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Inhaled braylin regulates Th2 response and induces relaxant effects in the airway muscles in a model of ovalbumin-induced asthma

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ABSTRACT

Background: Coumarins are compounds with wide and relevant pharmacological properties, being considered one of the most important chemical classes among natural compounds. Braylin (6-methoxyseselin) is a coumarin whose pharmacological properties have not yet been extensively explored. Previous studies have shown its anti-inflammatory and immunomodulatory activities, potentially associated with glucocorticoid receptors, plus it is a phosphodiesterase 4 inhibitor. Thus, the present study was designed to investigate the pharmacological potential of braylin for asthma treatment.

Methods: Mice induced to an asthma model using ovalbumin (OVA) were treated with vehicle, braylin, or dexamethasone via intraperitoneal injection or inhalation, and the bronchoalveolar lavage (BAL) was collected to evaluate infiltration of inflammatory cells, and cytokine levels. Histopathological and morphometric analysis of lung tissue were also conducted, while *ex vivo* isometric measurement assessed the effect of braylin on tracheal relaxation.

Results: Braylin (50 mg/kg) showed similar efficacy in reducing the total count of inflammatory cells in the BAL of asthmatic mice by inhalation or intraperitoneal route. Inhaled braylin reduced, in a dose-dependent manner (25 to 100 mg/kg), the total count of inflammatory cells in the BAL of OVA-induced mice, more specifically eosinophils and neutrophils. Plus, inhaled braylin reduced the BAL levels of IL-4, IL-5, and IL-13, cytokines involved in asthma Th2 response. It also reduced pulmonary inflammatory infiltrate and the occurrence of goblet cell metaplasia. In a set of *ex vivo* assays, braylin was able to induce concentration-dependent relaxation of the trachea from mice with or without OVA-induced asthma.

Conclusions: The present results suggest that braylin may be a promising candidate for the treatment of asthma by regulating the Th2 response, inducing relaxant effects in the airway muscles, and presenting efficacy by inhalation route.

1. Introduction

Asthma is a heterogeneous respiratory disease characterized by chronic airway inflammation. It includes a group of symptoms displayed by the patients that vary over time and in intensity, such as cough, shortness of breath, wheezing, and chest tightness (Asthma, 2021).

Abbreviations: ANOVA, Analysis of variance; BAL, Bronchoalveolar lavage; Dexa, Dexamethasone; HE, Hematoxylin and eosin; inh., Inhalation; ip., Intraperitoneal; OVA, Ovalbumin; PAS, Periodic acid-Schiff; PDE4, Phosphodiesterase 4; SD, Standard deviation; SEM, Standard error of the mean; PBS, Phosphate Buffered Saline.

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Genetic factors and environmental exposure influence the pathophysiology of asthma, which contributes to the heterogeneity of this disease, evidenced by the significant difference in outcomes in patients from different socioeconomic classes (Barnthouse and Jones, 2019). In the last 60 years, the prevalence of asthma has increased, which has made this chronic disease one of the most common in the world, mainly due to the population's transition to increasingly urbanized environments (Beasley et al., 2015). Currently, more than 240 million people worldwide have been diagnosed with asthma (Stern et al., 2020), and each year approximately 43 million new cases are diagnosed, which is also associated with a high mortality rate (Mattiuzzi and Lippi, 2020).

Options for the treatment of asthma are available. Although not curative, they can help control symptoms, allowing the asthmatic patient to have an active life and reducing mortality (Mattiuzzi and Lippi, 2020). The choice of therapeutic alternatives for the treatment of this respiratory disease depends on its severity and includes inhaled and oral corticosteroids (Busse et al., 2008), long-acting and short-acting β2 adrenergic agonists (Tee et al., 2007), and leukotriene receptor antagonists (Montuschi and Peters-Golden, 2010). More recently, phosphodiesterase 4 (PDE4)-inhibitors have been considered for the treatment of respiratory diseases such as chronic obstructive pulmonary disease (COPD) and asthma (Al-Sajee et al., 2019; Bodkhe et al., 2020; Luo et al., 2018), plus the potential additive effect with corticoids has been taken into account (Giembycz and Newton, 2015; Grundy et al., 2016). Despite the clear benefits of pharmacological therapy for asthmatic patients, the adverse effects of these drugs have a negative impact on their quality of life. These effects include reduced growth rate, osteoporosis, diabetes, suppression of the hypothalamic-pituitary-adrenal axis, cataracts, and respiratory infections due to the chronic use of glucocorticoids (Heffler et al., 2018), in addition to hypokalemia, cardiac chronotropic effects, nausea, and vomiting (Newnham, 2001). As a main consequence of the unwanted effects, a low rate of adherence to the prophylactic medication is observed, which increases the hospitalization rates (Dekhuijzen et al., 2018; Perry et al., 2019). One of the strategies to reduce adverse effects and increase the effectiveness of treatment is to combine more than one pharmacological class to obtain a synergistic effect, and the association between inhaled glucocorticoids and β2 agonists is one of the most common combinations for asthma (Lazarinis et al., 2014; O'Byrne et al., 2019; Sobieraj et al., 2018). Furthermore, an association that has been considered a good strategy in the treatment of respiratory diseases is the use of glucocorticoids with PDE4 inhibitors (Giembycz and Newton, 2015; Grundy et al., 2016).

Coumarins are compounds with wide and relevant pharmacological properties (Annunziata et al., 2020). This group is being considered one of the most important chemical classes among natural compounds (Malikov et al., 1998; Pereira et al., 2018). Within this group there are a few compounds with approved clinical use, for instance as anticoagulant (Wardrop and Keeling, 2008), anti-tumor (Kupeli Akkol et al., 2020), and anti-inflammatory (Annunziata et al., 2020). Braylin (6-methoxyseselin; Fig. 1) is a coumarin first described in 1949, whose pharmacological properties have not been extensively explored (Aneja et al., 1958). Until recently, anti-platelet aggregating activity (Tsai et al., 1998), relaxing vessel (Rakotoarison et al., 2003; Santos et al., 2021), and phosphodiesterase 4 inhibition (Chen et al., 2018; Lin et al., 2014) have been identified for this coumarin. Our research group was the first to describe the pharmacological properties of braylin in vivo, in a previous work that demonstrated its anti-inflammatory and immunomodulatory action, possibly mediated by glucocorticoid receptors (Espirito-Santo et al., 2017). Thus, considering the anti-inflammatory and immunomodulatory properties of braylin, as well as its potential to modulate glucocorticoid receptors and PDE4, the present study was designed to investigate the pharmacological potential of braylin as a candidate for the drug discovery process of new antiasthmatic drugs.

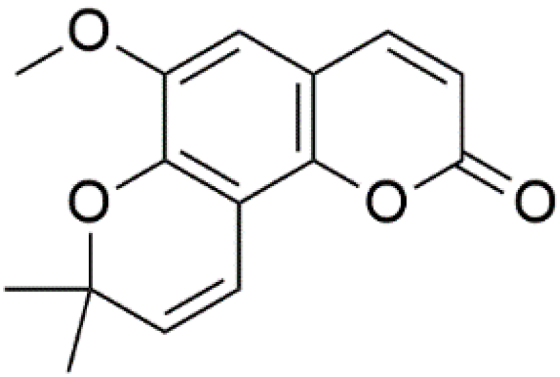

Fig. 1. Structural formula of braylin.

2. Results

2.1. Influence of the administration route on braylin bioactivity in the asthma model in mice

Inhalation is the main route of administration of medicines in asthma because it enables the delivery of drugs directly to the bronchial epithelium, reducing the adverse effects. Thus, it is important for antiasthmatic drugs and prototypes to be active via inhalation. In order to establish whether braylin presents pharmacological activity by inhalation route, the effect of inhalation or intraperitoneal (ip.) administration of this coumarin on the inflammatory cell count in bronchoalveolar lavage (BAL) of mice was initially compared in a single dose assay. The bar chart in Fig. 2 displays the number of inflammatory cells in each experimental group and compares the different routes of braylin's administration. Mice were exposed to the ovalbumin (OVA) administration protocol for induction of experimental asthma and treated with vehicle, braylin, or dexamethasone. OVA-induced mice treated with vehicle showed an increase in the number of total inflammatory cells in the BAL compared to naïve mice, confirming the development of asthma (Fig. 2). The inhalation or intraperitoneal administration of braylin (50 mg/Kg) significantly decreased, with similar efficacy, the number of inflammatory cells in the BAL ($p < 0.05$) of asthmatic mice. Significant inhibition of this parameter was also observed in mice treated intraperitoneally with the gold standard drug, dexamethasone, at a dose of 30 mg/Kg.

