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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ABSTRACT

Tiotropium is currently the only once-daily, long-acting muscarinic antagonist (LAMA) approved in the United States and other countries for the treatment of chronic obstructive pulmonary disease (COPD). Glycopyrronium has shown promise as a LAMA and was recently approved for once-daily maintenance treatment of 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]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-induced bronchoconstriction for up to 24 hours. In anesthetized rats, inhaled TD-4208 exhibited dose-dependent 24-hour bronchoprotection against methacholine-induced bronchoconstriction. The estimated 24-hour potency (expressed as concentration of dosing solution) was 45.0 μg/ml. The bronchoprotective potencies of TD-4208 and tiotropium were maintained after 7 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 with 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 (Rabe et al., 2007; Viegi 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 β₂-agonists or muscarinic antagonists (Qaseem et al., 2007; Vestbo et al., 2013). 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., 1989; 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, 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 (M₁, M₂, and M₃) 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 M₁ and M₃ muscarinic receptors (Bloom et al., 1987; Barnes, 1992, 1993).

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By contrast, prejunctional activation of M2 autoreceptors inhibits excessive release of acetylcholine (ACh) from the vagus nerve. Thus, blockade of M2 muscarinic receptors increases ACh-mediated contractions and compromises the bronchodilatory actions of nonselective anticholinergics (Barnes, 1993, 2004). Hence, anticholinergic drugs that preferentially antagonize M3, and potentially M1, receptors, should demonstrate improved efficacy compared with nonselective muscarinic receptor antagonists.

Tiotropium, currently the only once-daily, long-acting muscarinic antagonist (LAMA) approved for treatment of COPD in the United States and other countries worldwide, exhibits in vitro kinetic selectivity for M1 and M3 over the M2 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, 2002; Vincken et al., 2002; Tashkin et al., 2005). Although tiotropium is regarded as effective and generally safe (Barr et al., 2006; Oba et al., 2008; Tashkin 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, which was the most common adverse event in patients treated with tiotropium (Kesten et al., 2006, 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, 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 gland (Koumis and Samuel, 2005). Because salivation is likely mediated by activation of M1 and M3 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-carbamoylperidin-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 M3 receptor, and demonstrates in vitro kinetic selectivity for M3 over M2 muscarinic receptor subtype (Steinfeld et al., 2009) and no meaningful off-target activity (unpublished data). TD-4208 was designed to produce a 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 with tiotropium and glycopyrronium.

First, we determined the bronchoprotective potency and duration of action of TD-4208 in dogs and rats. Second, by determining its potency to antagonize systemic muscarinic M3 and M1 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 at the Lovelace Respiratory Research Institute (Albuquerque, NM). 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-hour 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; Harlan Laboratories, Madison, WI) 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 administration of a mixture of xylazine (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 hours. 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 and 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) and connected to a dual-phase respirator pump that was set at 3.0 l/min. This canine inhalation exposure system generates particle sizes with a median diameter of approximately 2–4 μ to ensure respirability of the aerosol (Johnson, 1989). Approximately 30 minutes before inhalation treatment with test article, increasing doses of ACh (1–1000 μg/kg i.v.) 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 the animal). At different time points after inhalation (5, 30, 60, 90, 120, 150, and 180 minutes postinhalation), the bronchoconstrictor response to ACh was re-evaluated. At 180 minutes postdosing, animals were allowed to recover from anesthesia, returned to their kennels, and fed. At 24 hours postdose, the animals were reanesthetized and instrumented. Persistence of bronchoprotective effect was evaluated by assessing the bronchoconstrictor response to ACh. Heart rate, blood pressure, O2 saturation, and body temperature were monitored throughout the experiment. Heart rate changes were analyzed during the period of 3 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, and 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). Bronchoprotective effects of test compounds were expressed as percent inhibition of pretreatment ACh–induced increase in pulmonary resistance. Statistical comparisons were performed using repeated-measures two-way analysis of variance (ANOVA) with Bonferroni post-test, where a value of $P < 0.05$ was considered significant (GraphPad Prism, La Jolla, CA). In addition, a 24-hour 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. $ID_{50}$ 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 acclimated 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-hour 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-minute period in a whole-body inhalation chamber (R+M 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.) driven by Biobland (5% CO2/85% atmospheric air; Praxair, Pittsburgh, PA) 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 7-day repeat-dosing regimen, animals were dosed every 24 hours 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 injection of thiobutabarbital (Inactin, 120 mg/kg i.p.). A supplemental dose of 40 mg/kg i.p. was given if animals were responsive to a physical stimulus (e.g., toe pinch) after the first dose. When complete anesthesia was achieved, as confirmed by the absence of response to a toe pinch stimulus, surgery was performed according to either protocol described later. For all studies conducted under anesthesia, body temperature was maintained at 37°C using a heating pad. Bronchoprotection was assessed in rats using the Einsthoven model of methacholine (MCh)-induced bronchoconstriction (McPhard et al., 2011). With rats under complete anesthesia, the jugular vein and trachea were catheterized. Each animal was ventilated using a respirator (model 683; Harvard Apparatus, Inc., Holliston, MA) 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 preamplifier (TSD 137C; BIOPAC, Goleta, CA). Changes in VP were recorded using the Acknowledge Data Collection Software (BIOPAC). Stable baseline VP was collected for at least 2.5 minutes, and then rats were challenged with noncumulative infusions of MCh (40 and 80 µg/kg i.v.) for 2.5 minutes 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 percent inhibition of peak MCh–induced bronchoconstriction at the 40 µg/kg i.v. dose (i.e., $ED_{50}$ dose) in the test compound treated versus 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). $ID_{50}$ was estimated as the concentration of nebulized solution of test compound that inhibited the MCh $ED_{50}$ 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 preweighed gauze pad was inserted into the animals’ mouth and the muscarinic agonist Pilo (3 mg/kg) was administered intravenously. Saliva produced for 10 minutes after Pilo injection was measured gravimetrically by determining the weight of the gauze pad before and after Pilo. The percent inhibition of Pilo-induced salivary 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 salivary response, was determined.

