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In vivo Pharmacological Characterization of TD-4208, a Novel Lung Selective Inhaled Muscarinic Antagonist with Sustained Bronchoprotective Effect in Experimental Animal Models

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Abbreviations: COPD, chronic obstructive pulmonary disease; LAMA, long acting muscarinic antagonist, LSI, lung selectivity index; ACh, acetylcholine, MCh, methacholine, Pilo, pilocarpine

Abstract

Tiotropium is currently the only once-daily, long-acting muscarinic antagonist (LAMA) approved in the US and other countries for the treatment of COPD. Glycopyrronium has shown promise as a LAMA and was recently approved for once-daily maintenance treatment for COPD in the European Union. Here, we describe the *in vivo* preclinical efficacy and lung selectivity of a novel inhaled muscarinic antagonist, TD-4208 (biphenyl-2-ylcarbamic acid 1-(2-{[4-(4-carbamoylpiperidin-1-ylmethyl)benzoyl]methylamino}ethyl)piperidin-4-yl ester) and compare its profile to tiotropium and glycopyrronium. In anesthetized dogs, TD-4208, along with tiotropium and glycopyrronium produced sustained inhibition of acetylcholine (ACh)-induced bronchoconstriction for up to 24 hr. In anesthetized rats, inhaled TD-4208 exhibited dose-dependent 24 hr bronchoprotection against methacholine (MCh)-induced bronchoconstriction. The estimated 24 hr potency (expressed as concentration of dosing solution) was 45.0 µg/ml. The bronchoprotective potencies of TD-4208 and tiotropium were maintained after seven days of once daily dosing, whereas glycopyrronium showed a 6-fold loss in potency after repeat dosing. To assess systemic functional activity using a clinically relevant readout, the antisialagogue effect of compounds was also evaluated. The calculated lung selectivity index (i.e. ratio of antisialagogue and bronchoprotective potency) of TD-4208 was superior to glycopyrronium after both single and repeat dosing regimens and was superior to tiotropium after repeat dosing. In conclusion, the *in vivo* preclinical profile suggests that TD-4208 has the potential to be a long-acting bronchodilator for once-daily treatment of respiratory diseases. Its greater functional selectivity for the lung in preclinical models

may translate to an improved tolerability profile compared to marketed muscarinic receptor antagonists.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease that is characterized by partially reversible and often progressive airflow limitation. COPD patients report symptoms of cough, increased sputum production and breathlessness upon exertion (Viegi, et al., 2007; Rabe, et al., 2007). Because current treatment options do not halt the progression of disease, management of COPD is focused on symptom relief and prevention of exacerbations mainly through use of corticosteroids and bronchodilators such as short- and long-acting β_2-agonists or muscarinic antagonists (Qaseem, et al., 2007; Vestbo, et al., 2012). Muscarinic receptor antagonists inhibit mucus hypersecretion in secretory glands and directly relax the airway smooth muscle by reversing the cholinergic tone of the bronchus (Eglen, et al., 1996; Barnes, 2000). Clinical evidence suggests that patients with COPD exhibit a higher basal cholinergic tone than normal subjects (Gross, et al., 1989). This increased tone contributes to persistent bronchoconstriction which is considered the major reversible component of the disease (Gross, et al., 1989; Barnes, 2000; Brusasco V., 2006).

The therapeutic utility of inhaled anticholinergic bronchodilators is governed by their selectivity for the muscarinic receptor subtypes and their distribution in the body. Of the five known muscarinic receptor subtypes, three (M_1, M_2 and M_3) have been identified in rat, dog and human pulmonary tissue (Gies, et al., 1989; Janssen and Daniel, 1990; Emala, et al., 1995). Anticholinergic drugs reduce bronchoconstriction and mucus secretion by blocking activation of M_1 and M_3 muscarinic receptors (Bloom, et al., 1987; Barnes, 1992; Barnes, 1993). By contrast, prejunctional activation of M_2 autoreceptors

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inhibits excessive release of acetylcholine (ACh) from the vagus nerve. Thus, blockade of M_2 muscarinic receptors increases ACh-mediated contractions and compromises the bronchodilatory actions of non-selective anticholinergics (Barnes, 1993; Barnes, 2004). Hence, anticholinergic drugs that preferentially antagonize M_3, and potentially M_1 receptors, should demonstrate improved efficacy compared to non-selective muscarinic receptor antagonists.

Tiotropium, currently the only once-daily long-acting muscarinic antagonist (LAMA) approved for treatment of COPD in the US and other countries worldwide, exhibits *in vitro* kinetic selectivity for M_1 and M_3 over M_2 muscarinic receptor subtype (Disse, et al., 1993; Barnes, 2000). In several clinical trials, tiotropium has been shown to improve lung function, reduce the frequency of exacerbations and enhance quality of life scores of patients with COPD (Casaburi, et al., 2000; Casaburi, et al., 2002; Vincken, et al., 2002; Tashkin, et al., 2008). While tiotropium is regarded as effective and generally safe (Barr, et al., 2006; Tashkin, et al., 2008; Oba, et al., 2008), its side effect profile is undesirable for some patients. For example, a meta-analysis of the adverse effects from several clinical trials indicated a 16% incidence of dry mouth and was the most common adverse event in patients treated with tiotropium (Kesten, et al., 2006; Kesten, et al., 2009). Beyond tolerability, dry mouth may limit the therapeutic dose of tiotropium since doses higher than the approved dose have been shown to be more efficacious in Phase 2 trials (Maesen, et al., 1993; Maesen, et al., 1995; Littner, et al., 2000). After inhalation, tiotropium is absorbed from the lung into systemic circulation and consequently antagonizes muscarinic receptors in tissues outside of the lung including the salivary

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gland (Koumis and Samuel, 2005). Because salivation is likely mediated by activation of M_1 and M_3 muscarinic receptors (Abrams, et al., 2006), minimizing dry mouth requires greater tissue selectivity than that offered by tiotropium. Thus, to maximize the therapeutic benefits already derived from inhaled muscarinic antagonists, we sought to identify a novel long-acting bronchodilator with greater lung selectivity than existing agents.

TD-4208, biphenyl-2-ylcarbamic acid 1-(2-{[4-(4-carbamoylpiperidin-1-ylmethyl)benzoyl]methylamino}ethyl)piperidin-4-yl ester, (Fig. 1) is a novel and potent muscarinic receptor antagonist that has a high affinity and long residence time at the M_3 receptor, demonstrates *in vitro* kinetic selectivity for M_3 over M_2 muscarinic receptor subtype (Steinfeld, et al., 2009) and no meaningful off-target activity (unpublished data). TD-4208 was designed to produce sustained and localized effect in the lung with minimal systemic exposure after inhalation dosing. The objective of the work reported herein was to characterize the *in vivo* pharmacological profile of inhaled TD-4208 in comparison to tiotropium and glycopyrronium. First, we determined the bronchoprotective potency and duration of action of TD-4208 in dog and rat. Second, by determining its potency to antagonize systemic muscarinic M_3 and M_1 receptors through measurement of the inhibition of pilocarpine (Pilo)-induced salivation (antisialagogue effect), we estimated the lung selectivity of TD-4208 in rats. Finally, we conducted studies to understand the concentration-effect relationship of the bronchoprotective and antisialagogue effects of TD-4208 in relevant tissues in rats.

