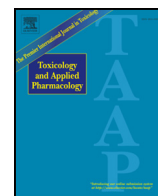




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Curine inhibits eosinophil activation and airway hyper-responsiveness in a mouse model of allergic asthma

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ABSTRACT

Allergic asthma is a chronic inflammatory airway disease with increasing prevalence around the world. Current asthma therapy includes drugs that usually cause significant side effects, justifying the search for new anti-asthmatic drugs. Curine is a bisbenzylisoquinoline alkaloid that modulates calcium influx in many cell types; however, its anti-allergic and putative toxic effects remain to be elucidated. Our aim was to investigate the effects of curine on eosinophil activation and airway hyper-responsiveness (AHR) and to characterize its potential toxic effects. We used a mouse model of allergic asthma induced by sensitization and challenge with ovalbumin (OVA) to evaluate the anti-allergic effects of oral treatment with curine. The oral administration of curine significantly inhibited eosinophilic inflammation, eosinophil lipid body formation and AHR in animals challenged with OVA compared with animals in the untreated group. The curine treatment also reduced eotaxin and IL-13 production triggered by OVA. Verapamil, a calcium channel antagonist, had similar anti-allergic properties, and curine pre-treatment inhibited the calcium-induced tracheal contractile response *ex-vivo*, suggesting that the mechanism by which curine exerts its effects is through the inhibition of a calcium-dependent response. A toxicological evaluation showed that orally administered curine did not significantly alter the biochemical, hematological, behavioral and physical parameters measured in the experimental animals compared with saline-treated animals. In conclusion, curine showed anti-allergic activity through mechanisms that involve inhibition of IL-13 and eotaxin and of Ca^{++} influx, without inducing evident toxicity and as such, has the potential for the development of anti-asthmatic drugs.

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Introduction

Allergic asthma is an important public health problem in terms of prevalence, morbidity and mortality, affecting approximately 20% of the world's population and requiring costly therapy (Chatila, 2004; Edwards et al., 2009). The pathophysiology of asthma is complex and results from an inappropriate immune response to common allergens; this response is characterized by chronic airway inflammation that is associated with intense leukocyte recruitment and activation at the site of injury and airway hyper-responsiveness (AHR) (Barnes, 2008; Paul and Zhu, 2010).

The current standard for the treatment of asthma involves the use of drugs that relieve and control the symptoms of the disease (Holgate and

Polosa, 2008). The medications that are broadly used in asthma management today include potent inhaled corticosteroids, such as budesonide and fluticasone; long-acting β -adrenergic agonists (LABAs), such as salmeterol and formoterol; and leukotriene modifiers, including zafirlukast, montelukast and zileuton; there are also combination therapies that include inhaled corticosteroids and LABAs in a single delivery device (Szefer, 2011). Corticosteroids are potent anti-inflammatory drugs that regulate the expression of cytokines, chemokines and adhesion molecules by modulating the activity of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein 1 (AP1). Inhaled corticosteroids are very effective at inhibiting airway inflammation, and they represent an important tool in asthma management (Ivancsó et al., 2013). However, these drugs are not effective under specific conditions, including virus- or smoke-induced exacerbations (Harrison et al., 2004). β_2 -Adrenergic agonists effectively promote bronchodilation through the production of cyclic adenosine 3'/5'-monophosphate (cAMP) and the activation of protein kinase A (PKA). Inhaled short- and long-acting β_2 -adrenergic agonists are

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currently most commonly used in association with inhaled corticosteroids as a supplementary therapy for asthma (Holgate and Polosa, 2008). Phosphodiesterase inhibitors also increase cAMP, but these treatments have been less commonly used of late because of the low therapeutic index of some drugs (Boswell-Smith et al., 2006). Antagonists of cysteinyl leukotriene receptor 1 (CysLTR1) are currently available and are important therapeutic tools in asthma. These drugs act by blocking many activities of CysLTs, including bronchoconstriction, and are mainly used as a supplementary therapy to inhaled corticosteroids (Polosa, 2007). Finally, mast cell inhibitors, cytokine-blocking monoclonal antibodies and allergen-specific immunotherapies are also important in the treatment of asthma and other allergic diseases (Edwards and Howell, 2000; Holgate and Polosa, 2008).

Despite the currently available array of anti-asthmatic therapies, the search for structurally novel chemicals and for the development of safe and effective drugs for the treatment of asthma and other allergic conditions remains an important field of investigation. Several studies have shown that bisbenzylisoquinoline alkaloids (BBAs) have immunomodulatory activity against allergy and inflammation (Seow et al., 1986; Teh et al., 1990). Our group has demonstrated that the alkaloid warifteine has anti-allergic properties, including the inhibition of anaphylaxis, the inhibition of eosinophil recruitment and activation, the ability to modulate airway hyper-responsiveness and remodeling

(Bezerra-Santos et al., 2005, 2006, 2012) and the ability to inhibit histamine release (Costa et al., 2008).

Curine (Fig. 1A) is a BBA that is the major constituent of the root bark of *Chondrodendron platyphyllum* (Menispermaceae). This compound has a molecular structure that is similar to that of warifteine. Recently, Medeiros et al. (2011) demonstrated that curine may have direct effects on L-type Ca^{2+} channels in vascular smooth muscle cells. However, despite curine's interesting pharmacological activity and its structural similarity to warifteine, this molecule's anti-allergic properties and potential for toxic effects remain to be investigated. Using a mouse model of allergic asthma and finding no detectable toxicity, our study is the first to report that curine is beneficial as an orally active anti-allergic compound.

Methods

Curine purification. *C. platyphyllum* Hil St. (Miers) was collected in the municipality of Santa Rita, Paraíba, Brazil. The voucher specimen of this plant is deposited in the Herbarium Prof. Lauro Pires Xavier, number 3631-P, and was identified by Prof. Dr Maria de Fatima Agra. Spectroscopically pure curine was isolated from the root bark of *C. platyphyllum* as described by Mambu et al. (2000). The curine solution was prepared using 1 mg of the crystal in 50 μl of 1 N HCl and 500 μl of distilled water.

