

RESEARCH ARTICLE

Treatment of allergic inflammation and hyperresponsiveness by a simple compound, Bavachinin, isolated from Chinese herbs

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Asthmatic inflammation is mediated by a type 2 helper T cell (Th2) cytokine response, and blocking Th2 cytokine production is proven to have a potent therapeutic effect against asthmatic inflammation. Using IL-4-green fluorescent protein (GFP) reporter mice, we demonstrated that Bavachinin, a single compound isolated from a Chinese herb, significantly inhibited Th2 cytokine production, including IL-4, IL-5 and IL-13. Notably, this compound almost completely blocked inflammation in the ovalbumin (OVA)-sensitized animal asthma model. Furthermore, we demonstrated that this chemical selectively affects the level of GATA-3, most likely by affecting the stability of GATA-3 mRNA. Our results demonstrate, for the first time, the potential therapeutic value of this single compound derived from Chinese herbs.

Cellular & Molecular Immunology advance online publication, 9 September 2013; doi:10.1038/cmi.2013.27

Keywords: asthma; bavachinin; IL-4

INTRODUCTION

Asthma is a common chronic inflammatory disease of the airways characterized by airway hyperresponsiveness, mucus overproduction, chronic eosinophilic inflammation, airway remodeling and episodic airway obstruction.^{1–6} Asthma is associated with an immune response biased toward type 2 helper T cells (Th2), which preferentially produce IL-4, -5, -9, -13 and -33.^{7–9} These cytokines in turn drive eosinophilic inflammation and tissue damage, leading to hyperresponsiveness and additional mediator release. The number of eosinophils in the lung is associated with disease severity and has been used to guide therapy in severe asthma models.

With much focus upon the importance of the T helper type 1 (Th1) *versus* Th2 balance, cytokines have become the central therapeutic targets in the development of biologics against asthma.^{10–13} The efficiency of these therapeutic approaches has been confirmed in a number of clinical trials.

Nebulized inhaled altrakincept (soluble, recombinant human IL-4 receptor) was shown to be effective in patients with mild to moderate asthma in phase I and II trials. Further phase II studies in progress use antibodies such as pascolizumab (SB240, 683) to block the interaction of IL-4 with the IL-4R α -chain (IL-4R α), which can bind either IL-4 or IL-13.^{14–19} Additionally, an IL-4 variant (pitrakinra) was used to inhibit the binding of IL-4 and IL-13 to IL-4R α in placebo-controlled allergen challenge studies and was shown to reduce the allergen-induced late-phase response and the need for rescue medication in asthmatic patients.⁴ Trials are now underway using an inhaled preparation. Humanized antibodies have also been developed to prevent the interaction of IL-4 and -13 with IL-4R α . Similarly, several other monoclonal antibodies against IL-13 have completed early safety trials in humans, including CAT-354 and AMG 317, and are undergoing clinical trials for asthma.²⁸

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Received: 19 January 2013; Revised: 1 April 2013; Accepted: 28 May 2013