Materials and Methods

Compounds. TD-4208, glycopyrronium bromide and tiotropium bromide were all synthesized at Theravance, Inc. and were dissolved and diluted in distilled water.

Bronchoprotection in Dogs. Studies were reviewed and approved by the Institutional Animal Care and Use Committee in Lovelace Respiratory Research Institute (Albuquerque, NM, USA). Adult (1.5 – 1.8 years of age) naïve Beagle dogs (10.3 - 11.7 kg) were housed in indoor-outdoor kennel runs with a 12:12-hr light–dark cycle and maintained at a temperature of 18 - 29°C and relative humidity of 30-70%. Dogs were fed a standard diet (2025 Teklad Global 25% Protein Dog Diet) once daily and drinking water was provided *ad libitum*. Dogs were fasted overnight prior to a study and were not fed until all procedures requiring anesthesia were completed. Dogs were anesthetized by intravenous (IV) administration of a mixture of valium (5 mg/kg) and ketamine (0.25 mg/kg). To avoid a rapid drop in body temperature due to anesthesia, dogs were placed on a water circulating heating pad during the experiment which did not exceed 4 hr. After placement of an endotracheal tube and a balloon catheter in the esophagus and while maintaining anesthesia using 2-3% isoflurane, animals were placed in a sling and artificially ventilated with a respirator set to deliver a volume between 210 – 249 ml at a rate of 15 strokes/min for the duration of the experiment. A PARI LC Plus nebulizer (PARI Respiratory Equipment, Inc. Midlothian, VA) pressurized with 20 psi compressed house air was mounted onto a two-way valve (Hans Rudolph, Inc.; Kansas City, MO, USA) and connected to a dual phase respirator pump that was set at 3.0 L/min. This canine inhalation (IH) exposure system generates particle sizes with a median diameter of approximately 2–4 microns to ensure respirability of the aerosol (Johnson, 1989).

Approximately 30 min before IH treatment with test article, increasing doses of ACh (1 – 1000 µg/kg, IV) were administered to determine the dose that produced a doubling of the baseline pulmonary resistance. This doubling dose was used in all subsequent exposures to ACh. After 15 minutes, ACh was again administered. Response to this challenge was considered 'pretreatment ACh response' to which all subsequent ACh challenges following inhalation treatment were normalized. After another 15 minutes, animals were dosed by inhalation with either test compound or vehicle. Exposure to test compound was carried out by running the nebulizer for 2-5 minutes, depending on the intended dose (expressed as amount of drug nebulized during the exposure time per body weight of animal). At different time points after inhalation (5, 30, 60, 90, 120, 150 and 180 min post- inhalation) the bronchoconstrictor response to ACh was re-evaluated. At 180 minutes post- dosing, animals were allowed to recover from anesthesia, returned to their kennels and fed. At 24 hr post- dose, the animals were reanesthetized and instrumented. Persistence of bronchoprotective effect was evaluated by assessing the bronchoconstrictor response to ACh. Heart rate, blood pressure, O_2 saturation and body temperature were monitored throughout the experiment. Heart rate changes were analyzed during the period of three hours after compound treatment when measurement was continuous. Airflow and tidal volume were measured using a differential pressure transducer located in front of the endotracheal tube while transpulmonary pressure was determined via the esophageal balloon catheter. Pulmonary resistance was calculated from the simultaneous measurement of transpulmonary pressure and respiratory flow using Labview software (National Instruments, Austin, TX, USA). Bronchoprotective effects of test compounds were expressed as % inhibition of pretreatment ACh-induced

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increase in pulmonary resistance. Statistical comparisons were done using repeated measures two-way analysis of variance (ANOVA) with Bonferroni post- test where value of $p < 0.05$ was considered significant (Graphpad Prism®, La Jolla, CA, USA). In addition, a 24 hr bronchoprotective potency (ID_{50}) was determined using a sigmoidal nonlinear regression analysis of the data points where the minimum and maximum were constrained to 0% and 100%, respectively. Bronchoprotective potency (ID_{50}) is the dose of test compound that inhibited ACh-induced bronchoconstriction by 50%.

Bronchoprotection in Rats. Studies were reviewed and approved by the Institutional Animal Care and Use Committee of Theravance, Inc. (South San Francisco, CA). Adult male Sprague-Dawley rats (200 - 350 g, Harlan, Indianapolis, IN) were acclimatized to their holding room for at least 1 week prior to any treatment. The holding rooms were kept at a temperature of 21±1°C with a 12:12-hr light–dark cycle. Standard rat diet (2018 Teklad) and drinking water were provided *ad libitum*. Animals were dosed via inhalation with test compounds or vehicle over a 10 min period in a whole body inhalation chamber (R+S Molds, San Carlos, CA). Aerosol was generated from 5 ml of dosing solution using a PARI-LC Star Nebulizer Set Model 22F51 (PARI Respiratory Equipment, Inc. Midlothian, VA) driven by Bioblend (5% CO_2/ 95% atmospheric air) at a pressure of 22 psi. With the duration of inhalation time constant between all treatment groups, doses were expressed as the concentration of drug solution nebulized. For the seven day repeat dosing regimen, animals were dosed every 24 hr and returned to their holding room after each treatment. At predetermined time points after inhaled dosing of either vehicle or test compound, rats were anesthetized with an intraperitoneal (IP) injection of Inactin (thiobutabarbital, 120 mg/kg). A supplemental dose of 40 mg/kg, IP was given if

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animals were responsive to physical stimulus (e.g., toe pinch) after the first dose. When complete anesthesia was achieved, as confirmed by the absence of response to toe pinch stimulus, surgery was performed according to either protocol described below. For all the studies conducted under anesthesia, body temperature was maintained at 37°C using a heating pad. Bronchoprotection was assessed in rats using the Einthoven model of methacholine- (MCh-) induced bronchoconstriction (McNamara, et al., 2010). Under complete anesthesia, the jugular vein and trachea were catheterized. Each animal was ventilated using a respirator (Model 683, Harvard Apparatus, Inc., Holliston, MA, USA) set at a stroke volume of 1 ml/100 g body weight but not exceeding 2.5 ml volume, and at a rate of 90 strokes per minute. A T-connector was placed along the respirator expiratory tubing to allow for measurement of changes in ventilation pressure (VP) using a Biopac transducer that was connected to a Biopac pre-amplifier (TSD 137C, Goleta, CA, USA). Changes in VP were recorded using the Acknowledge Data Collection Software (Biopac, Goleta, CA, USA). Stable baseline VP was collected for at least 2.5 minutes, and then rats were challenged with noncumulative IV infusions for 2.5 minutes of MCh (40 and 80 µg/kg) at a rate of 2 ml/kg/min with a 2 minute interval between the two doses of MCh. After completion of the study, animals were euthanized by carbon dioxide asphyxiation. Results were expressed as normalized % inhibition of peak MCh-induced bronchoconstriction at the 40 µg/kg, IV dose (i.e., ED_{80} dose) in test compound treated vs. vehicle control group. Inhibition curves were fitted to a sigmoidal nonlinear regression analysis where the minimum and maximum were constrained to 0% and 100%, respectively (GraphPad Prism®, La Jolla, CA, USA). Bronchoprotective potency (ID_{50}) was estimated as the concentration of nebulized solution of test compound that

inhibited the MCh ED_{80} response by 50%. The half-life of bronchoprotective effect was determined in time course studies as the time at which the initial bronchoprotective effect was reduced by 50%.