2.2. Dose-dependent profile of inhaled braylin in the OVA-induced mouse asthma model

The dose-dependency profile of braylin administered by inhalation in the dose range of 12.5 to 100 mg/Kg was next assessed (Fig. 3). The bar chart in Fig. 3 shows the number of inflammatory cells in each experimental group. When compared to vehicle-treated mice, inhaled braylin at doses of 25, 50, and 100 mg/Kg reduced, in a dose-dependent way, the number of inflammatory cells in the BAL of mice with experimental asthma ($p < 0.05$). There was no statistical difference between the doses of 100 and 50 mg/Kg, indicating that 50 mg/kg is the maximum effective dose. Dose dependence was evidenced by the difference in the magnitude of the effect induced by the doses of 50 and 25 mg/kg. Inhaled braylin exhibited no longer an effect at the dose of 12.5 mg/Kg. Importantly, the effect of braylin administered by inhalation had a similar efficacy to that of dexamethasone (30 mg/Kg/ip) administered by systemic route, the gold standard in this assay.

2.3. Braylin reduces the amount of eosinophils and neutrophils in the bronchoalveolar lavage

In addition to quantifying the total number of inflammatory cells in the bronchoalveolar lavage, the impact of inhaled braylin treatment on different types of leukocytes was investigated. The leukocyte differential

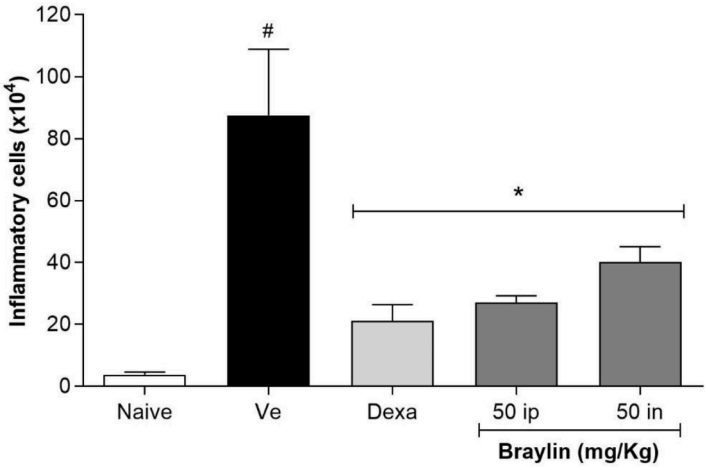

Fig. 2. Effect of braylin when administered by different routes on inflammatory cell count in the bronchoalveolar lavage (BAL) of asthmatic mice. The X-axis represents the tested groups: mice without experimental manipulation (naïve), mice induced to the asthma model and treated with vehicle (Ve; 10% propylene glycol in saline), dexamethasone intraperitoneally (Dexa; 30 mg/Kg; gold standard), braylin intraperitoneally (50 ip; 50 mg/kg), or braylin inhalation (50 in; 50 mg/kg). The Y-axis shows the amount of total inflammatory cells (x10^4) counted in the BAL. Treatments were carried out once daily for 5 consecutive days, 2 h before the challenge with ovalbumin. The bronchoalveolar lavage was collected for measurements 24 h after the last challenge. *Significantly different from the vehicle group (p <0.05). #Significantly different from naïve group (p <0.0001). Data represented as mean ± SD; n = 5 animals per group. One-way ANOVA test, followed by Tukey's test.

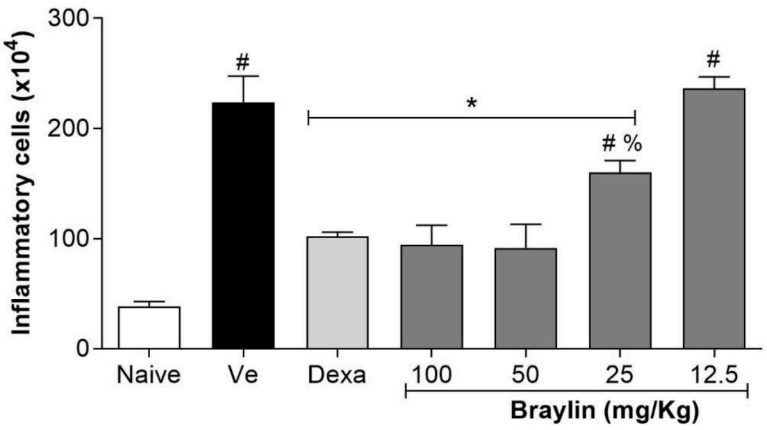

Fig. 3. Dose-response curve of inhaled braylin in the mouse asthma model. The X-axis represents the tested groups: mice without experimental manipulation (naïve), mice induced to the asthma model treated with inhaled vehicle (Ve; 10% propylene glycol in saline), inhaled braylin (12.5 to 100 mg/Kg), or systemic dexamethasone (Dexa; 30 mg/Kg/ip.; gold standard). The Y-axis shows the total amount of inflammatory cells (x10^4) obtained in the bronchoalveolar lavage. The treatments were carried out once daily for 5 consecutive days, 2 h before the challenge with ovalbumin. The bronchoalveolar lavage was collected for measurements 24 h after the last challenge. *Significantly different from the vehicle group (p <0.05). #Significantly different from the naïve group (p <0.05).%Significantly different from the braylin-50 mg/Kg group (p <0.05). Data represented as mean ± SD: n = 5 animals per group. One-way ANOVA test, followed by Tukey's test.

count identified and quantified the number of eosinophils, neutrophils, and mononuclear cells in the BAL samples (Fig. 4). Fig. 4 shows representative photomicrographs of inflammatory cells in the bronchoalveolar lavage of mice from different experimental groups. It also includes bar graphs reflecting the number of leukocytes in each experimental group. The treatment by inhalation with braylin (50 mg/Kg) lowers the proportion of eosinophils and neutrophils present in the BAL compared to the mice treated with vehicle (p <0.05) with similar efficacy to that of systemic dexamethasone (30 mg/Kg/ip), the gold standard. Accordingly, the percentage of mononuclear cells in the samples of mice treated with braylin was also comparable to the values observed for dexamethasone, which presented an increase compared to the vehicle (p <0.05).

2.4. Braylin modulates Th2 cytokines: IL-4, IL-5 e IL-13

The levels of cytokines that participate in the Th2 response were quantified in the bronchoalveolar lavage of mice from different experimental groups and are displayed in the bar graph in Fig. 5. OVA-induced mice showed elevated levels of IL4, IL-5, and IL13 cytokines in BAL compared to naïve mice (p <0.05). Inhalation of braylin at doses of 25 and 50 mg/Kg was able to reduce the levels of IL-4, IL-5, and IL-13 in the BAL of mice (p <0.05). Whereas braylin at a dose of 12.5 mg/Kg, induced a significant reduction of IL-5 and IL-13, but not of IL-4, in the BAL of mice. The animals treated with systemic dexamethasone (30 mg/Kg/ip.) showed a reduction in the levels of all cytokines quantified in BAL, with a magnitude similar to that obtained by the inhalation treatment with braylin.

2.5. Braylin reduces pulmonary inflammatory infiltrate and the occurrence of goblet cell metaplasia

To characterize the tissue changes caused by the induction of the asthma model and the possible effect of braylin on the migration of inflammatory cells, sections of lungs stained with hematoxylin-eosin (HE) were examined. Fig. 6 exhibits representative photomicrographs of histological sections of the lung of mice, in which both inflammatory cells (arrowheads) and mucus production by goblet cells (arrows) can be seen. It also includes bar graphs that quantify the different experimental groups' inflammatory cells and mucus production. A large cellular infiltrate containing lymphocytes, macrophages, and eosinophils was observed in the OVA-induced mice treated with vehicle (Figs. 6C and 6I). Mice that received braylin inhalation (50 mg/Kg) had a reduction in the pulmonary inflammatory cells infiltrate compared to mice treated with vehicle (p <0.05, Figs. 6G and 6I). Systemic treatment with dexamethasone (30 mg/Kg/ip.) was also able to reduce the pulmonary inflammatory infiltrate (p<0,05, Figs. 6E and 6I). Staining the pulmonary tissue with periodic acid-Schiff (PAS) enabled the observation of mucus production, characterizing the occurrence of metaplasia of goblet cells in the bronchiolar epithelium. The lungs of mice with experimental asthma treated with vehicle showed a superior area stained with PAS (p <0.05, Figs. 6D and 6J) compared to naïve mice (Figs. 6B and 6J), which indicates a greater mucus production. Inhalation treatment with braylin reduced the staining of goblet cells in the bronchiolar epithelium of asthmatic mice (p <0.05, Figs. 6H and 6J), which evidences the ability of this coumarin to modulate mucus production. As expected, systemic dexamethasone also reduced the presence of mucus in goblet cells

