytokines. But concerns are arising due to the side effects induced with chronic use of corticosteroids. Topical application of calcineurin inhibitors including pimecrolimus and FK506 have been approved by the FDA for treating AD. Both drugs inhibit the phosphatase activity of calcineurin which subsequently affects the translocation of NF-ATp into the nucleus to promote transcription of various Th2 and Th1 cytokines. However, systemic absorption of these compounds during long-term usage is still not studied. There is still a potential side effect of causing skin cancer or increasing viral skin infection. The future challenges will be the discovery and invention of more effective and safer agents for treating AD in long-term usage [9, 24]. Flavonoids, as a class of nature products extracted from plant pigments, comprise a large group of polyphenolic compounds that contain high level of anti-inflammatory or antioxidant activities. They have been used as pharmaceutical supplements. The risk of consuming flavonoids is very low through epidemiological studies. Therefore, the flavonoids may represent a new class of therapeutic agents for treating inflammatory-related skin disorders such as AD [25, 26]. Past studies have reported astragalin as flavonoid molecule can inhibit IgE elevation and development of dermatitis in NC/Nga mice [27, 28]. Our study demonstrated another flavonoid candidate, sulfuretin as effective therapeutic agent in AD mouse model. The resolution of AD lesion by sulfuretin probably mediates through the regulation on Th2 activity. First, these studies support the hypothesis that flavonoids may act as an alternative treatment approach for AD. Although we did not observe any side effects during the treatment, a risk assessment study on the long-term topical application of sulfuretin or other flavonoid candidates is necessary to ensure the safety on health. Second, different flavonoid molecules may exert the therapeutic effects through different mechanisms. It will be intriguing to test their synergistic effects on AD treatment. Third, the study on AD animal model has shown promising result. However, animal study may not always be a valid predictor of human response to the proposed treatment for AD. Bioavailability and metabolic reaction of the compound may vary between mouse and human. The safety and efficacy dose ranges need to be re-established in human subjects. The next step will be a randomized, double-blind, and placebo-controlled clinical study to confirm the

therapeutic functions of flavonoids such as sulfuretin in AD patients.

Conclusion

This is the first animal study to demonstrate that sulfuretin has the therapeutic function in DNCB-induced AD model. As a novel natural compound, sulfuretin could robustly suppress the immune response in Th2 cells and relieve the AD symptoms through targeting GATA3 pathway in Th2 cells. Since prolonged topical administration of steroid in AD patients is commonly associated with multiple side effects, sulfuretin will be a potent therapeutic agent to treat chronic AD.

Compliance with ethical standards

All experimental studies involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Wuxi No.2 People's Hospital.

Conflict of interest The authors declare that they have no conflict of interest.

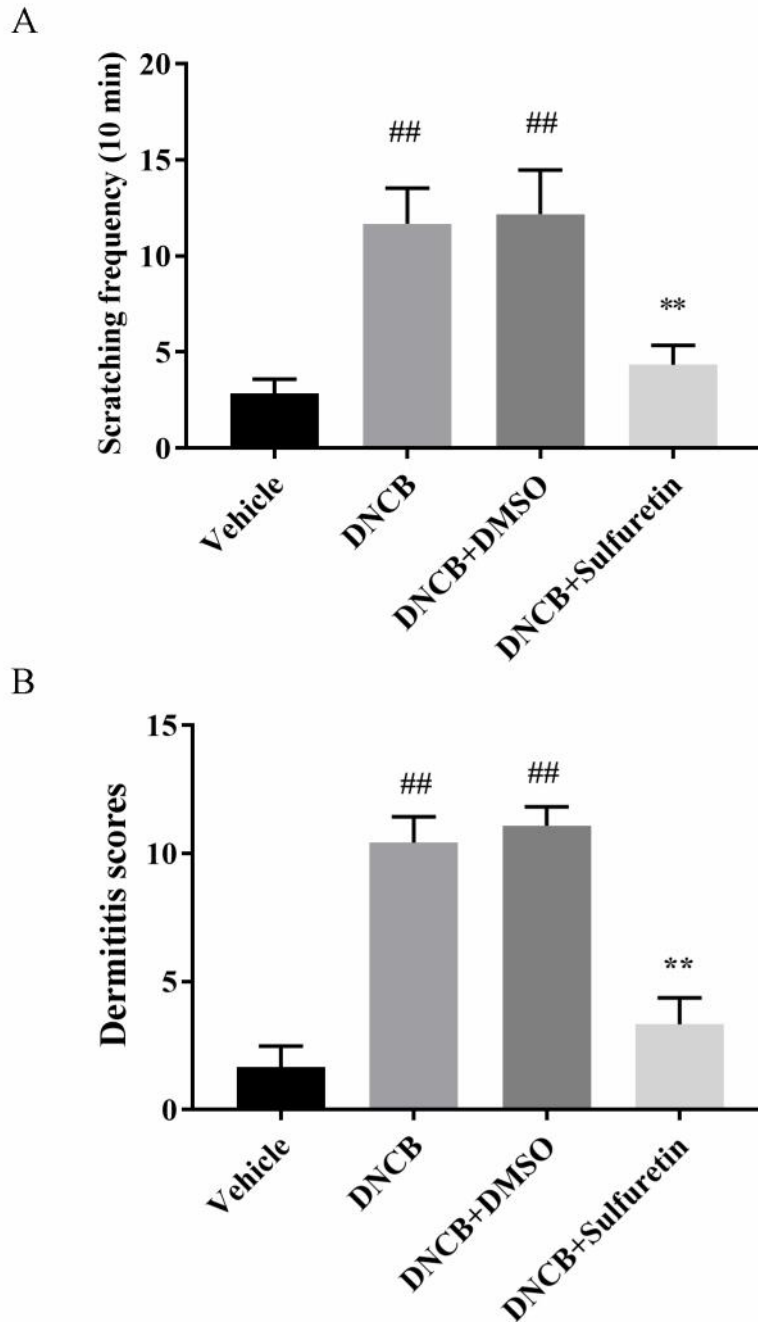
Research involving human participants and/or animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Not applicable.

