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Florfenicol inhibits allergic airway inflammation in mice by p38 MAPK-mediated phosphorylation of GATA 3

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Received 6 August 2010; accepted with revision 15 November 2010

KEYWORDS

Florfenicol;
Allergic asthma;
Airway inflammation;
MAPK;
GATA 3

Abstract Florfenicol has been shown to possess anti-inflammatory activity. However, its possible use for asthma has not yet been studied. First we investigated the anti-inflammatory properties of florfenicol using mice asthma model. BALB/c mice were immunized and challenged by ovalbumin. Treatment with florfenicol caused a marked reduction in inflammatory cells and three Th2 type cytokines in the bronchoalveolar lavage fluids of mice. The levels of ovalbumin-specific IgE and airway hyperresponsiveness were significantly altered after treatment with florfenicol. Histological studies using H&E and AB-PAS staining demonstrate that florfenicol substantially inhibited ovalbumin-induced inflammatory cells infiltration in lung tissue and goblet cell hyperplasia in the airway. These results were similar to those obtained with dexamethasone treatment. We then investigated which signal transduction mechanisms could be implicated in florfenicol activity. Our results suggested that the protective effect of florfenicol was mediated by the inhibition of the p38 MAPK-mediated phosphorylation of GATA 3.

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Abbreviations: H&E, hematoxylin and eosin; AB-PAS, alcian blue-periodic acid-Schiff; MAPKs, mitogen activated protein kinases; OVA, ovalbumin; AHR, airways hyperresponsiveness; IL, interleukins; IgE, immunoglobulin E.

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1. Introduction

Allergic asthma is characterized clinically by the hypersecretion of mucus, obstruction and inflammation of the airways and enhanced bronchial reactivity (airways hyperresponsiveness, AHR) to spasmogenic stimuli [1]. The T helper 2 (Th2)-type cytokines interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), produced by activated CD4⁺ T cells, play an important role in the pathogenesis of asthma by

controlling the process of immunoglobulin E (IgE) production and the differentiation and activation of eosinophils [2–4]. Th2 cytokines are regulated by the zinc finger transcription factor GATA-3, which determines Th2 cell differentiation and selectively activates the promoters of IL-4, IL-5, and IL-13 through chromatin remodeling [5–7]. In order for GATA-3 to regulate gene expression, it must translocate from the cytoplasm into the nucleus to access its target genes. A previous study has shown that p38 mitogen activated protein kinase (MAPK) play a critical role in phosphorylating GATA-3 to enhance its interaction with importin- α and subsequent transport into the nucleus [8]. In addition to p38, ERK pathway has also been implicated in the regulation of IL-5 and other cytokines [9].

Prevalence of allergic asthma is on the rise throughout the world, especially in western countries [10]. Despite a growing understanding of its pathophysiology, there is a need for innovative anti-inflammatory treatments and in this context antibiotics represent a potentially useful alternative. The doses of antibiotics used for the treatment of respiratory diseases are lower than the minimum inhibitory concentration for the most common respiratory pathogens, which suggests that the efficacy of antibiotics in asthma results not only from antimicrobial effects, but also from their immunomodulatory/anti-inflammatory activities [11]. In recent years, many reports suggest that some antibiotics also possess distinct anti-inflammatory properties in addition to antimicrobial effects [12]. Moreover, beneficial effects of antibiotics in asthma have been known for almost 50 years, especially with macrolides and ketolides [13].

Florfenicol is a new type of broad-spectrum antibiotic that is used in the veterinary clinic. It has been widely used to treat a broad array of infectious diseases, such as bovine and porcine respiratory tract infections [14], actinobacillus pleuropneumonia in pigs [15], and infectious bovine keratoconjunctivitis [16,17], and it has been shown to be highly effective and safe. In our previous study, we demonstrated that florfenicol has anti-inflammatory effects *in vitro* and *in vivo* in a mouse model of endotoxemia and acute lung injury [18,19]. However, it is unclear if the observed beneficial effects were related to the antimicrobial or anti-inflammatory properties considering that bacteria can often be isolated from the airways of patients with these diseases.

