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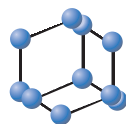


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A Novel Oral Glutarimide Derivative XC8 Suppresses Sephadex-Induced Lung Inflammation in Rats and Ovalbumin-induced Acute and Chronic Asthma in Guinea Pigs

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Abstract: Background: Corticosteroids are the preferred option to treat asthma, however, they possess serious side effects and are inefficient in 10% of patients. Thus, new therapeutic approaches for asthma treatment are required.

Objective: To study the efficacy of a novel glutarimide derivative XC8 in a Sephadex-induced lung inflammation in rats as well as in acute and chronic ovalbumin-induced allergic asthma in guinea pigs.

Method: Rats were treated with 0.18-18 mg/kg of XC8 intragastrically 4 times (24 h and 1 h prior to and 24 h and 45 h after endotracheal administration of Sephadex). The number of inflammatory cells in bronchoalveolar lavages (BAL) was determined. Guinea pigs were treated with 0.045 -1.4 mg/kg (acute asthma) or with 1.4 and 7.0 mg/kg of XC8 (chronic asthma) intragastrically following the sensitization with ovalbumin and during aerosol challenge. Lung inflammation, numbers of eosinophils (BAL and lung tissue), goblet cells, degranulating mast cells and specific airway resistance (sRAW) were determined. The comparator steroid drug budesonide (0.5 mg/kg for rats and 0.16 mg/kg for guinea pigs) was administered by inhalation.

Results: XC8 reduced influx of eosinophils into BAL in Sephadex-induced lung inflammation model in rats (by 2.6-6.4 times). Treatment of acute asthma in guinea pigs significantly reduced eosinophils in guinea pigs in BAL (from 55% to 30%-39% of the total cell count) and goblet cells in lung tissue. In a model of acute and chronic asthma, XC8 reduced significantly the number of eosinophils and degranulating mast cells in the lung tissue. Treatment with XC8 but not with budesonide decreased the specific airway resistance in acute and chronic asthma model up to the level of naive animals.

Conclusion: XC8 induced a profound anti-inflammatory effect by reducing eosinophils in BAL and eosinophils and degranulating mast cell numbers in the airway tissue. The anti-asthmatic effect of XC8 is comparable to that of budesonide. Moreover, in contrast to budesonide, XC8 was capable to reduce goblet cells and airway resistance.

Keywords: Allergic lung inflammation, airway hyperresponsiveness, asthma, allergic asthma, glutarimide derivatives, XC8.

1. INTRODUCTION

Asthma is a chronic lung disease, affecting over 300 million people and causing 0.30-0.35 million deaths annually worldwide [1]. Asthma is an inflammatory disorder characterized by bronchoconstriction and airway hyperresponsiveness, followed by inflammatory manifestations in the respiratory

system, hypersecretion of mucus, and reversible airway obstruction potentially leading to chronic disease with the permanent impairment of pulmonary function.

The frequent exacerbations of asthma occur mostly in autumn (for children) or in winter (for adults), the fact that is related to different factors. The most important of these factors include the female sex [2], obesity [3], psychopathology, allergens (house dust mites, animals, fungi), diseases (chronic sinusitis, gastroesophageal reflux, obstructive sleep apnea) [4],

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aspirin or nonsteroidal anti-inflammatory drugs (NSAID), viral respiratory infections, and some others. Allergy and viral infection are the most common factors that cause asthma exacerbations. The exacerbations induced by viruses are characterized by a larger deterioration in lung function and a longer recovery from disease [5]. The costs of asthma exacerbations treatment account for more than 80% of all asthma costs [6, 7].

Inhaled and systemic glucocorticosteroids (GCS) are the most effective drugs that can control the clinical manifestations of asthma. Another type of preparations like inhaled β_2 -agonists, short-acting inhaled anticholinergics, short-acting theophylline, and short-acting oral β_2 -agonists are known to act fast and are, therefore, used for symptoms relief. Although inhaled GCS may prevent allergen-induced asthma exacerbations, their use is limited because of known side effects [8]. Moreover, GCS were shown to be effective only in 50% of patients with asthma exacerbations induced by virus infections [9-11]. Therefore, there is a need for new therapeutic approaches for asthma treatment, which can replace or reduce the use of systemic and inhaled GCS and affect virus-induced exacerbations by the modulation of viral pathogenesis.

The derivatives of biogenic amines (peptidoamines) are known to be involved in multiple physiological processes in plants and animals [12-16] and believed to possess a certain potential to be used as novel drugs against a broad spectrum of health disorders, including asthma [17-19]. Several N-acyl derivatives of biogenic amines were synthesized and screened for their anti-inflammatory potential in the Sephadex-induced model of lung inflammation in rats (the results are not published). The most promising compound – a glutarimide derivative, named XC8 and revealing the best anti-inflammatory capacity – was selected for further pharmacological development.

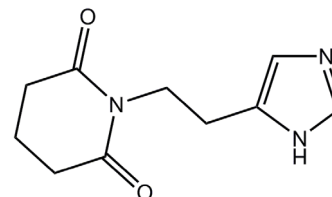
The proposed mechanism of XC8 action is associated with the suppression of glutaminyl cyclase – an enzyme that converts the immature form of chemokines of the monocyte chemoattractant protein (MCP) family (MCP-1, MCP-2, MCP-3, MCP-4) into the mature form by the reaction of pyroglutamination (data not published). Inhibition of this process by XC8 suppresses the chemokine-driven migration of eosinophils and other cells into the inflammation area and the degranulation of mast cells. Therefore, by inhibiting the activation of MCP chemokines XC8 could ultimately decrease the number of eosinophil chemoattractants which in turn would reduce infiltration of eosinophils into the lungs while preserving their plasma level. In order to confirm this hypothesis, we used animal models designed for the assessment of the migration of eosinophils and degranulation of mast cells.

Here we present the data on pharmacological activity of XC8 after repeated oral administration on the migration of inflammatory cells into the lung in a model of Sephadex-induced lung inflammation in rats, and on lung inflammation and airway hyperreactivity (AHR) in a model of acute and chronic asthma induced by ovalbumin in guinea pigs [20, 21].

2. MATERIALS AND METHODS

2.1. XC8 Compound

XC8 substance was produced and provided by Pharmenterprises LLC (Russia). XC8 is a 1 - (2 - (1H-Imidazol-4-yl) ethyl) piperidine-2, 6-dione based on IUPAC nomenclature with Molecular weight 207.23. The chemical structure of XC8 is shown below.