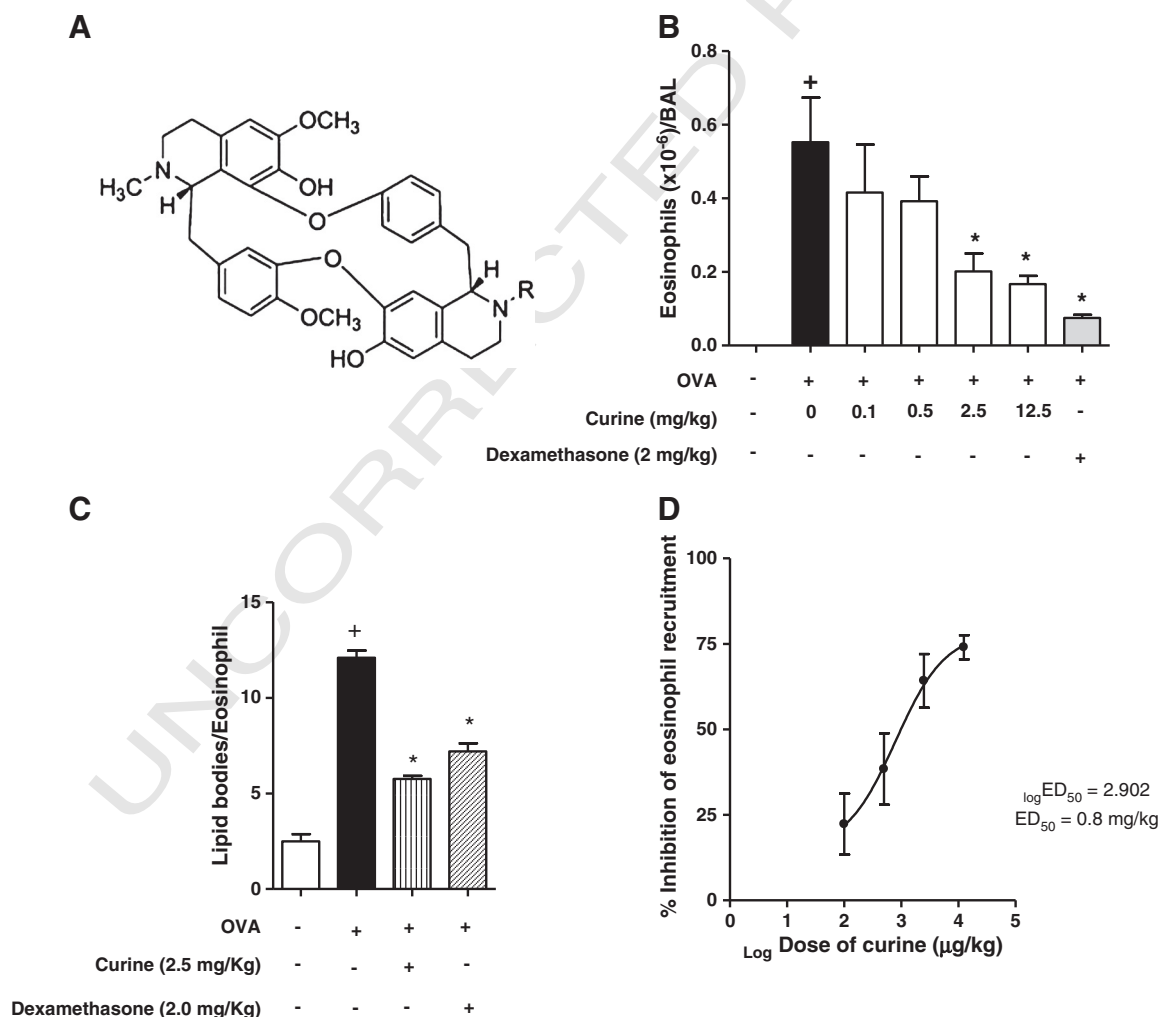


Fig. 1. The effect of curine on eosinophil recruitment and activation. BALB/c mice ($n = 5-7$) were treated orally with increasing doses of curine (0.1, 0.5, 2.5 or 12.5 mg/Kg), or dexamethasone (2.5 mg/kg) 1 h before each challenge. Twenty-four hours after the last challenge, the BAL was collected and the eosinophils and lipid bodies were counted using a light microscope. A) the chemical structure of curine; B) the number of BAL eosinophils; C) the number of lipid bodies per eosinophil; D) a dose-response curve of curine on the inhibition of eosinophil recruitment. These results are expressed as the mean $\pm$ SEM of at least 5 animals. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the untreated, OVA-challenged group.

The pH was adjusted to 7–8 with 1 N NaOH. The volume was adjusted to 1000 μ l, and the dilutions were made in phosphate-buffered saline (PBS).

Animals. Male BALB/c or Swiss mice weighing 20–30 g were obtained from the Oswaldo Cruz Foundation breeding unit. The animals were maintained with food and water *ad libitum* in a room with the temperature ranging from 22 to 24 °C and a 12 h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol # L-002/08).

Treatments. To perform the dose–response curve with eosinophil recruitment as a measured outcome, animals were treated orally with different doses of curine (0.1, 0.5, 2.5 or 12.5 mg/Kg) or dexamethasone (2 mg/Kg) orally (p.o.) 1 h before each challenge (pre-treatment protocol). Alternatively, the mice were treated with curine (2.5 mg/Kg) 1 h after the last challenge (post-treatment protocol). The dose of curine (2.5 mg/Kg) was chosen based on the results obtained from the pre-treatment dose–response curve. Verapamil (2.5 mg/Kg) was given as a control calcium channel antagonist. For the *ex-vivo* experiments, 9 μ M curine was used. This concentration was found to be the approximate IC₅₀ for the inhibition of calcium influx in the experiments performed by Medeiros et al. (2011). To evaluate the toxicological parameters, animals were treated orally once a day for 7 consecutive days with curine (2.5 or 8 mg/Kg) or dexamethasone (2 mg/Kg). The control group was treated orally with PBS. Importantly, the 8 mg/Kg dose of curine is 10-fold higher than its median effective dose (ED₅₀).

Toxicological evaluation. The male Swiss mice were treated as described above; their weight, behavior, and primary physical aspects (e.g., activity, breath, piloerection, convulsions and diarrhea) were monitored daily. Twenty-four hours after the last treatment, the animals were euthanized by exposure to an atmosphere of CO₂, and the blood was collected by cardiac puncture. EDTA was added to the blood samples for the hematological analyses of leukocytes, red blood cells (RBC), platelets, hematocrit, hemoglobin, the mean corpuscular hemoglobin concentration (MCHC), the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin (MCH). Blood samples were centrifuged at 6000 g for 8 min at 4 °C to separate out the serum. The serum concentrations of alkaline phosphatase, alanine transaminase (ALT), aspartate transaminase (AST), bilirubin, creatinine, creatinine kinase, cholesterol, glucose, total proteins and uric acid were determined. The relative weight and the macroscopic aspects of the stomach, liver, spleen, thymus, intestines, kidneys and lungs were analyzed, and the presence of gastric ulcer was assessed through macroscopic and histological analyses.

