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Original Article

A Physicochemical Assessment of Acid-induced Post-inhalation Cough in Guinea Pig Animal Model

Andrei Blasko[1]*, Nagaraja Rao[1], Philip J Kuehl[2], Thomas D Holmes[2], Matthew D Reed[2], Fadi Xu[2] and Jianguo Zhuang[2]

[1]Novartis Pharmaceuticals Corp., San Carlos, California, USA
[2]Lovelace Respiratory Research Institute, Albuquerque, NM, USA

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Address for Correspondence
Novartis Pharmaceuticals Corp., San Carlos, California, USA
E-mail: andrei.blasko@novartis.com

ABSTRACT

A study was conducted with a Guinea Pig (GP) cough model to demonstrate that post-inhalation (PI) cough can be correlated and predicted with the acidity constant of the chemical stimulant. The test animals were exposed to nebulized solutions of citric acid and maleic acid. Cough responses were measured by audio, video, and respiratory flow signals *via* whole-body plethysmography. Nonparametric one-way ANOVA (Kruskal-Wallis) of cough counts showed statistically significant dose dependence for both citric acid ($p<0.0001$) and maleic acid ($p<0.0001$). For citric acid, post-hoc Dunn's test indicated elevated cough response at the higher concentrations ($p \leq 0.0001$ for 200 mM, and 300 mM) compared to control, i.e., isotonic saline (SL). The cough count at 100 mM citric acid was not significantly different from SL ($p>0.05$). Similar results were obtained for maleic acid exposures, with cough response at the higher concentrations significantly different compared to SL ($p=0.0039$ for 50 mM, $p<0.0001$ for 100 mM), while cough counts at 30 mM were not different compared to SL. The GP model is able to differentiate the cough response of maleic acid *vs.* citric acid, and the threshold of cough (1-2 coughs in 2 min) for maleic acid (~30 mM) is lower than that for citric acid (~100 mM). The lower threshold of cough for maleic acid as compared to citric acid is consistent with the mechanism of action mediated by protons, in a dose dependent way. A prediction of the cough response from citric acid to maleic acid was confirmed by this study. Experiments with nebulized acids confirmed expectations that the threshold of cough for citric acid in GP is at 100 mM and for maleic acid at 10-30 mM. The lower cough threshold for maleic *vs*. citric acid is consistent with proton-mediated mechanism of action.

Keywords: Post-inhalation cough, Acid-induced cough, Whole body plethysmography, Nebulized aerosols.

INTRODUCTION

The literature on acid-evoked cough is extensive[1-6], and the subject matter is accessible for study *via* animal models. It was reported that citric acid-evoked coughing observed in anesthetized guinea pigs (GP) could be mimicked by administration of hydrochloric acid but not by sodium citrate[1]. Citric acid-evoked coughing in anesthetized GPs is mediated by direct activation of capsaicin-insensitive vagal afferent nerves, perhaps through sequential activation of acid-sensing ion channels and chloride channels[1]. Repetitive sub-threshold activation of the cough receptors or coincident activation of C-fibers and/or nTS (nucleus tractus solitaries) neurokinin receptors lead to sensitization of cough[2]. Chronic cough has often been considered to be caused by gastro-oesophageal reflux, post-nasal drip, or asthma. Nonetheless, new insights in chronic cough revealed two important mechanisms that

can initiate a cough: sensory-driven and protective cough reflex[3].

It has been demonstrated that chemical irritants bind to receptors on afferent nerves, opening ion channels on the terminals of the airway sensory nerves to activate nTS relay neurons via vagal sensory afferents, and ultimately activating motor neurons resulting in the cough reflex[4].

Citric acid is the gold standard for assessing the impact of H^+ ions on post-inhalation (PI) cough. The threshold of cough for citric acid in humans (i.e., cough in 2 out of 3 trials with a nebulizer running for 15 seconds at 8 L/min by face mask method) is ≤ 0.8 M[5]. Animal models for cough have been reported, and the conscious GP is the most useful laboratory animal for experimental studies of chemically induced cough, as compared with the rat and rabbit[6]. The threshold of cough for citric acid in anesthetized GPs was reported as 0.03 M[1].

