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ORIGINAL RESEARCH

The Effect of Anti-Chemokine Oral Drug XC8 on Cough Triggered by The Agonists of TRPA1 But Not TRPV1 Channels in Guinea Pigs

Julia Romanova · Anastasia Rydlovskaya · Stepan Mochalov · Oxana Proskurina · Yulia Gorokh · Vladimir Nebolsin

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ABSTRACT

Introduction: Chronic cough heavily affects patients' quality of life, and there are no effective licensed therapies available. Cough is a complication of severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) infection, asthma, and other diseases. Patients with various diseases have a different profile of tussive responses to diverse cough triggers, thereby suggesting sundry mechanisms of neuronal dysfunctions. Previously, we demonstrated that the small molecule drug XC8 shows a clinical anti-asthmatic effect. The objective of the present study was to investigate the effect of XC8 on cough.

Methods: We studied the antitussive effect of XC8 on cough induced by agonists activating human transient receptor potential (TRP) cation channels TRPA1 or TRPV1 in guinea pigs. We checked the agonistic/antagonistic activity of XC8 on the human cation channels TRPA1, TRPV1, TRPM8, P2X purinoceptor 2 (P2X2), and human acid sensing ion channel 3 (hASIC3) in Fluorescent Imaging Plate Reader (FLIPR) assay.

Results: XC8 demonstrated clear antitussive activity and dose-dependently inhibited cough in guinea pigs induced by citric acid alone (up to 67.1%) or in combination with IFN-γ (up to 76.4%). XC8 suppressed cough reflexes induced by the repeated inhalation of citric acid (up to 80%) or by cinnamaldehyde (up to 60%). No activity of XC8 against cough evoked by capsaicin was revealed. No direct agonistic/antagonistic activity of XC8 on human TRPA1, TRPV1, TRPM8, P2X2, or hASIC3 was detected.

Conclusions: XC8 acts against cough evoked by the activation of TRPA1 (citric acid/cinnamaldehyde) but not TRPV1 (capsaicin) channels. XC8 inhibits the cough reflex and suppresses the cough potentiation by IFN-γ. XC8 might be of significant therapeutic value for patients suffering from chronic cough associated with inflammation.

Keywords: Cough; TRPA1; TRPV1; IFN-γ; XC8; Citric acid; Capsaicin; Cinnamaldehyde

J. Romanova (✉) · A. Rydlovskaya · S. Mochalov · O. Proskurina · Y. Gorokh · V. Nebolsin
Pharmenterprises LLC, 42 Bolshoj Blvd., Building 1, office 771, 772, Skolkovo Innovation Centre, Moscow 121205, Russian Federation
e-mail: jr.romanova@gmail.com

Key Summary Points

XC8 acts against cough evoked by the activation of TRPA1 (citric acid/cinnamaldehyde) but not TRPV1 (capsaicin) channels.

XC8 does not have direct agonistic/antagonistic activity on human TRPA1, TRPV1, TRPM8, P2X2, or hASIC3 channels.

INTRODUCTION

Chronic cough (i.e., cough lasting > 8 weeks) is a common problem affecting approximately 2–18% of the general population [1, 2]. Patients with chronic cough suffer continuously, as they may experience 10–100 coughs per hour over weeks. This condition greatly affects the quality of life of patients and interferes with their daily activities, e.g., sleeping, working, and social life. In about half of cases, chronic cough is caused by other diseases such as viral infections, asthma, cystic fibrosis, bronchiectasis, and/or gastroesophageal reflux [3]. The underlying disease causes pathological processes affecting the neural pathways leading to the increased sensitivity/responsivity to coughing named cough hypersensitivity/hyperresponsivity syndrome (CHS) [4]. While the specific causes of chronic cough may vary, the underlying pathophysiologic mechanisms are quite similar. However, there are no effective licensed therapies available.

Human airways are innervated with vagal afferent Aδ- and C-fibers responsible for airway reflexes [5]. Autonomic and sensory nerve fibers in the airways express receptors for a variety of inflammatory mediators, including protons, neurotrophic factors, adenosine triphosphate (ATP), proinflammatory cytokines like interferons (IFNs), and tumor necrosis factor α (TNF-α), which can recruit nerve fibers in inflammation [6–9]. Chronic cough is often characterized by airway inflammation, which in turn induces airway remodeling and alters the plasticity of afferent vagus nerve C-fibers [10, 11]. In humans, cough can be evoked by the mechanical probing of tracheal mucosa, or by chemical stimuli, including hyper- and hypotonic solutions, acids, capsaicin, cinnamaldehyde, cigarette smoke, or others [12–16]. C-fibers respond to chemical stimuli by the activation of transient receptor potential (TRP) calcium permeable channels which act as sensors. In mammals, 28 TRP channels, belonging to six families are described. The most studied include TRP ankyrin (TRPA), TRP melastatin (TRPM), and TRP vanilloid (TRPV) channels. TRPV1 and TRPA1 channels are expressed at the nerve terminals and known to contribute to the pathogenesis of chronic cough [17]. Once activated, they start to release neuropeptides, such as calcitonin gene-related peptide (CGPR), substance P (SP), and neurokinin A (NKA), thereby contributing to local neurogenic inflammation characterized by cough, bronchoconstriction, vasodilation, and infiltration of immune cells [18]. The TRPV1 channel responds to high temperature, low pH, and capsaicin [19]. Capsaicin (an active component of chili pepper) is often used as a tussigenic stimulus to study the cough mechanisms [20, 21]. TRPA1 channel responds to cold temperature and a variety of irritants like acids, cigarette smoke, acrolein, and cinnamaldehyde [22]. Patients with various diseases were shown to have a divergent profile of tussive responses to stimuli such as cigarette smoke or capsaicin, thereby suggesting different mechanisms of evoked cough [23]. The response to capsaicin is known to differentiate a variety of neuronal dysfunctions [24].

Viral infections of the upper respiratory tract (URVIs) are one of the causes of chronic cough. URVIs induce inflammation accompanied by the release of such cytokines as interlukin-1 (IL-1), IL-6, IL-8, IL-11, GRO-α, TNF-α, RANTES, GM-CSF, and CCL11 (eotaxin-1) [25, 26]. An inflammatory response involving the epithelial and smooth muscle cells as well as infiltrating immune cells is associated with cough [27, 28]. Prolonged cough is one of the symptoms that many survivors of severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) infection suffer from [29].