It is a nearly white crystalline powder soluble in 96 % ethanol and water, pH: 8.2-9.3 (c = 1, water). The melting temperature of XC8 is 148-152°C (Koffler apparatus). Weighed XC8 substance was diluted in 1% solution of Tween-80, prepared in PBS immediately prior to administration.

2.2. Animals

Six to eight-week-old male Wistar rats (200-250 g) and 16-week-old male Agouti guinea pigs (250-260 g) were purchased from the Scientific Center of Biomedical Technologies (Andreevka, Moscow region). Animals were kept in cages with a controlled environment and provided with standard pelleted food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Ethics Committee. The experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals [22]. All painful procedures were performed under anesthesia for minimizing the animals' suffering.

2.3. Sephadex-induced Model of Lung Inflammation in Rats

Sephadex-induced model of lung inflammation in rats was based on the procedure described previously [20]. In a pilot study (data not shown), we found that the most pronounced eosinophilic inflammation in rats was observed 48 h after endotracheal administration of Sephadex G-200 (Sephadex). Rats (10 per group) were anaesthetized by intraperitoneal (i.p.) administration of 0.7 ml/kg Zoletyl-Xyla mixture in the ratio 1:5. Sephadex G-200 beads (Sigma, USA) were suspended in normal saline and soaked at 4°C for 72 h after autoclaving. Sephadex G-200 solution (5 mg/kg) was administered endotracheally to rats by High-Pressure Syringe (Model FMJ-250, Penn-Century, USA) in a volume of 50 μ l. The XC8 (0, 0.18, 0.54, 1.8, 5.4, or 18 mg/kg) dissolved in 1% solution of Tween-80, prepared in PBS was administered intragastrically (i.g.) by gavage four times: 24 and 1 h prior to and 24 and 45 h after the administration of Sephadex. The comparator drug, budesonide, was applied by inhalation at a dose of 0.5 mg/kg. The group of rats treated with 1% solution of Tween-80, prepared in PBS is designated as the diluent, the group of unsensitized and untreated animals designated as naïve.

2.4. Induction of Allergic Asthma and Treatment Protocol in Guinea Pigs

A standard method of acute allergic asthma was applied as previously described [21]. Briefly, guinea pigs were immunized twice i.p. on days 1 and 21 with 100 µg/ml of ovalbumin (Sigma) admixed with 100 mg/ml of aluminum hydroxide (250 mg/kg, ZHR Doneck-Reaktiv) in a volume of 0.5 ml. Then, the animals were challenged with increasing concentrations of 0.01, 0.03, 0.05, and 1% w/v of nebulized ovalbumin on days 29, 30, 31, and 32, respectively. Inhalation was carried out at least for 5 minutes to induce the acute onset of disease. The treatment of animals was performed i.g. with XC8 (0.045, 0.14, and 1.4 mg/kg) dissolved in 1% solution of Tween-80, prepared in PBS or with comparator drug budesonide (0.16 mg/kg) by daily inhalation between days 21 and 30. The group of animals treated with 1% solution of Tween-80, prepared in PBS is designated as the diluent, the group of unsensitized and untreated animals designated as naïve. Groups of naïve, XC8 and budesonide treated animals included 12 animals, the diluent-treated group contained 24 animals. The doses used in guinea pigs were recalculated from effective doses derived from the Sephadex lung inflammation model in rats. XC8 and the comparator were administered 30 min before ovalbumin aerosol challenge. Five experiments were performed, the results of more representative experiment are presented.

A standard method of chronic allergic asthma was applied as described previously [23, 24]. Guinea pigs were sensitized with injection of 1 ml of a solution containing 100 µg/ml of ovalbumin (Sigma) and 100 mg/ml of aluminum hydroxide (250 mg/kg, Doneck-Reaktiv ZHR): 0.5 ml i.p., and 0.5 ml intradermally (in the form of 7 injections in different parts of the body e.g. next to the lymph nodes of the legs, lumbar region, and neck). Four weeks after the sensitization (on the 29th study day), animals from the experimental groups were challenged with increasing concentrations of 0.05, 0.1, 0.3, 0.5, and 0.1% w/v nebulized ovalbumin once per week for 12 weeks. The inhalation was performed for 3 min each, with a 7-min interval. Ovalbumin inhalation was terminated when the first signs of respiratory failure were observed. The XC8 at doses of 1.4 and 7.0 mg/kg dissolved in diluent was administered i.g. daily once per day, starting from the first day of the 7th week (43d day) and until the end of the 16th week (112th day) of the experiment. Budesonide at a dose of 0.16 mg/kg was administered to animals by inhalation 2 times a week (24 and 1 h before inhalation of ovalbumin), starting from the 7th week and continuing until the 16th week (112th day) of the experiment. Groups of naïve, XC8 and budesonide treated animals included 8 animals. The results of more representative from 5 performed experiments are presented.

2.5. Broncho-alveolar Lavage (BAL) Fluid Collection and Cells Count

Rat BAL was collected under terminal anesthesia 48 h after the administration of Sephadex, whereas the BAL of guinea pigs (in acute asthma model) was collected 24 h after the inhalation of the last challenge of ovalbumin dose. Animals were anaesthetized by i.p. administration of 1 ml/kg Zoletyl-Xyla mixture in the ratio 1:5. After the semi-excision

of the trachea, a plastic cannula was inserted, and the air-space was washed with 5 ml of PBS pre-warmed to 37°C. The total number of cells in BAL was enumerated in a Neubauer hemacytometer. The percentage of inflammatory cells was determined by morphological examination of at least 300 cells in cytocentrifuged preparations (Hettich® Universal 320R centrifuge, Germany), stained with Kwik-Diff (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). Types of white blood cells were differentiated and quantified manually per 1 µl of BAL utilizing routine standard procedures [25]. All the differential cell counts were performed blind and analyzed by a one-way ANOVA test followed by Dunnett's multiple comparison test using GraphPad Prism 7 Software.

2.6. Histopathological Analysis

After animal euthanasia, the lungs were extracted from the thoracic cavity. Lung samples were fixed in formalin, dehydrated, impregnated with paraffin, cut, and stained with Luna stain for eosinophils enumeration [26]. Cuttings were made at the level of the main bronchi. The quantification of eosinophils was made in the mucosa of medium bronchial tubes at a magnification of x100 recalculated per 100 µm of the basement membrane (BM) in 3 fields of vision [27].

Goblet cells were stained with periodic Acid-Schiff stain (PAS) for mucopolysaccharide staining. Cell counting was carried out from the bronchial tree (intralobular bronchi) at a magnification of x100 in 3 fields of vision.

The preparations for lung mast cell quantification were stained with toluidine blue. The number of degranulating mast cells in the submucosa of large bronchial tubes was determined at a multiplication of x100 per 100 µm of the BM in 3 fields of vision.

