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ORIGINAL ARTICLE

Pharmacological properties of revefenacin (TD-4208), a novel, nebulized long-acting, and lung selective muscarinic antagonist, at human recombinant muscarinic receptors and in rat, guinea pig, and human isolated airway tissues

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Abstract

Revefenacin (TD-4208) is a novel, long-acting, and lung-selective muscarinic cholinergic receptor (mAChR) antagonist in development as a nebulized inhalation solution for the treatment of chronic obstructive pulmonary disease (COPD) patients. This study evaluated the pharmacology of revefenacin at human recombinant mAChRs and in airway tissues from rats, guinea pigs, and humans. At human recombinant mAChRs, revefenacin displayed high affinity (pK_I = 8.2-9.8) and behaved as a competitive antagonist (pKI, apparent = 9.4-10.9) at the five human recombinant mAChRs. Kinetic studies demonstrated that revefenacin dissociated significantly slower from the hM_3 ($t_{1/2}$ = 82 minutes) compared to the hM_2 ($t_{1/2}$ = 6.9 minutes) mAChR at 37°C, thereby making it kinetically selective for the former subtype. Similarly, in functional studies, revefenacin-mediated antagonism of acetylcholine (ACh)-evoked calcium mobilization responses were reversed less rapidly at hM_3 compared to the hM_2 mAChR. In isolated tracheal tissues from rat and guinea pig and isolated bronchial tissues from humans, revefenacin potently antagonized mAChR-mediated contractile responses. Furthermore, the antagonistic effects of revefenacin in rat, guinea pig, and human airway tissues were slowly reversible ($t_{1/2}$ of 13.3, >16, and >10 hours, respectively). These data demonstrate that revefenacin is a potent, high affinity, and selective functional mAChR antagonist with kinetic selectivity for the hM_3 receptor and produces potent and long-lasting antagonism of mAChR-mediated contractile responses in rat, guinea pig, and human airway tissue. These data

Abbreviations: ACh, acetylcholine; BA, beta2 agonist; CHO, chinese hamster ovary; COPD, chronic obstructive pulmonary disease; EFS, electrical field stimulated; FLIPR, fluorometric imaging plate reader; LAMA, long-acting muscarinic antagonist; mACh, muscarinic acetylcholine cholinergic receptor; MA, muscarinic antagonist; NMS, *N*-methyl scopolamine; $T_{1/2}$, antagonist offset time or time required for contractile response to recover by 50%; $t_{1/2}$, dissociation rate half-life; TD-4208, biphenyl-2-ylcarbamic acid 1-(2-[[4-(4-carbamoylpiperidin-1-ylmethyl)benzoyl] methylamino}ethyl)piperidin-4-yl ester.

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suggest that revefenacin has the potential to be a potent once-daily dosed inhaled bronchodilator in COPD patients.

KEYWORDS
Bronchodilator, COPD, LAMA, muscarinic, revefenacin, TD-4208

1 | INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease characterized by progressive and persistent airflow limitation and is symptomatically manifested as dyspnea, chronic cough, and increased sputum production.[1] Tobacco smoking is the most common cause of COPD, with a number of other factors such as air pollution and occupational chemical hazards playing a minor role. COPD is currently one of the leading causes of death in the United States and the rest of the world.[1]

The goal of pharmacological therapy in COPD is to alleviate symptoms, reduce the frequency and severity of exacerbations, and improve health status and exercise tolerance.[2] Inhaled bronchodilators, either mAChR antagonists (MA) or β_2 adrenoceptor agonists (BA), dosed alone or in combination with inhaled corticosteroids serve as the mainstay of COPD pharmacotherapy.[2] Inhaled MA evoke bronchodilation by suppressing the elevated cholinergic bronchoconstrictor tone mediated by mAChRs localized on parasympathetic ganglia and airway smooth muscle.[3] Based on duration of bronchodilation in patients, inhaled MA are classified as either short-acting muscarinic antagonists (SAMAs) or long-acting muscarinic antagonists (LAMAs).[4] SAMAs, which include molecules such as ipratropium, are dosed three to four times daily. LAMAs include twice-daily dosed drugs, such as aclidinium and glycopyrronium, or once-daily dosed drugs such as tiotropium and umeclidinium.