Antisialagogue Effect in Rats. Lung selectivity was determined by assessing the potential of muscarinic antagonists to inhibit Pilo-induced salivation (antisialagogue effect) which is a surrogate measure for dry mouth (Sanchez and Lembol, 1994; McNamara, et al., 2009). Rats were anesthetized and their jugular vein and trachea were catheterized. Rats were then placed on their dorsal side, on a board inclined at 20 degrees, with their heads oriented downward. A pre-weighed gauze pad was inserted into the animals' mouth and the muscarinic agonist Pilo (3 mg/kg) was administered intravenously. Saliva produced for 10 min after Pilo injection was measured gravimetrically by determining the weight of the gauze pad before and after Pilo. The percent inhibition of Pilo-induced sialagogue effect was calculated by dividing the weight of Pilo-induced saliva from each rat in a given treatment group by the mean Pilo-induced saliva in the vehicle control group and multiplying by 100. Inhibition curves were fitted to a sigmoidal nonlinear regression analysis where the minimum and maximum were constrained to 0% and 100%, respectively. From the fitted curve, the ID_{50} estimate or the dose required to inhibit 50% inhibition of the Pilo-induced sialagogue response was determined.

Study 1: Evaluation of bronchoprotective and antisialagogue effects after a single dose (single dosing). To determine the bronchoprotective and antisialagogue potency

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after a single dose, rats were exposed by inhalation to a nebulized solution of either TD-4208 (3 – 3000 µg/ml), tiotropium (0.3 – 300 µg/ml), glycopyrronium (1 – 1000 µg/ml) or vehicle (sterile water) as described above. Bronchoprotective activity was assessed 24 hr post-dose. For the antisialagogue effect, inhibition of Pilo was assessed 1 hr, 6 hr or 12 hr after inhalation of an efficacious dose of test compound to determine the time point at which peak effect occurred. All subsequent doses were measured at this time point.

Study 2: Evaluation of bronchoprotection and antisialagogue effects after seven once daily doses (repeat dosing). To evaluate the effect of repeated exposure, animals were exposed by inhalation to seven once-daily doses of either TD-4208 (3 – 1000 µg/ml), tiotropium (0.3 – 100 µg/ml), glycopyrronium (1 – 1000 µg/ml) or vehicle (sterile water). In different groups of animals, either bronchoprotective activity or antisialagogue effect was assessed 24 hr and 1 hr, respectively, after the last dose. This study was run concurrently with single dosing groups as control.

Study 3: Tissue concentration analysis. In a subset of animals from each group, blood, whole lung (without trachea and primary bronchi) and submaxillary gland (SMG) were collected immediately after completion of the bronchoprotective or antisialogogue assays. Blood, collected via the inferior vena cava under CO_2 narcosis, was placed in tubes containing 2.5-times the blood volume of either ice-cold methanol containing internal standard for TD-4208 or ice-cold acetone with internal standard for tiotropium and glycopyrronium. The blood-organic solvent mixtures were processed by centrifugation (10,000 rpm, 10 min, 4°C) and supernatant stored at -80°C until analysis. Lung and

SMG were homogenized in 3-times the volume of phosphate buffered saline (PBS) to generate a 25% w/w homogenate. On the day of analysis, 200 µl aliquots of each blood extract sample were dried under a stream of nitrogen and reconstituted in 200 µl of 5% acetonitrile in water. For lung and SMG samples, 50 µl aliquots of lung or SMG homogenate were extracted with 6 volumes of acetone containing internal standard and 250 µl of the supernatant was reconstituted in 200 µl of 5% acetonitrile in water. The concentrations of TD-4208, tiotropium and glycopyrronium in blood, lung, and SMG were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). TD-4208 samples were analyzed using a Hypurity C18 column (100 x 2.1 mm; 3 µM), and tiotropium and glycopyrronium samples were analyzed using a Betabasic C18 column (50 x 2.1 mm; 3 µM). Mobile phase A consisted of 0.2% formic acid in water and mobile phase B consisted of 0.2% formic acid in acetonitrile. The flow rate was 0.5 ml/min. For TD-4208 chromatography, the gradient started from 2% to 15% mobile phase B in 0.5 min followed by a 3.5 min gradient to 45% mobile phase B. For tiotropium chromatography, the gradient started from 2% to 10% mobile phase B in 0.5 min followed by a 3.5 min gradient to 60% B. For glycopyrronium, the chromatography used a gradient from 5% to 95% mobile phase B in 3.2 min. The sample injection volume was 25 µL for TD-4208 and tiotropium, and 20 µL for glycopyrronium. The mass spectrometer (API5000) was operated in positive ion multiple reaction monitoring mode (MRM).

Data Analysis. Data were expressed as mean ± standard error of the mean (S.E.M.) or mean with 95% confidence intervals (CI). Statistical differences between two or more

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groups were determined by Student's *t*-test (paired and unpaired) or two-way analysis of variance using post- hoc Bonferroni's test, p value set at $p<0.05$. The Lung Selectivity Index (LSI) was calculated as the ratio of antisialagogue ID_{50} (peak effect)/ 24 hr bronchoprotection ID_{50}.

Results

Potency and Duration of Action in Dogs. Prior to testing compounds, the sensitivity of each dog to cholinergic stimulation was evaluated by determining the dose of ACh (1 – 1000 µg/kg) that produced a doubling in baseline pulmonary resistance. Following randomization to different treatments, the mean doubling doses of ACh were not statistically different among groups (Table 1). Pretreatment with inhaled vehicle produced a modest but short-lasting inhibition of airway responsiveness to ACh. Inhaled TD-4208 (3, 10 and 30 µg/kg) markedly inhibited the bronchoconstriction response to ACh (Fig. 2A). A two-way ANOVA, comparing the three doses of TD-4208 with vehicle, revealed significant treatment ($F_{(3,72)} = 88.10$, $p<0.0001$) and time ($F_{(5,72)} = 4.52$, $p = 0.001$) effects. The onset of bronchoprotection was determined during the first 2 hr post- treatment. All three doses of TD-4208 (3, 10 and 30 µg/kg) produced greater than 75% inhibition of ACh-induced bronchoconstriction 5 min post- treatment. Responses at this time point were deemed maximal since the mean (± S.E.M.) bronchoprotective effects overlapped at all subsequent time points during the onset period. Thus, TD-4208 exhibited an onset of bronchoprotection of 5 min at all doses tested. The duration of bronchoprotection produced by the 3 µg/kg dose decreased from 76% ± 9% to 28% ± 10% by 24 hr; however, bronchoprotection was maintained for at least 24 hr following the two highest doses (57% ± 14% for 10 µg/kg and 80% ± 4% for 30 µg/kg; $p<0.05$ for both doses compared to vehicle).