2.7. Measurement of Specific Airway Resistance

Specific airway resistance (sRAW) in the acute asthma model was evaluated for 30 min immediately after the last ovalbumin inhalation. To evaluate the respiratory function, animals were placed in a two-chamber plethysmograph (Emka Technologies, France), consisting of a nasal and thorax chambers. The procedure was performed according to the standard method described previously [28]. The nasal chamber was equipped with a pneumotachograph. The airflow rate in the nasal chamber was set at 250 ml/min to ensure continuous air change and aerosol delivery at the time of inhalation. Isolation between the two chambers was achieved by placing a rubber membrane with a hole for the head of the guinea pig. The animal was placed in a plethysmograph in such a way that the head was in the nasal chamber, and the neck closed the hole in the membrane. Thus, the air did not penetrate between the chambers. Each plethysmograph chamber was connected to a laboratory system (DCRF analyzer, EMKA Technologies) using a DPT differential pressure sensor (± 2 cm) to determine the flow rate. All the data were recorded and analyzed using EMKA Technologies (iox2) laboratory system software. The measurements were carried out with a frequency of digitization of 1000 Hz.

In the chronic asthma model guinea pigs were anaesthetized by i.p. administration of 1 ml / kg Zoletyl-Xyla mixture

in the ratio 1: 5. Then the trachea was isolated, a cannula was inserted, connected to the sensor recording bronchospasm and a respirator (Ugo Basile, Italy). During the experiment, a certain breathing regimen was maintained: breathing volume – 7 ml, frequency – 70 respiratory movements per minute. The increasing methacholine (Sigma) doses of 31.25, 62.5, 125, 250, and 500 $\mu\text{g/kg}$ were injected into the jugular vein through intravenous catheters with an interval of 30 s. Changes in airway resistance were recorded using ADInstruments (Australia) laboratory equipment with the PowerLab 8sp recording station and LabCart software connected to sensors recording bronchospasms. The magnitude of methacholine induced hyperreactivity was expressed by the increasing degree of the airway resistance. To compare airway resistance areas under the curve (AUC) were calculated for each experimental animal and compared by One-way ANOVA followed by Dunnet's multiple comparison test.

3. RESULTS

3.1. XC8 Reduces Migration of Inflammatory Cells into the Lung in Sephadex-induced Inflammation Model

Administration of Sephadex to rats by inhalation caused a marked influx of leukocytes into the bronchoalveolar space. The measurement of the number of leukocytes in the BAL of animals showed that naïve animals had 865 ± 78 cells/ μl of BAL, whereas the animals of the control group receiving the diluent had a three times higher leukocyte count ($2,785 \pm 152$ cells/ μl , Fig. 1).

The analysis of the individual cell types revealed that the administration of Sephadex induced a marked influx of

neutrophils, eosinophils, macrophages, and lymphocytes. The maximum increase (34-fold) was observed for eosinophils. The administration of XC8 to rats resulted in a 2.6 - 6.4 times decrease in eosinophils in the BAL fluid. The pharmacological activity of XC8 was shown for all tested doses and was superior to the comparator steroid drug budesonide. It is noteworthy that the significantly reduced number of neutrophils was observed in animals treated with the highest dose (18 mg/kg) of XC8 when compared with the diluent-treated group. No reduction of neutrophils was revealed for the animals treated with budesonide.

The obtained data indicate that XC8 inhibits the migration of eosinophils and neutrophils to the lungs.

3.2. XC8 Reduces Eosinophils in the BAL in Acute Asthma Model

The model of acute asthma in sensitized guinea pigs allows for simulating bronchospasm and lung inflammation. Animals sensitized and challenged with ovalbumin and treated with the diluent developed acute airway inflammation characterized with an increase in the BAL inflammatory cell number presented mostly by eosinophils (Fig. 2A). Animals treated with XC8 at a dose range from 0.045 to 1.4 mg/kg once daily for 10 consecutive days showed a significant reduction of eosinophils from 55% to approx. 30% to 39% depending on the XC8 dose on the total BAL inflammatory cell count (Fig. 2A). The total number of eosinophils was 2.4 to 3.6 times lower in the XC8 treated groups when compared to the diluent-treated group (Fig. 2B). The obtained effect was comparable to that observed for the comparator drug budesonide.

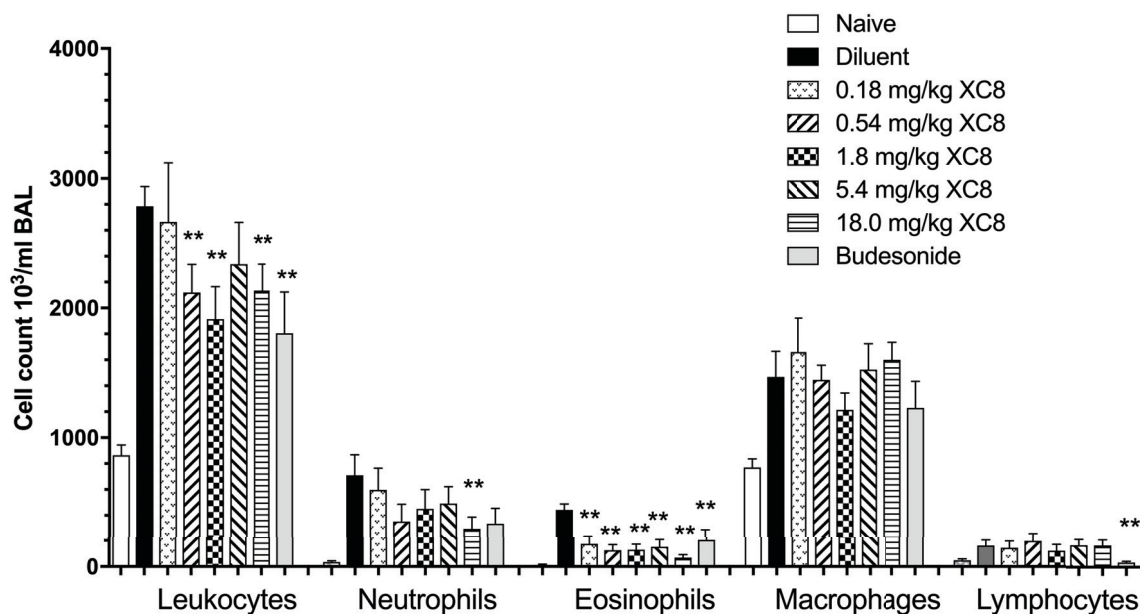


Fig. (1). The effect of XC8 on the number of inflammatory cells in BAL of rats after the administration of Sephadex. Sephadex was administered endotracheally. Animals (10 per group) were treated with XC8 (0, 0.18, 0.54, 1.8, 5.4, and 18 mg/kg) or by budesonide (by inhalation at a dose of 0.5 mg/kg) 24 and 1 h before and 24 and 45 h after the administration of Sephadex. BAL was collected 48 h after the inhalation of a Sephadex and monocytes/macrophages, neutrophils, eosinophils, and lymphocytes were differentiated and quantified. The results are expressed as mean $\pm$ SEM. One-way ANOVA followed by Dunnet's multiple comparison test.

