

MHTP, a synthetic alkaloid, attenuates combined allergic rhinitis and asthma syndrome through downregulation of the p38/ERK1/2 MAPK signaling pathway in mice

Laércia K.D. Paiva Ferreira^a, Larissa A.M. Paiva Ferreira^a, Grasiela C. Bezerra Barros^a, Talissa Mozzini Monteiro^b, Luiz A. de Araújo Silva^c, Ramon de A. Pereira^d, Pedro T. R. Figueiredo^e, Adriano Francisco Alves^f, Luís Cezar Rodrigues^c, Marcia Regina Piuvezam^{a,f,*}

^a Laboratory of Immunopharmacology, Postgraduate Program in Natural and Synthetic Bioactive Products, Federal University of Paraíba, João Pessoa, PB, Brazil

^b Paraíba Association of Renewed Education (ASPER), João Pessoa, PB, Brazil

^c Biotechnology Center, Federal University of Paraíba, Laboratory of Organic Synthesis, Postgraduate Program in Development and Technological Innovation in Medicines, Federal University of Paraíba, João Pessoa, PB, Brazil

^d Institute of Biological Sciences (ICB), Federal University of Minas Gerais, Department of General Pathology, Belo Horizonte, MG, Brazil

^e Drug Research Institute of the Federal University of Paraíba, Postgraduate Program in Natural and Synthetic Bioactive Products, Federal University of Paraíba, João Pessoa, PB, Brazil

^f Department of Physiology and Pathology, Federal University of Paraíba, João Pessoa, PB, Brazil

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ABSTRACT

The combined allergic rhinitis and asthma syndrome (CARAS) is a chronic airway inflammation of allergic individuals, with a type 2 immune response. Pharmacotherapy is based on drugs with relevant side effects. Thus, the goal of this study was to evaluate the synthetic alkaloid, MHTP in the experimental model of CARAS. Therefore, BALB/c mice were ovalbumin (OVA) -sensitized and -challenged and treated with MHTP by intranasal or oral routes. Treated animals showed a decrease ($p < 0.05$) of sneezing, nasal rubbings, and histamine nasal hyperactivity. Besides, MHTP presented binding energy and favorable interaction for adequate anchoring in the histamine H1 receptor. MHTP treatment inhibited the eosinophil migration into the nasal (NALF) and the bronchoalveolar (BALF) fluids. Histological analysis showed that the alkaloid decreased the inflammatory cells in the subepithelial and perivascular regions of nasal tissue and in the peribronchiolar and perivascular regions of lung tissue. The MHTP treatment also reduced the pulmonary hyperactivity by decreasing the smooth muscle layer hypertrophy and the collagen fiber deposition in the extracellular matrix. The immunomodulatory effect of the alkaloid was due to the decrease of cytokines like IL-5 and IL-17A (type 2 and 3), TSLP (epithelial), and the immunoregulatory cytokine, TGF- β . These MHTP effects on granulocytes were dependent on the p38/ERK1/2 MAP kinase signaling pathway axis. Indeed, the synthetic alkaloid reduced the frequency of activation of both kinases independent of the NF- κ B (p65) pathway indicating that the molecule shut down the intracellular transduction signals underlie the cytokine gene transcription.

1. Introduction

The combined allergic rhinitis and asthma syndrome (CARAS) is characterized as a single disease related to upper and lower airway inflammation in atopic individuals [1]. Currently, about 10 to 30% of the world population has allergic rhinitis (AR) and more than 300 million people have asthma. In this context, about 40% of individuals with rhinitis may develop asthma at some point in their lives and about

80% of asthmatic patients have concomitant rhinitis [2]. Several pieces of evidence are showing that both diseases are triggered by the same etiologic agent, presented the same pathophysiological profile, and shared the same therapeutic conduct [3].

The syndrome is a predominantly type 2 immune response that coordinates the inflammatory process. The TH2 cells and the type 2 innate lymphoid cells (ILC2) are responsible for the eosinophil migration in both airway compartments due to IL-5 and IL-13 production triggered by

* Corresponding author at: Universidade Federal da Paraíba Campus I, PO 5009, João Pessoa, PB 58-051-970, Brazil.

E-mail address: mrpiuvezam@ltf.ufpb.br (M.R. Piuvezam).

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alarmines such as thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 from airway activated epithelial [4]. This cell population produces a variety of molecules including chemokines, cytokines, and cytolytic enzymes as eosinophilic cationic protein (ECP) and main basic protein (MBP), which disrupt the airway epithelium promoting airway hyperactivity and tissue remodeling. The airway remodeling process is due to the TGF- β production, collagen fiber deposition on the extracellular matrix, increase of the number of subepithelial mesenchymal cells, and, mainly, increase of airway smooth muscle mass, which is the main determinant of airflow obstruction [5]. Besides, the destruction of the mucous epithelial layer of the upper airway compartment by eosinophil metabolism facilitates the entry of the allergen through the airways. The binding of the allergen with resident mast cells releases, mainly, histamine, which acts on sensory nerve endings, parasympathetic reflex stimulation of glandular secretions, vasodilation, and increased capillary permeability, promoting the rhinitis symptoms as nasal itching, sneezing, and rhinorrhea [6].

Currently, the therapies to treat AR and/or asthma promote several adverse reactions that compromise the quality of life of an affected individual as sedation, tachycardia, glaucoma, and/or bone resorption. These reactions are triggered by the use of antihistamines, bronchodilators, and corticosteroids, respectively. In addition, the immunobiological medication named Mepolizumab (anti-IL-5) induces a systemic immune dysregulation [7–9]. Therefore, research of molecules that play an immunoregulatory role in type 2 immune response with low side effects may be useful for the treatment of allergies.

In this context, several classes of alkaloids present an anti-allergic effect, for instance, milonine, a morphinamic alkaloid, inhibited the mast cell degranulation process and release of histamine [10]. Besides, warifteine, methylwarifteine, and curine, isoquinoline alkaloids, reduced the inflammatory condition in the upper and lower airways, as

well as the airway hyper-reactive in ovalbumin (OVA) sensitized mice [11,12].

In 2015 MHTP (Fig. 1A), a new alkaloid, synthesized by our research group, presented the base core 1,2,3,4-tetrahydroisoquinoline, being a structural analog of the cryptostilbin II, isolated from the *Cryptostylis fulva* [13]. The molecular formula and molecular weight of MHTP are C₁₇H₁₉NO₃ and 285 g/mol respectively, and its yield percentage of synthesis was 93.45% [13]. Studies in vivo, carried out by our research group, demonstrated that the alkaloid presented low acute toxicity (LD₅₀ > 1.000 mg/kg) without genotoxicity, anti-edematogenic effect, and inhibited the polymorphonuclear cell migration to the carrageenan-induced peritonitis [13]. Besides, oral or intranasal treatment with MHTP reduced the neutrophil migration to the lung in lipopolysaccharide (LPS) induced-acute lung injury (ALI) dependent on downregulation of the TLR4-p38MAPK/NF- κ B (p65) signaling pathway [14], and in LPS-stimulated macrophages, MHTP reduced the production of nitric oxide (NO) and IL-1 β and IL-6 [13]. In 2018, Paiva-Ferreira and collaborators [15] demonstrated that the alkaloid, intranasal administrated in ovalbumin-induced pulmonary inflammation decreased the serum level of IgE- allergen-specific and eosinophil migration to the lung. The molecule also promoted the immunomodulation of type 2 immune response by decreasing the CD4⁺T cells, IL-4, and IL-13 production in the bronchoalveolar fluid and diminished the mucus production.

Therefore, the goal of the present work was to deepen our scientific knowledge of the alkaloid MHTP in the context of the experimental model of the combined allergic rhinitis and asthma syndrome to define its mechanism of action and molecular target (s) to scientific support the molecule as a potential medicine to be tested in clinical trials.

