

## Full-length article

# Inhibitory effect of acetamide-45 on airway inflammation and phosphodiesterase 4 in allergic rats<sup>1</sup>

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## Key words

acetamide-45; 3',5'-cyclic nucleotide phosphodiesterase; respiratory function tests; eosinophils; asthma

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## Abstract

**Aim:** To determine the effects of acetamide-45 on respiratory function, airway inflammation, and the activity of phosphodiesterase 4 (PDE4) in allergic rats. **Methods:** Rats were sensitized by a single intramuscular injection with ovalbumin (OVA) and were challenged with ovalbumin applied by using an aerosol repeatedly for 7 d after 2 weeks. Acetamide-45 at concentrations of 5, 10, or 30 mg/kg was then administered by intraperitoneal injection. Changes in dynamic lung compliance and lung resistance, the accumulation of inflammatory cells in bronchoalveolar lavage, PDE4 activity, and the concentration of interleukin-4 in rat lung tissue were determined. **Results:** Seven days of treatment with acetamide-45 prevented eosinophil accumulation in allergic rats. At doses of 5, 10, and 30 mg/kg, acetamide-45 decreased lung resistance to  $0.20 \pm 0.04$ ,  $0.25 \pm 0.07$ , and  $0.22 \pm 0.05$   $\text{cmH}_2\text{O} \cdot \text{s}^{-1} \cdot \text{mL}^{-1}$ , respectively ( $P < 0.05$  vs OVA), and it also increased dynamic lung compliance to  $0.41 \pm 0.07$ ,  $0.39 \pm 0.06$ , and  $0.42 \pm 0.09$   $\text{mL/cmH}_2\text{O}$  ( $P < 0.05$  vs OVA). After being treated with different doses of acetamide-45, the PDE4 activities in lung tissue were  $281 \pm 55$ ,  $273 \pm 57$ , and  $238 \pm 36$   $\text{nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$  ( $P < 0.05$  vs OVA), and the concentrations of interleukin-4 in lung tissue were  $6.22 \pm 1.13$ ,  $5.95 \pm 1.20$ , and  $5.68 \pm 2.20$   $\mu\text{g/g}$  protein ( $P < 0.05$  vs OVA). **Conclusions:** Acetamide-45 was found to improve respiratory function and inhibit airway inflammation in this animal model, and the PDE4 activity of lung tissue was obviously inhibited. Acetamide-45 was an effective anti-inflammatory agent in respiratory inflammation, and the mechanism of its action might depend on inhibition of PDE4.

## Introduction

Asthma is a chronic inflammatory disease of the airways, of which lung inflammation and bronchial hyperactivity (BHR) are two distinct characteristics<sup>[1,2]</sup>. Eosinophils are considered to be the principal inflammatory leukocytes involved in the asthmatic reaction, due in part to the toxic granular proteins they secrete and the membrane products that induce airway epithelial pathology and mucus hypersecretion<sup>[3,4]</sup>. 3',5'-Cyclic-nucleotide (cAMP) has important regulatory roles in the process of inflammation. Phosphodiesterase 4 (PDE4), which catalyzes the hydrolysis of cAMP to the corresponding nucleotide, 5'-monophosphate, appears to be an attractive target for anti-inflammatory drugs<sup>[5,6]</sup>.

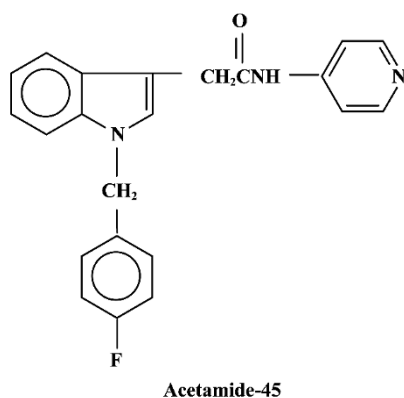
Inhibition of PDE4 results in increased levels of cAMP, which leads to functional inhibition of inflammatory cells such as eosinophils and lymphocytes, and reduced release of inflammatory cytokines such as interleukin-4 (IL-4)<sup>[7,8]</sup>. There has been significant interest in PDE4 inhibitors as potential therapeutic agents for asthma.