Tiotropium (0.3, 1 and 3 µg/kg) also produced significant bronchoprotection at all doses tested (Fig. 2B). A two-way ANOVA, comparing vehicle with the 3 doses of tiotropium,

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revealed significant treatment ($F_{(3,73)}$ = 81.03, p<0.0001) and time effects ($F_{(5,73)}$ = 6.57, p<0.001). Tiotropium inhibited ACh-induced bronchospasm by 58% ± 8% (0.3 µg/kg), 82% ± 5% (1 µg/kg) and 83% ± 8% (3 µg/kg) 5 min after inhalation. At this time point, peak effects for the two highest doses were observed. By contrast, the peak effect (79% ± 8%, n = 4) at the lowest dose (0.3 µg/kg) was not achieved until 2 hr after inhalation treatment [i.e., showed non-overlapping S.E.M.s with bronchoprotection at 5 min (58% ± 8%, n = 4)]. This indicates a slower onset of effect for the 0.3 µg/kg dose. Significant 24 hr activity was maintained for 1 and 3 µg/kg doses (60% ± 7% and 58% ± 14%, respectively, p<0.001 for both compared to vehicle) but not for the lowest dose of 0.3 µg/kg (22% ± 10% for 0.3 µg/kg).

Glycopyrronium (3, 10 and 30 µg/kg) also significantly inhibited the bronchoconstrictive effect of ACh (Fig. 2C). A two-way ANOVA, comparing vehicle with the 3 doses of glycopyrronium, revealed significant treatment ($F_{(3,73)}$ = 98.35, p<0.0001) and time effects ($F_{(5,73)}$ = 7.12, p<0.001). The 3 µg/kg dose of glycopyrronium inhibited ACh-induced bronchospasm by 65% ± 8%; whereas the two higher doses produced 82% ± 5% and 90% ± 3% inhibition, respectively, at 5 min. At this time point, responses were maximal for the 10 and 30 µg/kg doses, but response for the lowest dose (3 µg/kg) did not peak until 30 min after inhalation treatment (83% ± 5%). Significant 24 hr activity was observed only at the two highest doses (57% ± 9% for 10 µg/kg and 75% ± 7% for 30 µg/kg, p<0.05 compared to vehicle). Bronchoprotection at the lowest dose was not different from vehicle at 24 hr (22% ± 10% at 3 µg/kg).

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TD-4208, tiotropium and glycopyrronium produced dose-dependent inhibition of ACh-induced bronchoconstriction 24 hr after inhalation. Sigmoidal fit analysis of these data yielded mean 24 hr bronchoprotective potencies for each molecule of 7.9 µg/kg for TD-4208, 1.1 µg/kg for tiotropium and 9.0 µg/kg for glycopyrronium. Lastly, treatment with either of the three compounds was not associated with significant change in heart rate compared to the respective pretreatment levels (data not shown).

Bronchoprotective and Antisialagogue Activity in Rats. TD-4208 dose-dependently inhibited MCh-induced bronchoconstriction after either a single dose (Fig. 3A) or seven once daily doses (Fig. 3B). The estimated 24 hr bronchoprotective potency (ID_{50}) was 45.0 µg/ml after single dosing and 36.0 µg/ml after repeat dosing (Table 2). The peak antisialagogue effect after a single dose of 3000 µg/ml occurred 1 hr after inhalation; thus, the dose response curve was determined at this time point. TD-4208 (100, 300, 1000 and 3000 µg/ml) produced dose-dependent antisialagogue effects with an estimated potency of 1164.0 µg/ml after single dosing (Fig. 3A) and 794.0 µg/ml following repeat dosing (Fig. 3B). Table 2 shows that the lung selectivity index of TD-4208 was unchanged after repeat dosing (single dose LSI = 26 vs. repeat dose LSI = 22).

Tiotropium also inhibited MCh-induced bronchoconstriction in a dose-dependent manner after either single dosing (Fig. 4A) or repeat dosing (Fig. 4B). The 24 hr bronchoprotective potency of tiotropium after a single dose (ID_{50} = 3.2 µg/ml) was not significantly different from its 24 hr potency after seven repeat doses (ID_{50} = 3.7 µg/ml) (Table 2). To estimate the lung selectivity of tiotropium, we determined the

antisialagogue effect of a single 100 µg/ml dose of tiotropium at 1, 6 and 24 hr and observed that the peak antisialagogue effect occurred 6 hr after inhalation (Table 3). Next, we determined the antisialagogue ID_{50} of tiotropium (10, 30, 100 and 300 µg/ml) at both 1 and 6 hr after inhalation. Tiotropium's antisialogogue effects at 1 hr post-treatment were dose dependent with an estimated potency of 168.1 µg/ml. The antisialagogue activity of tiotropium increased at the 6 hr time point, with an estimated potency of 87.0 µg/ml. After seven days of dosing, tiotropium inhibited Pilo-induced salivation more potently than after a single dose at both the 1 hr (ID_{50} = 11.4 µg/ml) and 6 hr (ID_{50} = 38.0 µg/ml) time points. This shift in antisialagogue potency after repeat dosing is further exemplified by the comparison of effect after a single dose of 10 µg/ml tiotropium, which did not inhibit Pilo-induced salivation (-11 ± 16%; Fig. 5A), but inhibited salivation by 47 ± 10% after seven repeat doses (Fig. 5B). Thus, the calculated LSI of inhaled tiotropium based on peak antisialagogue effect diminished from 27 after single dosing to 3 after repeat dosing (Table 2).

Glycopyrronium also inhibited the bronchoconstrictive effect of MCh in a dose-dependent manner. The 24 hr bronchoprotective potency of glycopyrronium after a single inhaled dose was 52.9 µg/ml. (Fig. 5A, Table 2). After repeat dosing, glycopyrronium was 6-fold less potent (ID_{50} = 325.8 µg/ml) than after single dosing (Fig. 5B). The decreased bronchoprotective potency is illustrated by the 100 µg/ml dose which inhibited MCh by 78% ± 5% after a single dose (Fig. 5A), but only 42% ± 7% after seven daily doses (Fig. 5B). The peak antisialagogue effect of glycopyrronium (1000 µg/ml) was determined to occur at 1 hr after dosing (Table 3). At this time point,

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its antisialagogue potency following a single dose (ID_{50} = 228.2 µg/ml) was not significantly different from its potency after seven daily doses (ID_{50} = 384.2 µg/ml) (Fig. 5A and 5B). Thus, the calculated LSI of inhaled glycopyrronium was 4 after single dosing and 1 after repeat dosing. This difference was not statistically significant since the 95% confidence intervals overlap (values not shown) (Table 2).

Concentration-Effect Relationship for Bronchoprotective and Antisialagogue Activity in Rats.

Concentration-effect relationships were determined by comparing lung concentrations of TD-4208, tiotropium and glycopyrronium with their respective bronchoprotective effects; as well as concentrations of the compounds in SMG, a relevant peripheral tissue for the salivation response, with their respective antisialagogue effects. Following either single or repeat dosing, a positive correlation was observed between TD-4208 concentrations in the lung and its bronchoprotective effect (r^2 = 0.9; Fig. 6A). Similarly, TD-4208 levels in SMG also correlated (r^2 = 0.7) with its antisialagogue effects (Fig. 6B). TD-4208 levels in blood obtained from bronchoprotective studies were not measurable [Limit of quantitation (LOQ) = 0.01 ng/mL]; whereas blood samples from the antisialagogue studies, in which higher doses were tested, showed mean compound levels of 0.12 ± 0.02 ng/mL and 0.87 ± 0.20 ng/mL after single doses of 100 µg/mL and 1000 µg/mL, respectively. Repeat administration of either dose did not lead to a significant accumulation of compound in the blood (0.9-fold relative to single dose). At the time point measured, neither of the pharmacodynamic readouts correlated with blood concentrations (r^2 < 0.1; data not shown).