** $p < 0.01$ when compared with the diluent-treated control group.

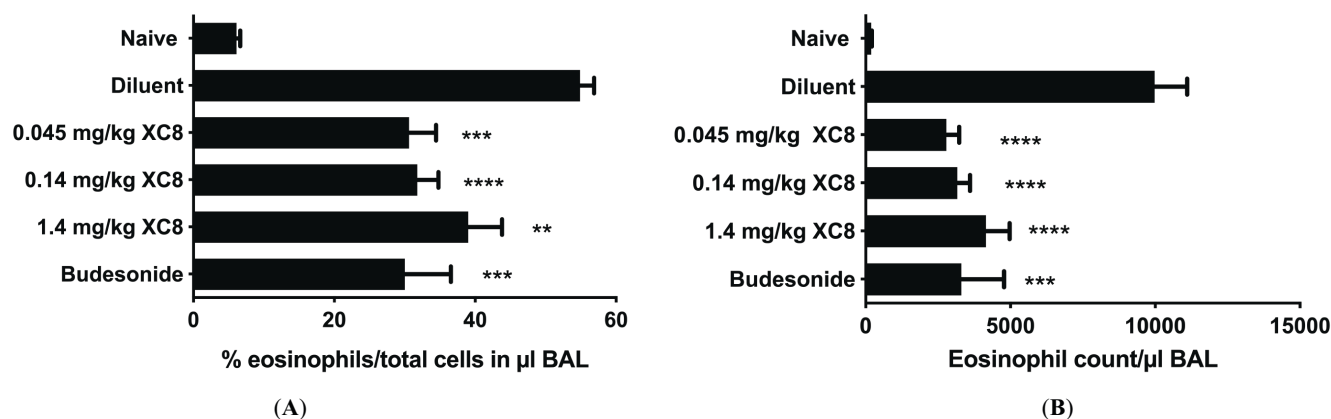


Fig. (2). The effect of XC8 on the number of eosinophils in the airways of guinea pigs in acute asthma model. Animals (12 per group) were treated daily i.g. with XC8 (0.045-1.4 mg/kg) or with nebulized budesonide (0.16 mg/kg) for 10 consecutive days (days from 21 to 30). The diluent-treated group included 24 animals. BAL was collected 24 h after the last inhalation dose of ovalbumin. **(A)** The percentage of eosinophils from the total number of cells in BAL. **(B)** The absolute number of eosinophils per 1 μ l of BAL. The results are expressed as mean $\pm$ SEM, *** p <0.001, **** p <0.0001 one-way ANOVA followed by Dunnet's multiple comparison test, compared with the group of animals treated with diluent.

3.3. XC8 Reduces Lung Inflammation, Mucus Secretion and Degranulation of Mast Cells in Acute and Chronic Asthma Model

The effect of XC8 was estimated on the severity of parenchymal allergic lung inflammation in the acute asthma model. Representative histological slides of lung tissue stained with Luna, PAS, and toluidine blue techniques were screened for the presence of eosinophils, goblet cells, and degranulating mast cells, respectively (Fig. 3).

Low eosinophil counts were detected in the lungs of naïve animals (Fig. 3A), whereas multiple inflamed areas with eosinophils were found in the lungs of diluent-treated animals (Fig. 3B, see also higher-power field insets). In contrast, i.g. treatment of animals with 1.4 mg/kg of XC8 and with the nebulized comparator drug budesonide for 10 days reduced overall inflammation and eosinophilic infiltration (Fig. 3C, D).

PAS-stained lung sections were screened for mucus producing goblet cells. The distribution of goblet cells in the airways is shown in Fig. (3E-H). The low number of mucus-producing goblet cells was noticed in the lungs of naïve animals (Fig. 3E), whereas ovalbumin sensitization and aerosol challenge caused the increased production of goblet cells in diluent-treated animals (Fig. 3F). The treatment of animals with XC8 (1.4 mg/kg) and with budesonide (Fig. 3G, H) reduced the number of goblet cells in the airways. The mean number of goblet cells in the bronchi of naïve animals was 7.3 cells/100 μ m of the mucosal wall. In the control diluent group, this number increased by more than 3 times (25.8 cells/100 μ m).

The enumeration of eosinophils, goblet cells and degranulating mast cells in the lung tissue revealed that the treatment of animals with XC8 had a broad therapeutic effect at tested doses. The treatment of guinea pigs with XC8 at a dose of 1.4 mg/kg significantly reduced the number of eosinophils at least by 28% compared to the diluent-treated

group and was superior to the effect of comparator drug budesonide (Fig. 4A). The number of goblet cells decreased significantly in the groups of animals treated with XC8 at 0.14, and 1.4 mg/kg, and the effect was superior to that of the comparator drug budesonide (Fig. 4B). The determination of degranulating mast cell numbers in toluidine blue stained lung tissue sections revealed the low numbers of degranulated mast cells, with an average of 0.2 cells/field in the lungs of naïve animals, while in the diluent-treated control group 0.9 cells/vision field of the degranulating mast cells were identified. The treatment of animals with XC8 at 0.14 and 1.4 mg/kg led to significantly reduced values for this parameter, that were practically up to the level found in naïve animals (Fig. 4C). The mean values of 0.25 cells/field (0.14 mg/kg of XC8), and 0.19 cells/field (1.4 mg/kg of XC8) were demonstrated in XC8-treated animals. Therefore, XC8 significantly reduced the degree of mast cells degranulation in a dose-dependent manner in acute asthma model. No significant effect on mast cell degranulation was shown for the comparator drug budesonide.

In the model of chronic asthma in guinea pigs we utilized the dose of 1.4 mg/kg of XC8 that showed the prominent treatment effect in the model of acute asthma and an additional higher dose of 7.0 mg/kg of XC8. The obtained results demonstrated that XC8 decreased significantly the eosinophil counts as well as degranulating mast cells in lung tissue at both doses and the effect was comparable to that shown for budesonide (Fig. 5).