Different results from evaluations of infectious and non-infectious mouse airway inflammatory models show a distinct anti-inflammatory role for florfenicol. Florfenicol treatment in mice also reduced the airway inflammation triggered by inhaled LPS [19]. However, there is no available study that evaluates the effects of florfenicol treatment on allergic airway inflammation in a mouse model of allergic asthma. Therefore, we have employed a non-infectious mouse airway inflammatory and bronchial asthma model to examine if there is a distinct anti-inflammatory activity of florfenicol and further investigated the mechanisms involved in MAPK pathway and nuclear translocation of GATA-3.

2. Materials and methods

2.1. Reagents

The IL-4, IL-5 and IL-13 ELISA kits were purchased from Biologend (California, USA). Mouse polyclonal anti-GATA-3,

rabbit anti-JNK polyclonal antibody, rabbit anti-ERK polyclonal antibody, rabbit anti-p38 polyclonal antibody, mouse anti-phospho-JNK monoclonal antibody, mouse anti-phospho-ERK monoclonal antibody, and mouse anti-phospho-p38 monoclonal antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). Florfenicol, dimethyl sulfoxide (DMSO) and OVA (Grade δ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Peroxidase-conjugated Affinipure Goat Anti-Mouse IgG (H+L) and Peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) were purchased from PTG (Chicago, IL, USA).

2.2. Animals

Female BALB/c mice, weighing approximately 18–20 g, were purchased from Shanghai Jingke Industrial Co., LTD (Certificate SCXK2003-0003) (Shanghai, China) and bred under specific pathogen-free conditions. The mice were housed in microisolator cages and received food and water *ad libitum*. The laboratory temperature was 24 ± 1 °C, and relative humidity was 40–80%.

All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

2.3. Sensitization and challenge with OVA and treatment

Groups of 10 animals were used for each experimental condition. Mice were sensitized by intraperitoneal injection of ovalbumin plus alum on days 0 and 14, followed by challenge with intranasal ovalbumin (or phosphate buffered saline, PBS, for controls, Con) on days 23, 24, 25 and 26, respectively. Lung cells were harvested on day 27. Mice were given an oral administration with PBS or florfenicol (5, 25, 100 mg/kg) or intraperitoneally injected with 2 mg/kg dexamethasone (DXM) on days 23, 24, 25 and 26, 1 h prior to each corresponding OVA administration.

2.4. Collection of blood and bronchoalveolar lavage

Twenty-four hours after the last inhalational exposure, mice were anesthetized and bled via the brachial plexus for collection of blood samples used to estimate IgE production. Bronchoalveolar lavage (BAL) was performed twice by intratracheal instillation of 500 μ L of PBS. The lavage fluid was centrifuged, and the supernatants were kept at 70 °C until use for cytokine measurements. The cell pellets were resuspended in 1 ml of PBS and used for total and differential cell counts. The total number of cells in bronchoalveolar lavage fluid was counted in a haematology counter (Sysmex SF 3000, Sysmex Co., Kobe, Japan). Eosinophils, neutrophils and macrophages were counted in BAL-fluid using cytopspins (centrifuged preparations) stained with the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). A total of 200 cells in each sample were counted.

2.5. Mouse anti-OVA IgE ELISA

To define serum levels of OVA-specific IgE, an ELISA analysis was performed using a mouse-specific anti-IgE

antibody. Briefly, microplate wells were coated with 1% OVA in coating buffer (0.05 M sodium carbonate–bicarbonate, pH 9.6) overnight at 4 °C. The wells were then incubated with blocking buffer (1% BSA in PBS, pH 7.2) at room temperature for 1 h and then washed. Diluted (1/10) serum samples were then introduced to the microplate, which was then incubated at room temperature for 2 h, washed and incubated with Biotin anti-mouse IgE, followed by extravidin-peroxidase at room temperature for 30 min and with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 30 min. The enzymatic reaction was stopped with 2 M H₂SO₄, and absorbance was read at 450 nm. Concentrations were determined in duplicates for each sample.

2.6. Measurement of cytokines

Levels of IL-4, IL-5 and IL-13 were quantified in BALF by enzyme immunoassays performed according to the manufacturer's protocol (BioLegend, Inc. Camino Santa Fe, Suite E San Diego, CA, USA).

2.7. Lung histology

Histopathological evaluations were performed on mice. Left lungs were removed by dissection and fixed in 4% paraformaldehyde. Lung tissues were sectioned, embedded in paraffin, and cut into 3- μ m sections. Tissue sections were then stained with H&E for general morphology [20] and with AB-PAS for the identification of goblet cells in the epithelium [21].