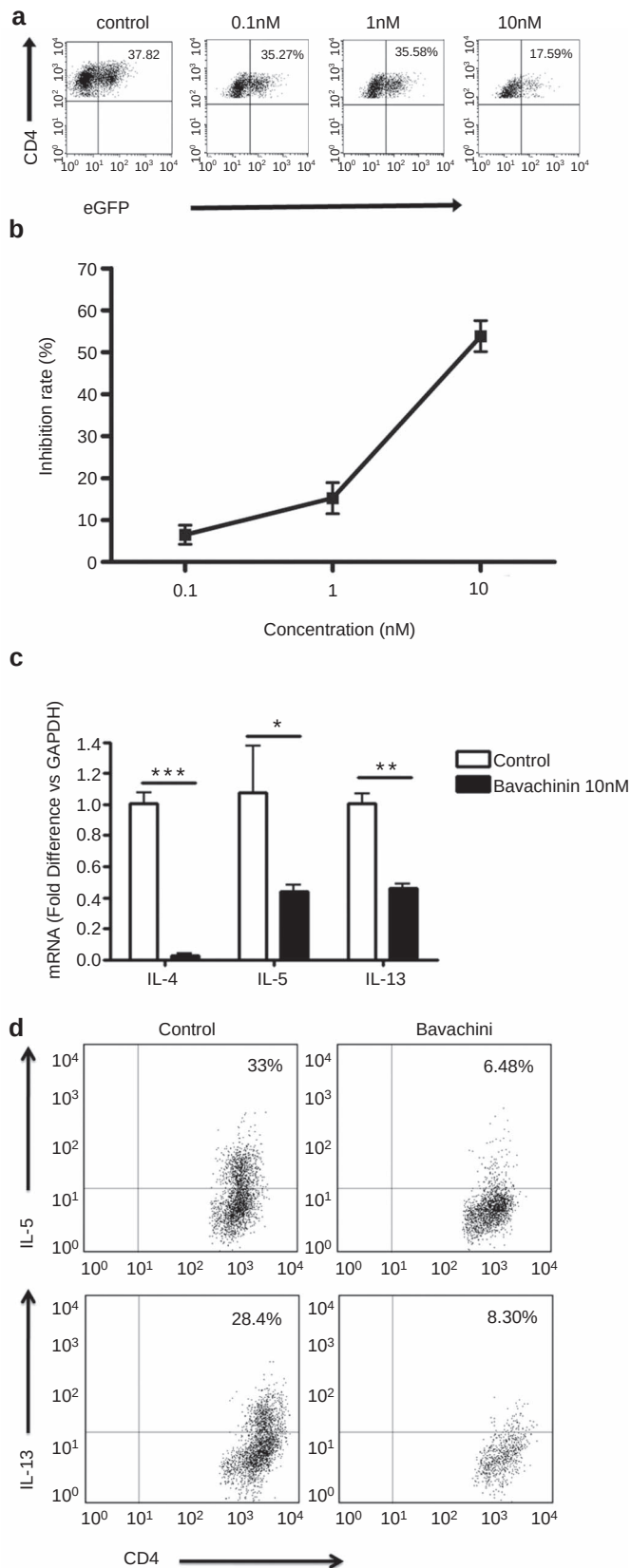


Figure 1 Bavachinin inhibits Th2 cells differentiation *in vitro*. Splenocytes from 4get mice were cultured under Th2 priming conditions for 48 h in the presence of the indicated concentration of

Bavachinin. One of at least three independent experiments is shown. **(a)** Representative FACS dot-plots show eGFP (IL-4)-positive cells within CD4⁺ T cells. **(b)** The dose curve of the inhibition rate of Bavachinin on IL-4 expression in CD4 T cells. Data represent the mean $\pm$ s.d. ($n=3$). **(c)** Expression of *IL-4*, *IL-5* and *IL-13* was quantified by real-time PCR. mRNA was extracted from splenocytes cultured under Th2 priming conditions in the presence of 10 nM Bavachinin for 48 h. Data represent the mean $\pm$ s.d. ($n=3$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$. **(d)** Representative FACS dot-plots show IL-5- and IL-13-positive cells within CD4⁺ T cells. eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; Th2, type 2 helper T cell.

Bavachinin is a natural small molecule from Chinese herb *Fructus Psoraleae*. It has been suggested to have an anti-inflammatory function. Recently, it has been reported that Bavachinin has anti-tumor activity. Using IL-4-green fluorescent protein-enhanced transcript (4get) mice,²⁹ we found that Bavachinin inhibits IL-4 expression as indicated by GFP expression as well as by quantitative PCR. Notably, we demonstrated that Bavachinin suppressed asthma in an animal model by inhibiting Th2 cytokine production.

MATERIALS AND METHODS

Mice

All experiments were performed with age- (6–8 weeks) and sex-matched mice (male or female mice were used). BALB/c wild-type mice were purchased from the Academy of Military Science (Beijing, China). IL-4-GFP mice, (4get mice, described previously) were kindly provided by Dr Richard Flavell from Yale University.

Reagents

Concanavalin A was purchased from Sigma Chemical (St Louis, MO, USA). Recombinant mouse IL-2 was purchased from R&D Systems (Minneapolis, MN, USA), and recombinant mouse IL-4 was purchased from Pepro Tech (Rocky Hill, NJ, USA). FITC-, APC- and PE-anti-mouse CD4 (clone GK1.5) were from Sungene Biotech (Tianjin, China); PE-conjugated anti-mouse GATA-3 (clone 4B10), APC-anti-mouse IFN- γ (clone XMG1.2), PE-conjugated anti-mouse IL-4 (clone 11B11), PE-conjugated anti-mouse/human IL-5 (Clone TRFK5), Alexa Fluor 647-conjugated anti-mouse IL-13 (clone eBioBA) and PE-conjugated anti-mouse STAT6 (clone J71-773.58.11) were all from BD Biosciences (San Jose, CA, USA).

Screening the effect of Bavachinin on IL-4 production using 4get mice

Spleen cells from 4get mice were cultured in a single-cell suspension in a 96-well plate at a density of 4×10^6 cells/ml (100 μ l per well) in RPMI medium. Concanavalin A (2.5 g/ml), IL-2 (2 ng/ml) and IL-4 (20 ng/ml) were used to differentiate the CD4⁺ T cells to a Th2 profile. Bavachinin was added at a concentration of 10 nM.²⁷

Experimental asthma model

Ages of 6 to 8-week-old female mice were sensitized and challenged essentially as described.³⁰ Sex- and age-matched mice

were injected intraperitoneally with grade V chicken OVA (Sigma-Aldrich, St Louis, MO, USA) mixed with 2 mg of aluminum hydroxide in saline once a week for two consecutive weeks, followed by a challenge with aerosolized OVA for 7 days 1 week after the second sensitization. Control groups were not sensitized or challenged. For Bavachinin treatment, the compound was given (50 mg/kg body weight) on day 22 for 7 days. Mice were then euthanized 24 h later for bronchoalveolar lavage for cytokine determination. Spleens were also collected for functional studies as indicated.