3.4. XC8 Improves Specific Airway Resistance in Acute and Chronic Asthma Models

Changes in the respiratory tract, including thickening of the mucous membrane and mucus in the lumen of the respiratory tract, can lead to the development of AHR in acute asthma. In chronic asthma, these changes cause irreversible structural changes in the respiratory tract, which may be associated with persistent AHR and a decrease in lung function.

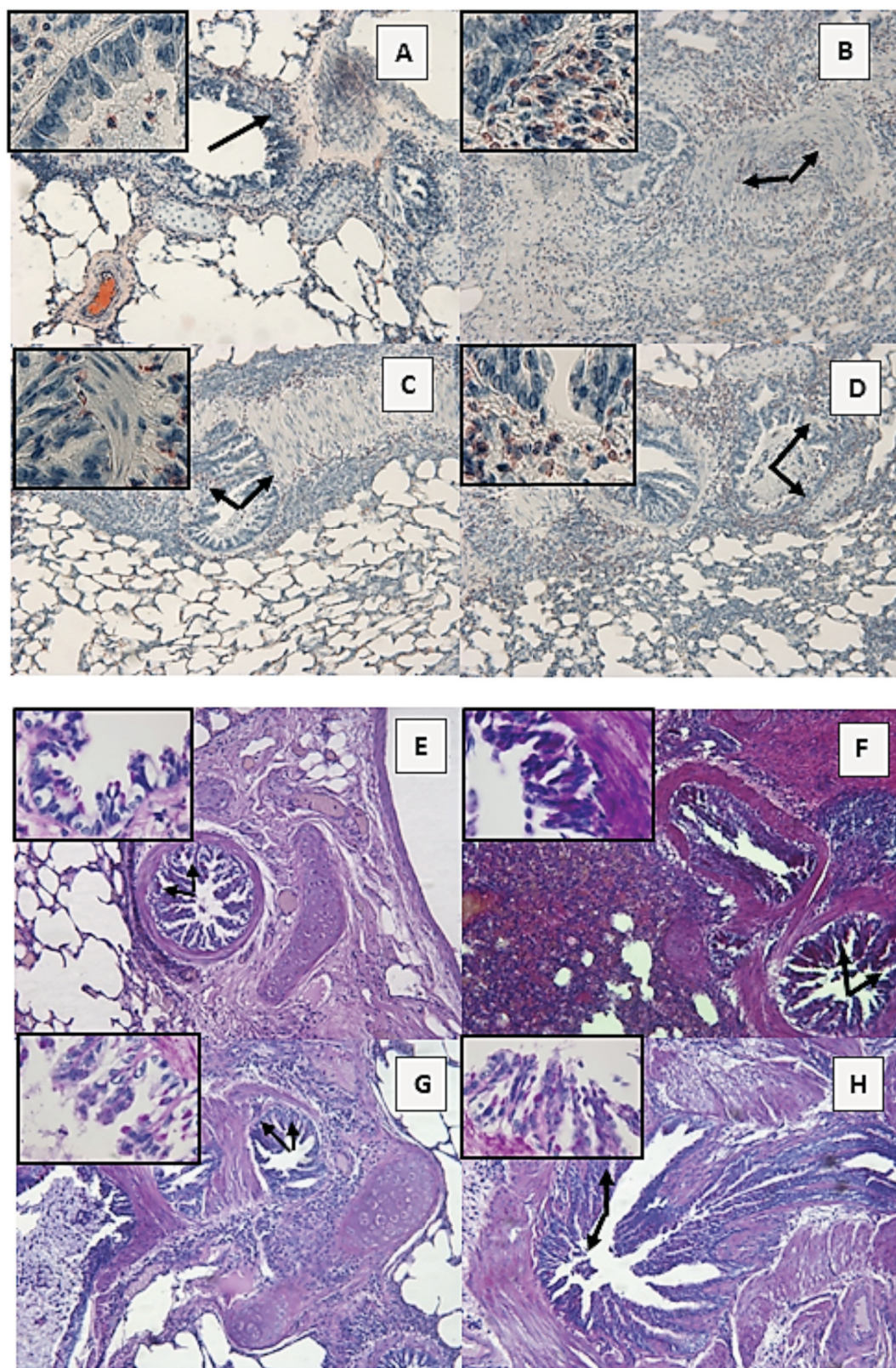


Fig. (3). Lung inflammation and mucus secretion in animals treated with XC8 or budesonide in acute asthma model. **A-D** and **E-H** are representative photomicrographs of Luna and PAS stained lung sections, respectively, from naïve guinea pigs (**A** & **E**), guinea pigs treated with diluent (**B** & **F**), treated by XC8 i.g. (1.4 mg/kg) (**C** & **G**), or by budesonide (0.16 mg/kg) administered by inhalation (**D** & **H**) for 10 days. Luna staining technique allows the visualization of inflamed areas with eosinophils. Eosinophils are identified by purple nuclei. PAS staining illustrates the presence of mucopolysaccharides. The arrows indicate the cell infiltrates or goblet cells filled with mucus.