**Study 1: Evaluation of Bronchoprotective and Antisialagogue Effects after a Single Dose (Single Dosing).** To determine the bronchoprotective and antisialagogue potency after a single dose, rats were exposed by inhalation to a nebulized solution of TD-4208 (3–3000 µg/ml), tiotropium (0.3–300 µg/ml), glycopyrronium (1–1000 µg/ml), or vehicle (sterile water) as described earlier. Bronchoprotective activity was assessed 24 hours after the last dose. For the antisialagogue effect, inhibition of Pilo was assessed 1, 6, or 12 hours 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 Bronchoprotective 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 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 hours and 1 hour, 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 antisialagogue assays. Blood, collected via the inferior vena cava under CO2 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 g, 10 minutes, 4°C), and the supernatant was stored at −80°C until analysis. Lung and SMG were homogenized in 3 times the volume of phosphate-buffered saline 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 acetonitrile 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. TD-4208 samples were analyzed using a Hypurity C18 column (100 × 2.1 mm; 3 µM; ThermoFisher Scientific, Inc., Waltham, MA), and tiotropium and glycopyrronium samples were analyzed using a Betabasic C18 column (50 × 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 minutes followed by a 3.5-minute gradient to

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45% mobile phase B. For tiotropium chromatography, the gradient started from 2 to 10% mobile phase B in 0.5 minutes followed by a 3.5-minute gradient to 60% mobile phase B. For glycopyrronium, the chromatography used a gradient from 5 to 95% mobile phase B in 3.2 minutes. The sample injection volume was 25 μl for TD-4208 and tiotropium, and 20 μl for glycopyrronium. The mass spectrometer (API5000; AB Sciex, Framingham, MA) was operated in positive ion multiple reaction monitoring mode.

Data Analysis. Data were expressed as the mean ± S.E.M. or the mean with 95% confidence intervals. Statistical differences between two or more groups were determined by Student’s t test (paired and unpaired) or two-way analysis of variance using post-hoc Bonferroni test. A *P* value set at 0.05.