Histology

Lung tissues were harvested, fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, stained with hematoxylin and eosin, and images were acquired on a Leica DM3000 microscope using a $\times 20$ objective. The severity of asthma was quantified by the total area using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Intracellular staining

For IL-5 staining, spleen cells were isolated 6 h after OVA treatment and stimulated with PMA (50 ng/ml; Sigma) and ionomycin (1 mg/ml; Sigma) in the presence of GolgiStop (BD Biosciences) for 4.5 h. Cells were stained with antibodies against surface molecules and then fixed and permeabilized as described previously (RW) for intracellular cytokine staining. Intracellular GATA-3 and T-bet staining were performed using the Foxp3 Staining Buffer Set (eBioscience, San Diego, CA, USA).

Real-time PCR for gene transcription

Total RNA was extracted from lung or spleen cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using the Quantscript RT Kit (Tiangen, Beijing, China). mRNA expression was quantified using SYBR

Premix Hotmaster Taq (Tiangen, Beijing, China), and ribosomal protein large P0 (RW) gene expression was used as an internal control. The primer sequences used were as follows:

IL-4 forward: 5'-GAA AAC TCC ATG CTT GAA GAA-3', reverse: 5'-TCT TTC AGT GAT GTG GAC TTG 3'; IL-5 forward: 5'-GAG CCA CAT ACA CCT CCA ATC-3', reverse: 5'-TGC CTA ATA ATG GCA TAT-3'; IL-13 forward: 5'-AAT CAG ACA GTC CCT GGA AAG-3', reverse: 5'-CCG CCT ACC CAA GAC ATT-3'; T-bet forward: 5'-TTT CAT TTG GGA AGC TAA AG-3', reverse: 5'-GGC TGG TAC TTG TGG AGA GA- 3'; and GATA-3 forward: 5'-AGA GGT GGA CGT ACT TTT TAA C-3', reverse: 5'-AGA GAT CCG TGC AGC AGA G-3'.

Measurement of airway responsiveness to acetylcholine chloride

To measure airway responsiveness to acetylcholine chloride, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) 24 h following the final challenge. A plastic tube of 2 mm internal diameter was inserted into the trachea. The mice were induced to breathe mechanically with an animal ventilator (AniRes2003; Beijing SYNOL High-Tech Co. Ltd, Beijing, China) to a tidal volume of 6 ml/kg and frequency of 75 breaths per minute. The airway expiration resistance (Re) was measured and recorded.

Cell count in lungs

Cells staining positive for B cell activating factor (BAF) on slides stained with Wright-Giemsa stain were counted (200 cells/slide) to determine the counts for total cells, eosinophils and neutrophils.

ELISA

Mouse IL-4 and IgE ELISA kits were purchased from Biolegend (San Diego, CA, USA), and ELISA was performed according to the manufacturer's protocol.

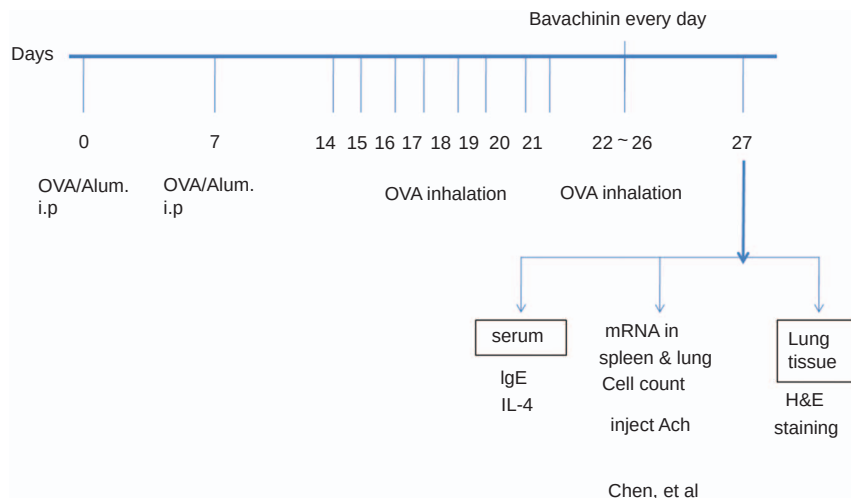


Figure 2 Experimental schematics of the asthma model. Mice received an i.p. injection of 100 μ g of grade V chicken OVA mixed with 2 mg of aluminum hydroxide in saline at days 0 and 7. Mice were then challenged with aerosolized OVA every day from days 14–21. Bavachinin was given every day from days 22–26. Mice were then euthanized at day 27 for analysis. i.p., intraperitoneal; OVA, ovalbumin.