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Lung and SMG concentrations of tiotropium also positively correlated (r^2 = 0.8 for both) with its bronchoprotective (Fig. 7A) and antisialagogue (Fig. 7B) effects, respectively. In contrast to TD-4208, levels of tiotropium after repeat dosing increased by an average of 8.4-fold in the SMG (Fig. 7B). From animals that were subjected to bronchoprotective studies, concentrations of tiotropium in blood were not measurable (LOQ = 0.005 ng/mL). However, blood samples obtained from antisialagogue studies yielded mean concentrations of 0.03 ± 0.01 ng/mL and 0.19 ± 0.05 ng/mL after single doses of 10 µg/mL and 100 µg/mL, respectively. These concentrations were maintained (0.9-fold) after repeat dosing treatment. At the time point measured, neither of the pharmacodynamic readouts correlated with blood concentrations ($r^2 \leq 0.1$; data not shown).

Lung and SMG concentrations of glycopyrronium also correlated (r^2 = 0.6 and 0.5, respectively) with its bronchoprotective (Fig. 8A) and antisialagogue effects (Fig. 8B), respectively. Blood concentrations of glycopyrronium were not measurable (LOQ = 0.01 ng/mL). However, blood samples obtained from antisialagogue studies yielded mean concentrations of 0.11 ± 0.03 ng/mL and 1.28 ± 0.34 ng/mL after a single dose of 100 and 1000 µg/mL, respectively. Blood levels of glycopyrronium after repeat dosing were 1.6-fold and 2.7-fold compared to blood levels after a single dose. Unlike TD-4208 and tiotropium, concentrations of glycopyrronium in the blood positively correlated (r^2 = 0.7) with its antisialagogue effect.

Discussion

TD-4208 is a novel long-acting muscarinic antagonist currently in clinical development for the treatment of respiratory diseases, including bronchospasm due to COPD. The purpose of our studies was to characterize the *in vivo* bronchoprotective and antisialagogue effect of this new agent. In two preclinical species, we showed that inhaled TD-4208 produces a sustained bronchoprotective activity up to 24 hr after dosing. The magnitude and duration of its bronchoprotective effect were similar to that of tiotropium and glycopyrronium. Moreover, after seven-day repeat dosing in rats, equieffective bronchoprotective doses of TD-4208 inhibited salivation to a much lesser extent than the other two muscarinic antagonists. Thus, TD-4208 exhibits greater lung selectivity than either tiotropium or glycopyrronium.

In anesthetized dogs, inhaled TD-4208 inhibited ACh-induced bronchoconstriction for up to 24 hr (the last time point measured). Previous *in vivo* duration studies in dogs showed that tiotropium produced long-lasting (>6 to >24 hr) bronchoprotection against ACh provocation (Disse, et al., 1993; Casarosa, et al., 2009;Gavalda, et al., 2009). We confirmed the 24 hr duration of tiotropium at doses producing initial bronchoprotection of more than 80% (Casarosa, et al., 2009). However, in contrast to published literature in which glycopyrronium showed no appreciable reversal of airway constriction as early as 12 hr post-dose (Casarosa, et al., 2009), we showed that the bronchodilatory effect of glycopyrronium was similar to tiotropium and sustained for 24 hr. While differences in our methodologies could have accounted for the apparent discrepancy, our findings are consistent with the sustained lung muscarinic receptor binding effects of glycopyrronium

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in rats (Ogoda, et al., 2011) and clinical reports on glycopyrronium wherein bronchodilation consistent with an extended duration of action was observed in patients with mild to moderate COPD (Vogelmeier, et al., 2010) or with asthma (Hansel, et al., 2005). In dogs, the 24 hr *in vivo* bronchoprotective potency of TD-4208 was about 10-fold less potent than that of tiotropium. The difference in potency is consistent with the lower *in vitro* affinity of TD-4208 for human muscarinic M_3 receptors relative to tiotropium (Steinfeld, et al., 2009). Lastly, we found no difference in the onset of action for the two highest doses tested for the three compounds. However, it is worth noting that the lowest tested dose of tiotropium reached maximum effect at a later time point (t_{max} = 2 hr) than the lowest tested dose of TD-4208 which achieved the same peak effect. Taken together, our findings in anesthetized dogs suggest that TD-4208 produced a potent bronchoprotective effect with duration of action similar to tiotropium; thus, supporting its potential as a once-daily bronchodilator.

By extending the *in vivo* characterization of TD-4208 from dogs to rats, we confirmed that inhaled TD-4208 is a potent and long acting bronchodilator in a second preclinical species and demonstrated its lung selectivity. Using the Einthoven model, we showed previously that the duration of bronchoprotection of marketed bronchodilators tiotropium and ipratropium matched the clinical duration of their bronchodilatory activity (McNamara, et al., 2010). In the current study, tiotropium (10 µg/ml) produced significant bronchoprotection in rat for up to 24 hr after a single administration. TD-4208 and tiotropium maintained their bronchoprotective potency after seven days of repeat dosing. Based on clinical data, the bronchodilator effect of tiotropium reaches

pharmacodynamic steady state within 48 hr after the first dose (van Noord, et al., 2002). Consistent with this, our results suggest that for both TD-4208 and tiotropium, pharmacodynamic steady state was achieved after one day of dosing and was maintained without loss of activity for up to seven days. By contrast, the bronchoprotective potency of glycopyrronium decreased by 6-fold after repeat dosing. The loss in potency of glycopyrronium was unexpected since the pharmacological and kinetic binding selectivity profiles for muscarinic M_2 and M_3 receptor are similar for the three compounds (Haddad, et al., 1994; Haddad, et al., 1999;Steinfeld, et al., 2009). This phenomenon may either be species-specific for glycopyrronium activity in the rat or a consequence of negative feedback mechanisms from blockade of M_2 autoreceptors at the highest dose tested (Aas and Maclagan, 1990; Celli, 2004). Although it is likely that postjunctional M_3 receptor antagonism drives the bronchoprotective activity of the three compounds in rat and dog, the possible involvement of postjunctional M_2 receptors cannot be excluded given that this receptor can also cause contraction of airway tissues through direct and indirect mechanisms (Hirshman, et al., 1999; Saria, et al., 2002).

In addition to pulmonary actions, antimuscarinics can also antagonize muscarinic M_1/M_3 receptors in salivary glands where blockade of these receptors promotes oral dryness. This anticholinergic action in the salivary glands underlies the clinical use of systemically administered glycopyrronium as an antisialorrheaic and preoperative antisecretory agent (Jongerius, et al., 2003). While the therapeutic window of an antimuscarinic bronchodilator improves significantly by topical delivery to the lung, by inhalation, compared to oral dosing (Lu, et al., 2006), the decreased systemic exposure at

therapeutically relevant doses may not be sufficient to prevent drug activity in extrapulmonary tissues (Ryberg, et al., 2008). We compared the inhaled doses required to inhibit bronchoconstriction and salivation in rat to provide a measure of lung selectivity. The lung-selectivity-index (LSI), which is the ratio between the antisialagogue and bronchoprotective potencies, serves as a sensitive preclinical functional measure of tolerability with respect to dry mouth. In our studies, we showed a 9-fold decrease in the LSI of tiotropium upon repeat dosing due largely to the potentiation of its antisialagogue effect. This observation appears consistent with findings reported by van Noord and colleagues who suggested that the clinical incidence of dry mouth with tiotropium is also delayed. In their study, the median onset of dry mouth occurred 4 weeks after starting treatment with tiotropium (van Noord, et al., 2000). In our models, glycopyrronium trended towards a narrowing of its LSI after repeat dosing but, unlike tiotropium, this was due to a significant decrease in bronchoprotective potency after repeat dosing. Thus, TD-4208 appears differentiated from the two drugs in that neither its bronchoprotective nor antisialagogue effects were influenced by repeat dosing. Thus, the LSI of TD-4208 was maintained and significantly greater than that of either tiotropium or glycopyrronium after repeated exposures.