### Results

**Potency and Duration of Action in Dogs.** Before 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 [F3,73 = 88.10, *P* < 0.0001] and time [F5,73 = 4.52, *P* = 0.001] effects. The onset of bronchoprotection was determined during the first 2 hours post-treatment. All three doses of TD-4208 (3, 10, and 30 μg/kg) produced greater than 75% inhibition of ACh-induced bronchoconstriction 5 minutes 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 minutes at all doses tested. The duration of bronchoprotection produced by the 3 μg/kg dose decreased from 76 ± 9 to 28 ± 10% by 24 hours; however, bronchoprotection was maintained for at least 24 hours following the two highest doses (57 ± 14% for 10 μg/kg and 83 ± 4% for 30 μg/kg; *P* < 0.05 for both doses compared with 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 three doses of tiotropium, revealed significant treatment [F3,73 = 81.03, *P* < 0.0001] and time [F5,73 = 6.57, *P* < 0.001] effects. Tiotropium inhibited ACh-induced bronchospasm by 58 ± 8% (0.3 μg/kg), 82 ± 5% (1 μg/kg), and 83 ± 8% (3 μg/kg) 5 minutes 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 hours after inhalation treatment [i.e., showed nonoverlapping S.E.M.s with bronchoprotection at 5 minutes (58 ± 8%, *n* = 4)]. This indicates a slower onset of effect for the 0.3 μg/kg dose. Significant 24-hour activity was maintained for 1- and 3 μg/kg doses (60 ± 7 and 58 ± 14%, respectively; *P* < 0.001 for both compared with vehicle) but not for the lowest dose of 0.3 μg/kg (22 ± 10%).

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 three doses of glycopyrronium, revealed significant treatment [F3,73 = 98.35, 1000:244 Pulido-Rios et al.](110x80)
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-hour 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 hour 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 versus 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-hour bronchoprotective potency of tiotropium after a single dose (ID_{50} = 3.2 μg/ml) was not significantly different from its 24-hour 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 hours, and observed that the peak antisialagogue effect occurred 6 hours 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 hours after inhalation. The antisialagogue effects of tiotropium at 1 hour post-treatment were dose dependent, with an estimated

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Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>In Vivo Potency ID_{50} (μg/ml)</th>
<th>LSI</th>
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</thead>
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<tr>
<td></td>
<td>Bronchoprotection (24 h)</td>
<td>Antisialagogue (1 h)</td>
</tr>
<tr>
<td></td>
<td>Single Dose</td>
<td>Repeat Dose</td>
</tr>
<tr>
<td>TD-4208</td>
<td>45.0 (34.6–58.7)</td>
<td>36.0 (24.4–53.2)</td>
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<tr>
<td>Tiotropium</td>
<td>3.2 (2.7–3.8)</td>
<td>3.7 (2.6–5.2)</td>
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<tr>
<td>Glycopyrronium</td>
<td>52.9 (43.1–65.0)</td>
<td>325.8 (77.6–1369)</td>
</tr>
</tbody>
</table>

IH, inhalation.

* All antisialagogue studies were conducted 1 hour after dosing unless otherwise indicated.

** Study conducted 6 hours after dosing.
potency of 168.1 µg/ml. The antisialagogue activity of tiotropium increased at the 6-hour time point, with an estimated potency of 87.0 µg/ml. After 7 days of dosing, tiotropium inhibited Pilo-induced salivation more potently than after a single dose at both the 1-hour (ID$_{50}$ = 11.4 µg/ml) and 6-hour (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. 4A), but inhibited salivation by 47 ± 10% after seven repeat doses (Fig. 4B). 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 at a dose-dependent manner. The 24-hour bronchoprotective potency of glycopyrronium after a single inhale 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 hour after dosing (Table 3). At this time point, 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. 5, A and B). 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 the 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 the 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 and 0.87 ± 0.20 ng/ml after single doses of 100 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).

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). In 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 and 0.19 ± 0.05 ng/ml after single doses of 10 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).

**TABLE 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (IH)</th>
<th>1 h Postdose</th>
<th>6 h Postdose</th>
<th>24 h Postdose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TD-4208</td>
<td>3000</td>
<td>81 ± 2 (6)</td>
<td>57 ± 4 (6)</td>
<td>32 ± 8 (6)</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>100</td>
<td>28 ± 4 (6)</td>
<td>64 ± 3 (5)</td>
<td>45 ± 8 (6)</td>
</tr>
<tr>
<td>Glycopyrronium</td>
<td>1000</td>
<td>90 ± 2 (5)</td>
<td>78 ± 5 (6)</td>
<td>51 ± 7 (6)</td>
</tr>
</tbody>
</table>