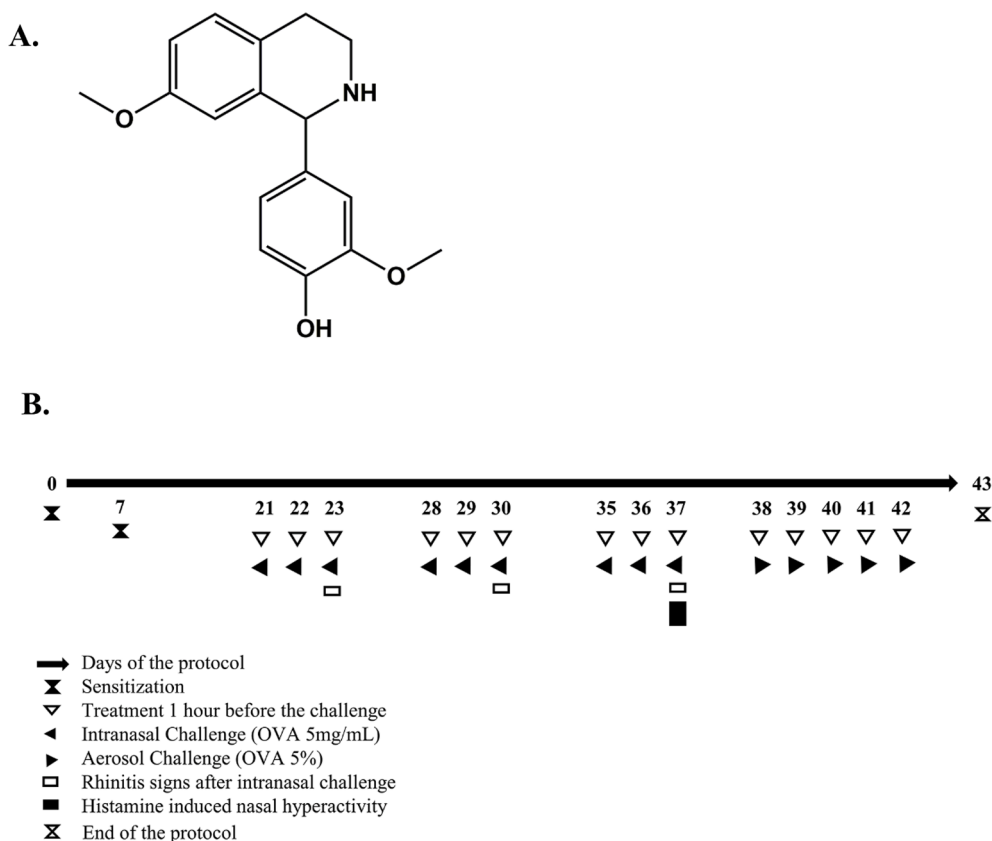


Fig. 1. Alkaloid chemical structure and the combined allergic rhinitis and asthma syndrome (CARAS) protocol. (A). The chemical structure of the synthetic isoquinolinic alkaloid MHTP (2-methoxy-4-(7-methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl) phenol). (B). Experimental protocol of CARAS-ovalbumin-induced and alkaloid treatment.

2. Material and methods

2.1. Mice

Female BALB/c mice (6–8-week-old, weighing 20–25 g) were used in the experimental protocols. The animals were kept in cages at a temperature of $25 \pm 2^\circ\text{C}$, 12/12-h light–dark cycle with free access to water and a controlled diet, based on pellets, throughout the trial period. The animals were from Prof. Dr. Thomas George at the Drug Research Institute of the Federal University of Paraíba (IPEFAR/UFPB), João Pessoa, PB, Brazil. The manipulation of the animals was performed according to the animal care guide [16]. The Committee on Ethics in Animal Use (CEUA/UFPB) approved the experimental procedures under certificate N° 3432290419. Euthanasia and anesthesia were performed by intramuscular administration of an anesthetic solution containing 29 mg/mL ketamine and 1.91 mg/mL xylazine in saline solution.

2.2. Material

Ovalbumin (OVA) and dexamethasone were purchased from Sigma (Chemical, St. Louis, MO, USA) and Achê® (Sao Paulo, SP, Brazil), respectively, and MHTP was synthesized by Prof. Dr. Luís Cezar Rodrique from Federal University of Paraíba, PB, Brazil [13].

2.3. Treatment

Animals ($n = 6$) were distributed as the following groups: Sal (healthy animals, non-ovalbumin (OVA)-sensitized and challenged animals, treated with saline), OVA (OVA control, OVA-sensitized and -challenged animals); MHTP (animals treated with 5.0 mg/kg of MHTP by intranasal (i.n.) or oral (p.o.) routes) and Dx (animals treated with 2.0 mg/kg of dexamethasone by p.o.). One hour before each OVA challenge animals were treated with MHTP or dexamethasone totalizing fourteen treatments/animal (Fig. 1B). For the nasal administration, the MHTP was at 3.125 mg/mL (40 μL /animal) and for the oral administration (by gavage) at 0.5 mg/mL (10 μL /10 g animal). The nasal instillation was performed on each awake animal by using a micropipette (20 μL) containing the MHTP solution. The 40 μL of MHTP was administrated in a period of 2 min. To solubilize the alkaloid, it was used 50 μL of 1 N Hydrochloric Acid (HCl) in 500 μL of saline solution (NaCl 0.9%) and to adjust the pH to 7.0 it was used the sodium hydroxide (NaOH).

2.4. Combined allergic rhinitis and asthma syndrome experimental model

The OVA-induced-combined allergic rhinitis and asthma syndrome was developed in mice as previously described (Fig. 1B). Briefly, mice were sensitized, by intraperitoneal injection (i.p.), with 50 μg /animal of OVA (grade V) and Al (OH) 3 (10 mg/mL) on days 0 and 7. The animals were challenged by nasal instillation with OVA (5 mg/mL, grade II) from day 21 to day 37 for three consecutive days, in three consecutive weeks. From the day, 38 to 42 the animals were OVA-challenged (5%, grade II, aerosol) for 30 min daily in a closed chamber. On days 23, 30 and 37 immediately after the OVA-challenge, rhinitis signs as sneezing and nasal frictions were quantified for a period of 15 min. Twenty-four hours (day 43) after the last OVA-challenge, the animals were euthanized with an anesthetic solution and nasal (NALF) and bronchoalveolar (BALF) fluids were collected. To obtain the bronchoalveolar lavage fluid (BALF), 1.5 mL of ice-cold HBSS+/- were injected into the animal's lung through the trachea, and to obtain the nasal lavage fluid (NALF), 1 mL of ice-cold HBSS+/- was injected into the animal's nasopharynx through the trachea. The fluids were stored at 4°C until the analyses of the cells and cytokines. The total cells and eosinophils were counted in a Neubauer chamber (40 $\times$) and in cytospin slides stained with Hematoxylin & Eosin, respectively, by oil-immersion objective (100 $\times$) [17].

2.5. Histamine-induced nasal hyperactivity and rhinitis signs

Nasal histamine hyperactivity was realized by nasal instillation of increasing doses of histamine (0.1, 1, 10, 100, and 1000 nmol/mice) in a period of five hours. Briefly, on day 37, one hour after the OVA challenge, 4 μL of histamine were consecutively instilled into the nasal cavity at intervals of 60 min in a period of five hours. The rhinitis signs as nasal friction and sneezing of each animal were quantified for a period of 10 min [12].

2.6. Molecular docking assays

The histamine H1 receptor was obtained from Protein Data Bank (<http://www.rcsb.org/>) together with its co-crystallized inhibitor, under PDB ID: 3RZE (3.1 Å). The chemical structures of MHTP and control drugs (dexamethasone, ketotifen, dexchlorpheniramine, loratadine, and cetirizine) had their geometry optimized by the Hyperchem v. 8.0.3 program, using the molecular mechanics' method (MM+) and the semi-empirical method AM1 (Austin Model 1). Molegro Virtual Docker (MVD) software (v 6.0.1, Molegro ApS, Aarhus, Denmark) was used to perform molecular docking analyzes. Standard software parameters were used, water molecules were removed and a template was generated in the PDB ligand crystallized in the enzyme [18,19].