Airway inflammation in actively sensitized mice. Allergic airway inflammation was induced as described by Lloyd et al. (2001). The animals were sensitized intraperitoneally (i.p.) with ovalbumin (OVA – 10 μ g/mouse) and Al(OH)₃ (10 mg/mL) in a 0.9% NaCl solution (saline 0.2 mL) on days 1 and 10. From day 19 to day 24 after sensitization, the mice were challenged daily for 20 min with OVA (5%) in PBS by aerosol. Aerosolized PBS was administered to some of the sensitized mice as a negative control. These procedures were performed in a 30 × 20 × 10 cm acrylic chamber, and the aerosol was generated by an ultrasonic nebulizer. Twenty-four hours after the last challenge, the animals were euthanized by exposure to an atmosphere of CO₂, and the trachea was surgically exposed and cannulated. The bronchoalveolar lavage (BAL) was collected from mice by washing the lungs with 1 mL of PBS.

Leukocyte counts and lipid body enumeration. Total leukocyte counts were performed using a Neubauer chamber under a light microscope after diluting the BAL samples in Turk fluid (2% acetic acid). Differential counts were performed. To enumerate lipid bodies, the cells, while still moist, were fixed in 3.7% formaldehyde (diluted in Ca²⁺/Mg²⁺-free HBSS; pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO₄ for 30 min, rinsed in distilled H₂O, immersed in 1.0% thiocarbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO₄ for 3 min, rinsed in distilled water and then dried and mounted (Bozza et al., 1997). The cell morphology was observed and the lipid bodies were enumerated by microscopy with an objective lens at 100× magnification. Twenty-five consecutively scanned eosinophils were evaluated, and the results were expressed as the mean numbers of lipid bodies per eosinophil.

Cytokine quantification. Twenty-four hours after the last challenge, the lungs of the mice were surgically removed. Each lung was treated with 800 μ l of a complete cocktail solution of protease inhibitors (Roche Diagnostics GmbH / Mannheim, Germany) containing both reversible and irreversible protease inhibitors and 20 μ M EDTA. The cocktail solution was prepared by dissolving 1 tablet in 50 ml of 100 mM phosphate buffer, pH 7.0. The lungs were then macerated, the samples were centrifuged at 6000 g for 20 min at 4 °C, and the supernatants were collected. The total protein concentration of these samples was determined using a kit for the determination of proteins (BCA, from Pierce, Rockford, IL). The concentrations of IL-13 and eotaxin were measured using DuoSet kits according to the manufacturer's instructions (R&D Systems).

Analysis of airway hyper-responsiveness. Airway hyper-responsiveness (AHR) in the mice was analyzed using non-invasive whole body plethysmography (Buxco, Sharon, CT) 24 h after the last OVA challenge. AHR was measured after the aerosolization of PBS followed by increasing concentrations of methacholine (0, 6, 12.5 and 25 mg/mL; Sigma-Aldrich), each of which was injected into the chamber for 2 min. The AHR data were expressed as an average enhanced pause (Penh). There was an interval of 10 min between each aerosol exposure, and within this period of time, the Penh values had returned to baseline.

Analysis of the trachea contractile response ex vivo. Tissue preparation. Male Wistar rats (8 weeks) were euthanized in a CO₂ chamber; the tracheas were removed and were immediately immersed in Krebs nutritive solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 24 mM NaHCO₃ and 11 mM glucose). The tracheas were dissected and cut into segments of 3–4 rings. The segments were mounted in isolated organ baths containing 10 mL of Krebs solution maintained at 37 °C and aerated with a carbogenic gas mixture (95% O₂ and 5% CO₂). To achieve a constant level of spontaneous tone, an initial tension of 1 g was applied. Contractions were measured isometrically with a force-displacement transducer (Ugo Basile, Comerio, Italy) and were recorded by an isolated organ data acquisition program (Proto5; Leticia Scientific Instruments, Barcelona, Spain).

Calcium-concentration response curve. Tissues were stabilized over a period of 60 min; the bath solution was changed every 10 min. At the end of the equilibration period, the response to carbachol (2.5 mM) was recorded. After the removal of the carbachol and the restoration of the tone to a stable baseline, the tissues were exposed to repeated cycles of stimulation with 60 mM KCl and washing in a calcium-free Krebs solution until there was complete desensitization of the contractile response evoked by 60 mM KCl. Then, the tracheal segments were immersed in Ca²⁺-free Krebs solution containing 60 mM KCl, and the concentration of extracellular Ca²⁺ was gradually increased by the cumulative addition of CaCl₂ (0.01 to 100 mM) in the presence or absence of 9 mM curine or vehicle. All of the responses were expressed as a percentage of the response to 2.5 mM carbachol (Foster et al., 1984).

Statistical analysis. Data were analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (GraphPad, San Diego, CA). The values were expressed as the means $\pm$ S.E.M. Differences with $p < 0.05$ were considered significant.

Results

Curine inhibits eosinophil recruitment and activation in a mouse model of allergic asthma

An allergic challenge with OVA (5%) in actively sensitized mice induced an intense influx of eosinophils into the BAL that was associated with the development of airway inflammation (Fig. 1B). To demonstrate the anti-allergic effects of pre-treatment with curine, the animals were pre-treated orally with curine (2.5 mg/Kg) or dexamethasone (2 mg/Kg) 1 h before each OVA challenge. This treatment significantly reduced both the total number of eosinophils in the BAL (Fig. 1B) and the numbers of cytoplasmic lipid bodies within those cells (Fig. 1C) compared with the untreated OVA-challenged group. In this model, curine had a dose-dependent effect, with a median effective dose (ED₅₀) of 0.8 mg/Kg (Fig. 1D).

Curine decreases AHR in a mouse model of allergic asthma

Airway hyper-responsiveness is a hallmark of asthma. Fig. 2 shows that an allergic challenge in actively sensitized BALB/c mice induced a significant increase in Penh values compared with the unchallenged group. Daily pre-treatment with curine or dexamethasone (2 mg/Kg) reduced the allergic reaction-triggered Penh values, indicating that curine, similarly to dexamethasone, has an inhibitory effect on the development of allergic AHR. Of note, when expressing data as % baseline the results were the same (not shown). Importantly, curine failed to inhibit the methacholine response in the non-allergic vehicle group (not shown), demonstrating that curine does not alter the bronchoconstriction induced by methacholine in the absence of inflammation.

Curine inhibits the production of eotaxin and IL-13 in vivo

Eotaxin and IL-13 play key roles in allergic responses, including the regulation of eosinophil migration and activation and the development of AHR (Lloyd et al., 2001). As shown in Fig. 3, the BAL fluid of actively sensitized OVA-challenged mice contained increased concentrations of these cytokines when compared with unstimulated mice. As shown in Figs. 3A and B, curine and dexamethasone pre-treatments both decreased the eotaxin (Fig. 3A) and IL-13 (Fig. 3B) BAL concentrations observed in the untreated OVA-challenged group, revealing a part of the mechanism by which curine inhibits eosinophil recruitment and activation and AHR.