2.8. Measurements of airway hyperresponsiveness

Mice were anesthetized, and tracheotomy was performed as described [22]. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Airway resistance (RI) and lung compliance (C_{dyn}) in response to increasing concentrations of methacholine were recorded using a whole-body plethysmograph chamber (Buxco, Sharon, CT) as described [22]. RI is defined as the pressure driving respiration divided by flow. C_{dyn} refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. Results are expressed as the percentage of the respective basal values.

2.9. Western blot analysis of MAPK and GATA-3

Lung tissues were added to lysis buffer and homogenized. Nuclear and cytoplasmic fractions of lung were prepared as previously described [23]. Whole cell lysates were prepared in 40 mL of lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6% NP-40, 1 mM Na₃VO₄, 20 mM β -glycerophosphate, 1 mM fluoride, 2 mM *p*-nitrophenyl phosphate and 1: 25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany)). After the lysates were incubated on ice for 30 min, they were centrifuged (12,000 $\times$ g at 4 °C) for 5 min to obtain the cytosolic fraction. Protein concentrations were determined using the Bradford

assay (Bio-Rad, Munich, Germany) before storage at –70 °C. Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were then washed in Tris–Tween buffered saline (TTBS, 20 mM Tris–HCl buffer, pH 7.6, containing 137 mM NaCl and 0.05% (vol/vol) Tween 20), blocked overnight with 5% (wt/vol) nonfat dry milk, and probed according to the method described by Towbin et al. [24] with specific antibodies against GATA-3, pJNK, p42/44ERK, p38 antibodies, monoclonal phospho-specific antibodies to pJNK, p42/p44 ERK, and p38 in 5% (wt/vol) BSA dissolved in TTBS. With the use of a peroxidase-conjugated secondary anti-mouse antibody, bound antibodies were detected by ECL plus (GE Healthcare Buckinghamshire, UK).

2.10. Statistical analysis

All values were expressed as mean $\pm$ standard error of the mean (SEM). Differences between mean values of normally distributed data were assessed by one-way analysis of variance (ANOVA; Dunnett's *t*-test) and Student's *t*-test. Statistical differences were considered significant if *P* < 0.05.

3. Results

3.1. Effect of florfenicol on the ovalbumin-specific IgE levels in serum and the release of IL-4, IL-5 and IL-13 in BALF

As expected, OVA-immunized mice had higher levels of serum anti-OVA IgE antibodies (0.37 $\pm$ 0.06) compared to control mice (0.026 $\pm$ 0.01). A significant reduction in OVA-specific IgE antibodies was observed in mice treated with florfenicol or dexamethasone in a dose-dependent manner (Fig. 1A). As shown in Fig. 1B, OVA sensitization and challenge induced significant elevation of cytokines (to 186.4 $\pm$ 24.47 pg/mL for IL-4 to 110.01 $\pm$ 12.1 pg/mL for IL-5 and to 85.3 $\pm$ 6.27 pg/mL for IL-13) in the BALF. Treatment with florfenicol significantly decreased the concentrations of IL-4, IL-5, and IL-13 in a dose-dependent manner.

3.2. Effect of florfenicol on inflammatory cell recruitment and airway hyperresponsiveness to methacholine

Inflammatory cell levels (i.e., total cells, eosinophils, neutrophils and macrophages) in bronchoalveolar lavage fluid were significantly elevated in OVA-challenged mice versus the negative control. Florfenicol treatment significantly reduced the number of total cells, eosinophils, neutrophils, and macrophages in a dose-dependent manner (Fig. 2A). Airway hyperresponsiveness was assessed by measuring RI and C_{dyn} in response to increasing doses of methacholine administration in mechanically ventilated mice. The RI of ovalbumin-challenged mice was dramatically increased by methacholine administration compared to that of negative control mice (Fig. 2B). However, this upregulation of RI in ovalbumin-challenged mice was effectively reduced by florfenicol (25 mg/kg or 100 mg/kg) pretreatment. C_{dyn}

was dramatically decreased in ovalbumin-challenged mice compared to negative control mice. This downregulation was also effectively prevented by florfenicol (25 mg/kg or 100 mg/kg) pretreatment (Fig. 2C).