The present study focused on the chemistry of the chemical irritants, particularly on the physicochemical properties of the acids as the main cause of cough as induced by changes in the local pH at the site of delivery, and how this knowledge can be used to mitigate PI cough for inhaled drug products.

This paper describes an animal exposure study conducted in conscious GPs to investigate the difference in cough response as a function of the acidity constant of the irritant. The test animals were exposed to nebulized solutions of citric acid and maleic acid, relatively straightforward to prepare and aerosolize. The study with nebulized aerosols were aimed at assessing if the conscious guinea pig model provided a suitable test system to study acid-induced cough in dry powder formulations for inhalation. Cough studies with dry powders administered intra-tracheally to anesthetized GPs has been reported in the literature[7], but not to conscious, unrestrained GPs placed within a whole-body exposure chamber.

MATERIALS AND METHODS

Test Materials

Citric acid, ACS reagent ≥ 99.0%, and maleic acid, ReagentPlus® ≥ 99.0%, were from Sigma-Aldrich (St. Louis, MO).

Preparation of Test Articles

Preparation of solutions: Dose-response characteristics were studied using nebulized solutions of citric acid and maleic acid, prepared over a range of solution strengths to achieve three dose levels of each treatment. The solutions (100, 200, and 300 mM citric acid and 25, 30, 50, 100, and 200 mM maleic acid) were prepared fresh daily. Prior to use, the pH of each formulation was measured and compared to the predicted pH. The pH of the solutions were measured with an Accumet XL150 pH meter (Fisher Scientific, Bridgewater, NJ) calibrated daily.

Animal Exposures

Dose-responses of cough to nebulized aerosols were studied in conscious GPs by using a whole body plethysmography system, and the cough characteristics were obtained by real-time monitoring and recording of audio, video, and respiratory flow signals.

Animals: A total of 30 Dunkin-Hartley GPs (250-350 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA) over the course of the study (nebulized citric and maleic acid exposures). The animals were housed in filter top cages with *ad libitum* access to water and food. The room was constantly ventilated and the temperature kept at 23°C. The animals were quarantined for two weeks before experiments. After quarantine, the GPs were individually placed in the aerosol exposure chamber for 45 min/day × 3 days for habituation.

Ethics statement: *In vivo* procedures were conducted under protocols approved by the Lovelace Respiratory Research Institute (Albuquerque, NM) Institutional Animal Care and Use Committee (IACUC). These facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Animals were examined twice per day (morning and afternoon) on each day of the study. No adverse clinical signs were noted during the course of these studies. Animals were fed a certified diet twice daily and allowed water *ad libitum*.

Aerosol exposures: A summary of the aerosol exposure treatments conducted in this study is shown in Table 1. The animals were exposed to liquid aerosols generated by nebulizing solutions of citric acid and maleic acid. These experiments were performed to confirm the suitability of the GP model for assessing cough challenge response, and to verify the soundness of the test methodology, as a prelude to further studies with inhaled dry powders (not reported here). Three solution strengths of each acid were selected so that a dose-response assessment could be conducted. Maleic acid solution strengths were lower compared to citric acid, based on the expectation that, as a stronger acid, maleic acid

should evoke a cough response at lower strengths, assuming comparable aerosol delivery. The solutions were made fresh on the day of exposure and the pH measurements recorded (Table 1).

A schematic diagram of the aerosol exposure system has been illustrated in Figure 1. The test aerosols were generated using an Aeroneb Lab vibrating mesh nebulizer (Aerogen Ltd., Galway, Ireland) attached to a whole-body plethysmography exposure chamber (Buxco, Wilmington, NC, USA). A bias air flow of 1 L/min was maintained for the duration of the experiment. For measurement of aerosol concentration, the chamber exhaust flow was sampled through a Pallfex filter (Pall Corp., Port Washington, NY 11050) at a flow rate of 0.6 L/min during nebulization. Measurement of "dose" of nebulized aerosol was done by drying the filter and weighing (i.e., gravimetric). The chamber pressure was maintained slightly positive, with 0.4 L/min allowed to vent out of the chamber for respiratory signal optimization. During dosing, each animal was individually exposed to nebulized isotonic saline (SL), and then randomly to the three selected concentrations (Table 1) of citric acid and maleic acid solution for two minutes, respectively, to assess cough response in the test animal. The animal was kept in the chamber for 30 min after the exposure to record the evoked responses and their recovery (Figure 1). At least two hours were allowed between two consecutive exposures. After completion of exposure runs and assessment of cough response, animals were euthanized by intraperitoneal injection of an overdose Euthasol solution (200 mg/kg)