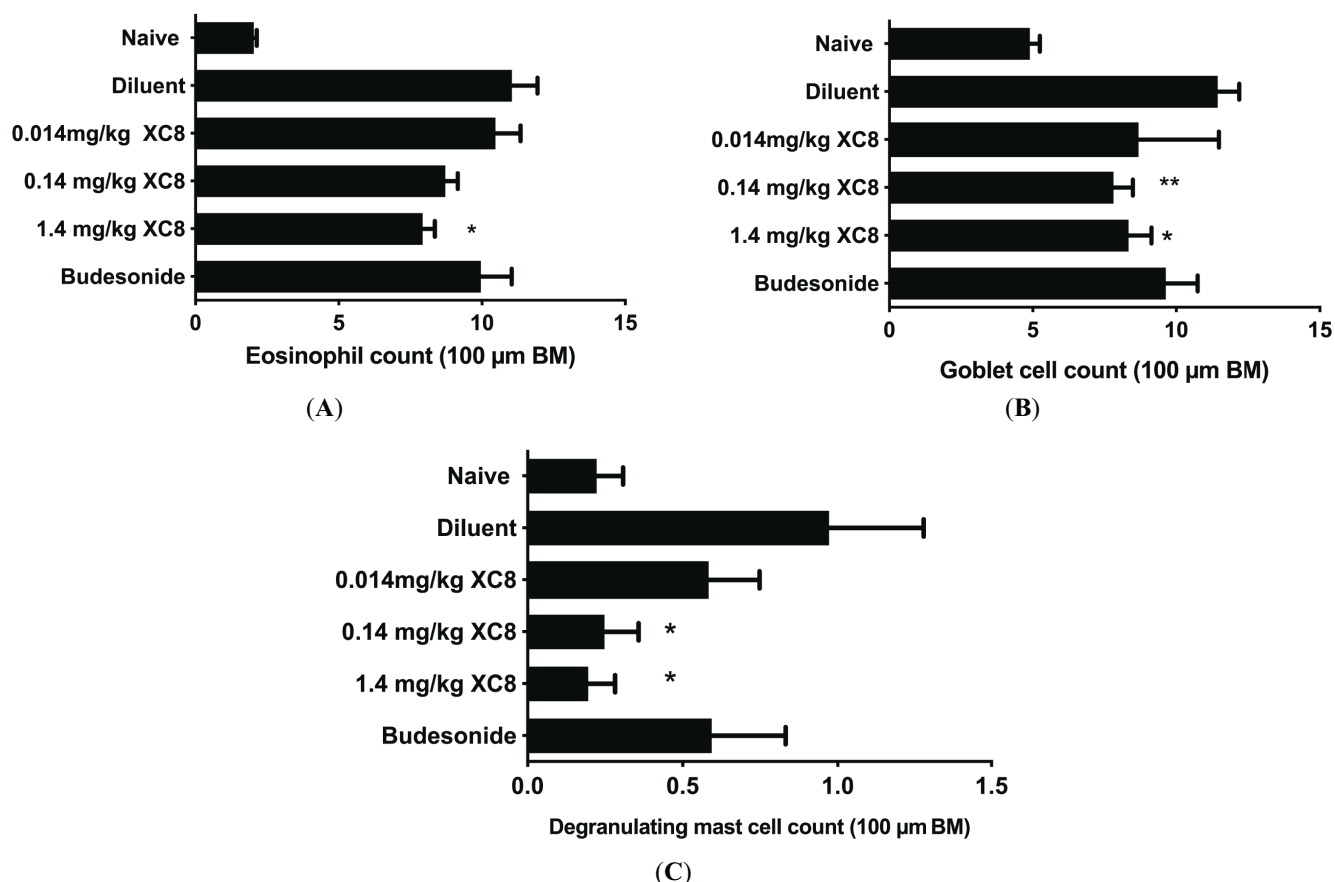


Fig. (4). The effect of XC8 on the number of eosinophils (A), goblet cells (B) and degranulating mast cells (C) in the lungs of guinea pigs in acute asthma model. Animals (8 per group) were treated daily i.g. with XC8 (0.014-1.4 mg/kg) or with nebulized budesonide (0.16 mg/kg) for 10 consecutive days (days 21-30). Lungs were collected on day 33. Counting was performed from three representative fields at a x100 magnification and normalized to 100 μ m of BM. The results are expressed as mean $\pm$ SEM. One-way ANOVA followed by Dunnet's multiple comparison test, compared with the group of animals treated with diluent, * $p < 0.05$, ** $p < 0.01$.

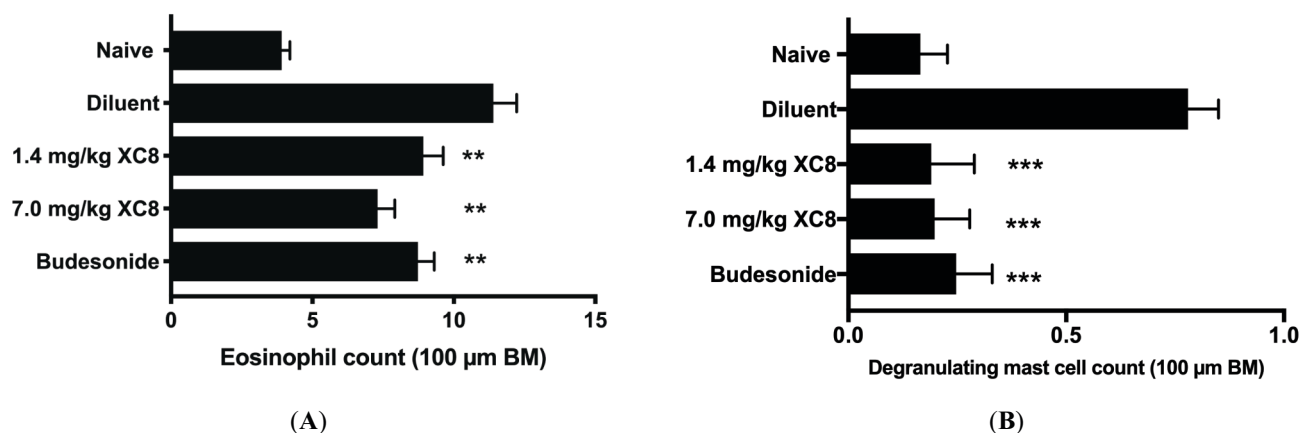


Fig. (5). The effect of XC8 on the number of eosinophils (A), and degranulating mast cells (B) in the lungs of animals in chronic asthma model. Guinea pigs (8 per group) were treated i.g. with XC8 (1.4 mg/kg and 7.0 mg/kg) daily once per day, starting from the first day of the 7th study week (day 43) and continuing until the end of the 16th week (day 112) of the experiment. Budesonide (0.16 mg/kg) was administered to animals by inhalation 2 times per week (24 and 1 h before inhalation of ovalbumin), starting from the 7th study week and continuing until the 16th study week (112th day) of the experiment. Lungs were collected 24 h after the last ovalbumin challenge. Eosinophils and degranulating mast cells counting was performed from three representative fields at a x100 magnification and normalized to 100 μ m of BM. The results are expressed as mean $\pm$ SEM. One-way ANOVA followed by Dunnet's multiple comparison test, compared with the group of animals treated with diluent, ** $p < 0.01$, *** $p < 0.001$.

Ovalbumin sensitization and subsequent challenge is known to lead to the development of airway hyperresponsiveness. To analyze the effect of XC8 on the airway responsiveness during the allergic asthma, guinea pigs were sensitized and challenged with ovalbumin in accordance to acute and chronic asthma protocols.