2.7. Morphological analysis of the upper and lower airway tissues

Each head and the largest lobe of the left lung of each euthanized animal was carefully scissored and fixed in buffered formalin for 48 h. The nasal and lung tissues were dehydrated and were embedded in paraffin. Four-micrometer thick sections of paraffin-embedded nasal and lung tissues were stained with hematoxylin-eosin (H&E), and Masson's Trichrome (MT) according to standard protocols. The photographs were captured by the MOTIC 5.0 MP camera attached to the optical microscope (Nikon®) and a computer. The images in the MOTIC PLUS program were calibrated in specific settings according to the light microscopy (Motic BA 410). Morphometric analysis of histological findings was performed using the KS400 software. The scores for H&E, stained tissue alterations were from 0 to 6 according to the lesions observed as vasodilation (present [1] or absent [0]), maintenance of bronchiolar or nasal integrity (present [1] or absent [0]), alveolar or nasal obstruction (present [1] or absent [0]). Finally, as part of the scores, cell migration was evaluated and zero (0) means no cell migration, (1) 0 to 9 cells per field, (2) more than 10 cells per field, and (3) perivascular and peribronchiolar cell rings or above 20 cells per field in the nasal cavity. The parameters were evaluated in all fields of each slide, double-blind in the 40x objective [17]. The morphometric analysis of MT, stained tissues was realized through the 40x objective and 20 random images scanned through the same microscope and micro camera, making a total area of 281,872 mm^2 of pulmonary or nasal parenchyma. The tissue area was calculated using algorithms built in the KS400 software. In each image were selected all pixels with shades of blue (MT) for the creation of a binary image, digital processing, and calculation of the area in μm^2 [17]. To calculate the smooth muscle area of the lung, the histological sections of the TM-stained lung were visualized through the 40x Olympus microscope objective (Tokyo, Japan) for the scanning of 20 random bronchiolar regions through the Q-Color3 microcamera. The entire pulmonary interstitium was excluded from the analysis using program tools. All pixel types of bronchiolar smooth muscle were selected for binary image creation and area calculation in μm^2 [17]. All morphometric analyzes were performed using the KS400 software contained in the Carl Zeiss image analyzer (Oberkochen, Germany).

2.8. Cytokine quantification

The cytokine production was analyzed on the bronchoalveolar lavage

fluid (BALF) of all animals the IL-5, IL-17, TSLP and TGF- β were measured by ELISA assay according to the manufactory instructions (eBioscience, San Diego, California).

2.9. Granulocyte analysis of p38 and ERK1/2 MAPK and NF- κ B (p65) by flow cytometry

The granulocytes (events) from the BALF were analyzed using flow cytometry methodology, where the direct scattered light (FSC) and lateral scattered light (SSC) distinguish the cell populations by size (light diffraction) and granularity (light diffusion), respectively. Granulocytes (mainly eosinophils) were identified as SSC^{hi} [20]. For granulocyte analysis, BALF was obtained from animals of all different groups and FSC and SSC parameters were applied [15]. In addition, 5×10^5 cells/mL were incubated with antibodies to anti-p-ERK1/2 (PE), anti-p-p38 (PE Cy7) and anti- NF- κ B(p65) (PerCP-Cy5.5). The specific experimental protocol for each marker was performed according to the manufacturer's kit standard used (BIOSCIENCE, Inc. Science Center Drive, San Diego, CA-USA). For each analysis, 10,000 events were analyzed using the flow cytometer (FACSCanto™ II, with the acquisition software being BD FACSDiva 6.1.) and the generated data were analyzed using the FlowJo software, version 10.

2.10. Statistical analyses

The data were analyzed by Graph Pad Prism © version 8.0 software (GraphPad Software, San Diego, CA, U.S.A.). Results were expressed as mean $\pm$ standard error of the mean (e.p.m.) and statistically analyzed by one-way ANOVA followed by the Tukey test, where values of $p < 0.05$ were considered significant. It was established (a) $p < 0.05$ compared to

the Saline group and (b) $p < 0.05$ compared to the OVA group.

3. Results

3.1. Effect of MHTP on rhinitis signs in OVA-sensitized and challenged mice

The animals of the OVA group showed an increase in sneezing and nasal frictions from the first week through the third week as compared to the animals of the Sal group (Fig. 2A and B). However, animals treated with MHTP (intranasal or oral routes) or dexamethasone (Dx group) showed a reduction ($p < 0.05$) of sneezing by 59%, 44%, and 69%, respectively, compared with OVA group in the third week of observation (Fig. 2A). Besides, similar to sneezing, the nasal frictions in animals treated with MHTP or Dx decreased ($p < 0.05$) mainly in the 3rd week as compared to OVA group. The percentage of reduction to the nasal friction was 44% for MHTP (i.n.), 52% for MHTP (p.o.), and Dx (Fig. 2B).

3.2. Effect of MHTP on histamine-induced nasal hyperactivity in OVA-sensitized and challenged mice

The animals of the OVA group showed sneezing and nasal rubbing as the histamine concentration increased (0.1–1000 nmol) (Fig. 3A and B). The animals treated with MHTP or Dx presented reduction ($p < 0.05$) in the rhinitis signs right after histamine nasal installation at the lowest dose (0.1 nmol) through the highest one (1000 nmol) as compared to the OVA group. The percentage of sneezing and nasal frictions reduction was about 84%, 89%, 84% and 47%, 53% and 58% for MHTP (i.n.), MHTP (p.o.) and Dx, respectively, through the increases of histamine concentration (Fig. 3A and B). These results indicate that the alkaloid

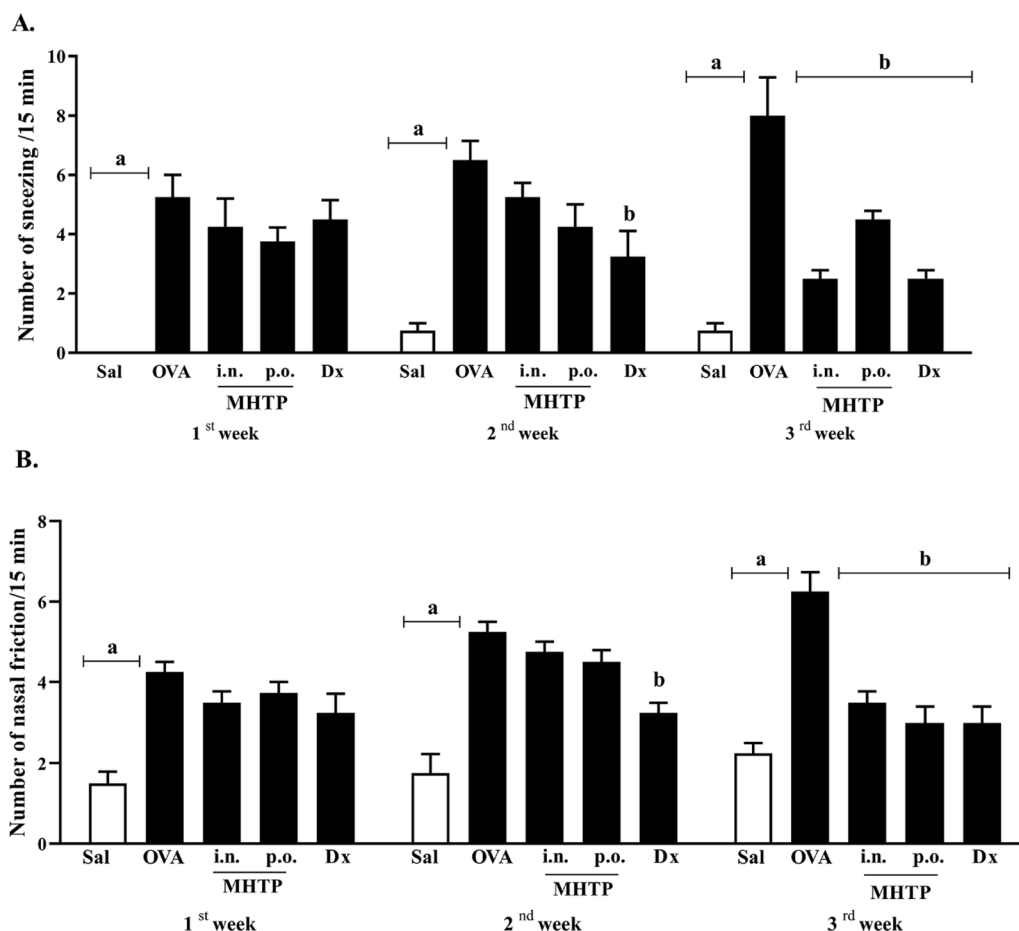


Fig. 2. Effect of MHTP on rhinitis signs in OVA-sensitized and challenged mice. The number of (A) sneezing and (B) nasal frictions were measured for a period of 15 min. right after the OVA-challenged on days 23, 30 and 37 of the CARAS experimental protocol. Sal: healthy animals; OVA: non-treated animals; MHT (i.n.) or (p.o.): animals treated with MHTP by intranasal or oral routes and Dx: animals treated with dexamethasone. Data are represented as mean $\pm$ e.p.m. analyzed by one-way ANOVA followed by Tukey test. (a) $p < 0.05$ when compared to the Saline group and (b) $p < 0.05$ when compared to the OVA group.