3.3. Effect of florfenicol on airway inflammation, goblet cell hyperplasia and mucus production

In order to assess the anti-inflammatory effect of florfenicol, histopathological studies were performed. The inflammatory cells infiltration into peribronchial and perivascular areas was observed in OVA-challenged mice (H&E staining). Treatment with florfenicol (25 mg/kg or 100 mg/kg) markedly reduced the degree of inflammatory cell infiltration in the peribronchial and perivascular areas (Fig. 3A). In order to evaluate goblet cell hyperplasia, we stained lung sections with AB-PAS stain. Overproduction of mucus, goblet cell hyperplasia and epithelial cell disruption were observed in the bronchial airways of OVA-treated mice but not in the PBS-treated mice (Fig. 3B). Florfenicol (25 mg/kg or 100 mg/kg) treatment significantly reduced mucus secretion, goblet cell hyperplasia and epithelial cell disruption.

3.4. Effect of florfenicol on MAPK and GATA-3 pathway activation

To evaluate the effect of florfenicol on MAPK and GATA-3 activation in a mouse asthma model, we homogenized the lung tissue of mice to obtain total, nuclear and cytoplasmic proteins. The levels of phosphorylated MAPKs were subsequently measured by Western blot analysis using three different phospho-specific antibodies. The phosphorylation levels of the p38 isoforms decreased dramatically in florfenicol-treated mice as compared to OVA-challenged mice; however, there was no change in the phosphorylation levels of the ERK and JNK isoforms between the different groups (Fig. 4A). Total protein levels of MAPKs were used as controls for possible fluctuations in overall protein levels. Th2 cytokines are regulated by the zinc finger transcription factor GATA-3. As shown in Fig. 4B, the lung tissue from OVA-challenged mice showed a notable increase in nuclear protein level and a decrease in cytoplasmic protein level, as compared to PBS-challenged mice. Florfenicol treatment significantly attenuated these alterations in a dose-dependent manner ($*p < 0.05$, $**p < 0.01$).