Asthma is another condition that causes cough. This cough can be resistant to treatment with classical anti-asthmatic drugs such as inhaled corticosteroids (ICS) or long-acting beta-agonists (LABA), but sensitive to leukotriene receptor antagonist (LTRA) or anticholinergics as tiotropium [30, 31]. Biopsies from patients with chronic non-asthmatic and asthmatic cough demonstrated that many signs of airway remodeling, such as the thickening of membrane, increase in vascularity, and hyperplasia of goblet cells, were common in both groups of patients, indicating a common pathophysiological process [10]. The elevated levels of prostaglandins or histamine in the sputum of patients with cough in combination with the infiltration of mast cells indicate the neuronal activation in the airways that could stimulate cough receptors [32].

The small molecule drug XC8 (glutarimide derivative) developed by Pharmenterprises LLC was shown to affect the influx of eosinophils into bronchoalveolar lavage (BAL) in a model of lung inflammation or asthma in rats and guinea pigs [33]. We demonstrated the complete safety of XC8 in healthy probands in phase 1 clinical trial [34]. In phase 2a trial, we have shown that the treatment of asthma patients with a high levels of blood eosinophils and serum IFN-γ with XC8 led to an improvement in force expiratory volume in 1 s (FEV_1) [35]. These results imply that XC8 can have the potential to affect cough. The objective of the present study was to investigate the effect of XC8 on cough induced by different stimuli in guinea pigs.

METHODS

Animals

The experiments were performed on 16-week-old male Agouti guinea pigs (250–260 g), which were purchased from the Scientific Center of Biomedical Technologies (Andreevka, Moscow region). The animals were kept in cages with a controlled environment with standard pelleted food and water ad libitum. All of the animal experiments were approved by the Animal Ethics Committee of LLC Pharmenterprises (protocols No. BEC 07/2020 dated 19.05.2020, and 09/2020 dated 14.07.2020). The experimental procedures with animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals [36]. All the procedures with animals were performed under anesthesia to minimize their suffering.

Cough Induced by Citric Acid

To study the effect of XC8 on cough induced by the inhalation of citric acid, guinea pigs were treated with XC8 intragastrically (p.o.) at doses of 0.7, 1.4, 2.8, 4.2, 5.6, 7.0, and 14.0 mg/kg diluted in distilled water with 1% Tween-80. In 7 h, all of the animals inhaled the citric acid solution (0.3 M in saline) for 8 min using an Omron compAIR NE-C28 compression nebulizer. The group of control animals received the vehicle (distilled water with 1% Tween-80) instead of XC8. Antitussive activity of XC8 was assessed manually by counting the number of coughs by trained personnel within 8 min after the start of citric acid inhalation and expressed as percentage of inhibition in comparison to control group of animals.

In order to assess the effect of IFN-γ on cough, animals inhaled IFN-γ at a dose of 10 μg/kg using an Omron compAIR NE-C28 compression nebulizer for 3 min. Then, after 7 h, all of the animals inhaled the citric acid solution (0.3 M in saline) for 8 min using an Omron compAIR NE-C28 compression nebulizer. XC8 was administered p.o., immediately after the inhalation of IFN-γ at doses of 0.7, 1.4, 2.8, 4.2, 5.6, 7.0, and 14.0 mg/kg. The group of control animals received the vehicle. Antitussive activity was assessed by counting the number of coughs within 8 min after the start of citric acid inhalation and expressed as percentage of inhibition in comparison to control group.

Chronic Cough Induced by Citric Acid

Guinea pigs inhaled a citric acid solution (0.4 M in saline) for 10 min daily from the 0th to 6th days. XC8 was given p.o. daily, 1 h before the inhalation of citric acid. Control animals received distilled water with 1% Tween-80

instead of XC8. Butamirate (Codelac Neo®, OTCPharm, Russia)—powerful centrally acting cough suppressant was used as a reference drug. Butamirate at a dose of 5 mg/kg was administered p.o. in the same regimen. Antitussive activity was evaluated by counting the number of coughs within 10 min after each inhalation of citric acid and expressed as percentage of inhibition in comparison to control group of animals. The results of the most representative experiment with XC8 (at doses of 1.4, 7.0, and 14 mg/kg) are presented.

Cough Induced by Cinnamaldehyde

Guinea pigs were treated p.o. with XC8 (at doses of 0.14, 1.4, or 14 mg/kg) 1, 3, 6, 8, and 12 h before the inhalation of cinnamaldehyde solution. Then, animals inhaled a solution of cinnamaldehyde (50 mM in saline with 3.5% Tween-80) for 10 min using an Omron compAIR NE-C28 compression nebulizer. The comparator drug butamirate was given p.o. at a dose of 5 mg/kg according to the same regimen. The evaluation of antitussive activity was carried out by counting the number of coughs within 20 min and expressed as percentage of inhibition in comparison to control group of animals, which received the vehicle instead of XC8.

Cough Induced by Capsaicin

Cough challenge with capsaicin (30 μM) was performed by inhalation for 5 min using an Omron compAIR NE-C28 compression nebulizer with subsequent measurements of coughs within 15 min. The treatment of guinea pigs with XC8 (at doses 0.14, 1.4, and 14 mg/kg) was done before the challenge at 0.5, 1, 3, 6, 12, or 24 h. Control animals received distilled water with 1% Tween-80 instead of XC8. Butamirate was used as a comparator drug at a dose of 5 mg/kg according to the same regimen. The evaluation of antitussive activity was carried out manually by counting the number of coughs within 15 min from the start of inhalation and expressed as percentage of inhibition in comparison to control group of animals.