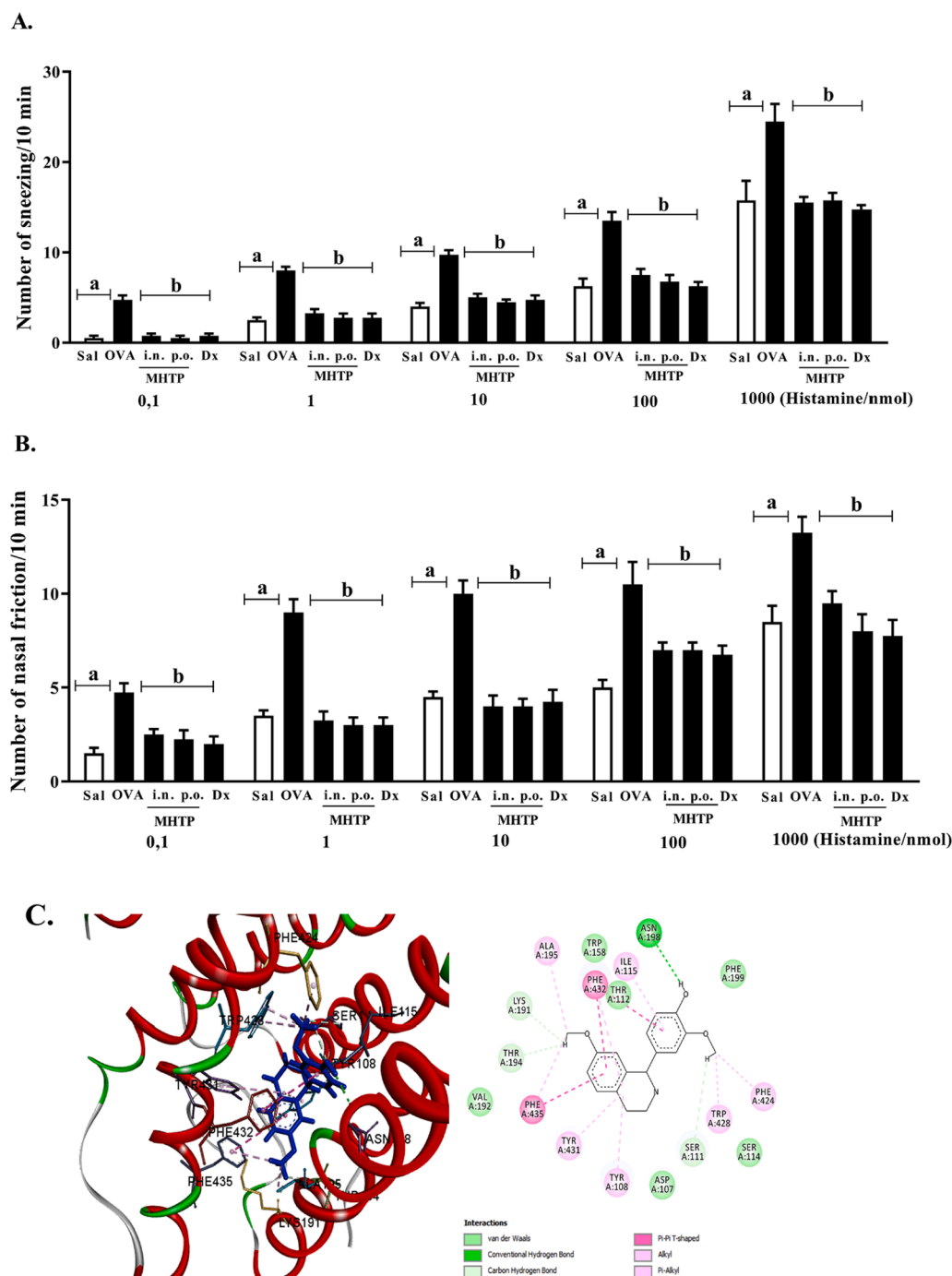


Fig. 3. Effect of MHTP on histamine-induced nasal hyperactivity in OVA-sensitized and challenged mice and molecular anchorage study of MHTP with the histamine H1 receptor. The 37th day of the CARAS experimental protocol, one hour after the OVA-challenged, arising doses of histamine (0.1; 1; 10; 100 and 1000 nmol) were intranasal administrated and, an interval of one hour among the doses, were measured the number of (A) sneezing and (B) nasal frictions. for 10 min immediately after increasing doses. of histamine by nasal instillation. Sal: healthy animals; OVA: non-treated animals; MHT (i.n.) or (p.o.): animals treated with MHTP by intranasal or oral routes and Dx: animals treated with dexamethasone (C). Molecular anchorage study of MHTP with the histamine H1 receptor. Interactions: light green-van der Waals, dark green - conventional hydrogen bond, pink-pi-alkyl, blue-hydrogen bond with carbon and pink-alkyl. Data are represented as mean $\pm$ e.p.m. analyzed by one-way ANOVA followed by Tukey test. (a) $p < 0.05$ when compared to the Saline group and (b) $p < 0.05$ when compared to the OVA group.

treatment of animals, regardless of the administration route, decreased the nasal hyperactivity to histamine.

3.3. Molecular anchorage study of MHTP with the histamine H1 receptor

For the validation of protocols in molecular docking, redocking was performed, and the RMSD (Root Mean Standard Deviation) value was used to analyze the accuracy of molecular docking. To be considered a valid docking, the RMSD values must remain in the range of 0–2 Å. For the 3RZE enzyme, the RMSD was 0.51 Å, remaining within the acceptable value, showing that the docking was accurate and validated. The MHTP showed interaction energy $-128.4 \text{ kcal.mol}^{-1}$, compared to the anti-inflammatory drugs; it showed higher energy than dexamethasone, dexchlorpheniramine, and ketotifen. Table 1 shows the interaction

Table 1

Interaction energies of MHTP and anti-inflammatory drugs in the histamine receptor 1 (H1).

Receptor H1 – 3RZE	
Compounds	Moldock score (kcal mol^{-1})
MHTP	–128.4
Dexamethasone	–82.8
Loratadine	–152.8
Cetirizine	–154.6
Dexchlorpheniramine	–124.6
Ketotifen	–108.8
Binder PDB – Doxepine	–144.8

energies of the tested compounds. The proper anchoring of MHTP in this receptor is explained by the interactions with tryptophan residues, mainly Trp428, Lys191, Lys179 and Thr112 that are essential for anchoring anti-inflammatory drugs in this receptor [21]. As seen in Fig. 3C, MHTP performed hydrophobic interactions with Thr112, Trp428 residues, and hydrogen bonding interaction with Lys191.