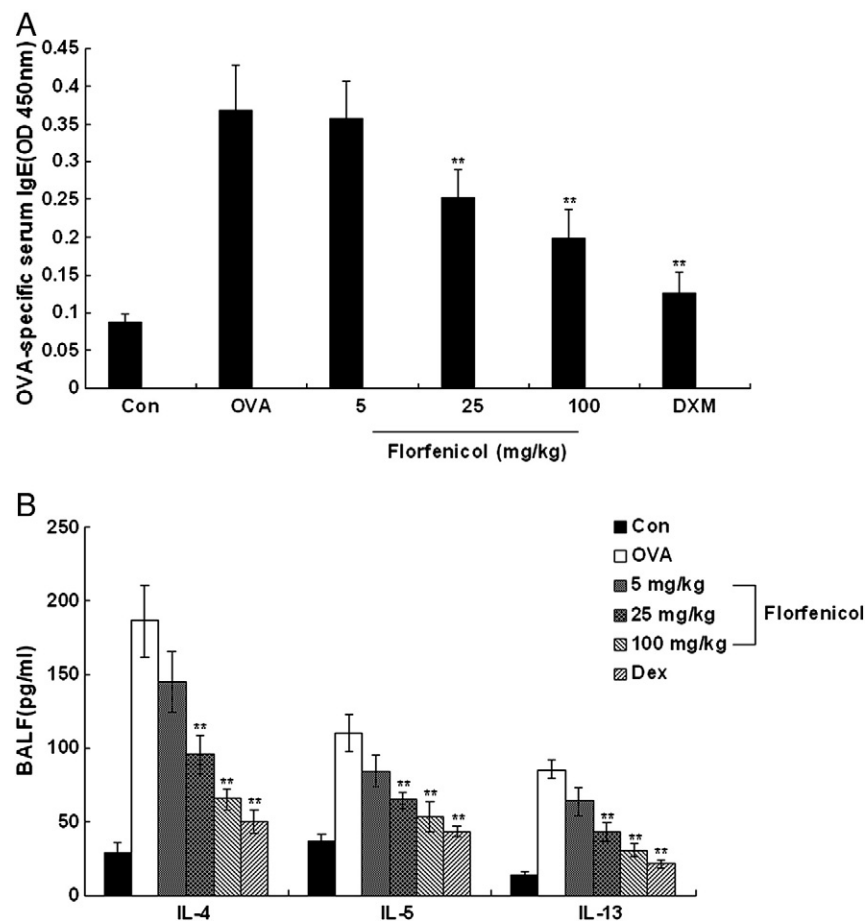


Figure 1 Effect of florfenicol on OVA-specific IgE in serum and the secretion of IL-4, IL-5 and IL-13 in BALF. OVA-specific IgE levels in the serum (A) and cytokines in BALF (B) were measured by ELISA. Results (mean $\pm$ SEM) are expressed as optical density values and are representative of at least three independent experiments. DXM=dexamethasone. $*p < 0.05$, $**p < 0.01$ vs. OVA.