Ion Channel Activity

P2X2 Receptor

The interaction of XC8 with the P2X2 ion channel was studied using the Fluorescent Imaging Plate Reader (FLIPR) assay by SB Drug Discovery (UK). Human P2X2 cells were trypsinized, counted, and seeded in black, clear-bottomed 96 well plates at a density of 50,000 cells per well in 100 μl volume and incubated overnight. Next day, media (DMEM containing 10% FBS, and 2 mM L-glutamine) was removed from cell plates and assay buffer (1.26 mM $CaCl_2$, 0.49 mM $MgCl_2$ $0.6H_2O$, 0.41 mM $MgSO_4$ 0.7H2O, 5.63 mM KCl, 0.44 mM KH_2 PO_4, 138 mM NaCl, 0.34 mM Na_2 HPO_4, 5.5 mM D-glucose, 20 mM HEPES) was added. Calcium 5 dye (Molecular Devices, USA, R8185) solution was then added to the wells and incubated at room temperature for 50 min. Calcium 5 Dye solution was prepared in assay buffer according to the manufacturer's instructions. For agonist testing: the plates were placed in the FLIPR, after incubation with dye, and fluorescence monitored every 1 s. After 20 s, test compound XC8 or reference agonist was added to the wells and the fluorescence monitored for 5 min at ex/emm: 488 nm/510–570 nm. For antagonist testing: the test compound XC8 and reference inhibitors were added to the wells and incubated at room temperature for 10 min. The plates were then placed in the FLIPR and fluorescence monitored every 1 s. After 20 s, the reference agonist, BzATP, was added and the fluorescence monitored for 5 min at ex/emm: 488 nm/510–570 nm.

hTRPA1 and hASIC3 Receptors

The interaction of XC8 with hTRPA1 and hASIC3 ion channels was studied using the $FLIPR^{TETRA}$ and Ion Flux platform, respectively, by Eurofins (France).

For the hTPRA1 FLIPR assay, the compound XC8 was prepared in assay buffer to the final concentration 50 μM. The compound wells, reference agonist allyl isothiocyanate (AITC), reference antagonist Ruthenium Red, and background vehicle controls were prepared in DMSO (0.3%). All wells were prepared using

FLIPR assay buffer. The reference agonist for each ion channel assay was prepared in a similar manner to serve as assay control. The reference agonist for each ion channel was included at E_{max} (the concentration where the reference agonist elicited a maximal response). The agonist assay was conducted on a FLIPR^{TETRA} instrument where the test compound XC8, vehicle controls, and reference agonist were added to the assay plate after a fluorescence baseline was established. The agonist assay was a total of 180 s and was used to assess the compound's ability to activate each ion channel assayed.

For the antagonist assay, compound XC8 was added on the FLIPR^{TETRA} and pre-incubated for 5 min at room temperature. Using historic EC_{80} potency values, all the pre-incubated sample compound wells were challenged with an EC_{80} concentration of reference agonist after the establishment of a fluorescence baseline. The antagonist assay was conducted on a FLIPR^{TETRA} instrument where vehicle controls and an EC_{80} concentration of reference agonist were added to the appropriate wells. The antagonist assay (total of 180 s) was used to assess the compound's ability to inhibit each ion channel assayed.

For the hASIC3 IonFlux HT Agonist Assay, all recordings were obtained from a holding potential of − 60 mV. Cells were exposed to a pH 7.4 external assay solution to establish a baseline response. One addition of the pH 5.5 external assay solution was added for 2 s to establish an ASIC3 peak current response and test concentration of compound XC8 in pH 7.4 was applied for 2 s to detect the agonist response. Results showing an inhibition or stimulation higher than 50% are considered to represent significant effects of the test compounds.

TRPM8 and TRPV1 Channels

The activity of XC8 was studied in cellular and nuclear receptor functional assays in a concentration of 50 μM according to Phelps et al. and Behrendt et al. [37, 38]. The study was performed by Eurofins. The cellular agonist effect was calculated as a percentage of the control response to a known reference agonist for each target, and the cellular antagonist effect was calculated as a percentage of the inhibition of control reference agonist response for each target. In each experiment, if applicable, the respective reference compound was tested concurrently with the test compounds, and the data were compared with historical values determined at Eurofins. The experiment was made in accordance with validation Standard Operating Procedure of Eurofins.

Statistics

Comparison of the data obtained from several experimental groups with one control group was performed by a one-way ANOVA or two--way ANOVA with Dunnett's multiple comparison test. Data are presented as mean (M) ± standard deviation (SD). The software GrafPadPrism version 8.0 was used for calculations.

RESULTS

The Effect of XC8 on Cough Induced by a Single Inhalation of Citric Acid with or Without IFN-γ

In humans, cough can be initiated by different irritants, such as cigarette smoke, acids, hyper- and hypotonic solutions, capsaicin, or prostanoids [13–15]. Patients exhibit a divergent response to various tussive stimuli suggesting the existence of several neurophenotypes of airway diseases evoked by distinct mechanisms [23]. We investigated the effect of XC8 on cough induced by several agonists activating various receptors in male guinea pigs. First, we estimated the effect of XC8 on cough induced by the inhalation of citric acid. Acid is known to activate the TRPV1 and TRPA1 channels, but the activation of the airway sensory nerve depends on the magnitude and rate of pH change [14]. Before we found that the most pronounced anti-asthmatic effect of XC8 on guinea pigs was observed in the dose range of 1.4–7.0 mg/ kg, which corresponds to 20–100 mg per day for humans [33]. In phase 2a

clinical trial, the highest anti-asthmatic effect of XC8 was observed for a dose of 100 mg per day [35]. Therefore, we chose the dose range of XC8 from 0.7 to 14.0 mg/kg for oral administration to animals. Control animals received the vehicle. In 7 h of XC8 dosing, all of the animals were inhaled with a 0.3 M citric acid and antitussive activity was assessed by counting the number of coughs (see experiment scheme, Fig. 1a). XC8 showed the pronounced dose-dependent effect on cough with the maximal inhibition of 67.1% at a dose of 5.6 mg/kg (Fig. 1b, d).

The IFN-γ was shown to enhance the cough response to citric acid in guinea pigs by the depolarization of membranes [6]. We were interested whether XC8 can inhibit the cough induced by citric acid and enhanced by IFN-γ. IFN-γ was administered to the animals just before the administration of XC8. As shown in Fig. 1b, the addition of IFN-γ to the citric acid increased the cough frequency by 42% (from 22.1 to 31.6 coughs/8 min). The maximal inhibition effect of XC8 on cough evoked by citric acid in combination with IFN-γ comprised 76.4% observed for the dose of XC8 4.2 mg/kg. The effect of XC8 on cough induced by citric acid combined with IFN-γ was more pronounced than on cough induced by citric acid alone, indicating that IFN-γ cough potentiation was successfully resolved by XC8.

The Effect of XC8 on Chronic Cough Induced by Multiple Inhalations of Citric Acid

Chronic cough in guinea pigs can be modeled by repeated inhalation of citric acid [39]. We aimed to assess the effect of tachyphylaxis by the tested drug XC8. To do this, the animals received XC8 (at doses of 1.4, 7.0, and 14 mg/kg p.o.), 1 h before the inhalation of the citric acid solution within 6 days every day (Fig. 2). The control animals received the vehicle instead of XC8. Butamirate was used as a comparator drug.