Detecting cough response: To detect cough, an omni-directional lavalier microphone system (UTX-B2, Sony, Japan) was mounted in the roof of the plethysmography chamber to record the sound, similar to previous reports[8-10]. To monitor animal behavior, a video monitor (Q2F-00001, Microsoft Corp, USA) was placed outside of the chamber. The digitized respiratory flow signals, the sound from microphone, and the video were recorded simultaneously with a data acquisition and analysis system (Powerlab 8/sp and LabChart 7 software, ADInstruments Inc., Colorado Springs, CO). The number of coughs was counted by two trained observers to ensure that only coughs but not sneezes or augmented breaths were counted.

A typical cough response, as reported before[8,11], was defined by the simultaneous appearance of: (1) A transient and great change in the respiratory airflow (a rapid inspiration followed by rapid expiration). (2) A typical cough sound with the peak power density at 1-2 kHz in the frequency spectrum (sneeze peaks at 3.5-6.5 kHz). (3) Characteristic animal body posture and movement (forward stretching of the neck) with opening mouth.

Data acquisition and statistics: The respiratory activities, including Respiratory Minute Ventilation (RMV), Respiratory Frequency (fR), Tidal Volume (VT), and cough and their responses to the aerosol exposures were continuously monitored before, during and after the aerosol exposures as indicated above.

A non-parametric one-way ANOVA analysis[12] was performed for each test article, with the animal ID treated as a blocking variable. Post-hoc multiple comparisons were performed using Dunn's test[13], with the isotonic saline exposure designated as the control, to assess cough response over the range of solution concentrations tested. Calculated P values of less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

The threshold of cough for citric acid is ≤ 800 mM in humans and 30 mM in GPs (*vide supra*). It is important to define experimental conditions for the reported threshold of cough for GP in order to relate this value to the data generated in the present study. For the 0.03 M reported value in GP, citric acid was applied topically in 100 µl aliquots directly to the tracheal mucosa of anesthetized GP[1]. The relationship between pH, pK_a and the concentration (C_a) of a weak acid is described by Eq. (1), and is valid for $3 < pK_a < 11$ and $C_a > 10$ mM[14].

$$pH = ½\left(pK_a - \log_{10} C_a\right) \qquad (1)$$

Citric acid has three acidity constants, pK_{a1}=3.13, pK_{a2}=4.76 and pK_{a3}=6.40[15]. There is a temperature dependence of the pK_a that is more significant for pK_{a1} than for pK_{a2} and pK_{a3} ($\Delta pK_{a1}/\Delta t$ = -0.0024)[16] and at 37°C, pK_{a1}=3.10. Ionic strength (µ=0.8 M) also has a significant contribution[14], decreasing the pK_{a1} from 3.10 to 2.93.

Upon dissolution of citric acid in water, most of the contribution to the (low) pH will be attributed to the first pK_a. By neglecting the other pK_a contributions, the pH of the threshold of cough in humans can be calculated from Eq. (1) and it is 1.51. The reported pH value in water for 0.8 M (15%) citric acid is 1.47[17]. Similarly, for GP the calculated threshold of cough is at pH 2.2 (for 0.03 M).