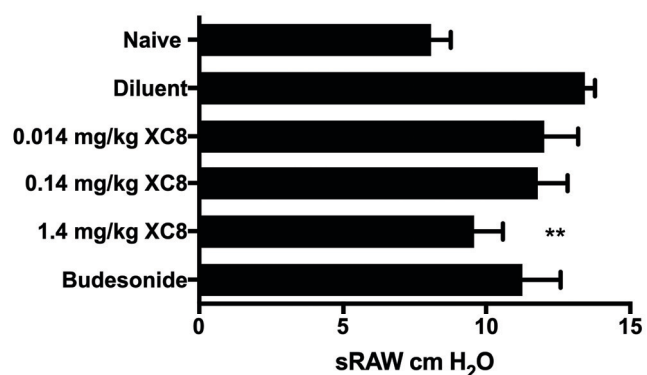


Fig. (6). The effect of XC8 on the airway resistance of guinea pigs in acute asthma model. Animals (12 per group) were treated i.g. with XC8 (0.014-1.4 mg/kg) or with nebulized budesonide (0.16 mg/kg) for 10 consecutive days (days 21-30). Diluent treated group included 24 animals. The sRAW was evaluated for 30 minutes after last inhalation with ovalbumin. The results are expressed as mean $\pm$ SEM, ** p <0.01 one-way ANOVA followed by Dunnet's multiple comparison test compared to the group of animals treated with diluent.

In acute asthma model the lung function was evaluated immediately after ovalbumin inhalation in a two-chamber plethysmograph (Emka Technologies, France). The immunization and sensitization of guinea pigs with ovalbumin in-

creased the sRAW in the diluent-treated animals compared to naïve animals (Fig. 6). Intragastric administration of XC8 to guinea pigs once for 10 consecutive days at a dose of 1.4 mg/kg significantly reduced sRAW compared to the diluent-treated group. The comparator drug budesonide was not capable to reduce significantly the airway resistance compared to the group of animals treated with diluent.

In the chronic asthma model AHR was evaluated 24 h after the last ovalbumin challenge in response to intravenous injections of methacholine. Changes in airway resistance in relation to airflow were recorded invasively using ADInstruments (Australia) laboratory equipment with the PowerLab 8sp recording station and LabCart software connected to sensor recording bronchospasms (Ugo Basile, Italy). As expected, sensitized guinea pigs that were challenged with ovalbumin exhibited increased sRAW responses to methacholine when compared to naïve guinea pigs (Fig. 7). Intragastric administration of XC8 to guinea pigs once per day for 6 consecutive weeks at doses of 1.4 and 7.0 mg/kg totally inhibited AHR up to the level of naïve animals. The comparator drug budesonide revealed no effect on AHR in response to injected methacholine when compared to the group of animals treated with diluent.

Taken together, these results indicate that XC8 at the doses 1.4 and 7.0 mg/kg improves specific airway resistance in acute and chronic asthma model in guinea pigs.

4. DISCUSSION

The anti-inflammatory effect of the novel glutarimide derivative XC8 was investigated in animal models of lung inflammation (rats) as well as in acute and chronic experimental allergic asthma (guinea pigs). The Sephadex-induced lung inflammation is a model of acute alveolitis

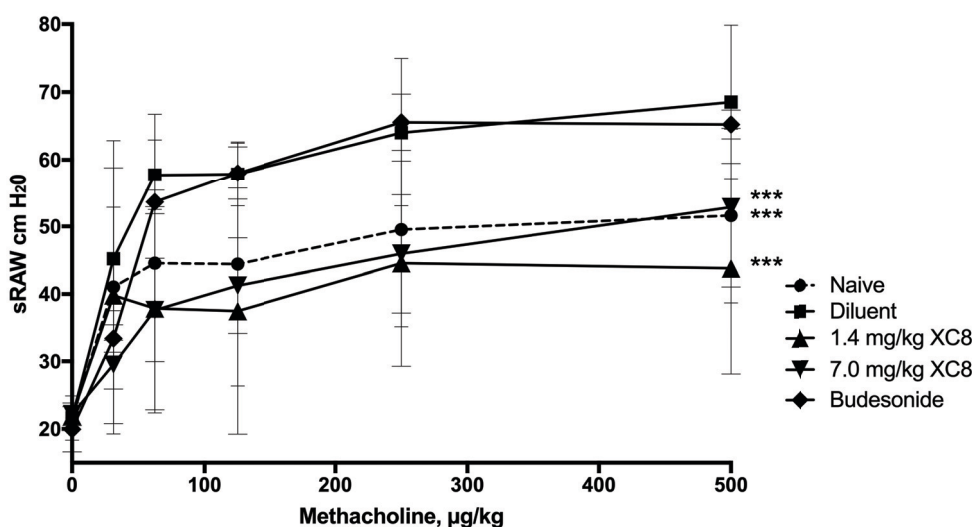


Fig. (7). The effect of XC8 on antigen-induced AHR to methacholine in chronic asthma model. Guinea pigs (8 per group) were treated i.g. with XC8 (1.4 mg/kg and 7.0 mg/kg) daily once per day, starting from the first day of the 7th week (day 43) and continuing until the end of the 16th study week (day 112). Nebulized budesonide (0.16 mg/kg) was administered to animals 2 times per week (24 and 1 h before inhalation of ovalbumin), starting from the 7th study week and continuing until the 16th study week (day 112). The increasing of methacholine (Sigma) concentrations (31.25, 62.5, 125, 250, and 500 µg/kg) were intravenously (i.v.) injected 24 h after the last ovalbumin challenge. The results are expressed as mean $\pm$ SEM. The AUC were calculated for each experimental animal and compared by One-way ANOVA followed by Dunnet's multiple comparison test compared to the group of animals treated with diluent, *** p <0.001.

and bronchiolitis leading to inflammatory cell infiltration and interstitial edema, which appears parallel to many of the pathophysiological features associated with human lung diseases including asthma [29]. The model of experimental asthma in guinea pigs has been known for decades and today guinea pigs are still the most widely used model of allergic respiratory disease [30, 31]. Guinea pigs control their airways autonomously and are capable of strong anti-allergen responses. It is known that sensitized guinea pigs develop airway inflammation and AHR upon a single exposure to an aerosol allergen, whereas remodeling of the airways occurs after repeated inhalations of allergens [23].

Endotracheal administration of Sephadex G-200 to rats caused a marked influx of eosinophils, neutrophils, macrophages, and lymphocytes into the bronchoalveolar space. Intragastric administration of XC8 in a broad dose range (0.18-18 mg/kg) to rats resulted in a 2.6-6.4 times decrease of eosinophils in BAL and was superior to the aerosol treatment of the comparator steroid drug budesonide. It is noteworthy that, at the highest dose (18 mg/kg), XC8 also significantly reduced the influx of neutrophils when compared with the diluent-treated animals. Neutrophils play a major role in severe neutrophilic asthma comprising approx. 5% of patients with asthma but turns to be an enormous burden on social health system. The underlying molecular and immunological mechanisms of neutrophilia in the cases of severe asthma are not clear and this variant of asthma is poorly controlled with the currently available drugs, including GCS [32, 33]. For this reason, further investigation of the effect of XC8 on severe neutrophilic asthma is very important.