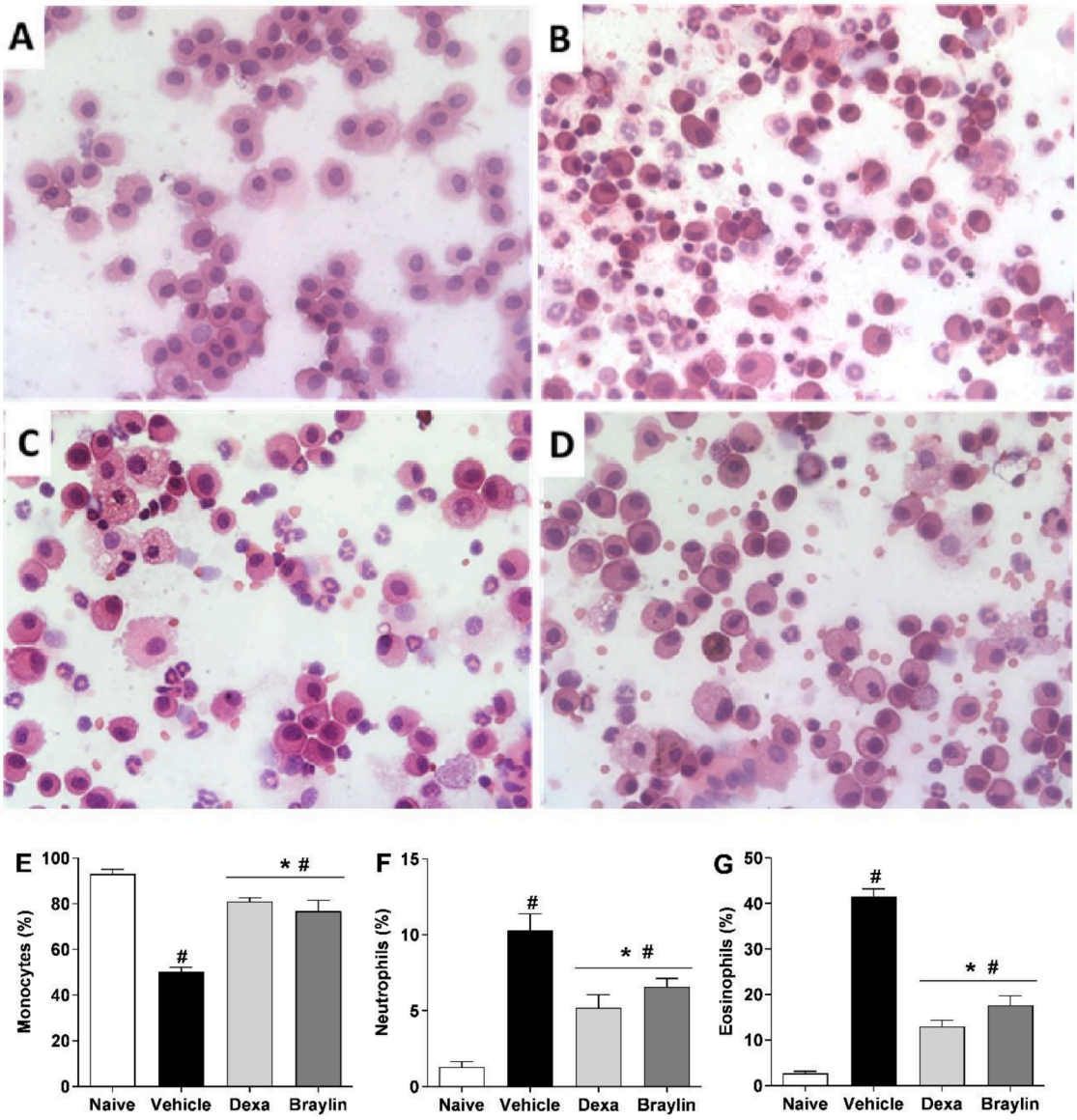

Fig. 4. Effect of inhaled braylin on the differential types of inflammatory cells in the bronchoalveolar lavage of asthmatic mice. Panels showcase representative photomicrographs of bronchoalveolar lavage cells from (A) naïve mice, (B) OVA-induced mice treated with inhaled vehicle, (C) OVA-induced mice treated with systemic dexamethasone (Dexa; 30 mg/Kg/ip.), and (D) OVA-induced mice treated with inhaled braylin (50 mg/ Kg). The treatments were carried out once daily for 5 consecutive days, 2 h before the challenge with ovalbumin. The samples were stained with hematoxylin and eosin (HE), 100X magnification. Panels E - G show the differential quantification of monocytes (E), neutrophils (F), and eosinophils (G) in bronchoalveolar lavage of the different experimental groups. Data represented as percentage in relation to the total count. #Significantly different from the naïve group (p <0.05). *Significantly different from the vehicle group (p <0.05). Data represented as mean ± SD; n = 5 animals per group. One-way ANOVA test, followed by Tukey's test.

stained with PAS (p<0,05, Figs. 6F and 6J).

2.6. Braylin induces tracheal relaxation

Fig. 7 shows recordings of the relaxing effect induced by braylin in trachea isolated from naïve (A) and asthmatic (B) mice precontracted with carbachol (10 μM), along with the scatter plot (C) showing a function of the percentage of relaxation and concentration of braylin, i.e., a concentration-response curve. As shown in Fig. 7, braylin (0.1 μM to 300 μM) induced a concentration-dependent relaxation in pre-contracted (carbachol 10 μM) tracheal segments with functional epithelium from both naïve and asthmatic mice ($E_{[300\ \mu M]}$:102.7 ± 2.8%, n = 5; $E_{[300\ \mu M]}$:102.6 ± 3.1%, n = 6, respectively).

3. Discussion

Braylin is a naturally occurring coumarin with previously related biological activity. The present work demonstrated, for the first time, the pharmacological properties of braylin administered by inhalation in an experimental model of asthma. Braylin modulated relevant parameters associated with the physiopathology of experimental asthma. It reduced the inflammatory cell infiltrate in the bronchoalveolar lavage and lung tissue, reduced the goblet cell metaplasia, and inhibited the production of key cytokines for the development of the Th2 immune response. Plus, in a set of *ex vivo* assays, braylin induced a relaxant effect on the airway muscle of the trachea of both naïve and asthmatic mice. These results show the potential of this natural coumarin as a drug candidate for the development of new drugs for the treatment of asthma.

Administration by inhalation was proven to be a good therapeutic strategy in the treatment of asthma because it allows reaching a high

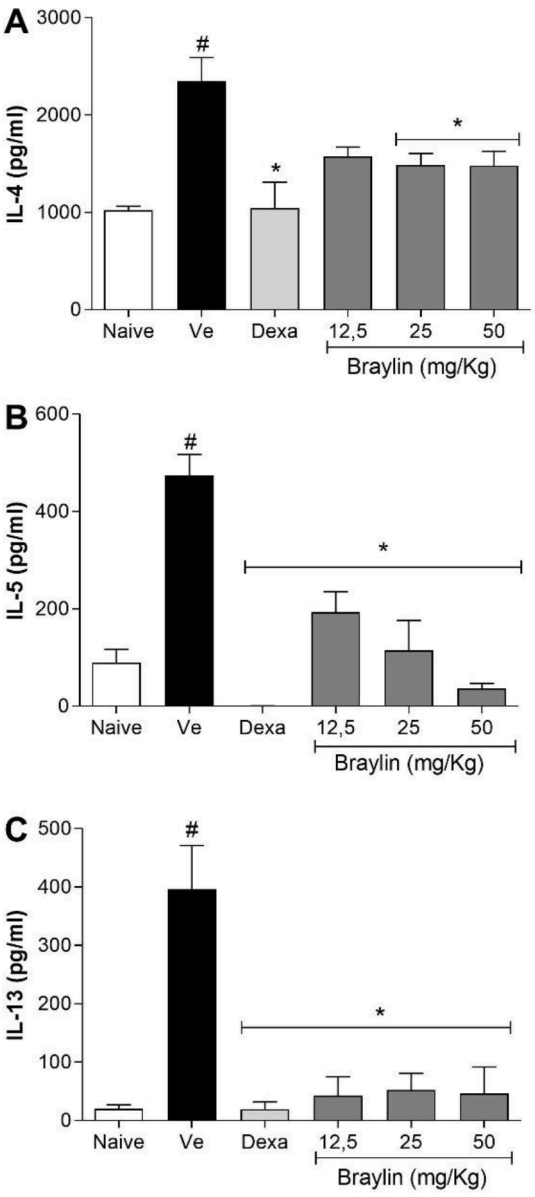

Fig. 5. Effect of inhaled braylin on cytokine levels in bronchoalveolar lavage in OVA-induced mice. Panels show the levels of cytokines IL-4 (A), IL-5 (B), and IL-13 (C) present in the bronchoalveolar lavage of mice, determined by ELISA. The X-axis represents the tested groups: mice without experimental manipulation (naïve), OVA-induced mice treated with inhaled vehicle (Ve; 10% propylene glycol in saline), systemic dexamethasone (Dexa; 30 mg/Kg/ip.; gold standard), or inhaled braylin (12.5, 25 and 50 mg/Kg). The treatments were carried out once daily for 5 consecutive days, 2 h before the challenge with ovalbumin. The bronchoalveolar lavage was collected for measurements 24 h after the last challenge. *Significantly different from the vehicle group ($p < 0.05$). #Significantly different from the naïve group ($p < 0.05$). Data represented as mean ± SD: $n = 6$ animals per group. One-way ANOVA test, followed by Tukey's test.