Effects of curine on the calcium-induced tracheal contractile response

Previous studies have shown that curine has an inhibitory effect on calcium influx in different cell types (Dias et al., 2002; Medeiros et al., 2011). Therefore, we investigated the involvement of calcium-dependent mechanisms on the anti-allergic effects of curine. In the experiments with trachea ring preparations maintained in Ca²⁺-free medium and depolarized with 60 mM KCl, the cumulative addition of Ca²⁺ (0.01 to 100 mM) produced a concentration-dependent contraction that was inhibited by up to 50% by pre-treatment with 9 mM curine (Fig. 4). This observation suggests that curine inhibits the influx of calcium by blocking the voltage-dependent Ca²⁺ channels in rat tracheal smooth muscle and is consistent with previously published data (Medeiros et al., 2011).

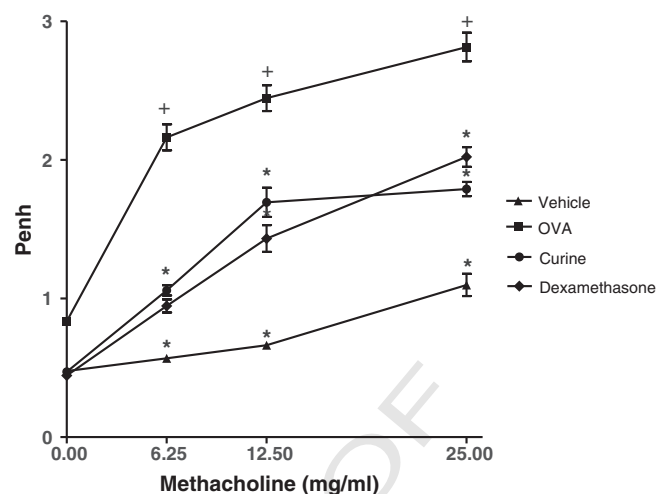


Fig. 2. The effect of curine on airway hyper-responsiveness (AHR). OVA-sensitized BALB/c mice ($n = 7-8$) were treated orally with curine (2.5 mg/Kg), or dexamethasone (2 mg/Kg) 1 h before each challenge. AHR was analyzed by aerosolization with PBS followed by increasing concentrations of methacholine, 24 h after the last allergic challenge. The AHR is expressed as the rate of enhanced pause (Penh). These results are expressed as the mean $\pm$ SEM. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

Comparison between the anti-allergic effects of curine and verapamil

In recent work, Khakzad et al. (2012) demonstrated the anti-inflammatory activity of verapamil, a calcium channel antagonist, using a model of allergic asthma. To confirm the role of calcium influx inhibition as an anti-allergic mechanism, we compared the effect of verapamil to that of curine on AHR and eosinophil recruitment, two important features of asthma. When administered under the same conditions (dose, time and pathway), verapamil and curine showed similar anti-allergic effects (Fig. 5), suggesting that the anti-allergic effects of curine may be due, at least in part, to the inhibition of calcium influx.

Curine post-treatment inhibits eosinophil recruitment and activation in OVA-challenged mice

To investigate the therapeutic effects of curine on ongoing airway inflammation, we administered a single dose of curine 1 h after the last allergic challenge (post-treatment). BALB/c mice post-treated with curine had a reduced number of eosinophils (Fig. 6A) and similarly reduced numbers of eosinophil lipid bodies (Fig. 6B) in the BAL compared with mice in the untreated group. Even when the treatment was given after the development of inflammation, curine inhibited eosinophil recruitment and activation; such findings demonstrate the therapeutic effectiveness and the clinical relevance of curine.

Curine fails to induce toxic effects in orally treated mice

Outbred Swiss mice treated orally with curine daily for seven days were evaluated for toxicological parameters. Outbred mouse stocks were used for the toxicological evaluation because of their genetic heterogeneity and their common use in toxicological studies (Faqi, 2012). We found that curine treatment did not induce significant alterations in biochemical (Table 1), hematological (Supplement Table S1) or physical and behavioral parameters and in gastric ulcer formation (Supplement Table S2) compared with saline treatment, even at a dose of 8 mg/Kg (a dose 10 times higher than the ED₅₀). These data indicate that curine is able to inhibit allergic inflammation at doses at which it does not induce significant toxicity. On the other hand, dexamethasone treatment induced an increase in the concentrations of total proteins, cholesterol and ALT compared with saline treatment, reflecting the side effects broadly described in the literature. Of note, no alterations in physical

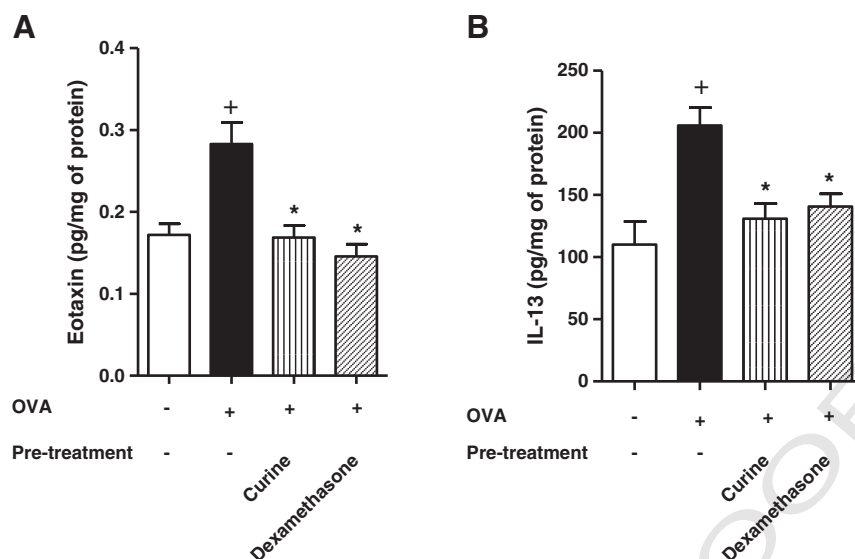


Fig. 3. The effect of curine on eotaxin and IL-13 production. BALB/c mice ($n = 5-6$) primed with OVA were treated orally with curine (2.5 mg/Kg), or dexamethasone (2 mg/Kg) 1 h before each challenge. Twenty-four hours after the last challenge, the lungs were collected and homogenized. The production of eotaxin (A) was analyzed using an EIA protocol, and the production of IL-13 (B) was analyzed by ELISA. These results are expressed as the mean $\pm$ SEM from at least 6 animals. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

and behavioral parameters or leukocyte counts were observed in the Balb/c mice treated with curine at 2.5 mg/kg (not shown).