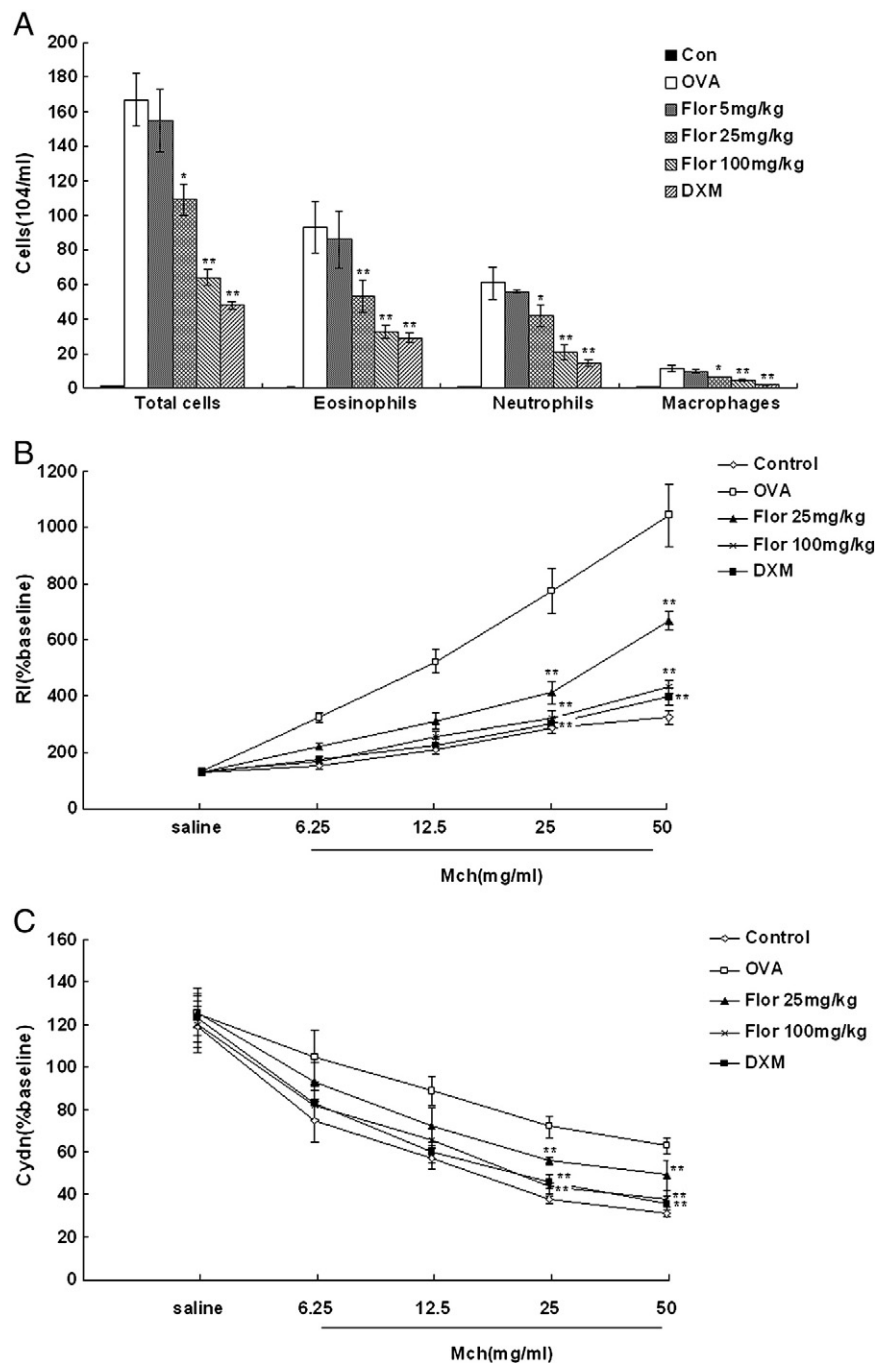


Figure 2 Effect of florfenicol on inflammatory cell recruitment and airway hyperresponsiveness to methacholine. (A) Effect of florfenicol on the recruitment of inflammatory cells in BALF. The lavage fluid was centrifuged, and the cell pellets were resuspended in 1 ml of PBS and applied to a slide by cytospinning to obtain differential cell counts. At least 200 cells were counted per slide. (B and C) Effect of florfenicol on ovalbumin-induced airway hyperresponsiveness in mice. Airway hyperresponsiveness was assessed by percentage change from the baseline level of (B) lung resistance (RI, $n=6$ mice per treatment group) and (C) dynamic compliance (Cdyn, $n=6$ mice per treatment group). The values represent the mean $\pm$ SEM of three independent experiments. DXM=Dexamethasone, Flor=florfenicol, Mch=methacholine. * $p<0.05$, ** $p<0.01$. vs. OVA.

4. Discussion

Extensive evidence suggest that antibiotics possess anti-inflammatory/immunomodulatory properties which are separate and distinct from their antibiotic actions [12,18]. These anti-inflammatory/immunomodulatory properties of

antibiotics are widely used or tested in the treatment of various inflammatory disorders of the respiratory tract, including asthma [25,26]. Florfenicol, an antibiotic used to treat infection, has previously been shown to modulate inflammatory responses in endotoxemia and acute lung injury [18,19]. In order to test whether the potentially