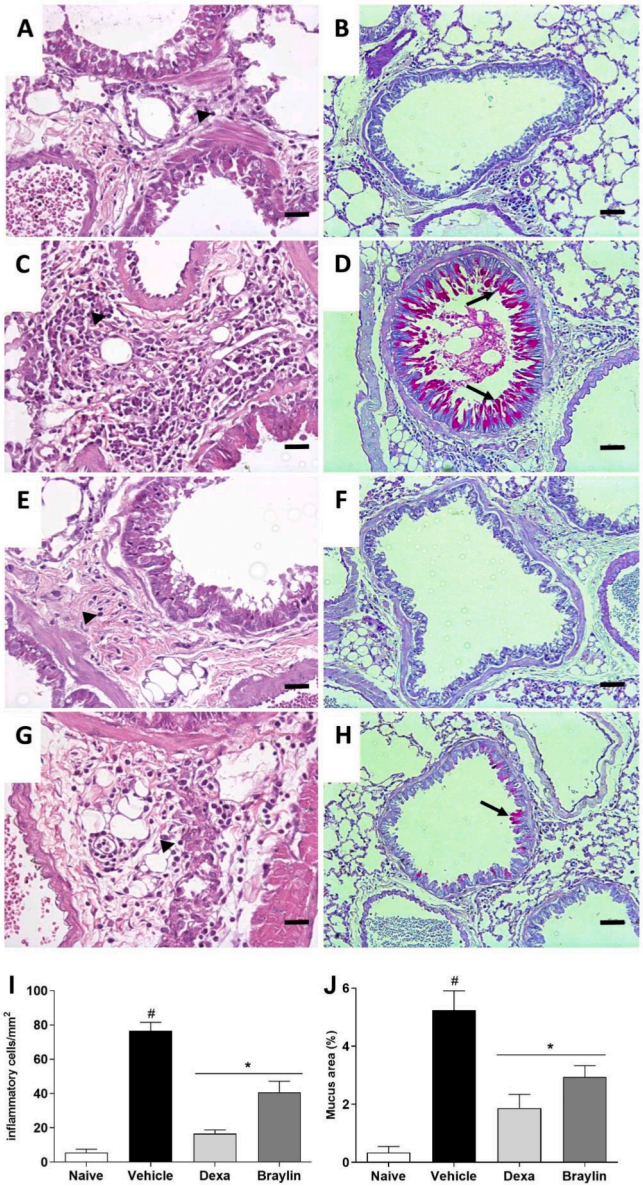

Fig. 6. Effects of braylin on pulmonary inflammatory infiltrate and mucus production. Panels show representative images of naïve mice (A-B), and asthmatic mice treated with inhaled vehicle (C-D), systemic dexamethasone (E-F; 30 mg/Kg/ip.), or inhaled braylin (G-H; 50 mg/Kg). Mice not experimentally manipulated comprise the naïve group. Lungs were stained with HE (A, C, E, G) or periodic acid-Schiff (B, D, F, H). Arrowheads indicate cells from the inflammatory infiltrate. Arrows indicate goblet cells marked with PAS. 40X magnification, 50 μm bar. Panel I shows the cell count in the inflammatory infiltrate of the different experimental groups, while panel J shows the quantification of the area occupied by mucus-producing goblet cells labeled with PAS. *Significantly different from the vehicle group ($p < 0.05$). #Significantly different from the naïve group ($p < 0.05$). Data represented as mean ± SD: $n = 5$ animals per group. One-way ANOVA test, followed by Tukey's test.

local concentration of the drug in the lungs, with reduced absorption and, consequently, fewer adverse effects (Lipworth, 1996). Indeed, the pulmonary route provides high drug deposition, leading to increased drug bioavailability in the airways and lungs (Ho et al., 2019). It is a major therapeutic advantage considering that the chronic use of corticosteroids can cause serious adverse effects such as osteopenia, hyperglycemia, cataracts, and hypertension, which limits their systemic use (Poetker and Reh, 2010; Shen and Young, 2012). In fact, the development of inhaled glucocorticoids revolutionized asthma pharmacotherapy in the 1950s. Even with reports of inhalation treatments in India 4000 years ago, it was only in 1956 that this method of administration was incorporated into the management of asthmatic patients, and today it is considered to have a central role in the treatment of this disease (Crompton, 2006). Therefore, it is an extremely desirable property of drug candidates for the treatment of asthma to have inhalation efficacy. Because of that, the effect of braylin administered by inhalation was initially compared to its effect after systemic administration. Inhaled braylin showed an efficacy comparable to that

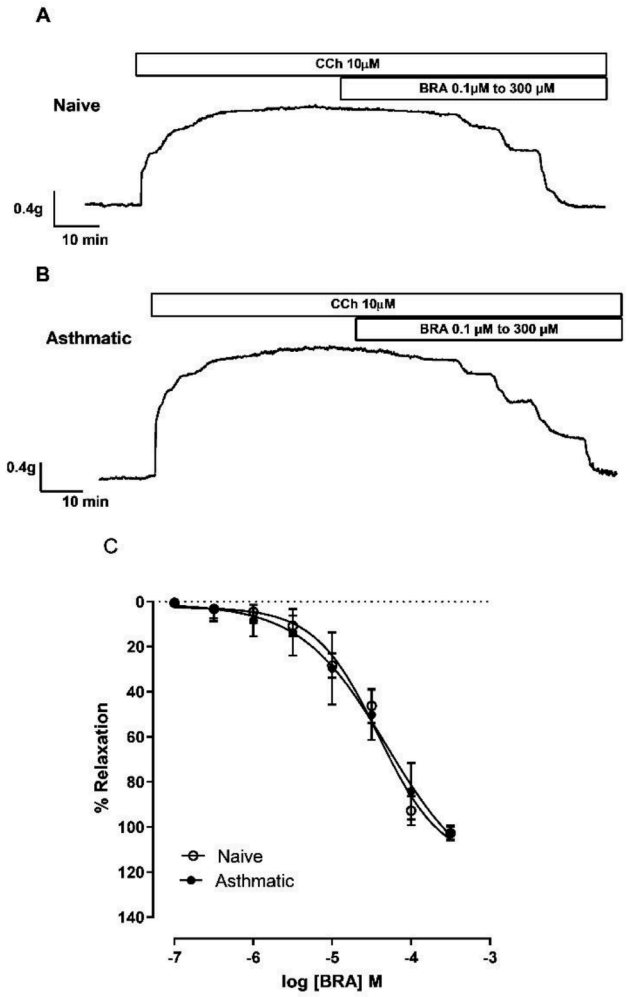

Fig. 7. Braylin induces a similar relaxing effect in the trachea isolated from naïve and asthmatic mice. Representative original recordings of the relaxing effect induced by braylin in trachea isolated from naïve (A) and asthmatic (B) mice precontracted with carbachol (10 μM). (C) Concentration-response curves for the relaxing effect induced by cumulative addition of braylin. Each point represents mean ± SD of isolated tracheal rings from 5 naïve and 6 asthmatic BALB/c mice. CCh: carbachol; BRA: braylin. Statistical analysis was performed using unpaired Student's *t*-test.

obtained by intraperitoneal administration in reducing the cellularity of the bronchoalveolar lavage in mice with induced asthma. The inhaled braylin effect was dose-dependent and had a similar efficacy to that of systemic dexamethasone, considered the gold standard in experimental asthma. From a perspective of drug development for asthma, it is interesting that a prototype drug exhibits the properties of dose-dependency and high efficacy by inhalation route, as it is one of the main routes of administration. Moreover, the dose-response relationship impacts on the determination of parameters such as the required dose, frequency of administration, and the therapeutic index for a drug in a population (Kenakin, 2017). These results highlight the potential of this coumarin to advance the drug discovery process.