Discussion

Allergic diseases, including asthma, have multifactorial etiologies, and their prevalence, morbidity and mortality have been increasing around the world (Edwards et al., 2009). Asthma is usually treated with a combination of anti-inflammatory and bronchodilator drugs. Inhaled corticosteroids are currently the most important drugs used to control airway inflammation. Because of their potent inhibitory effects on cell activation, cytokine and lipid mediator production and adhesion molecule and inflammatory receptor expression, they are simultaneously able to block several essential steps of the inflammatory

cascade (Barnes, 2006, 2011; Barnes and Adcock, 2009; Rhen and Cidlowski, 2005). However, corticosteroid therapy induces important side effects (Schacke et al., 2004), and some patients are completely corticoid resistant or fail to show clinical improvement after treatment with high doses of oral glucocorticoids (Barnes et al., 1995). This situation can limit the use of corticosteroids and impairs the patients' life quality. Some reports suggest that the ineffectiveness of corticosteroids in some severe forms of asthma is due to their failure to decrease the level of TH1 cytokines, such as tumor-necrosis factor (TNF) (Truyen et al., 2006). Classic bronchodilator drugs including β_2 -adrenergic agonists and phosphodiesterase inhibitors have long been used to control the shortness of breath that is characteristic of asthmatic attacks; however, these drugs are also not free from side effects (Boswell-Smith et al., 2006). Currently, leukotriene antagonists have been employed successfully in asthma therapy. Nevertheless, they are not effective as a monotherapy and are therefore mainly used as a supplementary therapy to inhaled corticosteroids (Polosa, 2007). Mast cell inhibitors, monoclonal antibodies, 5-lipoxygenase inhibitors and allergen-specific immunotherapy have also found a place in asthma therapy (Edwards and Howell, 2000; Holgate and Polosa, 2008). However, novel, safe and effective drugs are needed for the treatment of asthma, and the assessment of new compounds with potentially unique mechanisms of action remains an important area of research.

In this work, we have for the first time demonstrated the anti-allergic properties and toxicological characterization of curine, an orally active bisbenzylisoquinoline alkaloid. To investigate the anti-allergic properties of curine, we used a well-established mouse model of asthma induced by allergic sensitization and challenge with OVA. Eosinophilic infiltration into the airways is a hallmark of this model; allergic challenge induces eosinophil maturation and differentiation from precursors in the bone marrow and their migration to sites of inflammation in response to mediators such as eotaxin and IL-5 (Gleich, 2000). Therefore, we performed a dose-response experiment with oral treatment with curine, and we observed that such treatment dose-dependently inhibited the number of eosinophils in the BAL, supporting the inhibitory role that curine plays in eosinophil recruitment.

To further understand the additional effects of curine, we analyzed the formation of lipid bodies in eosinophils. Increased lipid body assembly within eosinophils is closely associated with cellular activation and LTC₄ production in an allergic response and therefore plays important roles in inflammatory conditions and may be used as a marker of

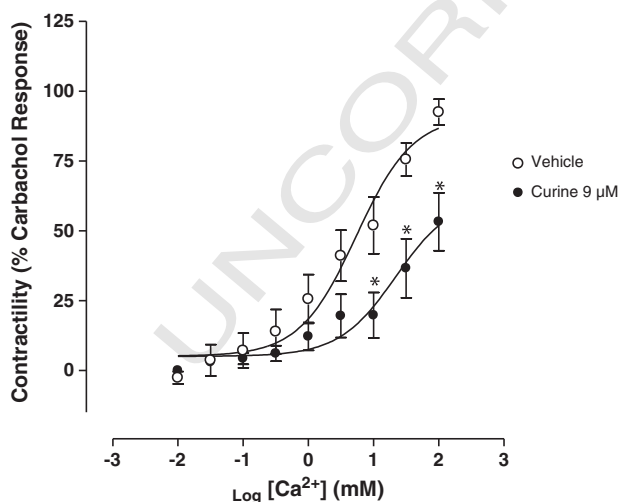


Fig. 4. The effects of curine on the calcium-induced tracheal contractile response. A contractile response was triggered after the cumulative application of calcium in tracheal segments maintained in Krebs solution rich in K⁺ (60 mM) and free of calcium. Pre-treatment with curine (9 μ M) led to a decrease in tension after the addition of calcium. Each point represents the mean $\pm$ S.E.M. from at least six segments. The magnitude of the voltage induced contraction of trachea calcium preparations was expressed as a percentage of the contractile response evoked by 2.5 mM carbachol. * Significantly different from the vehicle.

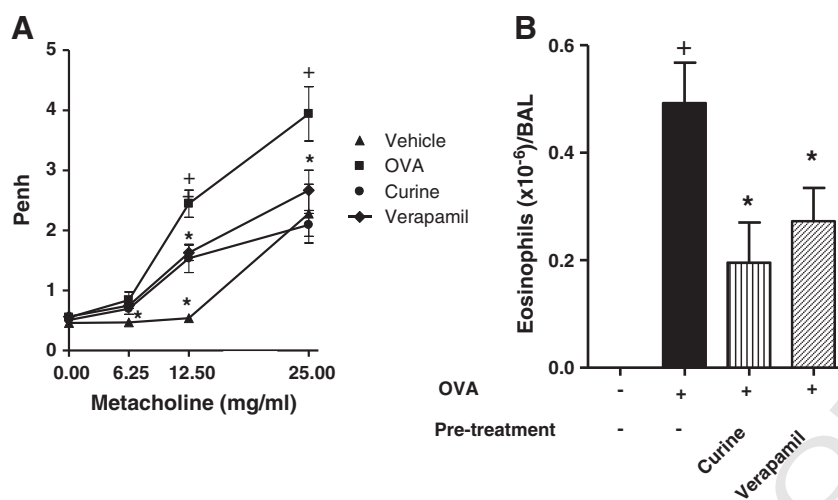


Fig. 5. A comparison between the anti-allergic effects of curine and verapamil. BALB/c mice ($n = 6-8$) primed with OVA were treated orally with curine (2.5 mg/kg) or verapamil (2.5 mg/kg) 1 h before each OVA challenge. A) AHR was analyzed by aerosolization with PBS followed by increasing concentrations of methacholine 24 h after the last allergic challenge. The AHR was expressed as the rate of enhanced pause (Penh). B) The eosinophils in the BAL were counted using a light microscope. These results are expressed as the mean $\pm$ SEM. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