The results showed that the administration of XC8 at doses of 7.0 and 14 mg/kg resulted in a statistically significant reduction of coughs induced by the repeated inhalation of citric acid from 1 to 5 consecutive days. The observed cough suppression ranged from 22 to 80%. The effect of XC8 observed after treatment at doses of 7.0 and 14 mg/kg was comparable to the reference antitussive drug butamirate. At lower dose (1.4, mg/kg), the effect of XC8 was less pronounced.

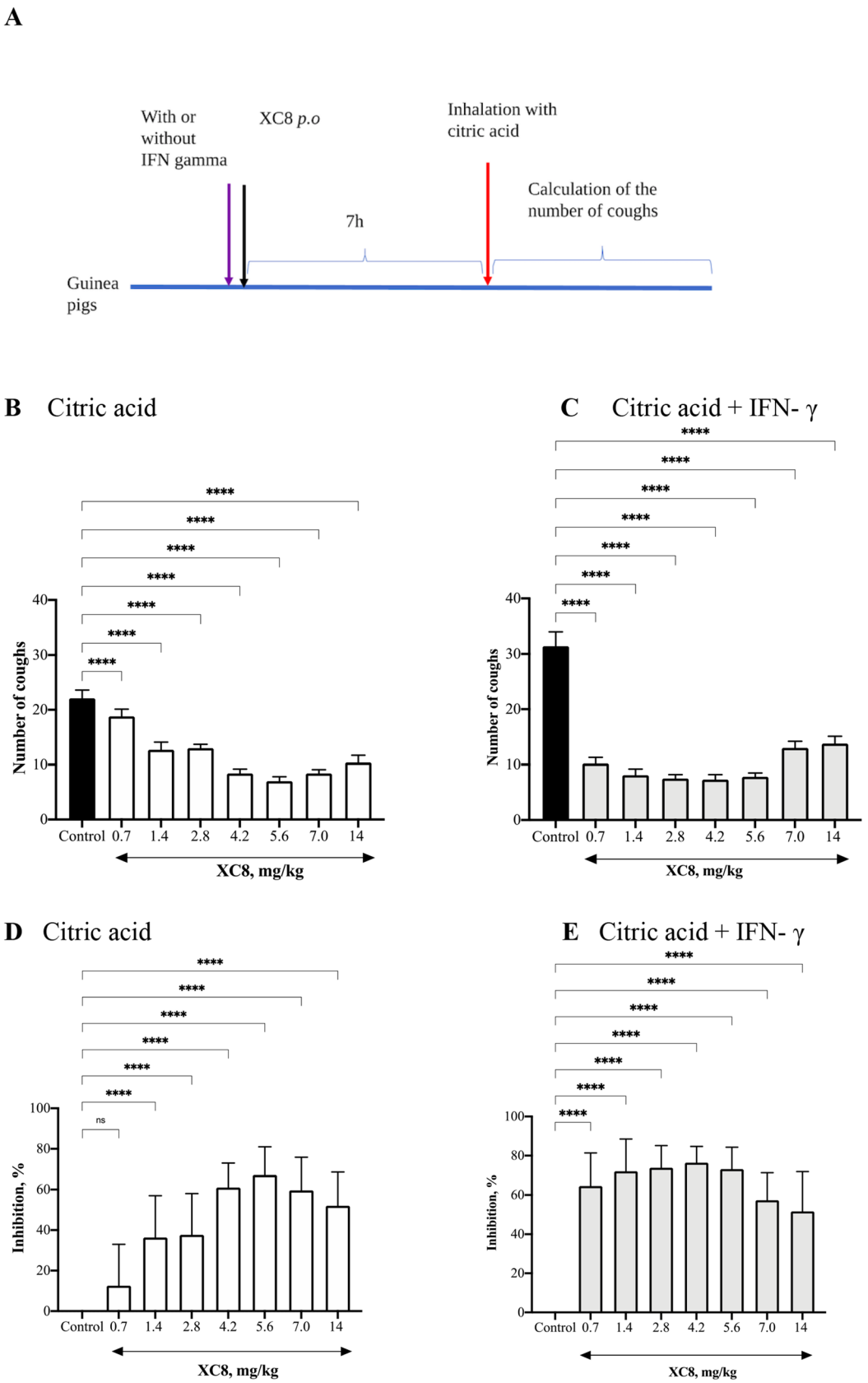
The Effect of XC8 on Cough Induced by the Inhalation of Cinnamaldehyde

Next, we assessed the effect of XC8 on cough induced by cinnamaldehyde, activator of TRPA1 channel. The TRPA1 channel is co-expressed with TRPV1 and is present on airway-innervating C-fibers [40]. TRPA1 is a chemosensitive receptor activated by cinnamaldehyde [41], nicotine [42], ozone, menthol [43], formalin, tear gases, and mustard oil. In addition, TRPA1 serves as a sensor for the cold temperature and early detection of bacterial lipopolysaccharide (LPS) [44, 45].

Animals were treated with XC8 (0.14, 1.4, or 14 mg/kg, p.o.) 1, 3, 6, 8, 12 h before the inhalation of 50 mM cinnamaldehyde solution. The group of control animals received the vehicle instead of XC8 (Fig. 3a). The results of the study show that XC8 significantly reduced the coughs in guinea pigs caused by the inhalation of cinnamaldehyde (Fig. 3b). The most pronounced effect of the XC8 was observed when it was administered 3 and 6 h before the inhalation at doses of 1.4 and 14 mg/kg, achieving up to 60% inhibition of the coughs. The comparator drug butamirate showed a similar effect on cough inhibition (up to 60%) with a maximal difference to control observed when administered 6 h before the inhalation of cinnamaldehyde.