In addition to the reduction in the total amount of inflammatory cells in the BAL, asthmatic mice treated with inhaled braylin showed a change in the proportion of inflammatory cell types. The BAL of naïve animals shows a greater proportion of monocytes than of eosinophils and neutrophils, whereas in asthmatic mice the percentage of granulocytes is increased, and monocytes is decreased. The treatment by inhalation with braylin shifted the proportion of inflammatory cells in the BAL of asthmatic mice to a profile closer to that observed in naïve mice. This suggests that this coumarin induces a beneficial phenotypic change in the population of inflammatory cells found in the bronchoalveolar lavage of animals with experimental asthma. The presence of neutrophils is common in some patients with chronic asthma, as these are the first cells to be recruited after the onset of the allergic reaction. When activated in lung tissue, neutrophils are able to release a myriad of inflammatory mediators, such as reactive oxygen species, myeloperoxidases, IL-8, thromboxanes, elastase, and adhesion molecules, which together promote bronchoconstriction, exudate formation, mucus overproduction, hyper-bronchial remodeling, and reactivity, that prolongs asthma symptoms (Cundall et al., 2003; Panettieri, 2018; Radermecker et al., 2018). In fact, most people with severe asthma exhibit an increase in the number of neutrophils in the BAL (Azim et al., 2021; O'Brien et al., 2015; Tanizaki et al., 1993). Similarly, the presence of eosinophils in the BAL has a direct relationship with the severity of asthma, as these cells play a central role in the pathogenesis of the disease. When recruited and activated via Th2 cytokines, such as IL-4, IL-5, and IL-13, eosinophils release leukotrienes and other arachidonic acid-driven inflammatory mediators, increasing the pulmonary inflammatory state, and inducing hyperresponsiveness and airway remodeling (Barrios et al., 2006; Possa et al., 2013).

In this context, the modulation/inhibition of Th2 cytokine production is an interesting target for asthma pharmacotherapy management. Importantly, braylin was able to negatively modulate the levels of IL-4, IL-5, and IL-13, cytokines that are directly involved in the signaling, recruitment, and differentiation of cells involved in the pathophysiology of asthma (Boyton and Altmann, 2004). It has been shown that the increase in IL-5 in asthmatic individuals is directly linked to the differentiation, recruitment, and activation of eosinophils in the lungs (Li and Wu, 2017). Likewise, IL-4 and IL-13 are responsible for the release of IgE via B cell activation, and act directly on the smooth muscles and epithelium of the airways, causing their hyperreactivity (Barrios et al., 2006). In line with the present data, the ability of coumarins to modulate the production of cytokines that are part of the Th2 response has already been described. Previous work has demonstrated that a group of coumarins were able to reduce the release of IL-4 and histamine by mast cells (Li and Wu, 2017). Moreover, the systemic administration of osthole, a coumarin derivative, also reduced the release of IL-4, IL-5, and IL-13, the inflammatory infiltrate, and the mucus production in lung tissue in an OVA-induced asthma model (Chiang et al., 2017).

The metaplasia of goblet cells and the consequent increase in mucus production are responsible for the first line of defense against toxic substances, allergens, or debris that access the airways (Curran and Cohn, 2010). Although mucus production is an important feature of innate immunity, within the pathological context of asthma, mucus secretion is increased, and mucociliary clearance is reduced. This state leads to mucus accumulation and airway plugging (Zhou-Suckow et al., 2017). Consequently, when mucosal metaplasia persists, mucus hypersecretion contributes to the increase in morbidity and mortality in asthmatic patients (Hays and Fahy, 2003), considering that mucus plugs have been found to be a common occurrence in severe forms of asthma (Dunican et al., 2018). Here, inhalation treatment with braylin was also able to reduce the production of mucus by goblet cells in lung tissue. Previous studies have correlated the reduction in mucus production with the reduction of the allergic response in an asthma model (Vasconcelos et al., 2009; Wang et al., 2017; Xiong et al., 2014). This correlation highlights the importance of modulating goblet cell metaplasia, a characteristic parameter of asthmatic conditions. Thus, the inhibitory effect of braylin on the production of mucus by goblet cells reinforces the therapeutic potential of this coumarin for asthma.

Along with goblet cell metaplasia and IgE production, bronchial hyperresponsiveness is one of the hallmarks of T2 driven allergic asthma. IL-4 and IL-13 are key mediators of hyperreactivity of smooth muscles and epithelium of the airways and were modulated by braylin. This modulation could indicate a possible role in airway muscle responsiveness, which was investigated in a set of *ex vivo* experiments on

trachea segments of mice. Effectively, braylin exhibited a potent relaxing effect in trachea segments in both naïve and asthmatic mice. To the best of our knowledge, this is the first work that shows the relaxant effect induced by braylin on an airway muscle of the trachea. As previously described, braylin has an anti-inflammatory property (Espirito-Santo et al., 2017) and may act as a phosphodiesterase inhibitor (Lin et al., 2014). It has also been described that braylin could participate in cyclic monophosphate nucleotides pathway, such as cAMP and cGMP, inducing vasorelaxation of the superior mesenteric artery (Santos et al., 2021; Singh et al., 2020). Although these pathways are also crucial for the regulation of airway muscle function, the exact mechanism of action underlying braylin-induced airway smooth muscle responses remains to be investigated.

In a previous work, evidence has demonstrated by using in vitro, *in silico*, and in vivo approaches that braylin induces anti-inflammatory effects that are mediated, at least in part, by glucocorticoid receptors (Espirito-Santo et al., 2017). Indeed, pharmacologically active molecules that exert an effect in asthma models by activating glucocorticoid receptors (Belvisi et al., 2005; Junchao et al., 2017) have shown effects similar to that demonstrated here for braylin, that is, reduction of mucus production, a decrease of inflammatory infiltrate in tissue and BAL, and reduction of Th2 cytokines production. Braylin, as a glucocorticoid agonist, might reduce the transcription of important Th2 inflammatory cytokines, such as IL-4, IL-5, and IL-13 (Payne and Adcock, 2001). As well as reduce the expression of mucin 2 and mucin 5A on goblet cells, resulting in decreased mucus production (Barnes, 1998). However, the contribution of glucocorticoid receptors to these effects of braylin in experimental asthma remains to be confirmed.

Additionally, Lin and coworkers (Lin et al., 2014) demonstrated that braylin was able to inhibit PDE_4 activity in an enzymatic assay with an IC_{50} lower than 10 μM. Likewise, the PDE_4 inhibitor piclamilast also has pharmacological effects in the OVA-induced asthma model (Sun et al., 2006) that are similar to that of braylin. PDE_4 inhibitors could enhance intracellular levels of cAMP, resulting in the activation of protein kinase A (Li et al., 2018). This activation can lead to increased calcium uptake by the sarcoplasmic reticulum in the smooth muscle cells, inactivation of contractile proteins, such as myosin light-chain kinase, and hyperpolarization by activating $K+$ channels and $Na+/K+$ ATPase (Delmotte et al., 2010; Nakamura et al., 2007). Thus, as a PDE_4 inhibitor, braylin could induce intracellular mechanisms that culminate in the relaxation of airway smooth muscle cells (Du et al., 2021), although this mechanistic hypothesis still needs confirmation.

Although PDE_4 inhibitors and glucocorticoid agonists are potent inhibitors of inflammation and remodeling of the airways, these two drug classes act by different mechanisms and may be complementary in the therapeutic effect on asthma (Kumar et al., 2003). In fact, the association of glucocorticoids with PDE4 inhibitors has been considered a good strategy in the treatment of inflammatory respiratory diseases, like chronic pulmonary obstructive disease (Giembycz and Newton, 2015; Grundy et al., 2016). Based on the pharmacological profile evidenced here and on literature data, it is possible to propose that the effects of braylin on experimental asthma are mediated by its dual action on glucocorticoid receptors and PDE_4, however this hypothesis has not been investigated here.

Nevertheless, braylin has a high efficacy by inhalation route, which was similar to systemic dexamethasone. This is a significant finding, considering that inhalation route provides the delivery of the drug straight to the lung, where it acts locally, achieving a higher concentration in the airways, a more rapid onset of action, and leading to a reduced potential for adverse effects than the systemic route (Asthma, 2021). Therefore, data in the present study indicate that braylin may represent an innovative prototype for the development of drugs for the treatment of asthma.

4. Materials and methods

4.1. Extraction, isolation, and identification of braylin

4.1.1. General experimental procedures

^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were acquired at room temperature on a VARIAN, Inova-500 spectrometer instrument with CD3OD as the solvent. The HPLC/ MS analysis was obtained with an HPLC Shimadzu 20A with Bruker micrOTOF II spectrometer using (N2) 10 eV for MS and 45 eV for MS/MS in positive ionization mode with a Phenomenex Luna C18 column (4.6 × 250 mm, 5 μm particle size, 0.6 mL·min−1 oven at 35 °C). Analytical HPLC analysis was carried out on a Shimadzu Prominence LC-6A instrument with a Kromasil® C18 column (4.6 × 250 mm, 100A 5 μm particle size, 0.6 mL·min−1), more guard column (4.6 × 20 mm, 5 μm particle size), all methods analysis were performed with an isocratic flow of solvent A (MeOH) and solvent B (H2O) in proportion 50:50. HPLC eluates were monitored using UV detection at wavelengths of 254 nm.