eosinophil activation (Mesquita-Santos et al., 2006; Vieira-de-Abreu et al., 2005). We demonstrated not only that the animals treated with curine showed a reduced number of eosinophils in the BA but also that these eosinophils had fewer cytoplasmic lipid bodies, indicating that curine plays an inhibitory role on both migration and activation of these cells. Our group has demonstrated that the inhibition of lipid bodies is an important parameter that can be used to evaluate the anti-inflammatory effects of drugs; furthermore, evaluating the inhibition of lipid bodies may be useful in understanding the pharmacology and therapeutic efficacy of new interventions in numerous conditions such as asthma, allergies, cancer, atherosclerosis, diabetes and other diseases in which the formation of lipid bodies is a negative clinical parameter (Bozza et al., 2009). We observed that treatment with curine as well as dexamethasone significantly reduced the production of eotaxin in the model we used. It has been shown that eotaxin plays a key role in eosinophil migration and activation (Vieira-de-Abreu et al., 2005),

which supports the effects of curine described herein. AHR is a particular feature of asthma, resulting from complex interactions between inflammatory and airway smooth muscle (ASM) cells, leading to recurrent episodes of shortness of breath, wheezing and coughing (Lauzon et al., 2012). Here, we showed that curine has an important inhibitory activity on AHR, decreasing the observed Penh values to levels similar to those observed in the animals treated with dexamethasone. To elucidate the mechanisms involved in the anti-allergic effects of curine, we analyzed the production of key mediators of allergic responses, such as IL-13. Previous studies have demonstrated that blocking IL-13 in mice prevents the development of AHR (Wills-Karp et al., 1998). Furthermore, Kuperman et al. (2005, 2002) demonstrated that the overexpression of IL-13 in mice was sufficient to cause AHR, possibly through a direct effect on the airway epithelium. Importantly, IL-13 is also involved in the modulation of various pathophysiological aspects of asthma, such as class switching to IgE, mucus production, inflammation,

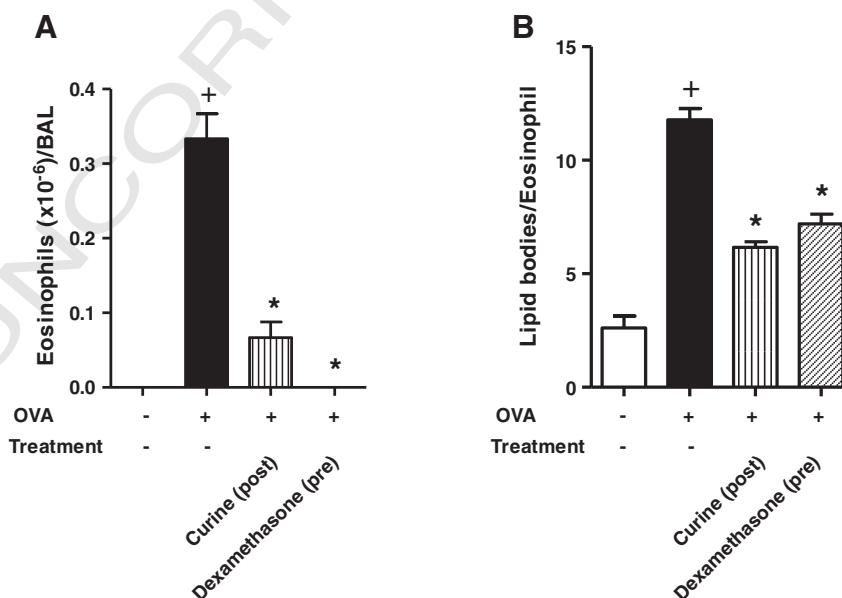


Fig. 6. The effect of post-treatment curine on eosinophil recruitment and activation. OVA-sensitized BALB/c mice ($n = 5-6$) received a single dose of curine (2.5 mg/Kg) 1 h after the last allergic challenge. Dexamethasone (2 mg/Kg) was administered 1 h before each challenge with OVA. The BAL was collected 24 h after the last challenge. The eosinophils (A) and lipid bodies (B) were counted using a light microscope. These results are expressed as the mean $\pm$ SEM. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

Table 1

The biochemical analyses of serum from male Swiss mice treated with curine (2.5 or 8 mg/kg) or dexamethasone (2 mg/kg) for 7 days.

Parameter (unit)	Vehicle	Curine (2.5 mg/kg)	Curine (8 mg/kg)	Dexamethasone (2 mg/kg)
Alkaline Phosphatase	222.40 ± 22.70	212.20 ± 32.80	218.90 ± 19.45	284.20 ± 38.13
ALT	79.50 ± 15.37	62.80 ± 4.25	82.67 ± 12.37	101.30 ± 13.37
AST	98.83 ± 15.91	94.10 ± 8.76	112.10 ± 11.17	175.10 ± 25.44*
Bilirubin	0.64 ± 0.03	0.67 ± 0.04	0.81 ± 0.04	1.15 ± 0.09*
Creatinine	0.20 ± 0.00	0.22 ± 0.01	0.22 ± 0.02	0.20 ± 0.00
Creatinine Kinase	289.00 ± 90.75	261.60 ± 60.55	267.40 ± 41.97	227.90 ± 26.16
Cholesterol	62.29 ± 2.50	65.67 ± 3.90	63.60 ± 4.52	106.60 ± 10.30*
Glucose	264.00 ± 25.26	249.60 ± 18.47	297.50 ± 15.29	292.80 ± 72.41
Total Proteins	5.29 ± 0.09	5.42 ± 0.08	5.54 ± 0.10	7.27 ± 0.17*
Uric acid	3.73 ± 0.47	3.90 ± 0.54	5.03 ± 0.42	2.90 ± 0.79

These values are expressed as the mean ± S.E.M. AST = aspartate transaminase. ALT = alanine transaminase.

* Significantly different from the vehicle group ($p < 0.05$).

airway remodeling and the contraction of ASM through an interaction with specific receptors on the cell surface (Bloemen et al., 2007).