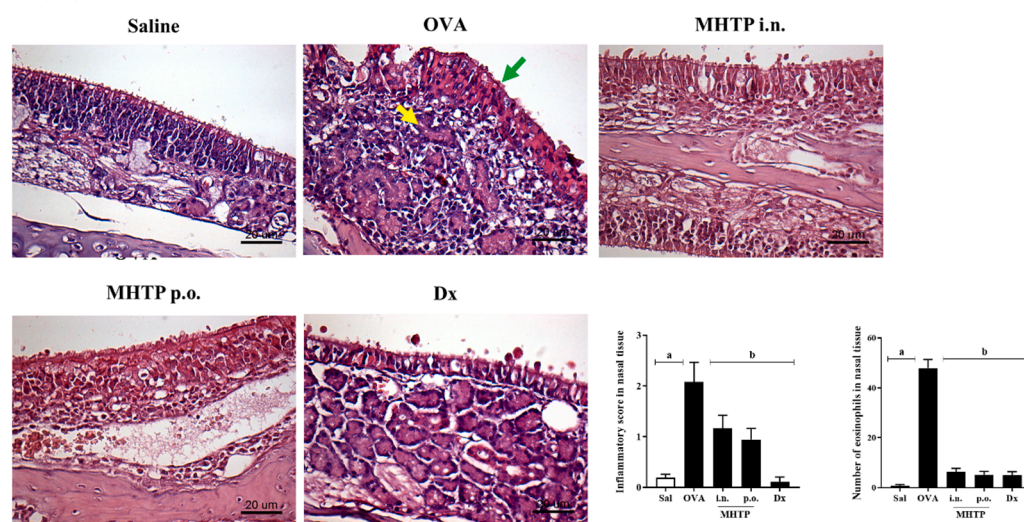
3.4. Effect of MHTP on nasal and lung tissues on eosinophil migration to both airway cavities of OVA-sensitized and challenged mice

The animals of the OVA group presented an important inflammatory process in both, upper and lower airway tissues as intense cell infiltration (yellow arrow), loss of epithelial integrity (green arrow), alveolar

obstruction (orange arrow) and vasodilation (blue arrow) as compared to Sal group (Fig. 4A and B). Animals treated with MHTP (MHTP i.n. or p.o.) or Dx showed a reduction ($p < 0.05$) in these inflammatory parameters in both airway tissues (Fig. 4A and B, see the color arrows). The percentage of reduction of the inflammatory parameters in the nasal and lung tissues was 44%, 55%, 95% ($p < 0.05$) and 18%, 48%, and 76% ($p < 0.05$), for the treated animals (MHTP i.n. or p.o. or Dx), respectively (Fig. 4A and B).

The OVA-sensitization and challenge were able to induce an eosinophilic infiltration to the nasal and lung tissues of animals of the OVA group (Fig. 4A and B). Treated animals with MHTP (i.n. or p.o.) or with Dx showed a reduction ($p < 0.05$) of eosinophils to the nasal tissue by 87%, 89%, and 90%, respectively, and for the lung tissue, the reduction

A. Nasal Tissue



B. Lung Tissue

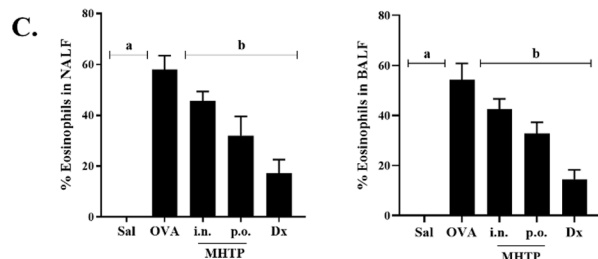
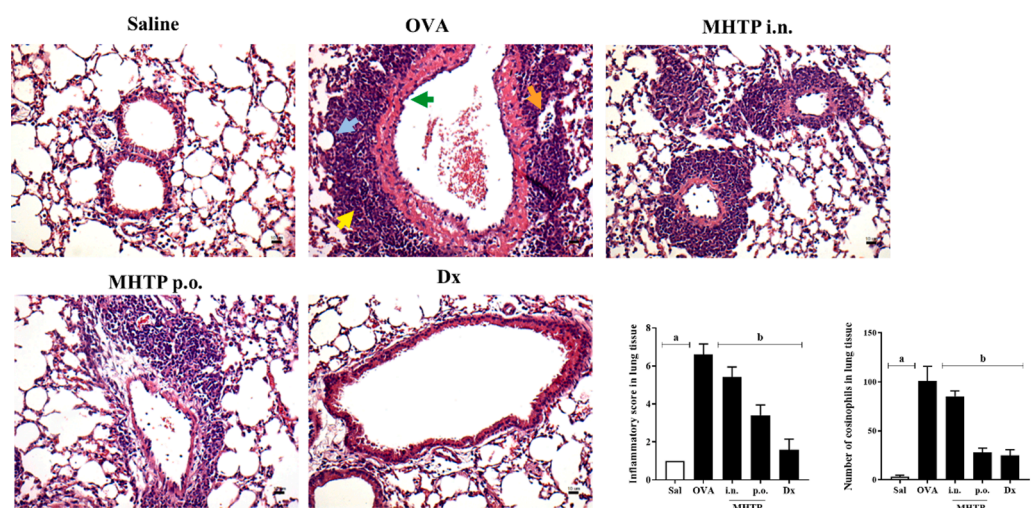


Fig. 4. Photomicrography, inflammatory scores and the eosinophil percentage of nasal and lung tissues of healthy, sick and MHTP or dexamethasone treated animals. (A). representative photomicrography of nasal tissues stained with H&E and the inflammatory parameters were intense cellular infiltration (yellow arrow) and loss of epithelial integrity (green arrow); inflammatory score (see material methods - Morphological analysis of the upper and lower airway tissues). (B). representative photomicrography of lung tissues stained with H&E and the inflammatory parameters were intense cellular infiltration (yellow arrow) and loss of epithelial integrity (green arrow), alveolar obstruction (orange arrow) and vasodilation (blue arrow); inflammatory score (material and methods). (C). the percentage of eosinophils into the nasal bronchoalveolar lavage fluid (BALF). Data are presented as mean $\pm$ e.p.m. analyzed by one-way ANOVA followed by Tukey test. (a) $p < 0.05$ when compared to the Saline group and (b) $p < 0.05$ when compared to the OVA group ($n = 6$ animals per group). The photomicrograph was captured under optical microscopy - AT 40x (nasal tissue) and AT 20x (lung tissue).

was 16%, 72%, and 75%, respectively (Fig. 4A and B). The nasal (NALF) and bronchoalveolar (BALF) lavage fluids of animals of the OVA group showed an increase in the number of eosinophils as compared to animals of the Sal group. However, the treatment of these animals with MHTP or dexamethasone showed a decrease in the number of this cell population, in both cavities. Indeed, the percentage of reduction in the NALF was about 21%, 45%, and 70% for MHTP (i.n.), MHTP (p.o.) and Dx groups, respectively. In the BALF, the reduction was 22%, 40%, and 73% for MHTP (i.n.), MHTP (p.o.) and Dx group, respectively, as compared to OVA group (Fig. 4C).

3.5. Effect of MHTP on airway hyperactivity and tissue remodeling in OVA-sensitized and challenged mice

The animals of the OVA group showed an increase in the extracellular matrix (ECM) compared to the animals of the Sal group (Fig. 5). Treatment of the animals with MHTP (i.n. or p.o.) or Dx reduced ($p < 0.05$) the bronchiolar smooth muscle area (area/ μm^2) in about 16%, 79% and 81%, respectively, and ECM in about 18%, 51%, and 67%, respectively, as compared to OVA group (Fig. 5A–C).

3.6. Effect of MHTP on cytokine production in OVA-sensitized and challenged mice

The animals treated with MHTP (i.n. or p.o.) or Dx presented reduction ($p < 0.05$) of all quantified cytokines compared to the animal of the OVA group. Indeed, the percentage of reduction for the three treated groups (MHTP i.n./p.o. or Dx) was about 46%, 50% and 46% for IL-5; 63%, 65% and 80% for IL-17A; 26%, 32% and 31%, for TSLP; and 32%, 38% and 47% for TGF- β , respectively (Fig. 6A–D).