If the cough is mediated through sequential activation

of acid-sensing ion channels and chloride channels[1], then the proton concentration is directly responsible for this mechanism. Let us consider the maleic acid at the lung pH (7.0-7.2)[18]. The first pK_a of maleic acid is $pK_{a1} = 1.93$ ($\Delta pK_{a1}/\Delta t \approx 0$)[19]. The simplified Eq. (1) in this case is no longer valid ($pK_{a1}<3$); instead, the initial concentration of the acid, C_a can be calculated from the dissociation constant of the weak acid, shown in Eq. (2)[14].

$$C_a = \frac{[H^+]^2 + K_a[H^+]}{K_a} \quad (2)$$

If the pH threshold of cough for citric acid (pH 1.51 for humans and pH 2.2 for GP) is considered to apply for maleic acid, the concentration threshold of cough (C_a) for maleic acid can be calculated as 112 mM and 9.7 mM for humans and GP, respectively (assuming equivalent modes of dose delivery).

The reported value for the threshold of cough in GP for citric acid (0.03 M) and the calculated value for maleic acid were used to guide the experimental design. Experimental data from nebulized acid exposures are presented in Figures 2 and 3.

A typical recording of the cough response for an animal exposed to nebulized citric acid. Panel A shows time traces of the respiratory flow signal (colored red), and the audio signal from the microphone (colored blue). Panel B is an enlarged segment of recordings marked with a thick bar in panel A. The arrows in panel B indicate cough events (Figure 2).

Dose response curves for animals exposed to three different concentrations of citric acid and maleic acid aerosols are plotted in Figure 3. Nonparametric one-way ANOVA (Kruskal-Wallis)[12] of cough counts showed statistically significant dose dependence for both citric acid ($p<0.0001$) and maleic acid ($p<0.0001$). For citric acid, post-hoc Dunn's test[13] indicated elevated cough response at the higher concentrations ($p \leq 0.0001$ for 200 mM, and 300 mM) compared to control (SL). The cough count at 100 mM citric acid was not significantly different from SL ($p>0.05$). Similar results were obtained for maleic acid exposures, with cough response at the higher concentrations significantly different compared to SL ($p=0.0039$ for 50 mM, $p<0.0001$ for 100 mM), while cough counts at 30 mM were not different compared to SL.

The data above demonstrate that the GP model is able to differentiate the cough response of maleic acid *vs.* citric acid, and that the threshold of cough (1-2 coughs in 2 min) for maleic acid (~30 mM) is lower than that for citric acid (~100 mM). The lower threshold of cough for maleic acid as compared to citric acid is consistent with the mechanism of action mediated by protons, in a dose dependent way. While comparison of dose response characteristics across studies is often clouded by differences in the mode of aerosol delivery, it should be noted that the dose-response relationship observed for nebulized citric acid is similar to that observed in a previous study[20] where nebulized citric acid was dosed to conscious guinea pigs.

Overall, the experiments with nebulized acid solutions helped establish confidence that the guinea pig model is well suited for evaluating acid-evoked cough response. This has practical relevance to the development of inhalation drug products, where there is interest in studying post-inhalation cough, an adverse event often observed during dose administration (Figure 3).

CONCLUSION

Acid-induced post-inhalation cough study was conducted in unrestrained Guinea Pigs placed within a whole-body exposure chamber to investigate the difference in cough response as a function of the acidity constant of the irritant. The test animals were exposed to nebulized solutions of citric acid and maleic acid. Nonparametric one-way ANOVA (Kruskal-Wallis) of cough counts showed statistically significant dose dependence for both citric acid ($p<0.0001$) and maleic acid ($p<0.0001$). For citric acid, post-hoc Dunn's test indicated elevated cough response at the higher concentrations ($p \leq 0.0001$ for 200 mM, and 300 mM) compared to control (SL). The cough count at 100 mM citric acid was not significantly different from SL ($p>0.05$). Similar results were obtained for maleic acid exposures, with cough response at the higher concentrations significantly different compared to SL ($p=0.0039$ for 50 mM, $p<0.0001$ for 100 mM), while cough counts at 30 mM were not different compared to SL. The data above demonstrate that the GP model is able to differentiate the cough response of maleic acid *vs.* citric acid, and that the threshold of cough (1-2 coughs in 2 min) for maleic acid (~30 mM) is lower than that for citric acid (~100 mM). The lower threshold of cough for maleic acid as compared to citric acid is consistent with the mechanism of action mediated by protons, in a dose dependent way. A prediction of the cough response from citric acid to maleic acid was confirmed by this study. Experiments with nebulized acids confirmed expectations that the threshold of cough for citric acid in GP is at 0.1 M

and for maleic acid at 0.01 - 0.03 M. The lower cough threshold for maleic *vs.* citric acid is consistent with proton-mediated mechanism of action.