IH, inhalation.
Lung and SMG concentrations of glycopyrronium also correlated ($r^2 = 0.6$ and 0.5, respectively) with its broncho-protective (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 $0.11 \pm 0.03$ and $1.28 \pm 0.34$ ng/ml after a single dose of 100 and 1000 μg/ml, respectively. Blood levels of glycopyrronium after repeat dosing were 1.6- and 2.7-fold compared with 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 sustained bronchoprotective activity up to 24 hours after dosing. The magnitude and duration of its bronchoprotective effect were similar to that of tiotropium and glycopyrronium. Moreover, after 7-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 hours (the last time point measured). Previous in vivo duration studies in dogs showed that tiotropium produced long-lasting (>6 to >24 hours) bronchoprotection against ACh provocation (Disse et al., 1993; Casarosa et al., 2009; Gavalda et al., 2009). We confirmed the 24-hour 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 hours postdose (Casarosa et al., 2009), we showed that the bronchodilatory effect of glycopyrronium was similar to tiotropium and was sustained for 24 hours. Although differences in our methodologies could

![Fig. 5. Bronchoprotective (24 hours) and antisialagogue (1 hour) effects of inhaled glycopyrronium after either single dosing (A) or 7-day repeat dosing (B) in rats. Data points represent the mean ± S.E.M.; n = 6–12 for bronchoprotective single and repeat dosing, n = 5–6 for antisialagogue single dosing, and n = 6 for antisialagogue repeat dosing.](image)

![Fig. 6. Concentration-effect relationships of TD-4208 concentrations in rat lung and bronchoprotective effect after single and 7-day repeat dosing (A) and of TD-4208 concentrations in rat SMG and antisialagogue effect after single and 7-day repeat dosing (B). Each data point represents an individual animal.](image)
have accounted for the apparent discrepancy, our findings are consistent with the sustained lung muscarinic receptor binding effects of glycopyrronium 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 asthma (Hansel et al., 2005). In dogs, the 24-hour 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 M3 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 ($t_{\text{max}} = 2$ hours) 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., 2011). In the current study, tiotropium (10 $\mu$g/ml) produced significant bronchoprotection in rats for up to 24 hours after a single administration. TD-4208 and tiotropium maintained their bronchoprotective potency after 7 days of repeat dosing. Based on clinical data, the bronchodilator effect of tiotropium reaches pharmacodynamic steady state within 48 hours 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 1 day of dosing and was maintained without loss of activity for up to 7 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 M2 and M3 receptors are similar for the three compounds (Haddad et al., 1994, 1999; Steinfeld et al., 2009). This phenomenon may be either species-specific for glycopyrronium activity in the rat or a consequence of negative feedback mechanisms from blockade of M2 autoreceptors at the highest dose tested (Aas and

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

Fig. 8. Concentration-effect relationships of glycopyrronium concentrations in rat lung and bronchoprotective effect after single and 7-day repeat dosing (A) and glycopyrronium concentrations in rat SMG after single and 7-day repeat dosing (B). Each data point represents an individual animal.
Maclagan, 1990; Celli, 2004). Although it is likely that postjunctional M₃ receptor antagonism drives the bronchoprotective activity of the three compounds in rats and dogs, the possible involvement of postjunctional M₂ receptors cannot be excluded given that this receptor can also cause contraction of airway tissues through direct and indirect mechanisms (Hirshman et al., 1999; Sarria et al., 2002).

In addition to pulmonary actions, antimuscarinics can also antagonize muscarinic M₁/M₃ 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 antisialorrhoeic and preoperative antisecretory agent (Jongerius et al., 2003). Although the therapeutic window of an antimuscarinic bronchodilator improves significantly by topical delivery to the lung, by inhalation, compared with 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 rats to provide a measure of lung selectivity. The 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 (2000) 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 toward 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 the SMG over time. Although the origin of drug levels in the 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, 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 the 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 continuous administration. In addition to dry mouth, central nervous system adverse effects, including cognitive dysfunction, can be a liability of antimuscarinic drugs. Although central nervous system 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 central nervous system 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 M₃ over M₂ 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 with either tiotropium or glycopyrronium after repeat dosing. The long pharmacodynamic duration and lung selectivity of TD-4208 results from its long M₃ 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 that of 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-hour period postdose (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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Contributed new reagents or analytic tools: Ji.
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Wrote or contributed to the writing of the manuscript: Pulido-Rios, Martin, Hegde, Obedencio.

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