Because asthma has been the focus of much study effort, treatment of the disease has been improved by the implementation of management guidelines in recent years<sup>[9]</sup>. Such treatments as corticosteroids,  $\beta_2$ -agonists, theophylline and leukotriene antagonists have been used as anti-inflammatory agents to control asthma<sup>[10]</sup>. However, the possible dose-related adverse systemic effects produced by long-term treatment with these drugs are not acceptable in

clinical practice<sup>[11,12]</sup>. Therefore, new anti-asthma agents are required.

A series of new *N*-(pyridin-4-yl)-(indol-3-yl) alkylamides (44-84) have been prepared in the search for novel anti-allergic drugs. Initial studies showed that acetamide-45 inhibited IL-4 and IL-5 biosynthesis and histamine release<sup>[13]</sup>. We have also previously reported that acetamide-45 has an inhibitory effect on histamine- and methacholine-induced contractions of isolated guinea pig trachea, and that it can inhibit PDE4 activity<sup>[14,15]</sup>. It was concluded that acetamide-45 was a new anti-allergic agent. However, little is known about its effects on eosinophil infiltration in airway and lung function in asthmatic rats. There have been no reports regarding the effect of acetamide-45 on PDE4 activity in the lungs in any animal model of asthma.

Therefore, in the present study, we examined the inhibitory effect of acetamide-45 on airway inflammation and lung function in a rat model of asthma, and indicated a possible mechanism by which the effect might be exerted.



## Materials and methods

**Animals** Male Sprague-Dawley rats weighing 140–160 g were obtained from the Laboratory Animal Center of the Medical School of Zhejiang University (Certificate No 220010027, conferred by the Zhejiang Medical Laboratory Animal Administration Committee).

**Chemicals** Aminophylline, cAMP, and ovalbumin were purchased from Sigma (St Louis, MO, USA). Acetamide-45 was kindly provided by the Department of Organic Chemistry and Medical Chemistry, Faculty of Pharmacy (Nantes University, France). The rat IL-4-specific enzyme-linked immunosorbent assay (ELISA) kit was purchased from Jingmei BioTech Co (Shenzhen, China).

**Sensitization and challenge procedure** Rats were sensitized by a single intramuscular injection of 10 mg ovalbumin

(OVA) mixed with 100 mg aluminum hydroxide in 1 mL of saline. After 2 weeks, these animals were then challenged by exposure for 20 min to aerosolize 1% OVA in saline generated by a jet nebulizer once a day for 7 d. The drugs were administered by intraperitoneal injection before challenge. The doses of acetamide-45 were 5, 10, or 30 mg/kg, respectively, and the dose of aminophylline was 10 mg/kg per day for 7 d. At d 21, the animals were killed, and the lung tissues were immediately removed, frozen in liquid nitrogen, and then stored at -80 °C until analysis.

**Measurement of lung function** At 24 h after the last antigen challenge, rats were anesthetized with urethane (1 g/kg, ip). The trachea was cannulated and placed in a whole body plethysmograph for the measurement of lung resistance ( $R_L$ ) and dynamic lung compliance ( $C_{dyn}$ )<sup>[16,17]</sup>.

**Cell counts in bronchoalveolar lavage** Bronchoalveolar lavage (BAL) was performed by flushing the airways with 10 mL/kg saline containing 1% bovine serum albumin and 1000 kU/L heparin sodium through a tracheal cannula. The BAL fluid was pooled and immediately centrifuged at 500 ×g at 4 °C for 10 min. The supernatant was removed and the cells were resuspended in 1 mL saline containing 10% bovine serum albumin. Counts of the total number of leukocytes recovered in the BAL fluid were carried out using a Neubauer chamber, and differential cell analysis was carried out under a light microscope after Wright-Giemsa staining.