Statistics

Statistical significance was evaluated by a two-tailed unpaired Student's *t*-test using InStat version 3.06 software for Windows (GraphPad, San Diego, CA, USA). Throughout the text, figures and legends, the following terminology is used to show statistical significance: **P*<0.05; ***P*<0.01 and ****P*<0.001.

RESULTS

Bavachinin inhibits Th2 cell differentiation

To investigate the effect of Bavachinin on Th2 cell differentiation, splenocytes from IL-4-GFP (4get) mice were activated with concanavalin A in the presence of IL-4 for 48 h, and the

percentage of GFP⁺ cells was analyzed by flow cytometry. We observed that Bavachinin significantly reduced the percentage of GFP⁺ cells in a dose-dependent manner (Figure 1a and b). This reduction reflected the inhibition of IL-4 expression, as analyzed by quantitative PCR. Similarly, Bavachinin inhibited the mRNA expression of both IL-5 and IL-13 (Figure 1c). These results indicate that Bavachinin inhibits Th2 cell cytokine expression.

Therapeutic effect of Bavachinin on asthma

Th2 cells and IL-4, IL-5 and IL-13 are critical in the pathophysiology of asthma. Based on the significant suppression of

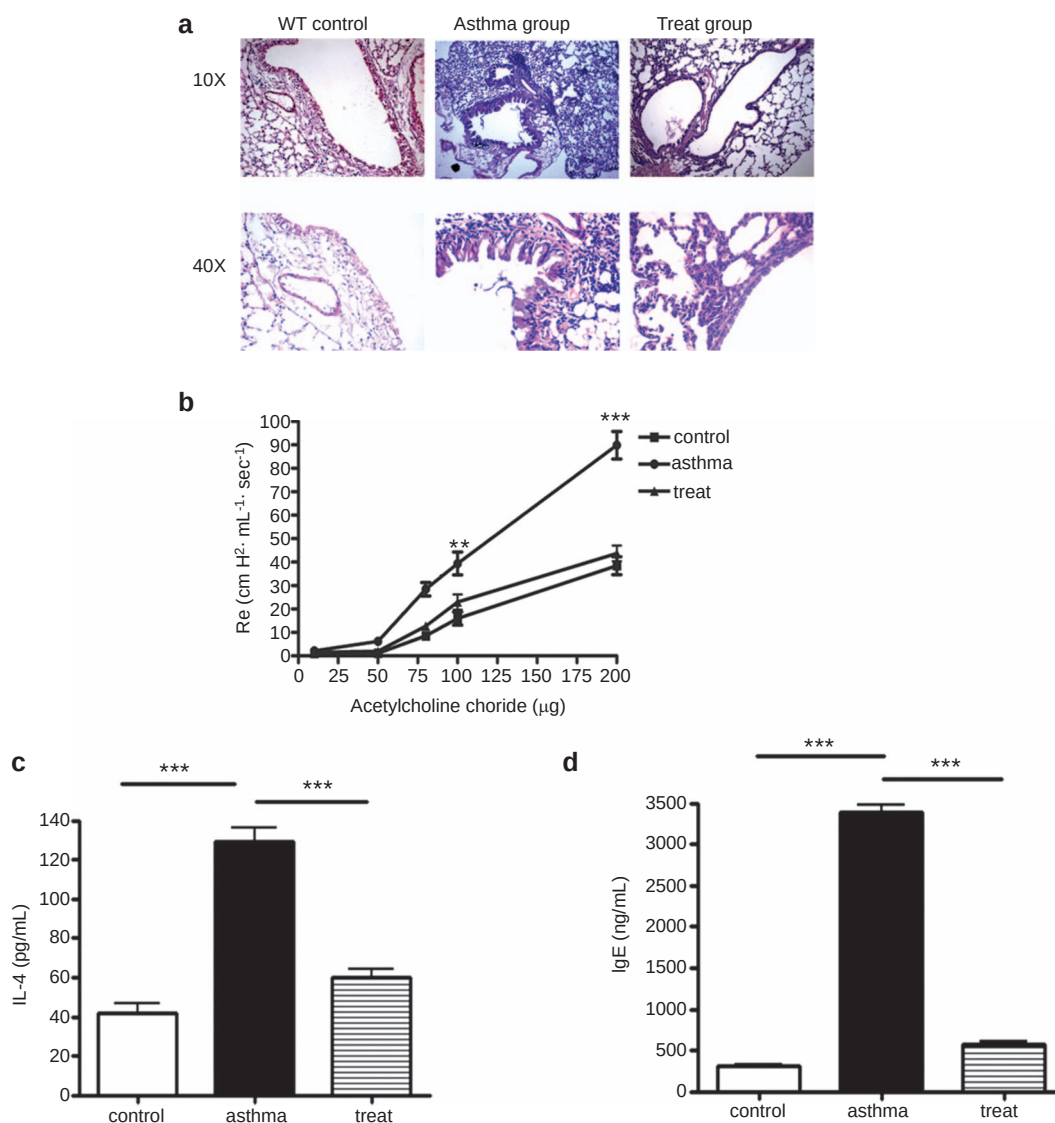


Figure 3 Bavachinin treatment ameliorates asthma in mice. One of at least three independent experiments is shown. **(a)** Histological features of airway inflammation in the control mice, asthmatic mice and Bavachinin-treated mice are shown by H&E staining. **(b)** Airway responsiveness to ACh in three different groups. Airway responsiveness was monitored by Re as described in the section on 'Materials and methods'. Statistical significance was determined between the Bavachinin-treated group and the asthmatic group. Data represent the mean $\pm$ s.d. (*n*=3). **(c)** Serum levels of IL-4 in the control mice, asthmatic mice and Bavachinin-treated mice. Data represent the mean $\pm$ s.d. (*n*=3). **(d)** Serum levels of IgE in the control mice, asthmatic mice and Bavachinin-treated mice. Data represent the mean $\pm$ s.d. (*n*=3). ***P*<0.01; ****P*<0.001. Ach, acetylcholine chloride; H&E, hematoxylin and eosin; Re, expiration resistance.