4.1.2. Plant material

Roots of Z. tingoassuiba A. St. Hil (Rutaceae) were collected in August 2009 in Jaiba district, localized in the city of Feira de Santana in Bahia State, 12°12′52.9″ S, 38°52′44.1″ W. A voucher specimen (n°. 88,005) was identified and stored at the Herbarium Alexandre Leal Costa (ALCB) of Federal University of Bahia, Brazil.

4.1.3. Extraction and isolation

Barks of roots (76,423 g) were extracted with hexane 1 L for seven days and repeated for four weeks. The same process was repeated using methanol, resulting in a methanol extract (12.716 g). 11,421 g of methanol extract was fractionated using a partition of CHCl3:MeOH:5% HCl (5:5:3), and the organic layer was dried under reduced pressure, resulting in fraction E1 (3.251 g). The fraction E1 was recrystallized using methanol, and 0,837 g of yellow powder was isolated. The powder was analyzed by NMR ^1H and ^{13}C (Supplementary Figures 1 and 2, respectively), and *m/z* was determined by analyses of HPLC/MS (see Supplementary Figure 3). After analyses and comparison with literature data (Randrianarivelojosia et al., 2005), the coumarin braylin was identified, as described elsewhere (Costa et al., 2018).

4.2. Animals

Experiments were performed on male BALb/c mice obtained from the Animal Facilities at Instituto Gonçalo Moniz (Salvador, Brazil). Mice (20 - 25 g) were housed in temperature-controlled rooms (22 ± 2 °C), under a 12:12 h light-dark cycle, with access to water and food ad libitum until experimental initiation. All behavioral tests were performed between 8:00 a.m. and 5:00 p.m. Animal care and handling procedures were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and Brazilian College of Animal Experimentation. The protocol was approved by the Institutional Animal Care and Use Committee of FIOCRUZ (CEUA/FIOCRUZ, permit number: L-IGM-013/2021) and the Institute of Health Sciences from the Federal University of Bahia (no. 1,867,180,621). Every effort was made to minimize the number of animals used in the experiments.

4.3. Ovalbumin-induced mice asthma model and experimental design

The mice were divided into groups of six animals and immunized with a subcutaneous injection of 10 μg ovalbumin (Sigma, St. Louis, MO) diluted in 2 mg/mL of aluminum hydroxide (AlumImject; Pierce, Rockford, IL), followed by an injection booster 14 days later. From the 28th day, the mice were placed in an acrylic box and subjected to the inhalation exposure of ovalbumin (1%) for 15 min a day, for five consecutive days. The ovalbumin solution was nebulized by an

ultrasonic inhaler (RespiraMax, Brazil). The protocol used to induce experimental asthma was performed as previously described (Possa et al., 2013). The groups were divided as follows: naïve (mice without experimental manipulation); ovalbumin + vehicle (mice induced to the asthma model and treated with vehicle; 10% propylene glycol in saline); ovalbumin + dexamethasone (mice induced to the asthma model and treated with dexamethasone intraperitoneally 30 mg/Kg; gold standard); ovalbumin + intraperitoneal braylin (mice induced to the asthma model and treated with braylin intraperitoneally 50 mg/kg); ovalbumin + inhaled braylin (mice induced to the asthma model and treated with braylin inhalation 12.5 to 100 mg/Kg). To perform the treatments, two hours before each challenge by inhalation, the mice were treated with braylin, dexamethasone or vehicle, according to each group.

4.4. Bronchoalveolar lavage collection

The animals were euthanized with a lethal dose of ketamine and xylazine (300 mg/Kg and 30 mg/Kg, respectively, via ip.) 24 h after the last challenge for the collection of bronchoalveolar lavage. The lungs of mice were instilled with 1 mL of cold PBS via intratracheal, followed by BAL collection, and the procedure was repeated. The first wash was centrifuged, and the supernatant was stored at −70 °C for later quantification of cytokines by ELISA. The second wash was centrifuged, the supernatant was discarded, and the pellets were resuspended in 1 ml of saline to perform the count of total leukocytes with the aid of the Neubauer chamber. To perform the differential cell count, 10,000 cells from the resuspension were collected, centrifuged in Cytospin®, and stained with hematoxylin and eosin (Vasconcelos et al., 2009).

4.5. Histopathological and morphometric analysis

After collection of the BAL, pulmonary perfusion was performed intratracheally with 1 ml formalin (4%) and, subsequently, the right lobe of the lungs of each animal was removed and fixed in the same solution for histological and morphometric analysis. The sections were stained with hematoxylin and eosin to quantify the inflammatory cells by optical microscopy according to the number of nuclei present in the fields with the largest population of cells. For the quantification of mucus production, staining was done with Periodic acid-Schiff (PAS), and the marked areas were quantified. The area of pulmonary tissue marked with PAS was considered positive for mucus produced by goblet cells (Southam et al., 2008). A total of 10 fields (400 x) per animal were analyzed in a total of 5 animals, and the data were used to calculate the average of cells per mm^2, or the area stained with PAS. The program used to assist in cell counting and area determination was Image-Pro-PLUS, version 4.5 (Media Cybernetics, Silver Spring, USA).

4.6. Quantification of cytokines from BAL

The bronchoalveolar lavage supernatant previously stored at −70 °C was thawed and used for the quantification of cytokines IL-4, IL-5, and IL-13, using specific enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minnesota, MN, USA) for mice, following the manufacturer's instructions (Vasconcelos et al., 2009).

4.7. Tissue preparation for isometric measurement

Male BALb/c mice naïve or asthmatic (10 - 14 weeks) were used in the studies. Mice were euthanized in a CO_2 chamber and had the trachea removed. The trachea was exposed and cleared of surrounding connective tissue before being isolated for functional studies. The rings were immediately immersed in Krebs solution of the following composition (mM): NaCl 117, $NaHCO_3$ 25, $CaCl_2 \times H_2O$ 2.5, KCl 5.36, KH_2PO_4 1.03, $MgSO_4 \times 7H_2O$ 0.57 and glucose 11.1. Then, the tracheas were dissected and cleaned from connective tissue and sectioned in 2 mm long ring. Isolated tracheal segments were mounted onto two stainless steel hooks suspended in an organ bath filled with 10 ml of Krebs solution, bubbled continuously with 5% CO_2 in O_2 (carbogen), and maintained at 37 °C. Changes in tension were recorded via an isometric force transducer (isometric transducer Insight©, Ribeirão Preto, São Paulo, Brazil) connected to an amplified recorder (Insight©, Ribeirão Preto, São Paulo, Brazil) and to a personal computer equipped with a data acquisition software (Windaq software, Dataq Instruments©, Akron, Ohio). The trachea was equilibrated under a resting tension of 0.5 g for at least 1 h with the replacement of the bath fluid every 15 min.

4.8. Experimental procedures in isolated mouse trachea

Following the equilibration period, the tracheal preparations were pre-contracted with a depolarizing solution containing KCl at 80 mM. After reaching a plateau, the solution was replaced with Krebs solution until it returned to the resting tension. Then, to confirm the epithelium function, the tracheal preparations were precontracted with carbachol (0.1 μM). When the plateau was reached, a relaxation response induced by arachidonic acid (10 μM) of at least 60% was obtained. To study the relaxation effect induced by braylin, preparations were first pre-contracted with carbachol (10 μM) and, upon reaching a plateau, were exposed to a single bolus concentration of braylin (0.1 μM to 300 μM). The relaxing response induced by braylin was expressed as a percentage of relaxation of contraction induced by carbachol (10 μM).

4.9. Statistical analysis

Data are presented as means ± standard deviation (SD) of measurements made on 6 - 9 animals in each group. Comparisons between three or more treatments were made using one-way ANOVA with Tukey's post hoc test. Comparison between two treatments was performed by unpaired Student's *t*-test. All data were analyzed using Prism 5 Computer Software (GraphPad, San Diego, CA, USA). Statistical differences were considered significant at $p < 0.05$.

5. Conclusions

In conclusion, the present study demonstrated that inhaled braylin has consistent pharmacological properties in experimental asthma. This coumarin reduced important pathophysiological events associated with asthma, with similar efficacy to systemic dexamethasone, considered the gold standard drug. These results suggest that braylin may be a promising candidate in the treatment of asthma by regulating the Th2 response, inducing relaxant effects in the airway muscles, and presenting relevant efficacy by inhalation. However, further studies, especially regarding its the mechanism of action, are still needed.

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CRediT authorship contribution statement

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Conceptualization, Resources. **Cristiane Flora Villarreal:** Supervision, Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2023.100435.