3.7. Effect of MHTP on granulocyte signaling pathways

The animals of the OVA group showed an increase in the frequency of granulocytes as compared to the animals of the Sal group. Treated animals with MHTP (i.n. or p.o.) or Dx showed a reduction of the frequency of this cell population into the BALF as compared to the OVA group (7.81%, 9.39% and 9.01%, respectively vs 21.1%) (Fig. 7A). The frequency of activation and the mean of the fluorescence intensity (MFI) of

the p-p38MAPK on cells from animals of the OVA group was significantly high as compared to the Sal group (Fig. 7B). However, the MHTP treated animals, independently of the route used, showed decrease in the frequency of activation of p-p38 MAPK and MFI as compared to the OVA group (17.09% - i.n., 11.80% - p.o. vs 54.02%; 44.3 ± 4.27 - i.n., 20.7 ± 0.99 - p.o. vs 63.5 ± 4.07 , respectively). Similar results were observed with Dx treatment (Fig. 7B). Also, MHTP treated animals presented a decrease of ERK1/2 MAPK and MFI as compared to the OVA group (37.7% - i.n., 34.6% - p.o. vs 45.9%; 490 ± 7.42 - i.n., 485 ± 3.06 - p.o. vs 529 ± 5.51 , respectively) independently of decreasing of NF- κB (p65) (Fig. 7C and D). Indeed, the statistical significance of the data described above is shown in figures aside from the dot blots.

4. Discussion

The combined allergic rhinitis and asthma syndrome (CARAS) is a concomitant chronic inflammation of the upper and lower airways. This syndrome is related to allergic rhinitis and asthma in a genetic allergic hypersensitivity individual and is considered a public health problem in global terms. Indeed the low quality of life of the patient interferes in the labor activities provoking economic losses [22]. The current pharmacotherapy for the syndrome is the same one to treat allergic rhinitis and asthma, although the drugs promote serious adverse effects, due to the treatment is being carried out in a long term [23]. Therefore, it is imperative the search for therapeutic alternatives that alleviate symptoms without causing serious adverse effects. Indeed, a series of molecules have been testing all over the world and some came up from alkaloid classes [12,24–29].

In this study, a synthetic tetrahydroisoquinoline alkaloid, 2-methoxy-4-(7-methoxy-1,2,3,4-tetrahydroisoquinoline-1-yl) phenol, coded as MHTP was investigated in an experimental model of CARAS. Our research group has been studied the alkaloid since 2015, and the data showed its anti-inflammatory and immunomodulatory effect in several *in vivo* experimental models related to airway inflammation pointing out the molecule might be a promising drug to treat diseases of the respiratory tract. [13–15].

Thereby, the intranasal and oral routes were chosen to administrate the alkaloid due to the classical treatment for airway diseases uses both routes, even with the same class of drug, for instance, the glucocorticoid

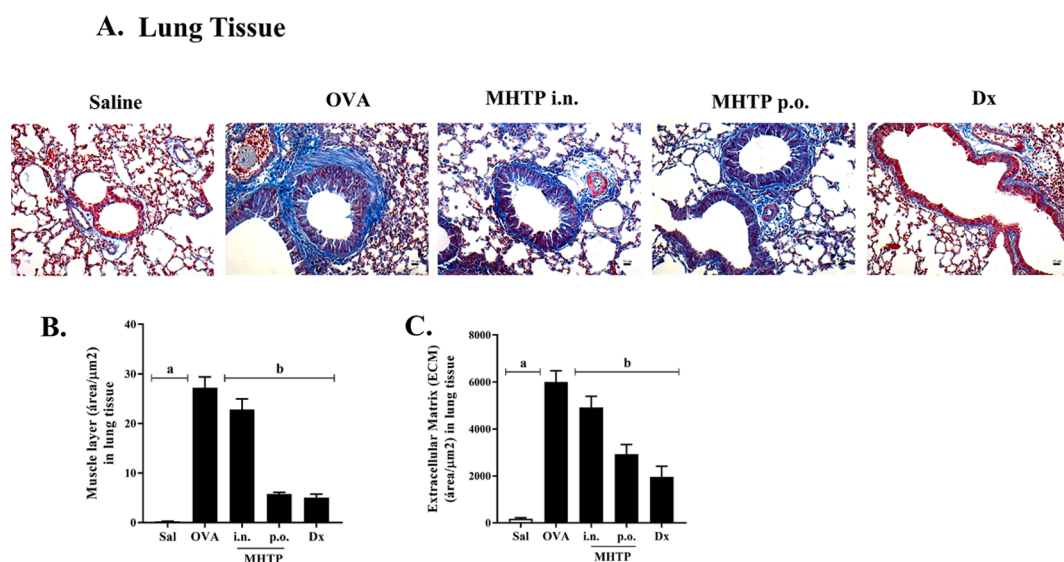


Fig. 5. Photomicrograph of the lung tissues of healthy, sick and MHTP or dexamethasone treated animals. (A). representative photomicrography of lung tissues stained with Masson's Trichrome (MT) thickening of the extracellular matrix (ECM) is observed by the bluish color. The morphometric analysis of smooth muscle layer (B) and extracellular matrix (C) of the lung tissues stained with MT (see material and methods- Morphological analysis of the upper and lower airway tissues). Data are represented as mean $\pm$ e.p.m. analyzed by one-way ANOVA followed by Tukey test. (a) $p < 0.05$ when compared to the Saline group and (b) $p < 0.05$ when compared to the OVA group ($n = 6$ animals per group). The photomicrograph was captured under optical microscopy - AT 20x.

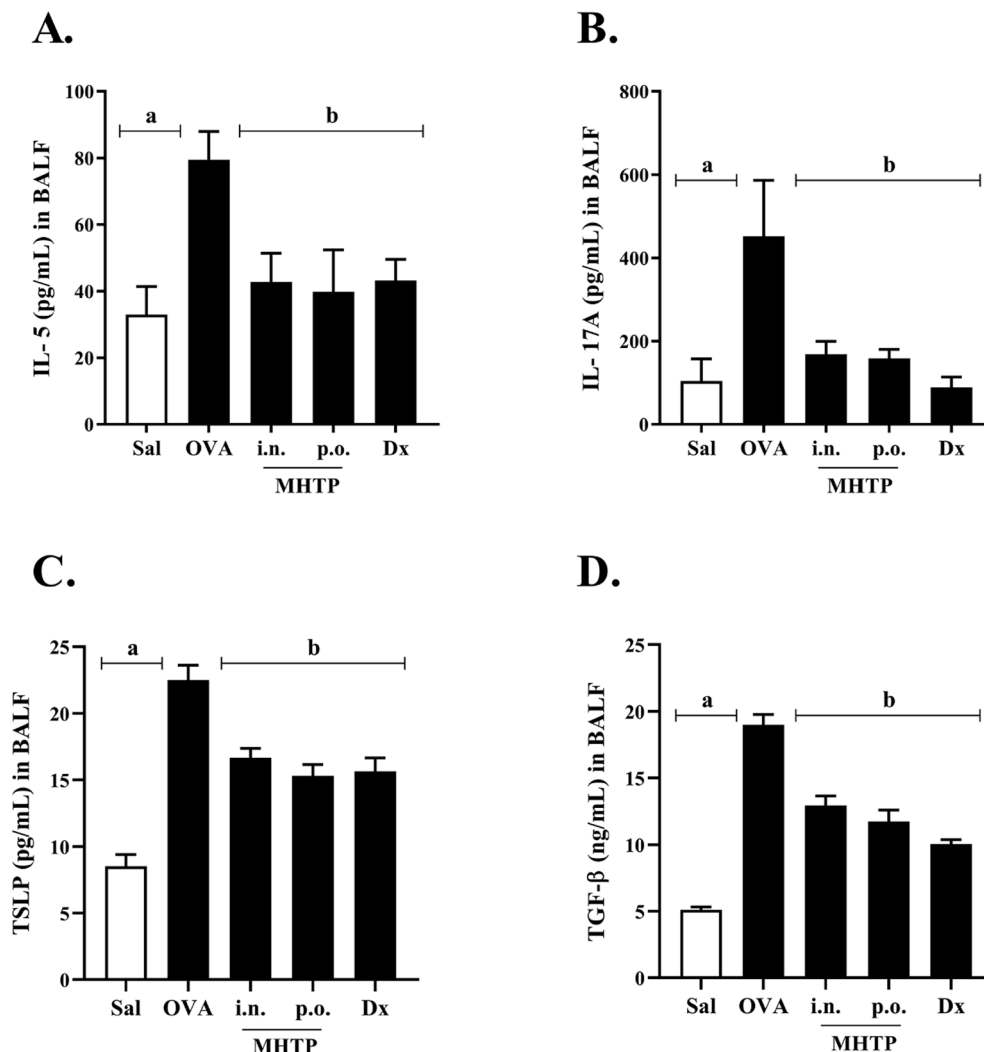


Fig. 6. Effect of MHTP on cytokine production in OVA-sensitized and challenged mice. The bronchoalveolar lavage fluids (BALF) from healthy, sick and MHTP or dexamethasone treated animals were harvested on day 43th to measure the amount of (A). IL-5, (B). IL-17, (C). TSLP and (D). TGF- β by ELISA. Data are represented as mean $\pm$ e.p.m. analyzed by one-way ANOVA followed by Tukey test. (a) $p < 0.05$ when compared to the Saline group and (b) $p < 0.05$ when compared to the OVA group.