The promising data obtained from the Sephadex-induced lung inflammation model in rats stimulated our efforts to investigate the anti-inflammatory effect of XC8 in the guinea pig model that mimics the acute and chronic onset of allergic asthma. We showed that i.g. treatment of animals with XC8 at a broad daily dose ranging from 0.045 to 1.4 mg/kg for 10 consecutive days reduced the eosinophil count in BAL in the case of acute asthma to the same extent as the comparator drug budesonide. Moreover, the analysis of stained histological tissue sections showed that i.g. treatment with XC8 at doses 0.14 and 1.4 mg/kg in the case of acute asthma reduced the goblet cell, the degranulated mast cell and eosinophil (1.4 mg/kg) counts in a dose-dependent manner up to the level shown in naive animals. The comparator drug budesonide did not reduce significantly the number of eosinophils, goblet cells, or degranulated mast cells in this model. However, in the model of chronic asthma the effect of XC8 at both studied doses was comparable to that of budesonide in reducing the eosinophil and degranulating mast cell counts in the lung tissues. This finding supports the anti-asthmatic effect of XC8 since mast cells are known to be effector cells during the inflammatory process and their degranulation correlates with the severity of allergic asthma [34, 35].

A similar dose-dependent effect was determined for specific airway resistance. The treatment of animals with XC8 at a dose of 1.4 mg/kg in the case of acute asthma and at 1.4 and 7.0 mg/kg doses in the case of chronic asthma reduced AHR up to the level revealed in naïve animals. The effect of XC8 was significantly more pronounced in contrast to that shown for nebulized comparator drug budesonide.

GCSs are currently the best drugs for treating but not curing allergic asthma and they are used as a treatment control when testing novel compounds [36]. However, in 10% of asthma cases, especially the cases of severe asthma this disease is resistant to corticosteroids [37]. Moreover, corticosteroids have limitations because of their potential side effects [8]. One of the serious complications of GCS is the alteration of the bone metabolism leading to osteoporosis [38]. Therefore, new and more effective drugs with the advantage of oral administration replacing or reducing the use of systemic and inhaled GCS are highly desired.

The dose range of 1.4-7.0 mg/kg which showed the prominent effect of XC8 in guinea pigs, corresponds to that of 20-100 mg a day for humans. In 6-month repeated dose oral toxicity study on dogs we showed that XC8 has a low toxicity (NOAEL is 25 mg/kg that corresponds to the 1000 mg a day for humans, data not shown), which might be important for the oral treatment of human allergic asthma.

The proposed mechanism of action of XC8 is the inhibition of glutaminyl cyclase. We previously demonstrated that XC8 inhibits human Golgi-resident glutaminyl cyclase *in vitro* (data not shown). Glutaminyl cyclase is known to catalyze the cyclization of N-terminal glutamyl residue [39] and therefore is involved in pyroglutamination reaction of MCP chemokines (MCP-1, MCP-2, MCP-3, MCP-4) as well as other substrates. As a result of this reaction, immature chemokines are converted into the mature form, which binds to receptors. Chemokines play a significant role in the pathogenesis of bronchial asthma. They are known to induce the migration of eosinophils and other cells to the site of inflammation and to trigger degranulation of mast cells and basophils that can lead to an increase of the bronchial hyper-reactivity [40]. The blockade of the chemokine receptor CCR2 by antibodies was shown to reduce the accumulation of macrophages and eosinophils in the BAL fluid and to decrease bronchial hyperreactivity in a model of allergic asthma [41, 42]. Therefore, inhibition of the glutaminyl cyclase and, as a consequence, reduction of chemokines activity can have anti-asthmatic effect. Unlike the effect of a recently approved anti-interleukin-5 receptor-specific monoclonal antibodies Benralizumab, which are strongly connected to the depletion of blood eosinophils [43], the effect of XC8 could be mostly based on the inhibition of activation of eosinophils, without depleting eosinophils plasma pool. This hypothesis is supported by the data discussed by Henriques and Penino, stating that MCP-1 activates $\gamma\delta$ T lymphocytes that secrete eosinophilic chemoattractant proteins [44]. Therefore, by inhibiting the activation of MCP chemokines XC8 could ultimately decrease the amount of eosinophilic chemoattractants, which in turn would reduce infiltration of eosinophils into the lungs while preserving its plasma level. We confirmed this hypothesis in rat and guinea pig models designed for the assessment of the migration of eosinophils and degranulation of mast cells.

CONCLUSION

We showed that daily i.g. treatment with XC8 and budesonide for ten consecutive days reduced eosinophilia and improved all of the disease parameters - both drugs in-

hibited allergic inflammation in the airways and parenchyma, and decreased mucus secretion. XC8 (but not the budesonide) improved the airway resistance in the cases of both acute and chronic asthma.

These results serve an impetus for further development of XC8 as an oral drug for the treatment of human allergic asthma. At the moment the phase I study to Assess Pharmacokinetics, Safety, and Tolerability of XC8 on humans (NCT02882217) is completed in Europe, and the phase II study Treatment of Patients with Bronchial Asthma (NCT03450434) has finished recently in Russia. The study of XC8 and in particular its effect on the presence of the cytokines specific for the Th1/Th2 response as well as on MCP/eotaxin subfamily of chemokines will continue.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were approved by the Institutional Animal Ethics Committee.

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. The experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals [22]. All painful procedures were performed under anesthesia for minimizing the animals' suffering. The animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

All experiments were financially covered by PHARMENTERPRISES LLC. Dr. B. Ferko, Dr. J. Romanova and H. Schmutz are employees of EURRUS Biotech GmbH. Dr. A. Renner is the employee of Karl Landsteiner Institute for Clinical and Experimental Pneumology. The authors declare no conflict of interest.

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Dr. V. Nebolsin is the founder and the Head of the company PHARMENTERPRISES LLC, which develops new medicinal preparations. Dr. V. Nebolsin is the author of the molecule XC8, the author of the idea and the initiator and head of the project. Dr. T. Kromova was responsible for the synthesis and quality control of the XC8 compound. Dr. A. Rydlovskaya was responsible for the development of the XC8 concept, design, planning and analyzes of all animal experiments. Dr. O. Proskurina was responsible for the performance of all animal experiments. Dr. A. Amelina was responsible for the performance of all histological images, their analysis and interpretation as well as analysis of BAL. EURRUS Biotech GmbH has the license for the clinical development of XC8 and holds the exclusive international marketing license. Dr. B. Ferko, Dr. J. Romanova, and Mag. H. Schmutz analyzed all preclinical data, selected data for publication and wrote the manuscript. Dr. A. Renner provided his

expertise in asthma research, contributed to the design of some selected experiments and edited the manuscript.

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