To gain insight on the observed differences in LSIs between the LAMAs, we explored the concentration-effect relationships of both pharmacodynamic endpoints. Combined tissue concentration data from the two dosing regimens showed that the bronchoprotective and antisialagogue effects of TD-4208, tiotropium and glycopyrronium correlated well with compound levels in the lung and SMG, respectively.

Thus, the enhancement in antisialagogue effect of tiotropium after repeat dosing was associated with corresponding increases in concentrations of tiotropium in SMG over time. Although the origin of drug levels in SMG is not completely understood, it is likely to be from systemic drug levels as opposed to local deposition of the aerosol, since rats are obligate nose breathers and will breathe from the mouth only when the nose is completely blocked (Schulz and Muhle H., 2000). Thus, although one cannot completely rule out that some proportion of the drug may be delivered to the SMG via a local rather than systemic route, a plausible mechanism for the greater increase in tiotropium concentration in SMG is through systemic exposure driven by its pharmacokinetic properties which includes high bioavailability following local administration to the lung, large volume of distribution (Leusch, et al., 2001) and a long terminal half-life (Tiotropium NDA-21-395, 2003), all of which could lead to preferential distribution to the SMG. Taken together, these data confirm that the pharmacodynamic readouts of bronchoprotection and antisialagogue effects correlate well with concentrations of the compounds in the respective target tissues and that the greater pharmacodynamic lung selectivity of TD-4208 is in line with this pharmacokinetic-pharmacodynamic relationship. If this profile translates in clinical settings, then TD-4208 would be expected to produce a lower incidence of dry mouth and potentially other systemic adverse effects following chronic administration. In addition to dry mouth, central nervous system adverse effects, including cognitive dysfunction, can be a liability of antimuscarinic drugs. Although CNS penetration of TD-4208 was not assessed in the present study, one may infer indirectly that the weak antisialagogue activity of TD-4208 is also suggestive of weak CNS activity since the latter is related to systemic exposure.

In summary, we showed that TD-4208, a novel muscarinic antagonist with *in vitro* kinetic selectivity for muscarinic M_3 over M_2 receptors, provides bronchoprotection in both rats and dogs with duration of effect consistent with once daily dosing. Moreover, TD-4208 achieves its bronchoprotective effects with superior functional lung selectivity compared to either tiotropium or glycopyrronium after repeat dosing. The long pharmacodynamic duration and lung selectivity of TD-4208 results from its long M_3 receptor residence time, maintained bronchoprotective potency after repeat dosing and its unique pharmacokinetic properties that allow preferential localization in the lung while maintaining low concentrations in systemic tissues such as the salivary gland. A more comprehensive characterization of the *in vitro* pharmacological and pharmacokinetic properties of TD-4208 will be the topic of separate manuscripts. Nonetheless, the preclinical *in vivo* pharmacological profile of TD-4208 is distinct from tiotropium and glycopyrronium and, as such, suggests this new agent may possess attributes that extend the clinical utility of the LAMA class of compounds. In a recently completed phase 2 clinical trial in patients with moderate to severe COPD, TD-4208 was well tolerated and demonstrated sustained bronchodilation over a 24 hr period post-dose (Potgieter, et al., 2012). Based on its preclinical profile and available clinical data, TD-4208 warrants further clinical development for the once-daily treatment of COPD and other respiratory indications.

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Authorship contributions:

Participated in research design: Pulido-Rios, Hegde, McNamara, Martin.

Conducted experiments: McNamara, Obedencio.

Contributed new reagents or analytic tools: Ji.

Performed data analysis: Pulido-Rios, McNamara, Obedencio, Jaw-Tsai.

Wrote or contributed to the writing of the manuscript: Pulido-Rios, Martin, Hegde, Obedencio.

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Legends for Figures

Figure 1. Chemical structure of TD-4208

Figure 2. Bronchoprotective effect of TD-4208 (A), tiotropium (B) and glycopyrronium (C) over a 24 hr period after a single inhaled dose in anesthetized dogs. Data points represent mean values ± S.E.M., n = 4 per dose for all treatments except for 30 µg/ml TD-4208, n = 2. Statistical differences between two groups (*) was determined by two-way ANOVA with post- hoc Bonferroni's test. A p value of <0.05 was considered significant (*); ns refers to $p>0.05$.

Figure 3. Bronchoprotective (24 h) and antisialagogue (1 h) effects of inhaled TD-4208 after either single dosing (A) or seven-day repeat dosing (B) in rats. Data points represent mean values ± S.E.M., n = 5 to 12 for bronchoprotective single dosing, n = 6 to 12 for bronchoprotective repeat dosing, n = 5 to 10 antisialagogue single dosing and n = 6 for antisialagogue repeat dosing.

Figure 4. Bronchoprotective (24 h) and antisialagogue (6 hr and 1 h) effects of inhaled tiotropium after either single dosing (A) or seven-day repeat dosing (B) in rats. Data points represent mean values ± S.E.M., n = 6 to 12 for bronchoprotective single and repeat dosing, n = 6 to 12 for antisialagogue single dosing (1 hr), n = 5 to 10 antisialagogue single dosing (6 hr) and n = 6 for antisialagogue repeat dosing.

Figure 5. Bronchoprotective (24 h) and antisialagogue (1 h) effects of inhaled glycopyrronium after either single dosing (A) or seven-day repeat dosing (B) in rats. Data points represent mean values ± S.E.M., n = 6 to 12 for bronchoprotective single and repeat dosing, n = 5 to 6 for antisialagogue single dosing and n = 6 for antisialagogue repeat dosing.

Figure 6. Concentration-effect relationships of TD-4208 concentrations in rat lung and bronchoprotective effect after single and seven-day repeat dosing (A) and of TD-4208 concentrations in rat submaxillary gland (SMG) and antisialagogue effect after single and seven-day repeat dosing (B). Each data point represents an individual animal.

Figure 7. Concentration-effect relationships of tiotropium concentrations in rat lung and bronchoprotective effect after single and seven-day repeat dosing (A) and of tiotropium concentrations in rat submaxillary gland (SMG) and antisialagogue effect after single and seven-day repeat dosing (B). Each data point represents an individual animal.

Figure 8. Concentration-effect relationships of glycopyrronium concentrations in rat lung and bronchoprotective effect after single and seven-day repeat dosing (A), glycopyrronium concentrations in rat submaxillary gland (SMG) after single and seven-day repeat dosing (B). Each data point represents an individual animal.

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Table 1

Mean doses of acetylcholine producing a doubling of baseline pulmonary resistance after assignment to the different treatment groups in the dog study. Data represent mean values (95% confidence interval), n = 4 per treatment group.