The Effect of XC8 on Cough Induced by the Inhalation of Capsaicin

Next, we investigated the effect of XC8 on cough induced by capsaicin. Capsaicin is known to activate the TRPV1 channel, which also can be activated by heat and low pH. Activation of TRPV1 induces calcium influx and membrane depolarization, which in turn leads to neurogenic inflammation [46]. Cough


A
With or without IFN gamma
XC8 p.o
Inhalation with citric acid
Calculation of the number of coughs
7h
Guinea pigs
B Citric acid
C Citric acid + IFN- γ
Number of coughs
Control 0.7 1.4 2.8 4.2 5.6 7.0 14
XC8, mg/kg
D Citric acid
E Citric acid + IFN- γ
Inhibition, %
ns

◀**Fig. 1** The effect of XC8 treatment on cough induced in guinea pigs by a single inhalation of citric acid alone or in combination with IFN-γ. **a** Experimental scheme. Animals (ten per group) were treated with XC8 at doses from 0.7 to 14 mg/kg p.o., or with vehicle (control group) 7 h before the inhalation of citric acid (0.3 M in PBS) alone (**b**), or in combination with IFN-γ (10 mg/kg) (**c**). The number of coughs was recorded from the start of inhalation and presented in absolute numbers (**b**, **c**) or as a percentage of inhibition (**d**, **e**). The data are presented as M ± SD. The *stars* (****) indicate the significant difference when compared to the control group determined by a one-way ANOVA followed by Dunnett's multiple comparison test, $p < 0.0001$; *ns* not significant

challenge was performed with capsaicin. Animals were treated with XC8 (0, 14, 1.4, and 14 mg/kg p.o.) before the challenge at 0.5, 1, 3, 6, 12, or 24 h. Group of control animals received the vehicle. Butamirate was used as a comparator drug (Fig. 4a). XC8 demonstrated very limited activity against cough evoked by capsaicin (Fig. 4b). Statistically significant reduction of coughs was shown only on late time point 12 h at 14 mg/kg of XC8. No statistically significant effect was shown for butamirate.

Effect of XC8 on the P2X2, hTRPA1, hASIC3, TRPV1, and TRPM8 Channels In Vitro

To determine the mechanism of action of XC8 in cough model in guinea pigs we evaluated the activity of XC8 on P2X2, hTRPA1, hASIC3, TRPV1, and TRPM8 ion channels in Fluorescent Imaging Plate Reader (FLIPR) assay by SB Drug Discovery (UK) and by Eurofins (France). No agonistic/antagonistic activity of XC8 on any of these receptors (at a concentration of 50 μM) was revealed.

DISCUSSION

XC8 has demonstrated a pronounced therapeutic effect on cough induced by the inhalation of citric acid or cinnamaldehyde, but not by capsaicin. It is believed that citric acid evokes a multimodal challenge affecting both TRPA1 and TRPV1 channels, expressed in the airway nerve endings of vagal C-fibers. However, Wang et al. have shown that weak organic acids activate the TRPA1 channel [47] because they diffuse into the cell causing intracellular acidification and changing the pH of the cytosol. In contrast, the TRPV1 channel reacts to extracellular protons from strong acids. Mukhopadhyay et al. demonstrated that the TRPA1 channel is a direct sensor for citric acid in three types of cells [48]. This observation was confirmed in experiments with the selective antagonist of the TRPV1 channel GRC 6211, which did not inhibit an increased Ca^{2+} influx induced by citric acid [49]. The effect of XC8 on cough caused by the single inhalations of citric acid was similar to that induced by the repeated inhalation of citric acid during 7 days. Moreover, XC8 reduced the cough caused by citric acid in combination with IFN-γ, which is known to enhance the cough reflex sensitivity [6]. The effect was even more pronounced (76.4%) than that induced by citric acid alone (67.1%). Another agonist of TRPA1 is cinnamaldehyde, which was shown to activate it specifically [40] while the activation can be inhibited by the HC030031 antagonist of TRPA1 [50]. The effect of XC8 on cough induced by cinnamaldehyde was comparable to that induced by the licensed drug butamirate.

Capsaicin acts specifically on TRPV1 channels. The effect of XC8 on cough induced by the inhalation of capsaicin did not show statistically significant difference compared to treatment with the vehicle. Therefore, XC8 acts exclusively against cough evoked by the activation of TRPA1, but not TRPV1 channels.

The TRPA1 ion channel is highly expressed on the C fibers innervating the entire respiratory tract as well as on the non-neuronal cells of respiratory tract including fibroblasts, epithelial cells (tracheal, bronchial, and alveolar), smooth muscle cells (SMC), and lymphocytes [51–56]. In addition to citric acid and cinnamaldehyde potential agonists of TRPA1 channel include cigarette smoke, reactive oxygen species (ROS), and hypochlorite, which are all known as asthma triggers. The increase in some of these stimuli has been observed in the lungs of