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Table 1. Overview of aerosol exposure treatments

Test article	Concentration (mM)	Solution pH		No. of Animals
		Predicted	Measured	
Citric acid solution	100	2.08	2.00	15
	200	1.93	1.89	
	300	1.84	1.81	
Maleic acid solution	30	1.87	1.88	13[a]
	50	1.73	1.69	
	100	1.55	1.55	

[a]For the maleic acid the dosing regimen (i.e., solution strength/aerosol concentration and exposure duration), were defined after conducting pilot runs with a small number of animals. The table reports only the number of animals that received the final treatment

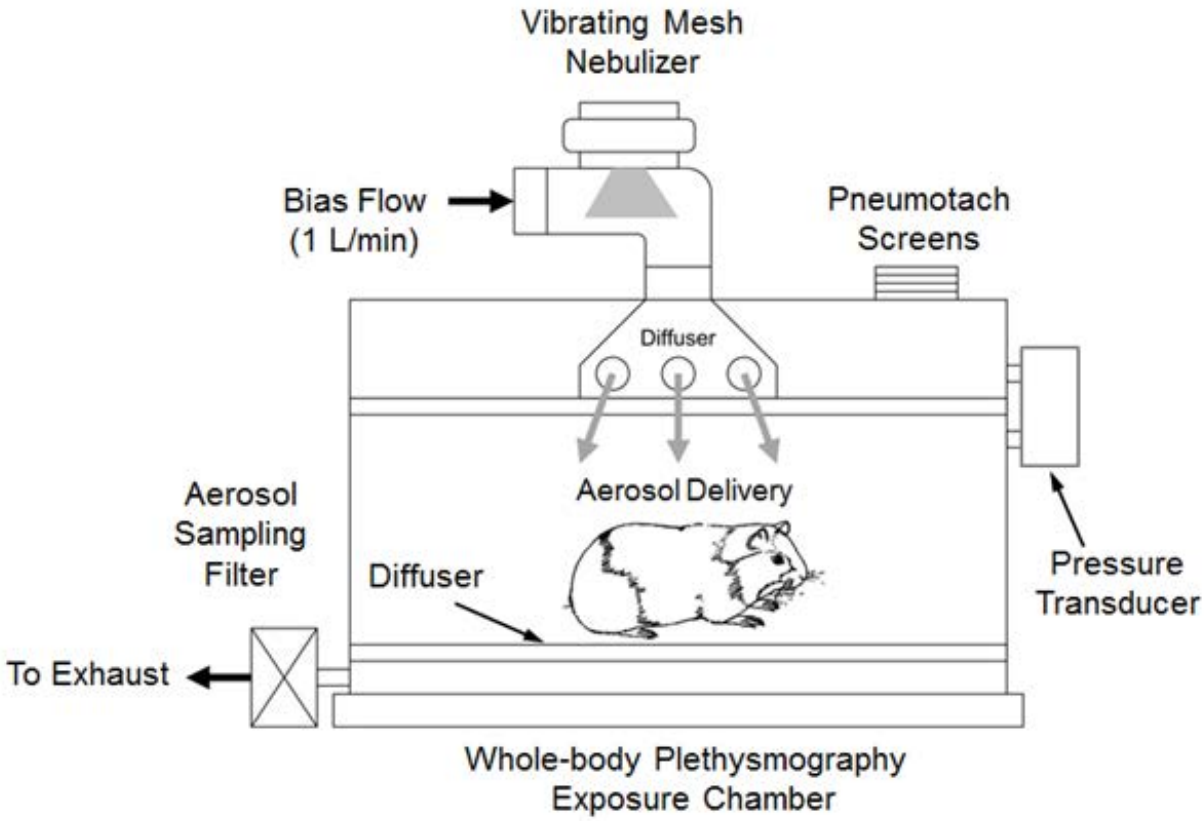

Figure 1. Schematic representation of aerosol exposure system used with nebulized aerosols