Th2 cell differentiation and cytokine production, we studied the potential effect of Bavachinin on asthma *in vivo*. Bavachinin was administered through oral gavage in PEG400 (50 mg/kg body weight) every day for 7 days starting on the last day of challenge (Figure 2). We observed that the thickness of the bronchial wall and the area of airway smooth muscle were significantly reduced in mice treated with Bavachinin compared with asthmatic mice treated with phosphate-buffered saline (Figure 3a). Treated asthmatic mice demonstrated decreased airway hyperreactivity compared with control mice after challenge (Figure 3a). Furthermore, administration of Bavachinin resulted in a significant downregulation of ACh responsiveness compared with asthmatic mice (Figure 3b). To further confirm the effect of Bavachinin *in vivo*, serum levels of IL-4 and IgE were also measured. Indeed, treatment by Bavachinin significantly inhibited the serum levels of both

IL-4 and IgE, which are hallmarks of asthma (Figure 3c and d). Taken together, our results demonstrate for the first time the therapeutic effect of Bavachinin in an animal model of asthma.

Bavachinin significantly reduces Th2 cytokine expression and cellular infiltration in the lung

To validate the therapeutic effect of Bavachinin, the lung tissues of asthmatic mice were further analyzed. We observed that Bavachinin suppressed Th2 cytokine expression in the lung tissues, with similar patterns for IL-4, IL-5 and IL-13 (Figure 4a). We also assessed the infiltration of eosinophils and neutrophils in the lung. The numbers of eosinophils and neutrophils in the lung interstitium were significantly reduced after administration of the compound compared with the asthmatic mice (Figure 4b). Our results further support the