is presented in formulations that are administrated by oral, intranasal, and, in some cases, by intramuscular injection [30,31]. Also, the administration of the MHTP, regardless of the route, in healthy animals did not alter the physiological lung parameters as the loss of epithelial integrity, increase in the smooth muscle layer, or deposition of ECM (complementary data). Besides, the dose of the MHTP used in this study (5 mg/kg) is 200 times lower than the LD50 providing therapeutic safety, and, it is worth mentioning that MHTP is an alkaloid synthesized by the Pictet-Spengler chemical reaction and has a yield of 93.45% making it a viable molecule for large-scale production [13].

The data of this study showed that animals of the OVA group treated with MHTP, regardless of the route, presented a reduction in the granulocyte migration to both airway tissues (upper and lower) as well as into the nasal and bronchoalveolar fluids. In the lower airway, the alkaloid reduced the hyperplasia and/or the hypertrophy of the smooth muscle layer and, thereby, bronchial hyper-reactivity and deposition of the extracellular matrix (ECM), inhibiting tissue remodeling. The MHTP treatment also alleviated the severity of the rhinitis signs as sneezing and nasal frictions, after the OVA challenge or histamine-induced nasal hyper-reactivity mainly in the third week after the intranasal OVA-challenge. The clinical signs as sneezing, nasal rubbing, itching, and rhinorrhea characterize allergic rhinitis in human, and in the murine model, sneezing and nasal frictions are easy to measure.

Histamine is known to be one of the main mediators responsible for the clinical signs of allergic rhinitis, food allergy, and systemic anaphylaxis and one of the first mediators to be released during the

activation of mast cells and basophils by allergen [6,32]. Histamine activates sensory nerves, causing itching (or nasal friction) and several motor reflexes, provoking sneezing and parasympathetic reflexes, stimulating nasal gland hypersecretion with consequent rhinorrhea [33]. Using the molecular coupling analysis in granulocytes, it was observed the anchoring of the MHTP with considering binding energy to the histamine receptor (H1) indicating an active site for MHTP binding. Besides, the energy of interaction between the alkaloid and the receptor was higher than that of dexamethasone, chlorpheniramine, and ketotifen.

The MHTP treatment also decreased the number of sneezing and nasal frictions after exogenous histamine-induced nasal hyperactivity regardless of the doses (0.1–1000 nmol). These data indicate a reduction of histamine effect by MHTP in these animals, probably due to the H1 receptor blockage and/or the mucosal and nasal epithelial barrier protection and lack of neural activation. The interaction between MHTP and the H1 receptor may indicate that the synthetic alkaloid has an inverse agonist or neutral antagonist activity. A reverse agonist ligand may have the ability to reduce H1 receptor activity constitutively. A ligand with neutral antagonist activity is not capable of altering the constitutive activation of the H1 receptor, however, it interferes with the binding of its agonists (histamine) [34]. Given the results presented, it can be inferred that MHTP has an H1 antihistamine activity. Indeed, the production of nerve growth factor (NGF) associated with TNF- α production in allergic animals leads to the extension of sensory innervation to the nasal dermis, causing nasal hyperactivity [35]. The previous study

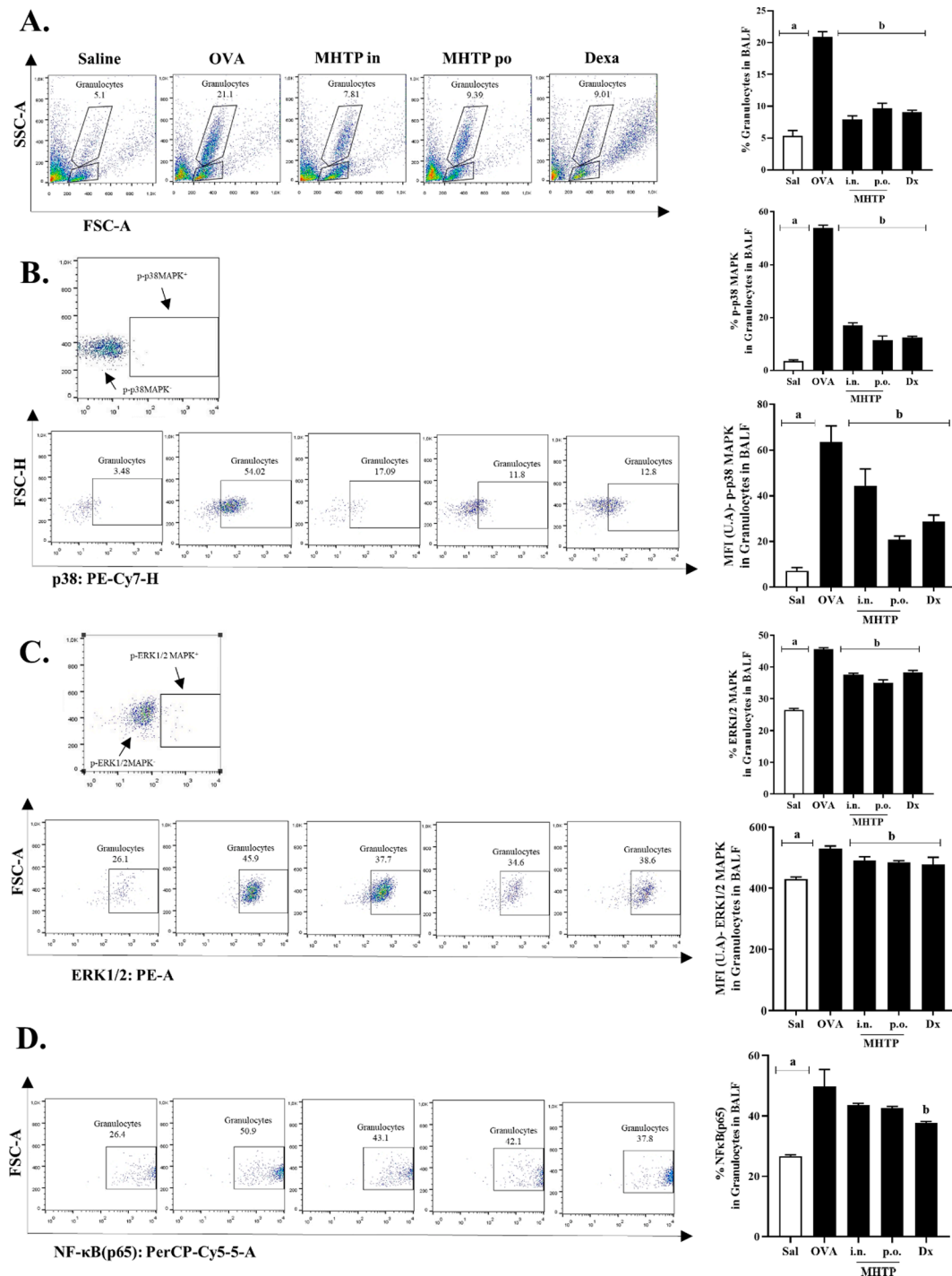


Fig. 7. Effect of MHTP on the frequency of activation of kinases and transcription on granulocytes of OVA-sensitized and challenged mice. The granulocytes (10,000 events). (A). from bronchoalveolar lavage fluids (BALF) of healthy, sick and MHTP or dexamethasone treated animals were analysed for the (B). frequency of activation of p-p38, (C). p-ERK1/2 MAPK and (D). NF-κB(p65) as well as the percentage of granulocytes (bar charts). The granulocytes were identified as SSC^{hi} parameters by flow cytometry. Data are represented as mean $\pm$ e.p.m. analyzed by one-way ANOVA followed by Tukey test. (a) $p < 0.05$ when compared to the Saline group and (b) $p < 0.05$ when compared to the OVA group.

showed that one of the effects of MHTP treatment of animals with LPS induce acute lung injury was a decrease of TNF- α production, thus the airway epithelial barrier protection may be associated with the TNF- α inhibition [14]. Besides, clinical signs of rhinitis are also triggered by the cross-link between the allergen and the IgE-allergen specific bound to Fc ϵ RI in mast cells that release histamine. Paiva-Ferreira and collaborators [15] demonstrated that MHTP intranasal treatment in OVA-sensitized and -challenged animals showed decreasing in IgE-OVA

specific serum titer indicating an immunomodulatory activity of the alkaloid.