**Determination of IL-4 in lung tissue** At a ratio of 1 g to 10 mL, homogenized lung tissue was added to 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHARPS). The homogenates were centrifuged twice at 19 851 ×g, each for 30 min<sup>[17]</sup>. IL-4 in the supernatants was quantified using a rat IL-4-specific ELISA kit.

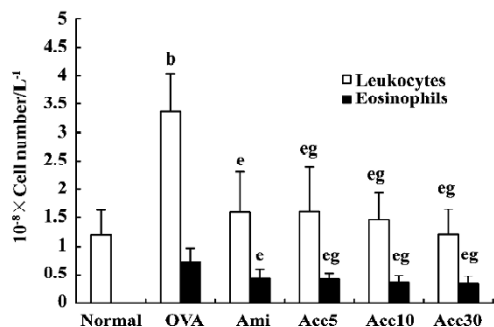
**PDE4 activity assay** Frozen 25-mg pieces of lung tissue were homogenized and PDE4 activity was determined by high performance liquid chromatography (HPLC) as described elsewhere<sup>[15,18]</sup>.

**Statistical analysis** Data are expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance.

## Results

**Effects of acetamide-45 on inflammatory cells in the airways of allergic rats** In our rat model of allergic asthma, ovalbumin sensitization and challenge caused an obvious increase in inflammatory cells in BAL. As shown in Figure 1, the total number of leukocytes and eosinophils in the OVA group was significantly greater than that in the normal group

( $P < 0.05$ ), but the number of inflammatory cells was depressed when aminophylline (Ami) was administered ( $P < 0.05$  vs OVA). Seven days of treatment with acetamide-45 (Ace) at 5, 10, or 30 mg/kg significantly decreased the number of inflammatory cells in BAL caused by sensitization and challenge ( $P < 0.05$  vs OVA; Figure 1). There was no statistical difference between the Ace group and the Ami group ( $P > 0.05$ ; Figure 1).



**Figure 1.** Effect of acetamide-45 on inflammatory cells in rat airway after ovalbumin challenge. Rats were sensitized and challenged with ovalbumin (OVA). Acetamide-45 at concentrations of 5 mg/kg (Ace5), 10 mg/kg (Ace10), or 30 mg/kg (Ace30), or aminothiophylline at a concentration of 10 mg/kg (Ami) were administered once a day for 7 d during challenge.  $n=6$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs normal. <sup>e</sup> $P < 0.05$  vs OVA. <sup>eg</sup> $P > 0.05$  vs Ami.

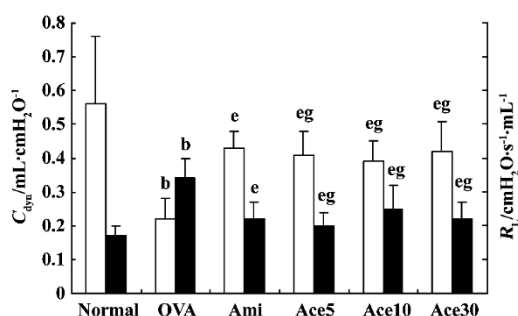
#### Effects of acetamide-45 on $R_L$ and $C_{dyn}$ in allergic rats

After rats were given 7 aerosolized OVA challenges, a significant decrease in  $C_{dyn}$  was induced. However, through sensitization and challenge,  $R_L$  increased to a level that was significantly greater than that in the normal group. The administration of aminophylline decreased the  $R_L$  and increased the  $C_{dyn}$  of sensitized and challenged animals. At a dose of 5, 10, or 30 mg/kg, acetamide-45 improved both  $C_{dyn}$  and  $R_L$  (Figure 2).