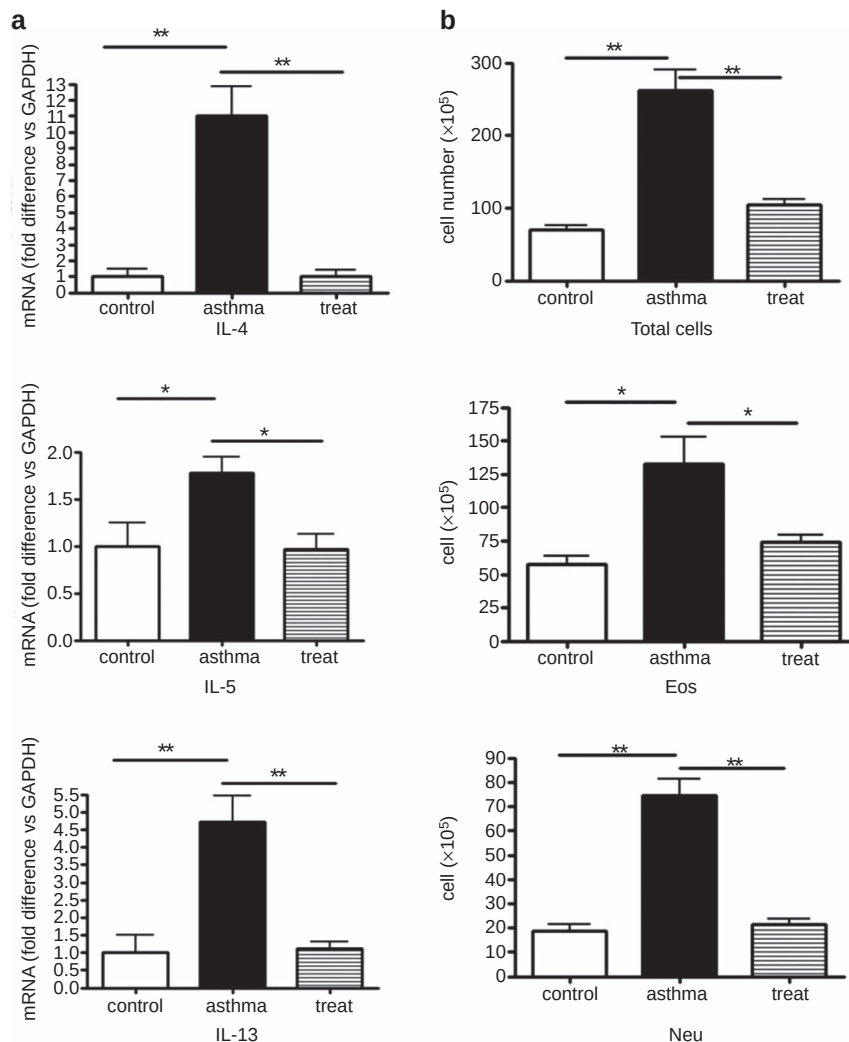


Figure 4 Bavachinin treatment inhibits lung inflammation in a mouse asthma model. One of three independent experiments is shown. (a) Histogram showing the expression levels of *IL-4*, *IL-5* and *IL-13* in the lungs of mice in the three different groups. mRNA was extracted from the lung tissues, and gene expression was quantified by real-time PCR. Data represent the mean $\pm$ s.d. ($n=3$). (b) The number of total cells, Eos and Neu was counted in the lungs of mice in the three different groups. Data represent the mean $\pm$ s.d. ($n=3$). * $P<0.05$; ** $P<0.01$. Eos, eosinophils; Neu, neutrophils.

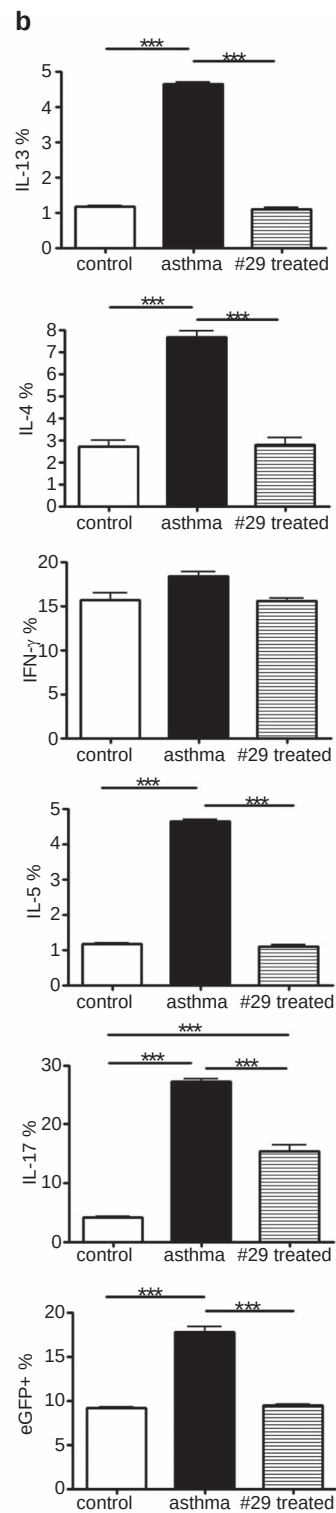


Figure 5 Bavachinin treatment inhibits Th2 cell generation in a mouse asthma model. (a) Representative FACS dot-plots showed IL-5, IL-13, IL-4, IFN- γ , IL-17 and eGFP (IL-4) positive cells in CD4⁺ T cells. Splenocytes from mice receiving Bavachinin treatment were *ex vivo* stimulated with PMA/ionomycin before staining. Cell percentages are summarized in histograms (b). Data represent the mean $\pm$ s.d. ($n=3$). One of three independent experiments is shown. *** $P<0.001$. eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; Th2, type 2 helper T cell.

therapeutic effect of Bavachinin on the pathogenesis of asthma through altering the local cytokine expression.

Treatment with Bavachinin selectively reduces systemic Th2 cytokine levels

To test the effect of Bavachinin on the global impact of CD4⁺ T-cell differentiation, splenocytes from asthmatic mice were activated with PMA/ionomycin for intracellular cytokine staining. Treatment with Bavachinin significantly reduced IL-4, IL-5 and IL-13 production from CD4⁺ T cells, whereas IFN- λ and IL-17 production was not affected (Figure 5). In summary, administration with Bavachinin selectively altered the Th2 differentiation induced by OVA immunization.