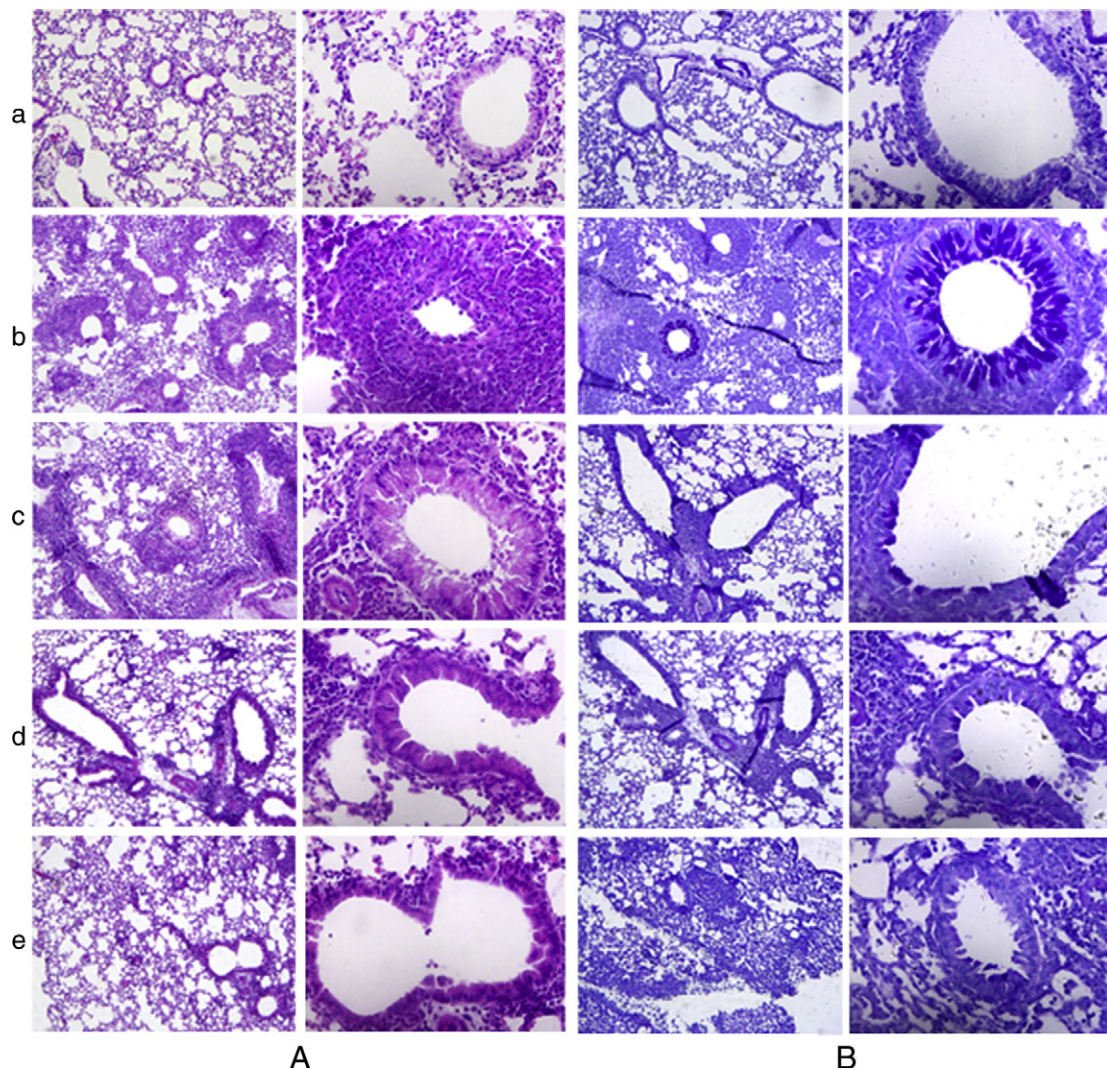


Figure 3 Effect of florfenicol on airway inflammation, airway goblet cell hyperplasia and mucus production. Representative haematoxylin–eosin (A) and alcian blue–periodic acid–Schiff (B) stained sections of lung from (a) PBS-challenged mice; (b) OVA-challenged mice; (c) OVA-challenged mice treated with florfenicol (25 mg/kg); (d) OVA-challenged mice treated with florfenicol (100 mg/kg); (e) OVA-challenged mice treated with dexamethasone (2 mg/kg). The left panel is magnified $\times 100$, the right panel is magnified $\times 400$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

beneficial effect of florfenicol in asthma could be attributed to its anti-inflammatory/immunomodulatory properties, we have investigated the ability of florfenicol to suppress inflammation and airway hyperresponsiveness in models of asthma. Th2 cytokines play an essential role in the pathogenesis of allergic airway inflammation [2,3]. Allergen exposure in the airway produces a Th2-dominant response by activating inflammatory cells and up-regulating concentrations of IL-4, IL-5 and IL-13 [27]. Our experiments were conducted on specific pathogen-free (SPF) female BALB/c mice referring to previous studies [28–30]. Using SPF mice, we can exclusively attribute the beneficial effects of florfenicol to its anti-inflammatory/immunomodulatory properties (thereby excluding the antimicrobial effects). In our study, treatment with florfenicol significantly decreased concentrations of IL-4, IL-5 and IL-13 both in vivo and in vitro. IL-4 causes B cells to synthesize IgE, which are

responsible of mast cell degranulation following cross-linking of their antigen-binding sites when they are bound to basophil type-1 receptors [31]. In our study, treatment with florfenicol significantly decreased concentrations of IL-4 and IgE. These findings suggest that IL-4 could be involved in the reduction of blood IgE levels by decreasing the expression of adhesion molecules and altering IgE class switching. It has also been shown that IL-5 enables terminal differentiation and proliferation of eosinophil precursors and the eosinophil activation effect [32]. The reduction of eosinophil infiltrates found in the florfenicol-treated group may be due to IL-5 reduction as it promotes the differentiation to and activation of eosinophils. IL-13 also has a pivotal role in causing asthma symptoms such as airway hyperresponsiveness, airway inflammation, goblet cell hyperplasia, airway luminal narrowing and subepithelial fibrosis [33]. We can conclude that the effect of florfenicol on airway hyperresponsiveness,