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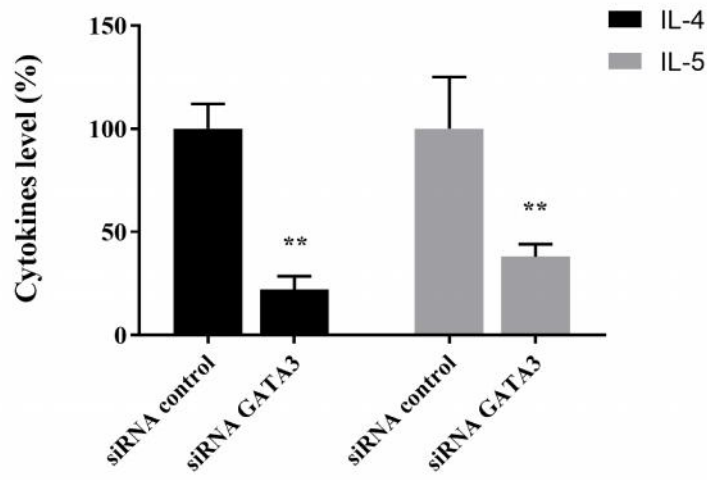
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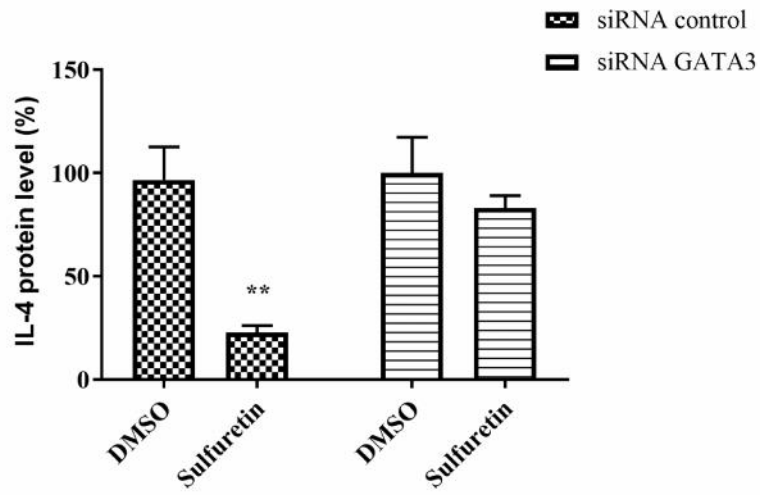


Supplementary Figure S1: Effect of DMSO or sulfuretin on DNCB-induced AD-like symptoms in mice. **A)** The scratching behavior was assessed on the last day of the study (day 18). Briefly, the number of scratching behaviors was counted for 10 min. Measurement was repeated for six times (60 min in total). **B)** The severity of clinical symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scarring/dryness) of the AD-like skin lesions was evaluated macroscopically as the dermatitis scores. The data are presented as the mean $\pm$ SD (n=6). ## p<0.01, significantly different from control group; *p<0.05, ** p<0.01, significantly different from DNCB treated group.

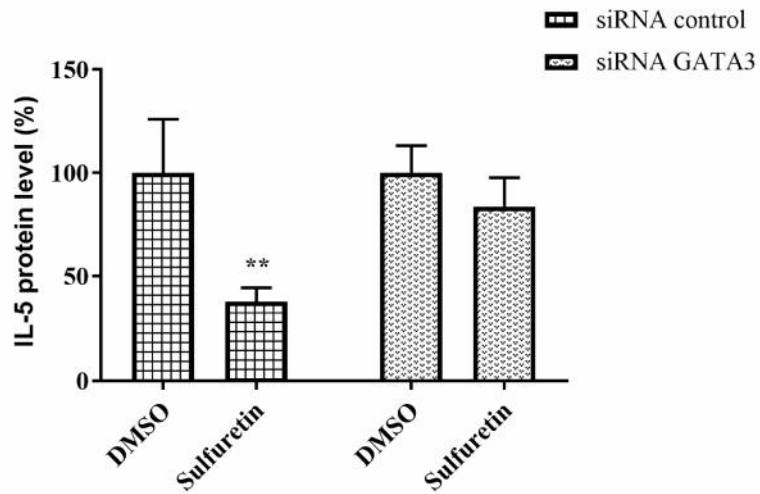
A



B



C



Supplementary Figure S2: GATA3 mediated the biological effect of Sulfuretin in Th2 cells.

A) Primary naïve CD4⁺ cells were differentiated into Th2 cells in vitro. Then the cells were transfected with either scramble siRNA or siRNA against GATA3 with Amaxa® Human T Cell Nucleofector® Kit. At 72h post transfection, IL4 and IL5 protein expression levels secreted from transfected Th2 cells were determined with ELISA. **B) & C)** Th2 cells transfected with either scramble siRNA or siRNA against GATA3 were treated with 20 µM sulfuretin for 48 respectively. Then, IL4 and IL5 cytokines secretion in the media were determined with ELISA. *p<0.05, ** p<0.01, significantly different from control group.