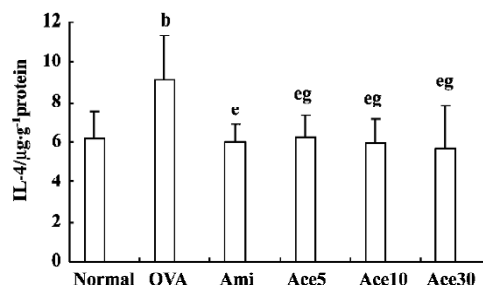
#### Effect of acetamide-45 on IL-4 in lung tissue in allergic rats

As shown in Figure 3, IL-4 levels in the lungs of rats from the various groups were measured. Sensitization and challenge increased the concentration of IL-4 to a level significantly different from that of the normal group ( $P < 0.05$ ). Seven days of treatment with acetamide-45 at concentrations of 5, 10, or 30 mg/kg, or with aminophylline at a concentration of 10 mg/kg, obviously reduced the increase in the concentration of IL-4 ( $P < 0.05$ ).

**Effect of acetamide-45 on PDE4 activity in lung tissue in allergic rats** Sensitization and challenge increased the PDE4 activity of lung tissue to  $503 \pm 125$  nmol $\cdot$ g $^{-1}$  $\cdot$ min $^{-1}$ , which was greater than that of the normal group ( $P < 0.05$ ). Acetamide-45 at concentrations of 5, 10, or 30 mg/kg inhibited the hy-



**Figure 2.** Effects of acetamide-45 on lung resistance ( $R_L$ , filled bars) and dynamic lung compliance ( $C_{dyn}$ , open bars) in allergic rats. Rats were sensitized and challenged with ovalbumin (OVA). Acetamide-45 at a concentration of 5 mg/kg (Ace5), 10 mg/kg (Ace10) or 30 mg/kg (Ace30), and aminothiophylline at a concentration of 10 mg/kg (Ami) were administered once a day for 7 d during challenge, and then lung function was measured.  $n=6$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs normal. <sup>e</sup> $P < 0.05$  vs OVA. <sup>eg</sup> $P > 0.05$  vs Ami.

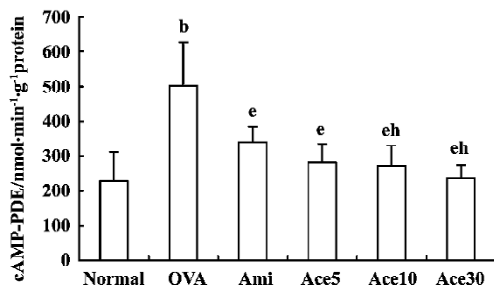


**Figure 3.** Effect of acetamide-45 on IL-4 concentration in lung tissue in allergic rats. Rats were sensitized and challenged with ovalbumin (OVA). Acetamide-45 at concentrations of 5 mg/kg (Ace5), 10 mg/kg (Ace10) or 30 mg/kg (Ace30), and aminothiophylline at a concentration of 10 mg/kg (Ami) were administered once a day for 7 d during challenge, and then the IL-4 concentrations of lung tissue were measured.  $n=6$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs normal. <sup>e</sup> $P < 0.05$  vs OVA. <sup>eg</sup> $P > 0.05$  vs Ami.

drolysis of cAMP by the PDE4 extracted from lung tissue. The PDE4 activities of the lung tissue were  $281 \pm 55$ ,  $273 \pm 57$ , and  $238 \pm 36$  nmol $\cdot$ g $^{-1}$  $\cdot$ min $^{-1}$ , respectively (for acetamide-45 at concentrations of 5, 10, or 30 mg/kg). As a non-selective inhibitor of PDE4, aminophylline suppressed PDE4 activity to  $341 \pm 44$  nmol $\cdot$ g $^{-1}$  $\cdot$ min $^{-1}$  at a dose of 10 mg/kg, which was weaker than the activity produced by treatment with acetamide-45 at concentrations of 10 and 30 mg/kg ( $P < 0.05$ , Figure 4).

## Discussion

Acetamide-45, *N*-(pyridin-4-yl)-[1-(4-fluorophenyl) indol-3-yl] acetamide, is a new anti-inflammatory drug that inhibits the release of cytokines and the activation of eosinophils<sup>[12]</sup>.