Compound	Dose of Acetylcholine (µg/kg, IV)
Vehicle	57.5 (27.2 – 172.9)
TD-4208	44.3 (25.9 – 82.3)
Tiotropium	52.2 (29.4 – 111.9)
Glycopyrronium	47.2 (31.3 – 75.4)

Table 2

Bronchoprotective and antisialagogue potency and lung selectivity index (LSI) of inhaled TD-4208, tiotropium and glycopyrronium after either single dosing or seven- repeat dosing in anesthetized rats. Data represent mean values (95% confidence interval), n = 2-4.

Compound	***In vivo* Potency ID_{50} (μg/mL, IH)**				**Lung Selectivity Index (LSI)**	
	Bronchoprotection (24 h)		**Antisialagogue (1 h)[a]**			
	Single dose	**Repeat dose**	**Single dose**	**Repeat dose**	**Single dose**	**Repeat dose**
TD-4208	45.0 (34.6 - 58.7)	36.0 (24.4 - 53.2)	1164.0 (881.2 - 1538.0)	794.0	26 (15 - 45)	22 (15 - 33)
Tiotropium	3.2 (2.7 – 3.8)	3.7 (2.6 – 5.2)	168.1 (131.1 – 215.7) 87.0 (6 hr) (59.5 - 127.3)	11.4 38.0 (6 hr)	27 (6 hr) (17 – 47)	3 (2 - 5)
Glycopyrronium	52.9 (43.1 – 65.0)	325.8 (77.6 – 1369)	228.2 (101.3 – 514.1)	384.2 (198.3 – 744.4)	4	1

[a]All antisialagogue studies were conducted 1 hr after dosing except otherwise indicated.

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Table 3

Time course of antisisalagogue effect of inhaled TD-4208, tiotropium and glycopyrronium. Data represent mean values ± S.E.M. (n-value).

Compound	Dose (µg/mL, IH)	Inhibition of pilocarpine (%)		
		1 hr post dose	6 hr post dose	24 hr post dose
TD-4208	3000	81 ± 2 (6)	57 ± 4 (6)	32 ± 8 (6)
Tiotropium	100	28 ± 4 (6)	64 ± 3 (5)	45 ± 8 (6)
Glycopyrronium	1000	90 ± 2 (5)	78 ± 5 (6)	51 ± 7 (6)