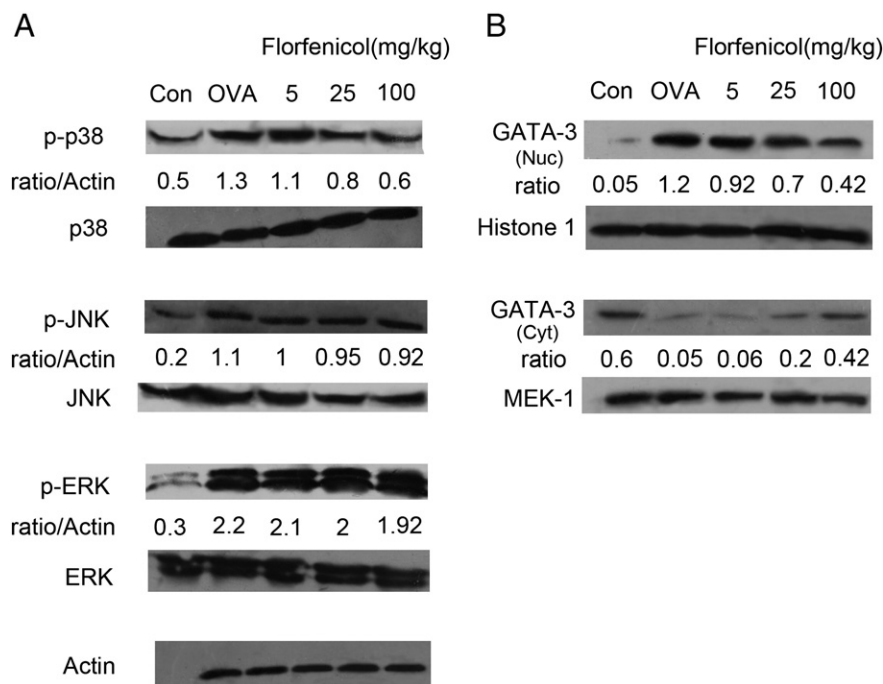


Figure 4 Effect of florfenicol on MAPK and GATA-3 pathway activation. (A) Effect of florfenicol on the activation of MAPK pathway. Total protein samples were analyzed by Western blot with phosphospecific antibodies. β -Actin was used as an internal control. Experiments were repeated three times, and similar results were obtained. (B) Effect of florfenicol treatment on nuclear translocation of GATA-3. Nuclear and cytoplasmic proteins from lung were analyzed by Western blot with specific antibodies. Histone 1 and MEK-1 were used as internal control. Experiments were repeated three times, and similar results were obtained.

airway inflammation and goblet cell hyperplasia may be attributed to an inhibition of IL-13.

The previous study has shown that p38 mitogen activated protein kinase (MAPK) play a critical role in phosphorylating GATA-3 to enhance its interaction with importin- α and subsequent transport into the nucleus [8]. MAPK and GATA-3 plays a critical role in regulating the expression of the cytokines interleukin (IL)-4, IL-5, and IL-13 from T helper-2 (Th2) cells and therefore is a key mediator of allergic diseases. In trying to understand the mechanisms by which florfenicol regulates Th2 cytokines, we investigated the effect of florfenicol on the MAPK and the activation of GATA-3. Using Western blot analysis, we found that florfenicol can inhibit the activation of p38 and the translocation of GATA-3 from the cytoplasm into the nucleus activation in a time-dependent manner. These results suggested that florfenicol prevent the activation of p38 MAPK to prevent the phosphorylation of GATA-3 and subsequent nuclear transport.

A necessary concern for the long-term use of florfenicol are its potential side effects as an antibiotic. A prolonged use of florfenicol may cause several side effects, for example gastrointestinal side effects, and bacterial resistance to the drug [34,35]. As a result, further studies are needed to determine its safety and effectiveness in humans. However, in veterinary clinics where respiratory inflammation is associated with bacterial infection, florfenicol exhibits an advantage both as an antimicrobial and as an anti-inflammatory, making the use of florfenicol acceptable despite its side effects. However, in humans with asthma viruses rather than bacteria trigger exacerbations.

In summary, treatment with florfenicol in a murine asthma model successfully downregulated OVA-induced Th2 cytokines production, allergen-specific IgE production and infiltration of inflammatory cells in the airways, all effects which are compatible with the reduction of lung tissue damage. This activity appeared to be mediated by the inhibition of the activation of p38 MAPK and the phosphorylation of GATA-3. Previously we have demonstrated florfenicol's anti-inflammatory effect on an infectious model, and now we are further observing florfenicol's anti-inflammatory effect on a non-infectious model. florfenicol presents anti-inflammatory effect on both levels, which shows that this is an ideal medicine to treat some respiratory diseases in veterinary clinic.

Acknowledgments

This study was financed by the National Science and Technology Supporting Plan of China (2006BAD31B03-4).

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