Bavachinin blocks GATA-3 function by reducing the stability of GATA-3 mRNA

Given the critical role of T-bet and GATA-3 in Th1/Th2 differentiation, we wanted to analyze the effect of Bavachinin on these two transcription factors. CD4⁺ T cells isolated from splenocytes of wild-type mice were primed using Th1/Th2 differentiation conditions in the presence or absence of Bavachinin, as described previously.²⁴ We observed that Bavachinin selectively inhibited IL-4 production without affecting secretion of IFN- γ (Figure 6a). Consistently, Bavachinin had no effect on the expression level of T-bet. In sharp contrast, Bavachinin significantly reduced the level of GATA-3 upon Th2 priming conditions (Figure 6b). Given the critical roles of T-bet and GATA-3 in Th1/Th2 differentiation,²⁴ we measured T-bet and GATA-3 expression under Th1 and Th2 priming conditions with Bavachinin by intracellular staining *in vitro*. At day 2 post-priming, GATA-3 was significantly inhibited by Bavachinin (Figure 6b). However, Bavachinin could not inhibit T-bet in either Th1 or Th2 culture conditions (Figure 6b), indicating that T-bet may not be involved in the mechanisms of Th2 inhibition by Bavachinin. To study the inhibition of GATA-3 expression by Bavachinin, we measured the effect of Bavachinin on GATA-3 mRNA levels at different time points under Th2-differentiating conditions. Bavachinin suppressed GATA-3 mRNA at the 2–4 h time points, consistent with the subsequent reduction of GATA-3 protein synthesis (Figure 6C).

STAT6 is known to be an important transcription factor for GATA-3 expression. To study the mechanisms underlying Bavachinin-mediated GATA-3 suppression, we then postulated that Bavachinin might suppress STAT6 function. However, we did not observe any change in pSTAT6 expression levels by intracellular staining. Thus, these results collectively suggest that Bavachinin suppresses GATA-3 mRNA levels. Bavachinin may suppress pSTAT6 binding or co-activating functions independent of pSTAT6 protein levels (Figure 6d).

DISCUSSION

Asthma is a threat against human health. Although great efforts have been made to study the molecular mechanisms of asthma, an effective and economic therapy is still out of reach. Several Chinese herbs have been used for treating asthma in ancient China, and one of them, Bavachinin, has been indicated for the treatment of asthma and other diseases, such as hypertension and cardiovascular disease.²¹ Bavachinin is a constituent of Bavachini; however, the effect of Bavachinin on asthma is still unknown. In this report, we demonstrate that Bavachinin significantly reduces asthmatic inflammation and hyperresponsiveness through the selective inhibition of Th2 cytokine production.

It is well known that a Th2 response is responsible for the pathogenesis of asthmatic inflammation, and blocking Th2 cytokine production or interfering with their interaction with their receptors shows promising clinical benefits for patients with asthma. Using our 4get mice, we observed that Bavachinin significantly inhibited Th2 cytokine production by CD4⁺ T cells at both the transcription and protein levels (Figures 1 and 6). Based on these results, we hypothesized that this chemical might have a therapeutic effect on asthmatic inflammation. To test our hypothesis, an OVA-sensitized animal model of asthma was adopted. Indeed, Bavachinin significantly inhibited inflammation in the bronchial tubes, reduced cellular infiltration and increased resistance against airway expiratory (Figure 3). The therapeutic effects of this compound were most likely mediated by inhibition of local Th2 cytokines (Figure 4) as well as systemic Th2 cytokine production (Figure 5). In our preliminary studies, the therapeutic effect was dose-dependent, and at the dose of 50 mg/kg body weight, no signs of toxicity (weight loss, elevated levels of ALT and urine protein) were observed (data not shown). Further studies are in progress to study the bioabsorbance and the pharmacokinetics of this compound.

One potential mechanism for the effect of this compound on Th2 cytokine production is through GATA-3. GATA-3 is a critical transcription factor for Th2 differentiation, and the expression levels of GATA-3 can be regulated at both the transcription and post-transcription levels. Notably, Bavachinin significantly reduced the GATA-3 protein level, most likely through reducing the stability of GATA-3 mRNA (Figure 6). In contrast, this compound had no effect on T-bet, which may explain the observed lack of effect on IFN- γ production (Figure 6). In our experiments, Bavachinin had little effect on the level of p-STAT6 (Figure 6), further supporting the effect of this chemical on the stability of GATA-3 mRNA. Notably, our group recently defined a small molecule, derived from the tylophorine analog NK-007, which significantly reduces TNF- α production by affecting the stability of