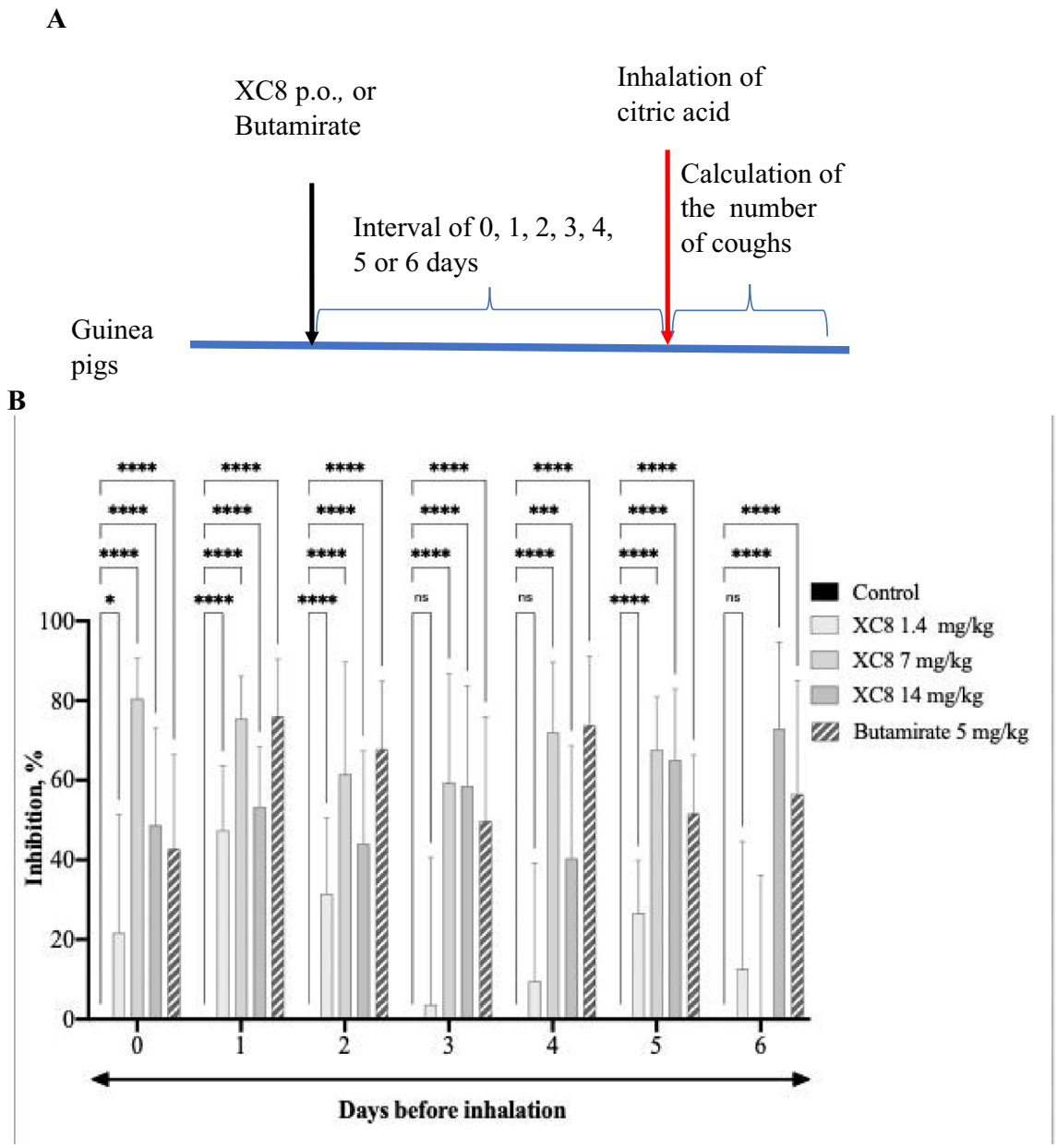


Fig. 2 The effect of XC8 on the cough induced by the repeated inhalation of citric acid to guinea pigs. **a** Experimental scheme. Groups of 15 animals were treated with XC8 at doses of 1.4, 7, or 14 mg/kg p.o. before the inhalation (0, 1, 2, 3, 4, 5, or 6 days) with citric acid (0.3 M in PBS). Control animals received the vehicle. Butamirate was administered in the same way at a dose of 5 mg/kg. **b** The number of coughs was measured from the start of inhalation and expressed as percentage of inhibition. Data are presented as M ± SD. The *stars* indicate the significant difference compared to the control group determined by two-way ANOVA followed by Dunnett's multiple comparison test, (****) indicates $p < 0.0001$, (*) indicates $p < 0.05$; *ns* not significant

asthma patients [57–60]. Neuronal ion channel TRPA1 plays a tremendous role in the induction of allergen-induced airway inflammation. The activation of the TRPA1 channel by different chemical irritants triggers the release of CGRP, SP, or NKA, known as mediators of neurogenic