References

Al-Sajee, D., Yin, X., Gauvreau, G.M., 2019. An evaluation of roflumilast and PDE4 inhibitors with a focus on the treatment of asthma. Expert Opin. Pharmacother. 20 (5), 609–620.

Aneja, R., Mukerjee, S.K., Seshadri, T.R., 1958. Synthesis of benzo-furan derivatives-I. Tetrahedron 2 (3–4), 203–210.

Annunziata, F., Pinna, C., Dallavalle, S., Tamborini, L., Pinto, A., 2020. An overview of coumarin as a versatile and readily accessible scaffold with broad-ranging biological activities. In. J. Mol. Sci. 21 (13).

G.I.f. Asthma, 2021. www.ginasthma.org.

Azim, A., Green, B., Lau, L., Rupani, H., Jayasekera, N., Bruce, K., Howarth, P., 2021. Peripheral airways type 2 inflammation, neutrophilia and microbial dysbiosis in severe asthma. Allergy 76 (7), 2070–2078.

Barnes, P.J., 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin. Sci. (Lond) 94 (6), 557–572.

Barnthouse, M., Jones, B.L., 2019. The impact of environmental chronic and toxic stress on asthma. Clin. Rev. Allergy Immunol. 57 (3), 427–438.

Barrios, R.J., Kheradmand, F., Batts, L., Corry, D.B., 2006. Asthma: pathology and pathophysiology. Arch. Pathol. Lab Med. 130 (4), 447–451.

Beasley, R., Semprini, A., Mitchell, E.A., 2015. Risk factors for asthma: is prevention possible? The Lancet 386 (9998), 1075–1085.

Belvisi, M.G., Bundschuh, D.S., Stoeck, M., Wicks, S., Underwood, S., Battram, C.H., Haddad, E.-.B., Webber, S.E., Foster, M.L., 2005. Preclinical profile of ciclesonide, a novel corticosteroid for the treatment of asthma. J. Pharmacol. Experimental Therapeutics 314 (2), 568.

Bodkhe, S., Nikam, M., Sherje, A.P., Khan, T., Suvarna, V., Patel, K., 2020. Current insights on clinical efficacy of roflumilast for treatment of COPD, asthma and ACOS. Int. Immunopharmacol. 88, 106906.

Boyton, R.J., Altmann, D.M., 2004. Asthma: new developments in cytokine regulation. Clin. Exp. Immunol. 136 (1), 13–14.

Busse, W.W., Pedersen, S., Pauwels, R.A., Tan, W.C., Chen, Y.Z., Lamm, C.J., O'Byrne, P. M., Group, S.I., 2008. The inhaled steroid treatment as regular therapy in early asthma (START) study 5-year follow-up: effectiveness of early intervention with budesonide in mild persistent asthma. J. Allergy Clin. Immunol. 121 (5), 1167–1174.

Chen, L., Tang, G.H., Guo, F.L., Li, W., Zhang, J.S., Liu, B., Yin, S., 2018. (P)/(M)-corinepalensin A, a pair of axially chiral prenylated bicoumarin enantiomers with a rare C-5C-5' linkage from the twigs of Coriaria nepalensis. Phytochemistry 149, 140–145.

Chiang, C.-.Y., Lee, C.-.C., Fan, C.-.K., Huang, H.-.M., Chiang, B.-.L., Lee, Y.-.L., 2017. Osthole treatment ameliorates Th2-mediated allergic asthma and exerts immunomodulatory effects on dendritic cell maturation and function. Cell. Mol. Immunol. 14 (11), 935–947.

Costa, R.S., Souza Filho, O.P., Dias Júnior, O.C.S., Silva, J.J., Hyaric, M.Le, Santos, M.A. V., Velozo, E.S., 2018. In vitro antileishmanial and antitrypanosomal activity of compounds isolated from the roots of Zanthoxylum tingoassuiba. Revista Brasileira de Farmacognosia 28 (5), 551–558.

Crompton, G., 2006. A brief history of inhaled asthma therapy over the last fifty years. Prim. Care Respir. J. 15 (6), 326–331.

Cundall, M., Sun, Y., Miranda, C., Trudeau, J.B., Barnes, S., Wenzel, S.E., 2003. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. J. Allergy Clin. Immunol. 112 (6), 1064–1071.

Curran, D.R., Cohn, L., 2010. Advances in mucous cell metaplasia: a plug for mucus as a therapeutic focus in chronic airway disease. Am. J. Respir. Cell Mol. Biol. 42 (3), 268–275.

Dekhuijzen, R., Lavorini, F., Usmani, O.S., van Boven, J.F.M., 2018. Addressing the impact and unmet needs of nonadherence in asthma and chronic obstructive pulmonary disease: where do we go from here? J. Allergy Clin. Immunol. Pract. 6 (3), 785–793.

Delmotte, P., Ressmeyer, A.R., Bai, Y., Sanderson, M.J., 2010. Mechanisms of airway smooth muscle relaxation induced by beta2-adrenergic agonists. Front. Biosci. (Landmark Ed) 15 (2), 750–764.

Du, X., Zhi, J., Yang, D., Wang, Q., Luo, X., Deng, X., 2021. Research progress in the mechanism of calcium ion on contraction and relaxation of airway smooth muscle cells. J. Recept Signal Transduct. Res. 41 (2), 117–122.

Dunican, E.M., Watchorn, D.C., Fahy, J.V., 2018. Autopsy and imaging studies of mucus in asthma. lessons learned about disease mechanisms and the role of mucus in airflow obstruction. Ann. Am. Thorac. Soc. 15 (Suppl 3), S184–S191.

Espirito-Santo, R.F., Meira, C.S., Costa, R.D.S., Souza Filho, O.P., Evangelista, A.F., Trossini, G.H.G., Ferreira, G.M., Velozo, E.D.S., Villarreal, C.F., Pereira Soares, M.B., 2017. The anti-inflammatory and immunomodulatory potential of braylin: pharmacological properties and mechanisms by in silico, in vitro and in vivo approaches. PLoS ONE 12 (6), e0179174.

Giembycz, M.A., Newton, R., 2015. Potential mechanisms to explain how LABAs and PDE4 inhibitors enhance the clinical efficacy of glucocorticoids in inflammatory lung diseases. F1000Prime Rep. 7, 16.

Grundy, S., Plumb, J., Kaur, M., Ray, D., Singh, D., 2016. Additive anti-inflammatory effects of corticosteroids and phosphodiesterase-4 inhibitors in COPD CD8 cells. Respir. Res. 17, 9.

Hays, S.R., Fahy, J.V., 2003. The role of mucus in fatal asthma. Am. J. Med. 115 (1), 68–69.

Heffler, E., Madeira, L.N.G., Ferrando, M., Puggioni, F., Racca, F., Malvezzi, L., Passalacqua, G., Canonica, G.W., 2018. Inhaled corticosteroids safety and adverse effects in patients with asthma. J. Allergy Clin. Immunol. Pract. 6 (3), 776–781.

Ho, D.K., Nichols, B.L.B., Edgar, K.J., Murgia, X., Loretz, B., Lehr, C.M., 2019. Challenges and strategies in drug delivery systems for treatment of pulmonary infections. Eur. J. Pharm. Biopharm. 144, 110–124.

Junchao, Y., Zhen, W., Yuan, W., Liying, X., Libin, J., Yuanhong, Z., Wei, Z., Ruilin, C., Lu, Z., 2017. Anti- trachea inflammatory effects of diosgenin from dioscorea nipponica through interactions with glucocorticoid receptor alpha. J. Int. Med. Res. 45 (1), 101–113.

Kenakin, T.P., 2017. Chapter 1 - pharmacology: the chemical control of physiology. Pharmacol. Drug Discovery and Dev. 1–20.

Kumar, R.K., Herbert, C., Thomas, P.S., Wollin, L., Beume, R., Yang, M., Webb, D.C., Foster, P.S., 2003. Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. J. Pharmacol. Experimental Therapeutics 307 (1), 349.

Kupeli Akkol, E., Genc, Y., Karpuz, B., Sobarzo-Sanchez, E., Capasso, R., 2020. Coumarins and coumarin-related compounds in pharmacotherapy of cancer. Cancers (Basel) 12 (7).

Lazarinis, N., Jorgensen, L., Ekstrom, T., Bjermer, L., Dahlen, B., Pullerits, T., Hedlin, G., Carlsen, K.H., Larsson, K., 2014. Combination of budesonide/formoterol on demand improves asthma control by reducing exercise-induced bronchoconstriction. Thorax 69 (2), 130–136.

Li, D., Wu, L., 2017. Coumarins from the roots of Angelica dahurica cause anti-allergic inflammation. Exp. Ther. Med. 14 (1), 874–880.