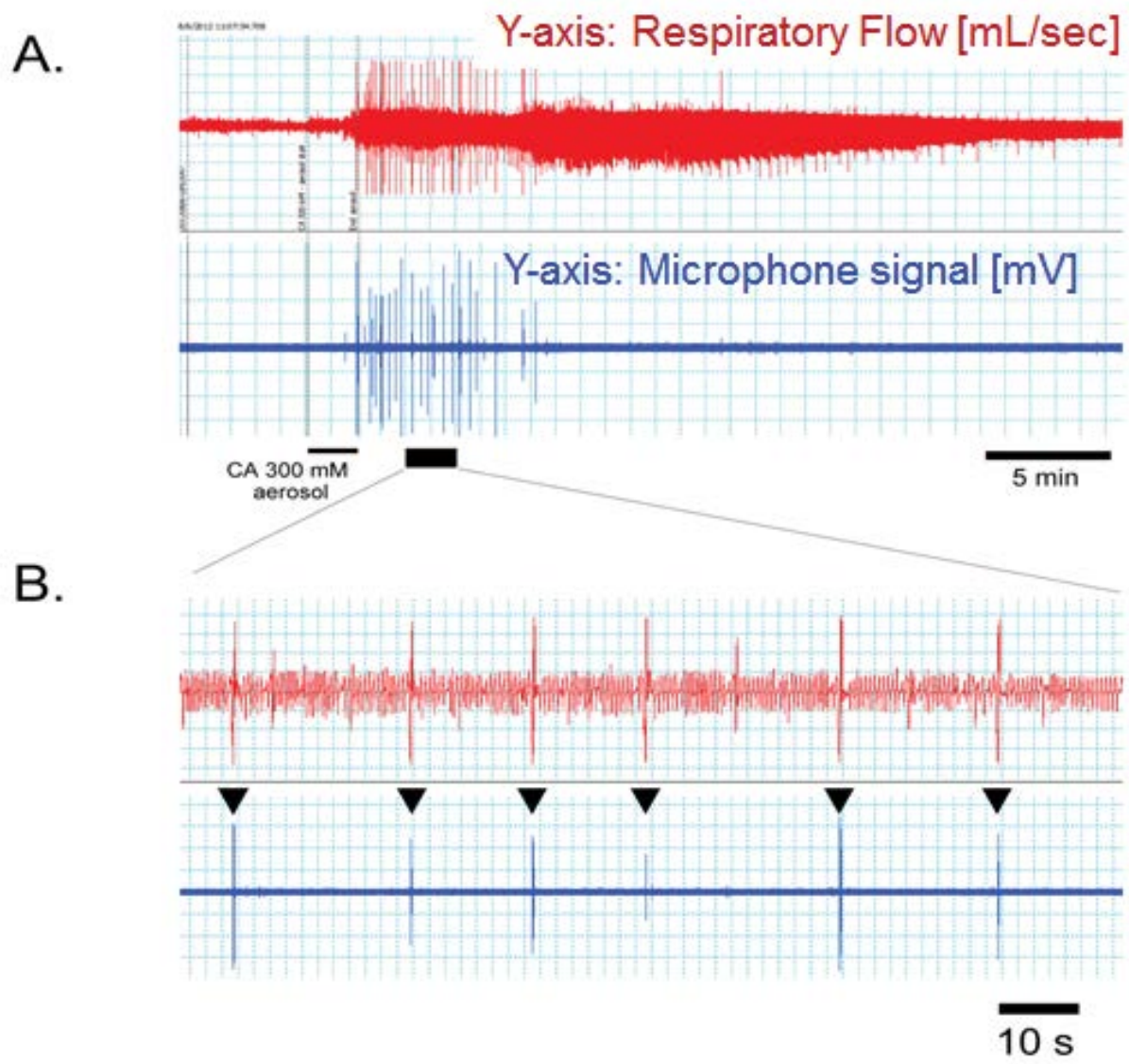

Figure 2. Representative recording of GP cough events before, during, and after exposure to nebulized citric acid (CA)