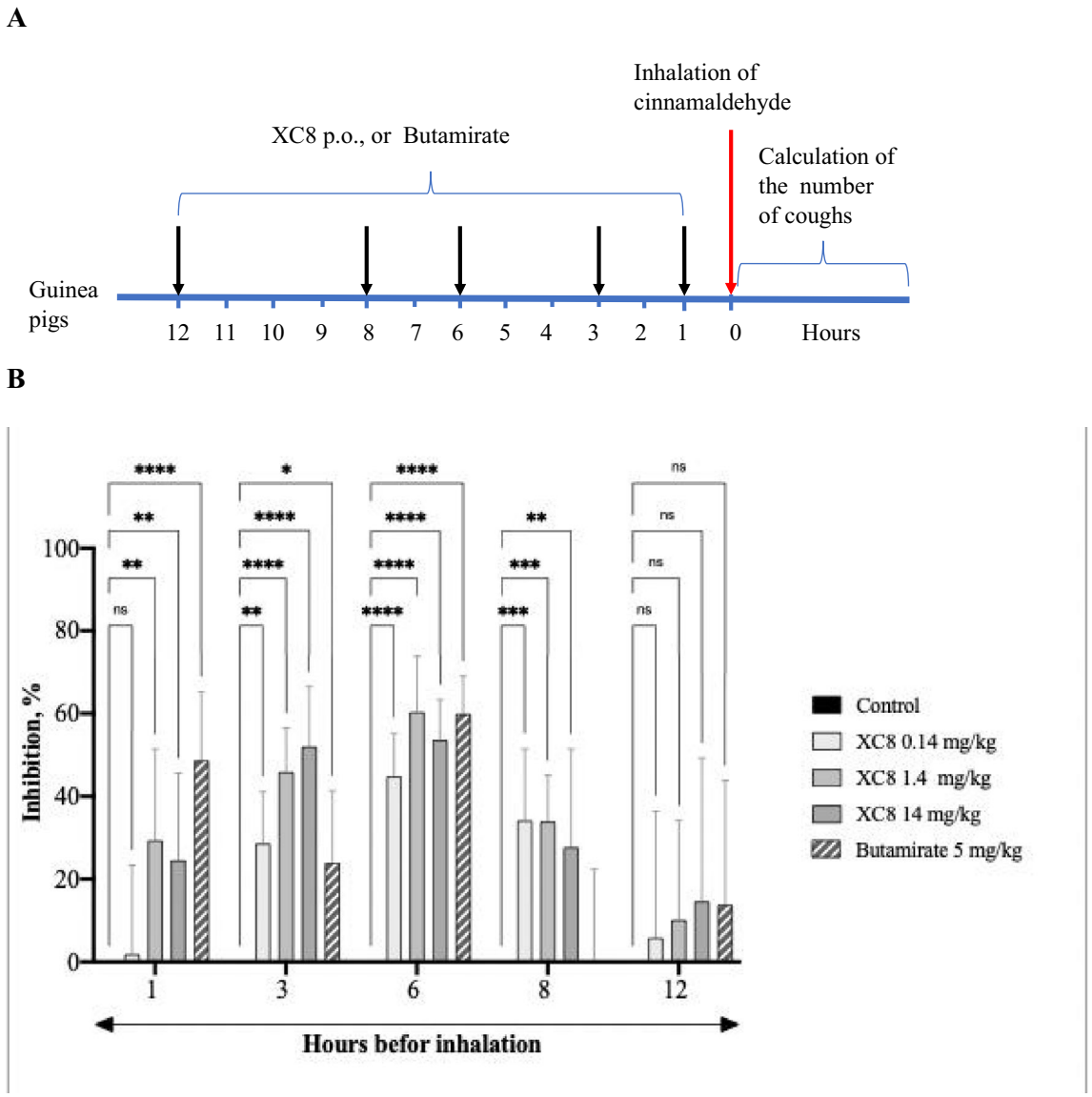


Fig. 3 The effect of XC8 on the of cough hypersensitivity induced by inhalation of cinnamaldehyde to guinea pigs. **a** Experimental scheme. Animals (ten per group) were treated with XC8 (0.14, 1.4 or 14 mg/kg p.o.) on 1, 3, 6, 8, or 12 h before inhalation of cinnamaldehyde (50 mM) for 10 min. Group of control animals received the vehicle. **b** The number of coughs was measured from the start of inhalation and expressed as percentage of inhibition. Data are presented as M ± SD. The *stars* indicate the significant difference in comparison to the control group (treated with vehicle) determined by two-way ANOVA followed by Dunnett's multiple comparison test, (****) indicates $p < 0.0001$, (***) indicates $p < 0.001$, (**) indicates $p < 0.01$, (*) indicates $p < 0.05$; *ns* not significant

inflammation, and induce an inflammatory response with mucus production, local release of cytokines IL-5, IL-13, TNF-α, CCL11, and finally airway hyperreactivity (AHR) [61]. The blockade of TRPA1 channel by specific antagonist was shown to reduce the released neuropeptides and subsequent infiltration of airway eosinophils, mucus production, and AHR [61]. These data indicate that the TRPA1 channel is involved in the interaction of

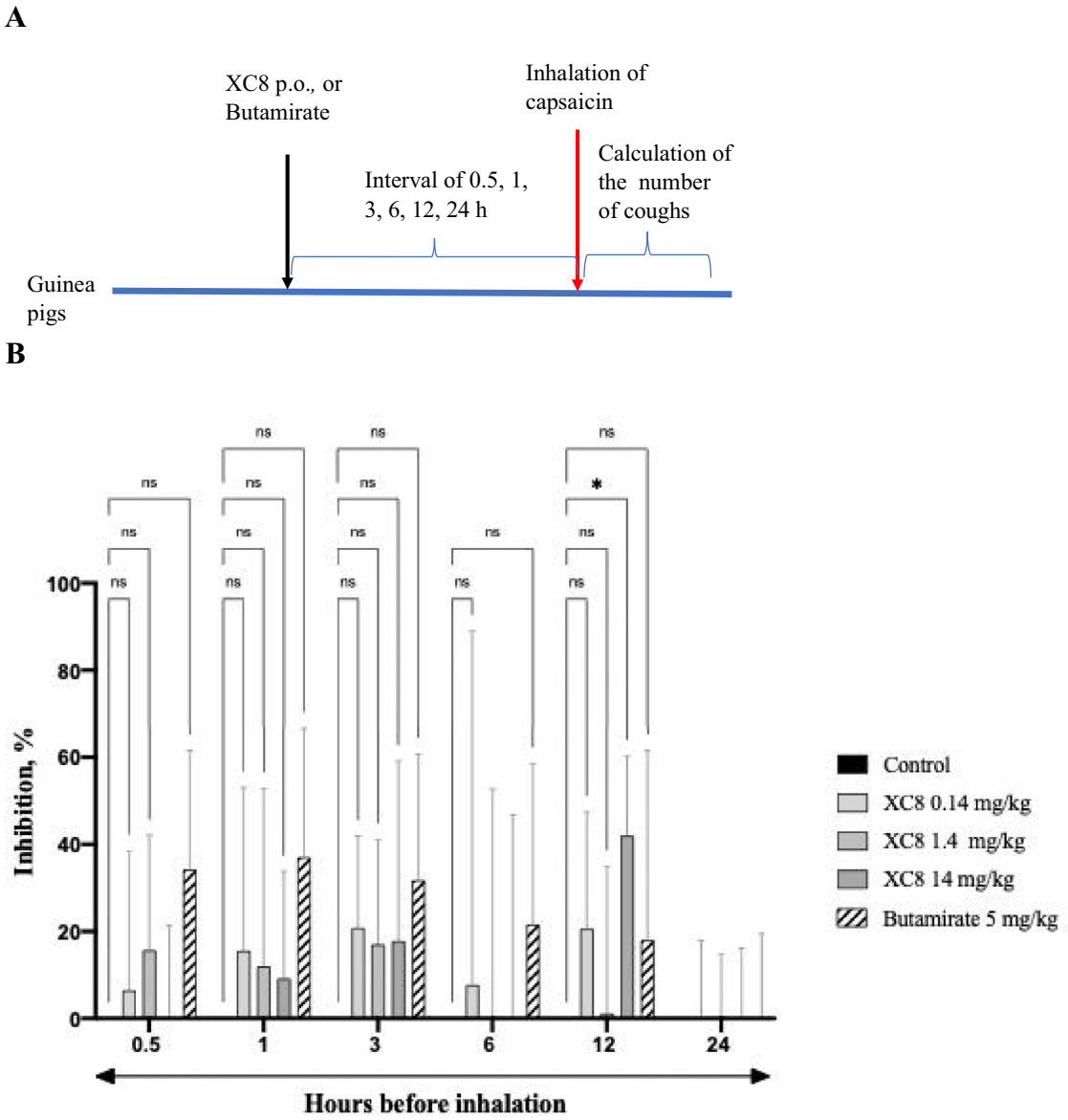


Fig. 4 The effect of XC8 on the cough induced by the inhalation of capsaicin in guinea pigs. **a** Experimental scheme. Animals (10 per group) were treated with XC8 at doses of 0.14, 1.4, or 14 mg/kg at 0.5, 1, 3, 6, 12, or 24 h before the inhalation of capsaicin (30 µM in PBS) within 5 min. Group of control animals received the vehicle. **b** The number of coughs measured within 15 min from the start of inhalation and expressed as percentage of inhibition. Data are presented as M ± SD. The *stars* indicate the significant difference in comparison to the control group (treated with vehicle) determined by two-way ANOVA followed by Dunnett's multiple comparison test, (*) indicates $p < 0.05$; *ns* not significant

nervous and immune systems in the airways, causing asthmatic inflammation of airways after an exposure to an inhaled allergen.