**Figure 4.** Effect of acetamide-45 on PDE4 activity in lung tissue in allergic rats. Acetamide-45 at concentrations of 5 mg/kg (Ace5), 10 mg/kg (Ace10), or 30 mg/kg (Ace30) and aminothiophylline at a concentration of 10 mg/kg (Ami) were administered once a day for 7 d during challenge, and then the PDE4 activity of lung tissues was measured. *n*=6. Mean±SD. <sup>b</sup>*P*<0.05 vs normal. <sup>e</sup>*P*<0.05 vs OVA. <sup>eh</sup>*P*<0.05 vs Ami.

It has also been reported that acetamide-45 inhibits the contraction of isolated guinea pig trachea induced by histamine and methacholine<sup>[14]</sup>. However, the mechanism by which acetamide-45 acts remains unknown. The present study shows that acetamide-45 can suppress the accumulation of inflammatory cells in airways and improve the lung function of allergic rats. We also found that acetamide-45 could inhibit PDE4 activity and reduce the IL-4 level in the lung tissue of allergic rats. To our knowledge, this study represents the first investigation of the effect of acetamide-45 on lung function and PDE4 concentration in the lung in an animal model of asthma.

Asthma is a syndrome characterized by eosinophil infiltration and airway hyperresponsiveness, which occurs via a mechanism that is very complex. It is well known that eosinophils play a critical role in airway damage and dysfunction in asthma. We found that acetamide-45 had an inhibitory effect on eosinophil infiltration in the airways of allergic rats. Not only eosinophils but also other inflammatory cells were decreased in concentration, so that inflammation of the airway in allergic rats was inhibited by acetamide-45.

Inflammation of the airway leads to spasms of the bronchial smooth muscles, mucus hypersecretion, and airflow limitations, which result in pulmonary dysfunction in asthma. The data obtained by a whole body plethysmograph in this study indicate that acetamide-45 improves the lung function of allergic rats produced by sensitization and challenge. Its effectiveness has been demonstrated in two ways. First, the *C*<sub>dyn</sub> of allergic rats increased obviously relative to normal rats at a dose of 5 mg/kg acetamide-45. Second, the *R*<sub>L</sub> of allergic rats was reduced significantly by acetamide-45 in the same way. The improvement in lung function produced by acetamide-45 is similar to that produced by aminophylline.

The experiments presented here show that both acetamide-45 and aminophylline repress IL-4 production in allergic rats, but that there is no difference between the two agents. IL-4 is a key cytokine in the development of allergic inflammation. It is associated with the secretion of IgE by B lymphocytes and the expression of eotaxin and other inflammatory cytokines that contribute to inflammation and lung remodeling in asthma<sup>[18,19]</sup>. A previous study shows that acetamide-45 inhibits IL-4 biosynthesis and release, which is identical to our result<sup>[12]</sup>. This indicates that acetamide-45 can reduce the release of IL-4 and reduce its concentration in the lung, which results in the inhibition of airway inflammation.

Our study of PDE4 activity shows that acetamide-45 has an inhibitory effect on cAMP hydrolysis of PDE4 in the lung tissue of allergic rats. In a previous study, we demonstrated that acetamide-45 could inhibit the hydrolysis of cAMP by PDE4 extracted from transfected yeast, and that its inhibitory effect was stronger than that of theophylline<sup>[15]</sup>. The present study also shows that at the same dose (10 mg/kg), the inhibitory effect of acetamide-45 is greater than that of aminophylline, which is a non-selective PDE inhibitor. PDE4 is specific for cAMP, and is predominantly expressed in inflammatory cells. It plays an important role in the regulation of cellular functions in inflammatory and immune cells, and inhibition of PDE4 may be one of the anti-inflammatory mechanisms of acetamide-45.

In conclusion, we have shown that acetamide-45 could improve the lung function of allergic rats produced by sensitization and challenge, and that production of eosinophils in those animals could also be repressed. Acetamide-45 had an inhibitory effect on PDE4 activity in the lung tissue of allergic rats, and the inhibition was stronger than that produced by aminophylline. The present research may provide an experimental basis for further study of this agent.

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