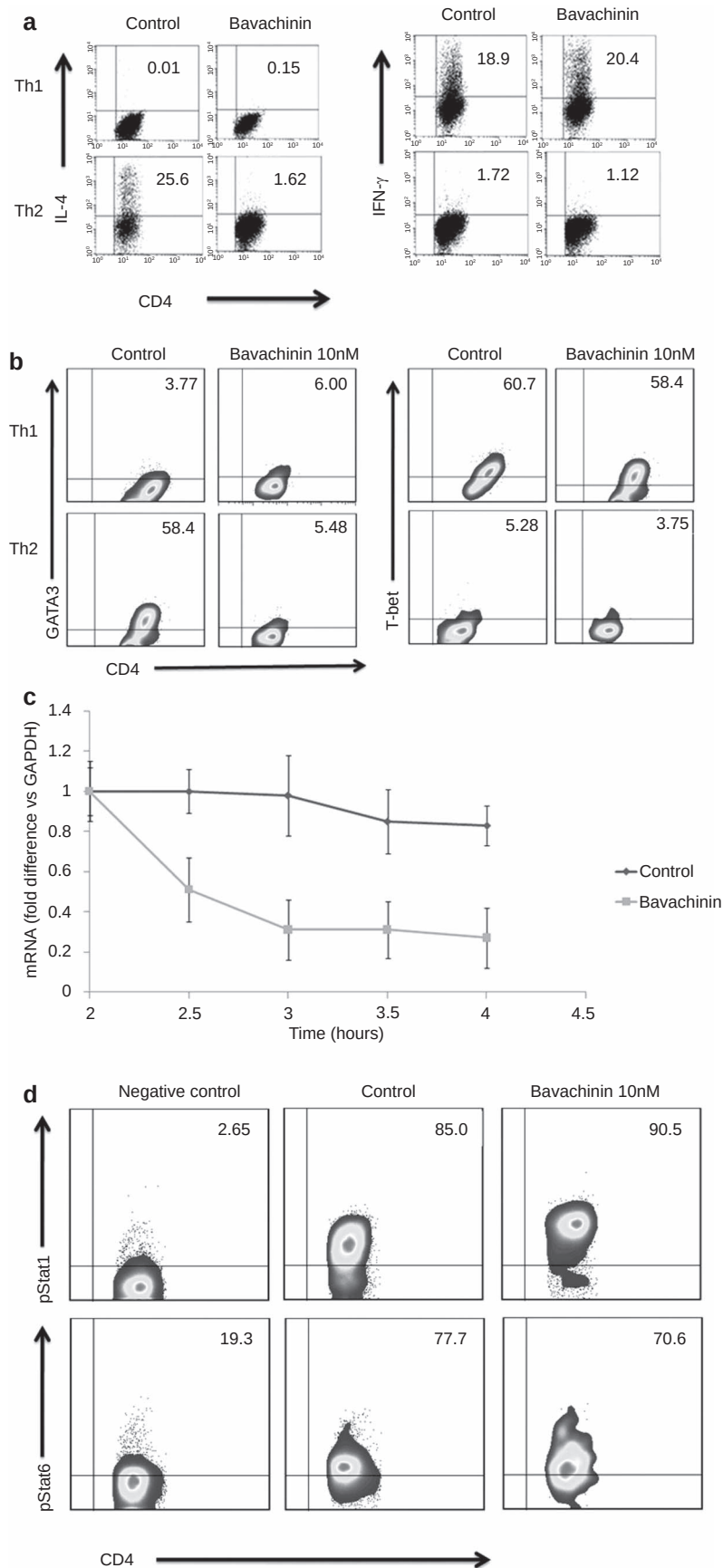


Figure 6 Bavachinin inhibits the expression of Th2-related transcriptional factors. One of three independent experiments is shown. CD4⁺ T cells were cultured under Th1 or Th2 priming conditions for 48 h in the presence or absence of 10 nM Bavachinin. (a) Representative FACS dot-plots show IL-4 and IFN- γ expression. (b) Representative FACS dot-plots show GATA-3 and T-bet expression. (c) The mRNA level of *GATA-3* in cultured cells. Data represent the mean $\pm$ s.d. ($n=3$). (d) Representative FACS dot-plots show the phosphorylation levels of STAT1 and STAT6. FACS, fluorescence-activated cell sorting; Th2, type 2 helper T cell.

TNF- α mRNA through p38/p38 MAP kinase, its downstream MAPK-activated protein kinase 2 and tristetraprolin, a well-characterized RNA binding protein.^{22–24} NK-007 significantly reduced the levels of p-p38 and MAPK-activated protein kinase 2, which in turn enhanced the expression of tristetraprolin, which might be the possible cause of the instability of the TNF- α mRNA in NK-007-treated cells.^{25,26} Further studies are needed to see whether Bavachinin affects *GATA-3* mRNA through this pathway.

Bavachinin has been shown to have potent anti-tumor and anti-inflammation effects in animal models.²⁰ Our study demonstrates, for the first time, the therapeutic effect of this compound in Th2-mediated asthmatic inflammation. Given the potency and enriched source of this compound, further studies to explore the pharmacological application of this compound to Th2-mediated inflammation might provide a lead candidate for a novel therapeutic agent against asthma.

ACKNOWLEDGEMENTS

This work was funded by the National Natural Science Foundation of China (30890143), the Key Program for International S&T Cooperation Projects of China (2010DFB34000) and the National Basic Research Grant of China (2010CB529104) to ZY.

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