The TRPV1 ion channel, which is activated by capsaicin is not involved in allergic airway inflammation in ovalbumin-induced asthma [61], but plays a role in the induction of chronic

cough when stimulated by TRPV1-specific stimuli [62]. Non-asthmatic coughers were shown to have an increased expression of TRPV1 in epithelial nerves when compared to normal volunteers [20]. The inflammation induced by the activation of the TRPV1 channel was shown to be histamine-dependent, while the TRPA1 channel mediates a histamine-independent response [63]. The effect of XC8 on coughs induced by TRPA1 agonists suggests that pathological allergy resulting from neuro-immune interactions of TRPA1 with inflammatory factors may be a target for XC8 treatment.

Respiratory viruses such as human rhinovirus (HRV) and respiratory syncytial virus (RSV) infect human respiratory epithelial cells and cause about 75% of all asthma exacerbations in adults [64]. The infection of epithelial cells with viruses is known to induce the upregulation of mRNA of both TRPV1 and TPRA1 channels. The upregulation of the TRPA1 channels in neuronal cells was demonstrated for HRV-16 [65, 66]. The expression of TRPA1 in the membrane of peripheral nerves does not require the virus replication and can be induced by released IL-6 and IL-8 proinflammatory cytokines [65]. The neutralization of IL-6 and IL-8 blocks the upregulation of both TRRV1 and TPRA1 channels [67]. These data indicate that TRPA1 ion channel activation could be involved in asthma exacerbations induced by respiratory viruses. Cough is also the most common symptom (79%) in COVID-19 patients [68]. It was shown that 34% of recovering patients have persistent cough [69]. Therefore, XC8 can have a beneficial effect in treating persistent cough and asthma exacerbations caused by respiratory viruses including SARS-CoV-2.

The exact mechanism of action of XC8 in the treatment of asthma and cough is not clear. The cough reflex is a complex biological process that involves multiple steps, including airway receptors, vagal nerve, CNS, muscles, and antitussive agents may interfere at any step of this process [70]. We've evaluated the activity of XC8 across most commonly affected cough targets and did not find any agonistic or antagonistic activity of XC8 on TRPV1, TRPA1, hASIC3, TRPM8, or P2X2 ion channels. This result suggests that the effect of XC8 is other than direct blocking of these channels.

One of the discovered actions of XC8 is the inhibition of the Golgi resident human glutamyl cyclase (gQC), the enzyme that catalyzes the pyroglutamination of chemokines of C– C motif family involved in many inflammatory disorders including asthma (unpublished data). Pyroglutamination is required for the potent chemotactic activity of CCL2, CCL7, CCL8, and CCL13 chemokines. Being released at the site of allergic inflammation, these chemokines act as chemoattractants and activators of eosinophils [71–74]. Binding of these chemokines to CCR receptors present on human eosinophils can induce their degranulation with the subsequent release of inflammatory mediators from specific granules, which are involved in tissue remodeling [75–77]. The CCR3 receptor was shown to play the major role in eosinophil chemotaxis [77]. By inhibiting gQC, XC8 can reduce the activity of CCL2, CCL7, CCL8, and CCL13 chemokines and finally suppresses the eosinophil-driven inflammation.

According to O'Connell et al. asthma resistance to treatment with glucocorticoids (GC) is caused by IFN-γ induced JAK/STAT signaling [78]. We have demonstrated that patients with an increased level of IFN-γ are sensitive to treatment with XC8, resulting in an improvement in FEV_1 [35]. IFN-γ is known as a non-specific inducer of cough [79], which enhances the cough response when administered together with citric acid [6]. In our experiments, cough induced by citric acid combined with IFN-γ was sensitive to treatment with XC8, as XC8 completely abolished the IFN-γ enhancement effect and reduced cough frequency to the same level as without IFN- γ. We can assume that one mechanism by which XC8 suppresses cough is inhibition of IFN-γ induced signaling pathways. Such a mechanism could be relevant for cough conditions like asthma, COPD, and post-viral cough in patients with severe COVID-19 [67].

It is currently believed that TRPV1 and TRPA1 channels play a complex regulatory role in airway function and inflammatory diseases [80, 81]. The TRPV1 channel is likely involved in the pathophysiological condition of cough hypersensitivity [82]. However, the treatment of

patients having refractory chronic cough with TRPV1 antagonists did not show a promising result, indicating more complex mechanism of this disease [83, 84]. The TRPA1 channel is considered as a more promising target for the treatment of cough or asthma with TRPA1-dependent neuronal inflammation [85]. Besides asthma, the upregulation of TRPA1 channel is implicated in the pathogenesis of such respiratory diseases as COPD, allergic rhinitis, and cystic fibrosis [52]. The TRPA1 channel is shown to be a major neuronal sensor of oxidative stress, present in patients with COPD [86]. The activation of TRPA1 channel on nerve endings in the skin causes local inflammation leading to dermatitis or psoriasis [87]. XC8 being effective in the treatment of cough induced by the activation of the TRPA1 channel can have significant potential in the treatment of other diseases mediated by this mechanism.

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Author Contributions. Dr. VN is the founder and CEO of the Pharmenterprises LLC, which develops new drugs. Dr. VN is the author of the XC8 molecule, the author of the idea, the initiator and the head of the project. Dr. AR is responsible for the development of the XC8 concept, design, planning, and analyzes of all animal experiments. Dr. OP is responsible for the performance of animal experiments. Dr. SM analyzed animal experiments and performed statistical analysis of the data. YG took part in analysis and interpretation of the results. Dr. JR analyzed all preclinical data, selected data for publication, and wrote the manuscript.

Disclosures. Vladimir Nebolsin is the author of the project, the founder, and the CEO of the company Pharmenterprises LLC. Julia Romanova, Anastasia Rydlovskaya, Stepan Mochalov, Oxana Proskurina, and Yulia Gorokh are employees of Pharmenterprises LLC and have nothing to disclose.

Compliance with Ethics Guidelines. All of the animal experiments were approved by the Animal Ethics Committee of LLC Pharmenterprises (protocols No. BEC 07/2020 dated 19.05.2020, and 09/2020 dated 14.07.2020). The experimental procedures with animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals [36]. All the procedures with animals were performed under anesthesia to minimize their suffering.

Data Availability. All data are available from the corresponding author upon request.

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