Allergic mechanisms involve signaling and the activation of many cell types, including leukocytes and epithelial and airway muscle cells (Paul and Zhu, 2009). Eosinophilic inflammation and AHR are important features of asthma, and both processes require calcium-dependent mechanisms. Accordingly, recent work has demonstrated that verapamil, a calcium channel antagonist, significantly inhibited goblet cell hyperplasia, mucus hypersecretion and inflammation in a model of allergic asthma (Khakzad et al., 2012). Medeiros et al. (2011) demonstrated that curine might have a direct effect on L-type Ca^{2+} channels in vascular smooth muscle cells. In this work, we investigated the involvement of calcium-dependent mechanisms on the anti-allergic effects of curine. We demonstrated that curine pre-treatment significantly inhibited a calcium-induced trachea contractile response *ex vivo*, suggesting that curine inhibits the influx of calcium by blocking voltage-dependent Ca^{2+} channels in rat tracheal smooth muscle. Interestingly, in non-allergic mice, curine did not alter the bronchoconstriction induced by methacholine (*in vivo*), which is mediated by calcium release from intracellular stores (Foster et al., 1984). These data support our hypothesis that curine inhibits calcium channels in the cell membrane. Additionally, *in vivo* treatments with curine or verapamil (an L-type calcium-channel antagonist) using the same dose, time and method of administration had similar effects on AHR and eosinophilic inflammation, suggesting that the anti-allergic effects of curine may be, at least in part, dependent on the inhibition of calcium influx. Indeed, calcium-dependent signaling plays key roles both in asthma-related cytokine production and in their function. Antigen-triggered cytokine and chemokine expression is largely dependent on the activation of NFAT, a transcription factor that requires Ca^{2+} for its activation (Macian, 2005). Rothenberg et al. (1996) demonstrated that eotaxin signaling through its receptor, CCR3, effectively induced a calcium influx into eosinophils *in vitro*. Additionally, Moynihan et al. (2008) demonstrated that IL-13 modulated AHR by inducing SOCS expression and a calcium influx into human airway smooth muscle cells.

Finally, we investigated the possible toxicity of curine by oral treatment because this parameter has not previously been addressed experimentally. To that end, Swiss mice were treated daily for seven days with curine at doses of 2.5 and 8 mg/Kg. The dose of 2.5 mg/Kg was chosen because it had a significant anti-allergic effect throughout the experiments and the dose of 8 mg/Kg is 10 fold higher than the ED_{50} . We also included a group treated with dexamethasone (2 mg/Kg, *p.o.*) here as we did for other experiments. All of the treatments were compared with the oral treatment with PBS with HCl and NaOH added (pH 7), which was used as a vehicle for the dilution of the curine. The toxicological evaluation showed that the treatment with curine induced no changes in the hematologic or biochemical parameters. Additionally, the treatment did not induce the formation of gastric ulcers, and there were no physical or behavioral changes, indicating that under these conditions, curine showed no evident toxicity.

Aiming to provide additional therapeutic information concerning the anti-allergic properties of curine, we have demonstrated that curine post-treatment was effective in the inhibition of eosinophil recruitment and activation; this observation highlights the potential of curine as an anti-allergic compound, as post-treatment more accurately replicates what happens in clinical situations.

In conclusion, orally administered curine showed potent anti-allergic activity without inducing evident toxicity and, as such, has the potential for the development of anti-asthmatic drugs.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2013.08.015>.

Disclosure of conflict of interest

The authors state that they have no conflict of interest.

Uncited references

Bezerra-Santos et al., 2004
Kuperman et al., 2002

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References

- Barnes, P.J., 2006. How corticosteroids control inflammation. *Br. J. Pharmacol.* 148, 245–254.
- Barnes, P.J., 2008. Immunology of asthma and chronic obstructive pulmonary disease. *Nat. Rev. Immunol.* 8, 183–192.
- Barnes, P.J., 2011. Glucocorticosteroids: current and future directions. *Br. J. Pharmacol.* 163, 29–43.
- Barnes, P.J., Adcock, I.M., 2009. Glucocorticoid resistance in inflammatory diseases. *Lancet* 373, 1905–1917.
- Barnes, P.J., Greening, A.P., Crompton, G.K., 1995. Glucocorticoid resistance in asthma. *Am. J. Respir. Crit. Care Med.* 152, 125–140.
- Bezerra-Santos, C.R., Balestieri, F.M., Rossi-Bergmann, B., Peçanha, L.M., Piuvezam, M.R., 2004. *Cissampelos sympodialis* Eichl. (Menispermaceae): oral treatment decreases IgE levels and induces a Th1-skewed cytokine production in ovalbumin-sensitized mice. *J. Ethnopharmacol.* 95, 191–197.
- Bezerra-Santos, C.R., Peçanha, L.M.T., Piuvezam, M.R., 2005. *Cissampelos sympodialis* Eichl. (Menispermaceae) inhibits anaphylactic shock reaction in murine allergic model. *Braz. J. Pharmacogn.* 15, 287–291.
- Bezerra-Santos, C.R., Vieira-de-Abreu, A., Barbosa-Filho, J.M., Bandeira-Melo, C., Piuvezam, M.R., Bozza, P.T., 2006. Anti-allergic properties of *Cissampelos sympodialis* and its isolated alkaloid warifetine. *Int. Immunopharmacol.* 6, 1152–1160.
- Bezerra-Santos, C.R., Vieira-de-Abreu, A., Vieira, G.C., Ribeiro-Filho, J., Barbosa-Filho, J.M., Pires, A.L., Martins, M.A., Souza, H.S., Bandeira-Melo, C., Bozza, P.T., Piuvezam, M.R., 2012. Effectiveness of *Cissampelos sympodialis* and its isolated alkaloid warifetine in airway hyperreactivity and lung remodeling in a mouse model of asthma. *Int. Immunopharmacol.* 13, 148–155.