Li, H., Zuo, J., Tang, W., 2018. Phosphodiesterase-4 inhibitors for the treatment of inflammatory diseases. Front. Pharmacol. 9, 1048.

Lin, T.T., Huang, Y.Y., Tang, G.H., Cheng, Z.B., Liu, X., Luo, H.B., Yin, S., 2014. Prenylated coumarins: natural phosphodiesterase-4 inhibitors from toddalia asiatica. J. Nat. Prod. 77 (4), 955–962.

Lipworth, B.J., 1996. Pharmacokinetics of inhaled drugs. Br. J. Clin. Pharmacol. 42 (6), 697–705.

Luo, J., Yang, L., Yang, J., Yang, D., Liu, B.C., Liu, D., Liang, B.M., Liu, C.T., 2018. Efficacy and safety of phosphodiesterase 4 inhibitors in patients with asthma: a systematic review and meta-analysis. Respirology 23 (5), 467–477.

Malikov, V.M., Saidkhodzhaev, A.I., Aripov, K.N., 1998. Coumarins: plants, structure, properties. Chem. Nat. Compounds 34 (2), 202–264.

Mattiuzzi, C., Lippi, G., 2020. Worldwide asthma epidemiology: insights from the global health data exchange database. Int. Forum Allergy Rhinol. 10 (1), 75–80.

Montuschi, P., Peters-Golden, M.L., 2010. Leukotriene modifiers for asthma treatment. Clin. Exp. Allergy 40 (12), 1732–1741.

Nakamura, K., Koga, Y., Sakai, H., Homma, K., Ikebe, M., 2007. cGMP-dependent relaxation of smooth muscle is coupled with the change in the phosphorylation of myosin phosphatase. Circ. Res. 101 (7), 712–722.

Newnham, D.M., 2001. Asthma medications and their potential adverse effects in the elderly: recommendations for prescribing. Drug Saf. 24 (14), 1065–1080.

O'Brien, C.E., Tsirilakis, K., Santiago, M.T., Goldman, D.L., Vicencio, A.G., 2015. Heterogeneity of lower airway inflammation in children with severe-persistent asthma. Pediatr. Pulmonol. 50 (12), 1200–1204.

O'Byrne, P., Fabbri, L.M., Pavord, I.D., Papi, A., Petruzzelli, S., Lange, P., 2019. Asthma progression and mortality: the role of inhaled corticosteroids. Eur. Respir. J. 54 (1).

Panettieri Jr., R.A., 2018. The role of neutrophils in asthma. Immunol. Allergy Clin. North Am. 38 (4), 629–638.

Payne, D.N., Adcock, I.M., 2001. Molecular mechanisms of corticosteroid actions. Paediatr. Respir. Rev. 2 (2), 145–150.

Pereira, T.M., Franco, D.P., Vitorio, F., Kummerle, A.E., 2018. Coumarin compounds in medicinal chemistry: some important examples from the last years. Curr. Top Med. Chem. 18 (2), 124–148.

Perry, R., Braileanu, G., Palmer, T., Stevens, P., 2019. The economic burden of pediatric asthma in the United States: literature review of current evidence. Pharmacoeconomics 37 (2), 155–167.

Poetker, D.M., Reh, D.D., 2010. A comprehensive review of the adverse effects of systemic corticosteroids. Otolaryngol. Clin. North Am. 43 (4), 753–768.

Possa, S.S., Leick, E.A., Prado, C.M., Martins, M.A., Tiberio, I.F., 2013. Eosinophilic inflammation in allergic asthma. Front. Pharmacol. 4, 46.

Radermecker, C., Louis, R., Bureau, F., Marichal, T., 2018. Role of neutrophils in allergic asthma. Curr. Opin. Immunol. 54, 28–34.

Rakotoarison, O., Rabenau, I., Lobstein, A., Um, B.H., Schott, C., Anton, R., Randriantsoa, A., Andriantsitohaina, R., 2003. Vasorelaxing properties and bio-guided fractionation of Cedrelopsis grevei. Planta Med. 69 (2), 179–181.

Randrianarivelojosia, M., Mulholland, D.A., Mc Farland, K., 2005. Prenylated coumarins from Cedrelopsis longibracteata (Ptaeroxylaceae). Biochem. Syst. Ecol. 33 (3), 301–304.

Santos, W.A., Dourado, K.M.C., Araujo, F.A., Jesus, R.L.C., Moraes, R.A., Oliveira, S., Alves, Q.L., Simoes, L.O., Casais, E.S.L.L., Costa, R.S., Velozo, E.S., Silva, D.F., 2021. Braylin induces a potent vasorelaxation, involving distinct mechanisms in superior mesenteric and iliac arteries of rats. Naunyn Schmiedebergs Arch Pharmacol 394 (3), 437–446.

Shen, J.Z., Young, M.J., 2012. Corticosteroids, heart failure, and hypertension: a role for immune cells? Endocrinology 153 (12), 5692–5700.

Singh, S., Agarwal, K., Iqbal, H., Yadav, P., Yadav, D., Chanda, D., Tandon, S., Khan, F., Gupta, A.K., Gupta, A., 2020. Synthesis and evaluation of substituted 8,8-dimethyl-8H-pyrano[2,3-*f*]chromen-2-one derivatives as vasorelaxing agents. Bioorg Med. Chem. Lett. 30 (1), 126759.

Sobieraj, D.M., Weeda, E.R., Nguyen, E., Coleman, C.I., White, C.M., Lazarus, S.C., Blake, K.V., Lang, J.E., Baker, W.L., 2018. Association of inhaled corticosteroids and long-acting beta-agonists as controller and quick relief therapy with exacerbations and symptom control in persistent asthma: a systematic review and meta-analysis. JAMA 319 (14), 1485–1496.

Southam, D.S., Ellis, R., Wattie, J., Glass, W., Inman, M.D., 2008. Goblet cell rebound and airway dysfunction with corticosteroid withdrawal in a mouse model of asthma. Am. J. Respir. Crit. Care Med. 178 (11), 1115–1122.

Stern, J., Pier, J., Litonjua, A.A., 2020. Asthma epidemiology and risk factors. Semin Immunopathol. 42 (1), 5–15.

Sun, J.G., Deng, Y.M., Wu, X., Tang, H.F., Deng, J.F., Chen, J.Q., Yang, S.Y., Xie, Q.M., 2006. Inhibition of phosphodiesterase activity, airway inflammation and hyperresponsiveness by PDE4 inhibitor and glucocorticoid in a murine model of allergic asthma. Life Sci. 79 (22), 2077–2085.

Tanizaki, Y., Kitani, H., Okazaki, M., Mifune, T., Mitsunobu, F., Kimura, I., 1993. Changes in the proportions of bronchoalveolar lymphocytes, neutrophils and basophilic cells and the release of histamine and leukotrienes from bronchoalveolar cells in patients with steroid-dependent intractable asthma. Int. Arch. Allergy Immunol. 101 (2), 196–202.

Tee, A.K., Koh, M.S., Gibson, P.G., Lasserson, T.J., Wilson, A.J., Irving, L.B., 2007. Long-acting beta2-agonists versus theophylline for maintenance treatment of asthma. Cochrane Database Syst. Rev. (3), CD001281

Tsai, I.-.L., Wun, M.-.F., Teng, C.-.M., Ishikawa, T., Chen, I.-.S., 1998. Anti-platelet aggregation constituents from formosan Toddalia asiatica. Phytochemistry 48 (8), 1377–1382.

Vasconcelos, J.F., Teixeira, M.M., Barbosa-Filho, J.M., Agra, M.F., Nunes, X.P., Giulietti, A.M., Ribeiro-Dos-Santos, R., Soares, M.B., 2009. Effects of umbelliferone in a murine model of allergic airway inflammation. Eur. J. Pharmacol. 609 (1–3), 126–131.

Wang, J., Fu, Y., Wei, Z., He, X., Shi, M., Kou, J., Zhou, E., Liu, W., Yang, Z., Guo, C., 2017. Anti-asthmatic activity of osthole in an ovalbumin-induced asthma murine model. Respir. Physiol. Neurobiol. 239, 64–69.

Wardrop, D., Keeling, D., 2008. The story of the discovery of heparin and warfarin. Br. J. Haematol. 141 (6), 757–763.

Xiong, Y., Wang, J., Yu, H., Zhang, X., Miao, C., Ma, S., 2014. The effects of nodakenin on airway inflammation, hyper-responsiveness and remodeling in a murine model of allergic asthma. Immunopharmacol. Immunotoxicol. 36 (5), 341–348.

Zhou-Suckow, Z., Duerr, J., Hagner, M., Agrawal, R., Mall, M.A., 2017. Airway mucus, inflammation and remodeling: emerging links in the pathogenesis of chronic lung diseases. Cell Tissue Res. 367 (3), 537–550.