The MHTP treatment downregulated the production of an eosinophil-dependent cytokine, the IL-5. This immunomodulatory activity was implied in the inhibitory eosinophil migration to the upper and lower airways of animals of the OVA group. It is well described that the type 2 immune response with the T_H2 polarization profile in allergic airways is associated with ILC2 (innate class 2 lymphoid cells) and the

production of IL-5 and IL-13 as well as the eosinophil migration to the inflamed site. The ILC2 is activated by alarmins as the TSLP [36,37] that was inhibited by the MHTP treatment corroborating to its down regulatory effect on the type 2 immune response. Indeed, studies have shown that allergen proteases induce the production of TSLP dependent on the PAR-2 receptor coupled to G protein in epithelial cells. The recruitment of G protein increases the intracellular phospholipase C (PKC) and Ca^{2+} levels that activates the MAPK pathway and the transcription factor NF- κ B that, in turn, induces the production of TSLP [37].

Therefore, to clarify the MHTP mechanism of action in inflammatory cells from OVA-sensitized and challenged animals, the frequency of activation of the MAPK and NF- κ B was analyzed. The MHTP decreased the frequency of MAPK activation (p-ERK1/2 and p-p38) regardless of NF- κ B (p65) on granulocytes of the BALF. This data surprised us due to the previous study showed that MHTP treatment of LPS-induced acute lung injury (ALI) in mice decreased the TLR4-p38MAPK/NF- κ B (p65) signaling pathway in neutrophils [14]. These controversial data may be explained by the difference between both experimental protocols (CARAS vs ALI) as the triggering agent (OVA vs LPS), the cell population of the BALF (eosinophil vs neutrophil), and the inflammatory process (chronic vs acute) demonstrating a selective role of the alkaloid in inhibiting intracellular signaling pathways in different cell populations.

Another important issue addressed in this study was the production of TGF- β , one of the mediators responsible for the bronchial hyper-responsiveness (BHR) and tissue remodeling [38]. MHTP, regardless of the route, decreased this cytokine level at the BALF and ameliorated the histopathological parameters associated with its effect as hyperplasia and/or hypertrophy of the bronchiolar smooth muscle, extracellular matrix (ECM), and collagen fiber deposition/fibrosis. Besides, the alkaloid also decreases the IL-17A level. This type 3 cytokine profile is also associated with the production of pro-fibrotic cytokines as IL-6 and IL-11 that promote the proliferation of fibroblasts [4].

Therefore, this study demonstrated that the synthetic isoquinoline alkaloid MHTP treatment in animals with combined allergic rhinitis and asthma syndrome (CARAS), regardless of the administration route, ameliorated the rhinitis signals dependent on histamine receptor, the upper and lower airway inflammatory process by inhibiting the granulocyte migration, the type 2 cytokine production, fibrotic cytokines, and histopathological parameters. These effects were dependent on the downregulation of MAPK pathway (p38 and ERK1/2) regardless of NF- κ B activation on granulocytes from the BALF. Thus, the downregulation of MAPK signaling pathway by MHTP on this cell population puts the molecule as a powerful tool to be considered in clinical trials.

CRedit authorship contribution statement

Laércia K.D. Paiva Ferreira: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Larissa A.M. Paiva Ferreira:** Conceptualization, Data curation, Methodology, Formal analysis, Software, Validation. **Grasiela C. Bezerra Barros:** Data curation, Methodology. **Talissa M. Monteiro:** Formal analysis, Software, Validation. **Luiz A. de Araújo Silva:** Formal analysis, Methodology, Validation. **Ramon de A. Pereira:** Formal analysis, Software, Validation. **Pedro T.R. Figueiredo:** Formal analysis, Software, Validation. **Adriano Francisco Alves:** Supervision, Formal analysis, Software, Validation. **Luís Cezar Rodrigues:** Supervision, Formal analysis, Methodology, Validation. **Marcia Regina Piuvezam:** Conceptualization, Data curation, Visualization, Resources, Funding acquisition, Project administration, Supervision, 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.

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Appendix A. Supplementary material

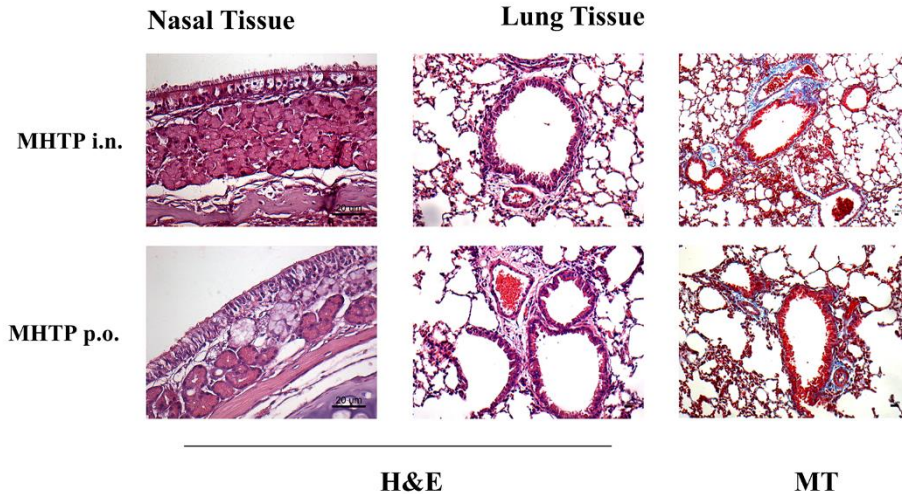
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2021.107590>.

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A.



B.

	Saline	MHTP i.n.	MHTP p.o.
% Eosinophils in NALF	0 ± 0	0.250 ± 0.250	0 ± 0
% Eosinophils in BALF	0 ± 0	0 ± 0	0.250 ± 0.250
Nasal tissue			
Inflammatory score	0.195 ± 0.337	0.106 ± 0.0208	0.453 ± 0.0180
Number of eosinophils	0.750 ± 0.250	3 ± 0.408	3.25 ± 0.479
Pulmonary tissue			
Inflammatory score	1 ± 0	1 ± 0	1 ± 0
Number of eosinophils	3 ± 0.707	10.6 ± 0.927	6.60 ± 0.678
Muscle layer (area/μm ²)	0.276 ± 0.0103	0.290 ± 0.114	0.284 ± 0.0117
Extracellular Matrix (ECM) (area/μm ²)	184 ± 18.2	220 ± 38	156 ± 17.6

Complementary figure. Effect of intranasal or oral MHTP treatment on healthy animals. (A). Representative photomicrographs of nasal and lung tissues stained with Hematoxylin and Eosin (H&E) and lung tissue stained with and Masson's Trichrome (MT). **(B).** Table of histological parameters and nasal (NALF) and bronchoalveolar (BALF) lavage fluid analysis and extracellular matrix (ECM). The photomicrographs were captured under optical microscopy - AT 40x (nasal tissue) and AT 20x (lung tissue) for the H&E stain and 20x for the MT stain. Data are represented as mean ± e.p.m. analyzed by one-way ANOVA followed by Tukey test.