- Bloemen, K., Verstraelen, S., Van-Den-Heuvel, S., Witters, H., Nelissen, I., Schoeters, G., 2007. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunol. Lett.* 113, 6–18.
- Boswell-Smith, V., Cazzola, M., Page, C.P., 2006. Are phosphodiesterase-4 inhibitors just more theophylline? *J. Allergy Clin. Immunol.* 117, 1237–1243.
- Bozza, P.T., Yu, W., Penrose, J.F., Morgan, E.S., Dvorak, A.M., Weller, P.F., 1997. Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. *J. Exp. Med.* 186, 909–920.
- Bozza, P.T., Magalhães, K.G., Weller, P.F., 2009. Leukocyte lipid bodies – biogenesis and functions in inflammation. *Biochem. Biophys. Acta* 1791 (6), 540–551.
- Chatila, T.A., 2004. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends Mol. Med.* 10, 493–499.
- Costa, H.F., Bezerra-Santos, C.R., Barbosa-Filho, J.M., Martins, M.A., Piuvezam, M.R., 2008. Warifteine, a bisbenzylisoquinoline alkaloid, decreases immediate allergic and thermal hyperalgesic reactions in sensitized animals. *Int. Immunopharmacol.* 8, 519–525.
- Dias, C.S., Barbosa-Filho, J.M., Lemos, V.S., Cortes, S.F., 2002. Mechanisms involved in the vasodilator effect of curine in rat resistance arteries. *Planta Med.* 68, 1049–1051.
- Edwards, A.M., Howell, J.B., 2000. The chromones: history, chemistry and clinical development. A tribute to the work of Dr R. E. C. Altounyan. *Clin. Exp. Allergy* 30, 756–774.
- Edwards, M.R., Bartlett, N.W., Clarke, D., Birrell, M., Belvisi, M., Johnston, S.L., 2009. Targeting the NF- κ B pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol. Ther.* 121, 1–13.
- Faqi, A., 2012. *A Comprehensive Guide to Toxicology in Preclinical Drug Development*, First ed. Academic Press, New York.
- Foster, R.W., Okpalugo, B.I., Small, R.C., 1984. Antagonism of Ca²⁺ and other actions of verapamil in guinea-pig isolated trachealis. *Br. J. Pharmacol.* 81, 499–507.
- Gleich, G.J., 2000. Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* 105, 651–663.
- Harrison, T.W., Osborne, J., Newton, S., Tattersfield, A.E., 2004. Doubling the dose of inhaled corticosteroid to prevent asthma exacerbations: randomised controlled trial. *Lancet* 363, 271–275.
- Holgate, S.T., Polosa, R., 2008. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* 8, 116–124.
- Ivancsó, I., Böcskei, R., Müller, V., Tamási, L., 2013. Extrafine inhaled corticosteroid therapy in the control of asthma. *J. Asthma Allergy* 6, 69–80.
- Khakzad, M.R., Mirsadraee, M., Mohammadpour, A., Ghafarzadegan, K., Hadi, R., Saghari, M., Meshkat, M., 2012. Effect of verapamil on bronchial goblet cells of asthma: an experimental study on sensitized animals. *Pulm. Pharmacol. Ther.* 25, 163–168.
- Kuperman, D.A., Huang, X., Koth, L.L., Chang, G.H., Dolganov, G.M., Zhu, Z., Elias, J.A., Sheppard, D., Erle, D.J., 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* 8, 885–888.
- Kuperman, D.A., Lewis, C.C., Woodruff, P.G., Rodriguez, M.W., Yang, Y.H., Dolganov, G.M., Fahy, J.V., Erle, D.J., 2005. Dissecting asthma using focused transgenic modeling and functional genomics. *J. Allergy Clin. Immunol.* 116, 305–311.
- Lauzon, A.M., Bates, H.T., Donovan, G., Tawhai, M., Sneed, J., Sanderson, M.J., 2012. A multi-scale approach to airway hyperresponsiveness: from molecule to organ. *Front. Physiol.* 3, 1–25.
- Lloyd, C.M., Gonzalo, J.A., Nguyen, T., Delaney, T., Tian, J., Oettgen, H., Coyle, A.J., Gutierrez-Ramos, J.C., 2001. Resolution of bronchial hyperresponsiveness and pulmonary inflammation is associated with IL-3 and tissue leukocyte apoptosis. *J. Immunol.* 166, 2033–2204.
- Macian, F., 2005. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* 5, 472–484.
- Mambu, L., Martin, M.T., Razafimahefa, D., Ramanitrahasimbola, D., Rasoanaivo, P., Frappier, F., 2000. Spectral characterization and antiparasitodal activity of bisbenzylisoquinolines from *Isolona ghesquiereina*. *Planta Med.* 66, 537–540.
- Medeiros, M.A.A., Pinho, J.F., De-Lira, D.P., Barbosa-Filho, J.M., Araújo, D.A.M., Cortes, S.F., Lemos, V.S., Cruz, J.S., 2011. Curine, a bisbenzylisoquinoline alkaloid, blocks L-type Ca²⁺ channels and decreases intracellular Ca²⁺ transients in A7r5 cells. *Eur. J. Pharmacol.* 669, 100–107.
- Mesquita-Santos, F.P., Vieira-de-Abreu, A., Calheiros, A.S., Figueiredo, I.H., Castro-Faria-Neto, H.C., Weller, P.F., Bozza, P.T., Diaz, B.L., Bandeira-Melo, C., 2006. Cutting edge: prostaglandin D₂ enhances leukotriene C₄ synthesis by eosinophils during allergic inflammation: synergistic *in vivo* role of endogenous eotaxin. *J. Immunol.* 176, 1326–1330.
- Moynihan, B.J., Tolloczko, B., Bassam, S.E., Ferraro, P., Michoud, M., Martin, J.G., Laberge, S., 2008. IFN- γ , IL-4 and IL-13 modulate responsiveness of human airway smooth muscle cells to IL-13. *Respir. Res.* 9 (1), 84.
- Paul, W., Zhu, J., 2010. How are TH₂-type immune responses initiated and amplified. *Nat. Rev. Immunol.* 10, 225–235.
- Polosa, R., 2007. Critical appraisal of antileukotriene use in asthma management. *Curr. Opin. Pulm. Med.* 13, 24–30.
- Rhen, T., Cidlowski, J.A., 2005. Antiinflammatory action of glucocorticoids – new mechanisms for old drugs. *N. Engl. J. Med.* 353, 1711–1723.
- Rothenberg, M.E., Robert-OWNbey, T., Mehlhop, P.D., Loiselle, P.M., Van de Rijn, M., Bonventre, J.V., Oettgen, H.C., Leder, P., Luster, A.D., 1996. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice. *Mol. Med.* 2 (3), 334–348.
- Schacke, H., Schottelius, A., Docke, W.D., Strehlke, P., Jaroch, S., Schmees, N., Rehwinkel, H., Hennekes, H., Asadullah, K., 2004. Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. *Proc. Natl. Acad. Sci. U. S. A.* 101, 227–232.
- Seow, W.K., Li, S.T.Y., Thong, Y.H., 1986. Inhibitory effect of tetrandrine on human neutrophil and monocyte adherence. *Immunol. Lett.* 13, 83–88.
- Szefer, S.J., 2011. Advancing asthma care: the glass is only half full. *J. Allergy Clin. Immunol.* 128 (3), 485–494.
- Teh, B.S., Seow, W.K., Li, S.Y., Thong, Y.H., 1990. Inhibition of prostaglandin and leukotriene generation by the plant alkaloids tetrandrine and berberine. *Int. J. Immunopharmacol.* 12, 321–326.
- Truyen, E., Coteur, L., Dilissen, E., Overbergh, L., Dupont, L.J., Ceuppens, J.L., Bullens, D.M., 2006. Evaluation of airway inflammation by quantitative TH1/TH2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 61, 202–208.
- Vieira-de-Abreu, A., Assis, E.F., Gomes, G.S., Castro-Faria-Neto, H.C., Weller, P.F., Bandeira-Melo, C., Bozza, P.T., 2005. Allergic challenge-elicited lipid bodies compartmentalize *in vivo* leukotriene C₄ synthesis within eosinophils. *Am. J. Respir. Cell Mol. Biol.* 33, 254–261.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258–2261.