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Figure 1

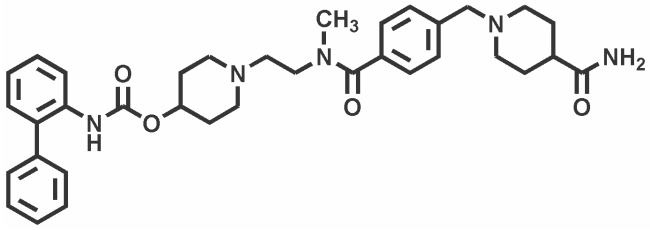

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Figure 2

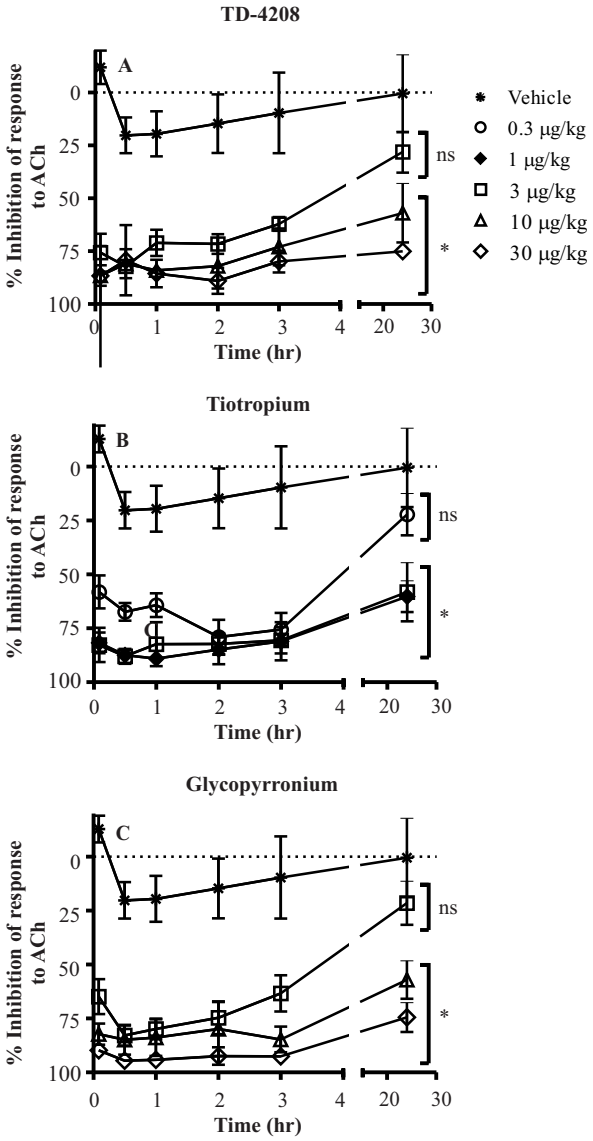

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Figure 3

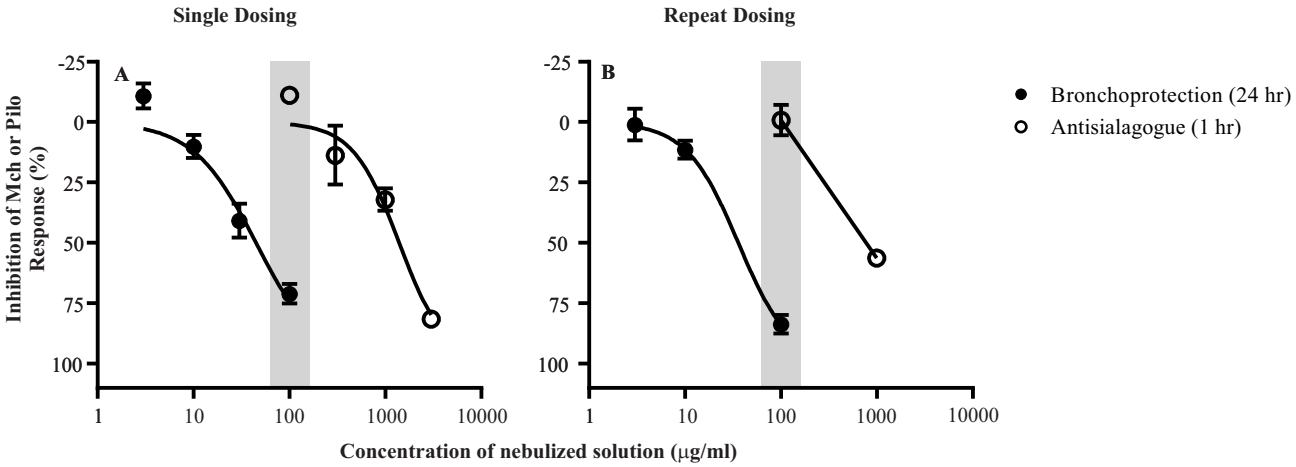

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Figure 4

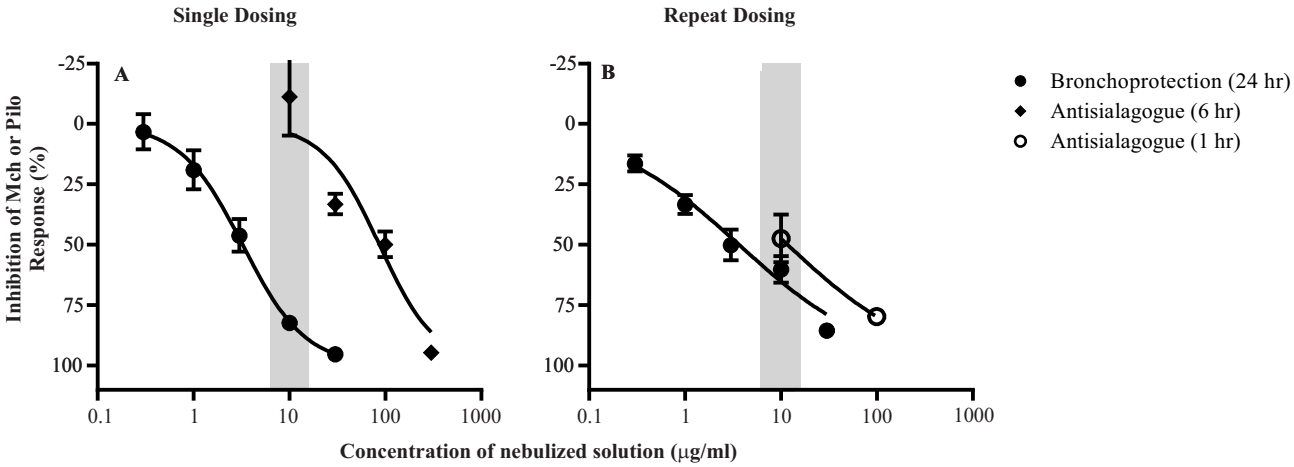

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Figure 5

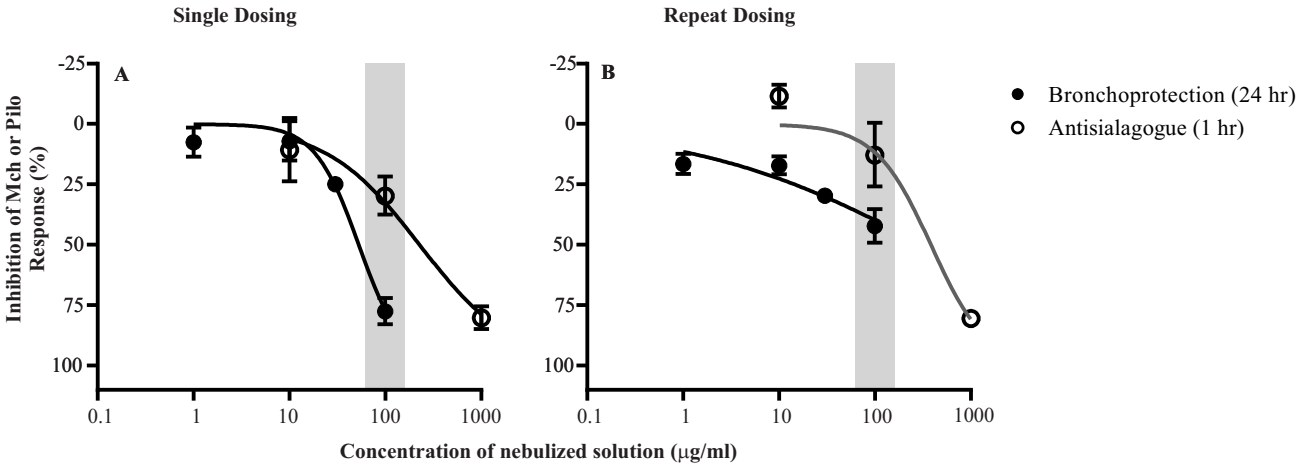

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Figure 6

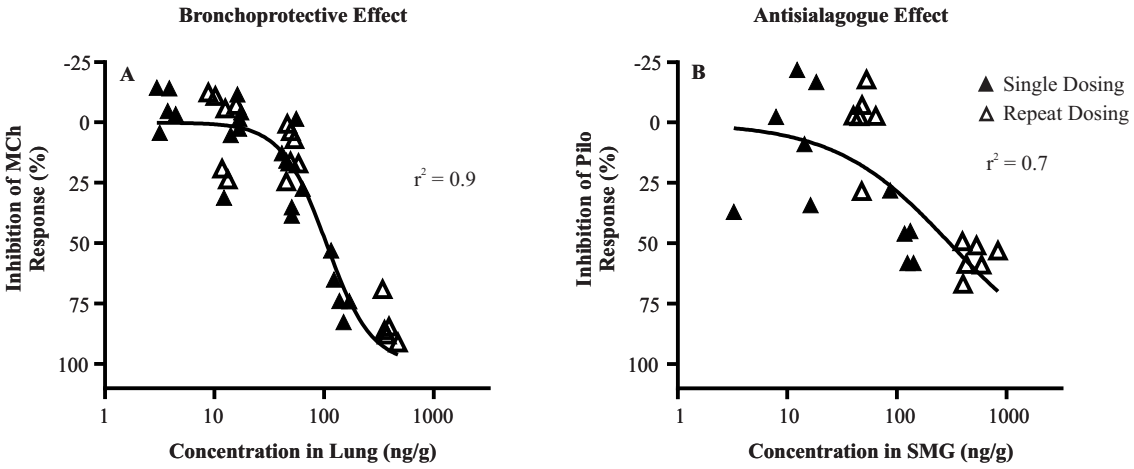

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Figure 7

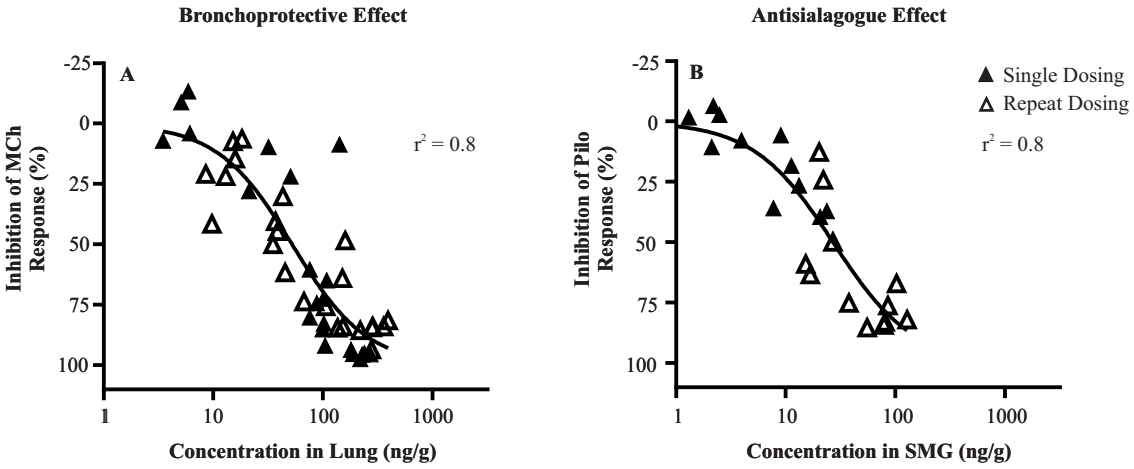

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Figure 8