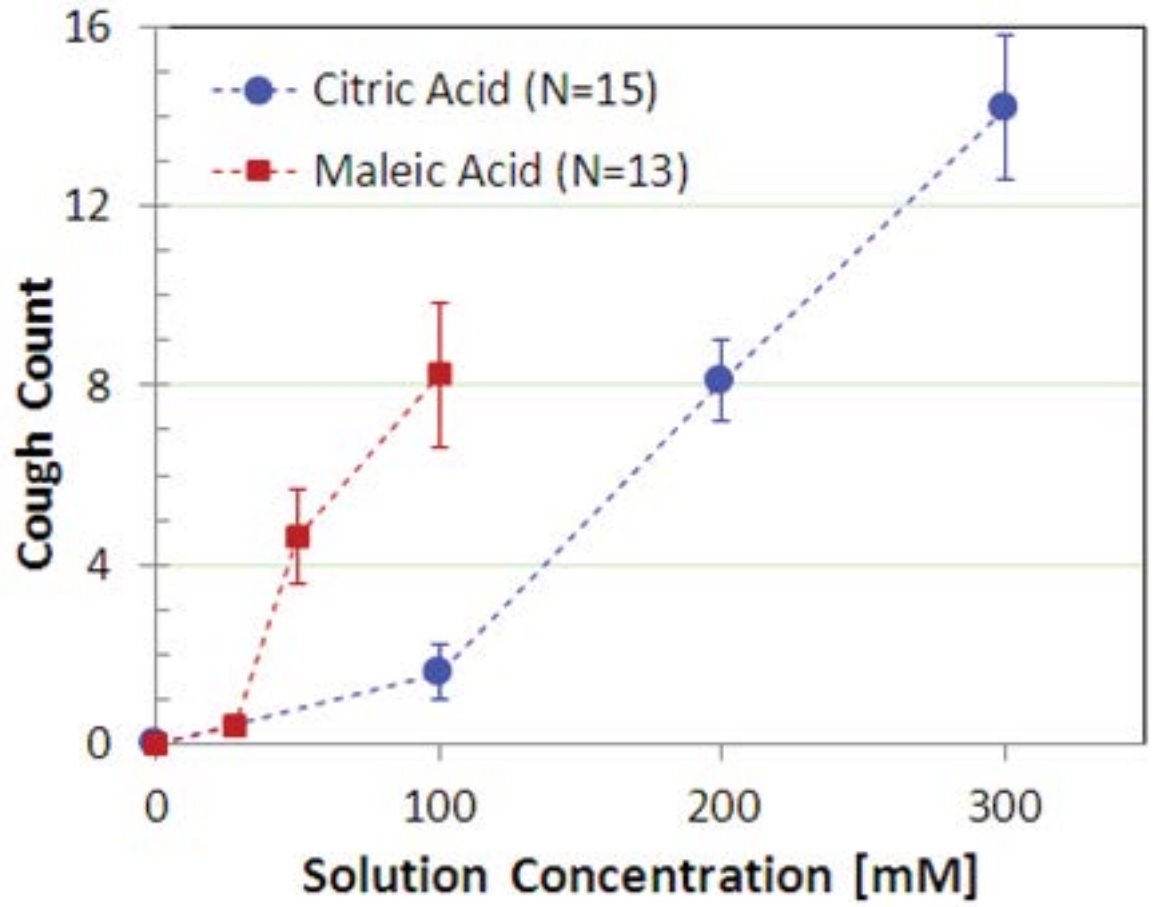

Figure 3. Dose response to nebulized citric (solid circles) and maleic acids (solid squares). The lower cough threshold for maleic *vs.* citric acid is consistent with a proton-mediated mechanism of action. The error bars represent ±1 standard deviation around the mean