Inhaled therapies for COPD are marketed as either dry-powder inhalers, pressurized metered dose inhalers or nebulizers. Although nebulizers can be less portable than hand-held inhalers, they are the preferred delivery device for a proportion of COPD patients who have severe disease, frequent exacerbations, those with physical and/or cognitive limitations [5] and those with compromised peak inspiratory flow rates.[6] At present, a once-daily dosed nebulized LAMA is not commercially available for COPD patients.

Revefenacin (TD-4208; biphenyl-2-ylcarbamic acid 1-(2-{[4-(4-carbamoylpiperidin-1-ylmethyl)benzoyl]methylamino}ethyl)piperidin-4-yl ester) (Figure 1) is an investigational LAMA, in late-stage development as a nebulized inhalation solution, that was designed to produce long-acting bronchodilation, consistent with once-daily dosing, and with a high degree of lung-selectivity to avoid systemic antimuscarinic adverse effects such as dry mouth, urinary retention, and constipation.[7–10] Following inhalation dosing to rats and dogs, revefenacin produced potent protection against the bronchoconstrictor response to ACh or methacholine (MCh).[10] In both species, the bronchoprotective effect was long-acting (≥24 hours) and comparable in duration to tiotropium. The functional lung-selectivity index of revefenacin in rats, derived from its relative potency to antagonize muscarinic bronchoconstrictor versus sialagogue effects, was superior to that of tiotropium after either a single dose or seven repeated inhaled doses. Here, we report the in vitro pharmacological properties of revefenacin at recombinant mAChRs and in rat, guinea pig, and human airway tissues expressing native mAChRs. The findings from these translational studies are consistent with emerging clinical data showing 24 hours bronchodilatory activity of nebulized revefenacin in COPD patients.[7,9]

2 | MATERIALS AND METHODS

2.1 | Materials

[*N*-methyl-^{3}H]Scopolamine methyl chloride ([^{3}H]NMS; specific activity 82 Ci mmol^{-1}) was obtained from GE Healthcare (Piscataway, NJ). Atropine, acetylcholine (ACh), carbachol (CCh), and oxotremorine-M were purchased from Sigma Chemical Co. (St. Louis, MO). Revefenacin (Figure 1) tiotropium, ipratropium, and glycopyrrolate were prepared at Theravance Biopharma (South San Francisco, CA); tritium labeling of these compounds was performed at ViTrax (Placentia, CA). Sprague-Dawley rats and Dunkin-Hartley guinea pigs were obtained from Harlan (Livermore, CA). Fresh human lung from organ donors were obtained from the National Disease Research Interchange (Philadelphia, PA).

2.2 | Studies with animals and human tissue

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Theravance Biopharma US, Inc. (South San Francisco, CA) and were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals. Human biological samples were sourced ethically and their

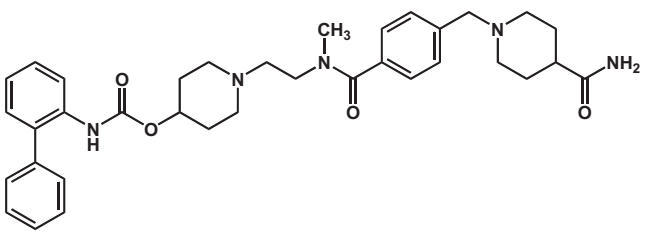

FIGURE 1 Revefenacin (TD-4208; biphenyl 2 ylcarbamic acid 1-(2-{[4 -4 carbamoylpiperidin-1-ylmethyl)benzoyl]methylamino}ethyl) piperidin-4-yl ester)

research use was in accord with the terms of the informed consents. All procedures with human tissue were performed in accredited facilities in accordance with Universal Precautions for Handling Human Blood, Body Fluids, and Tissue.

2.3 | Competition radioligand binding studies at human hM_1, hM_2, hM_3, hM_4, and hM_5 mAChRs

These studies were conducted using Chinese hamster ovary (CHO)-K1 cell membrane fractions stably expressing human recombinant M1, M2, M3, M4, or M5 mAChRs. Assays were conducted with 1 nmol·L^{-1} [^{3}H]NMS in a 10 mmol·L^{-1} HEPES buffer containing 100 mmol·L^{-1} NaCl, 10 mmol·L^{-1} $MgCl_2$, and 0.025% bovine serum albumin, pH 7.4 at 37°C. Nonspecific binding was defined in the presence of 10 μmol L^{-1} atropine.

2.4 | [^{3}H]Revefenacin saturation binding to human hM_2 or hM_3 mAChRs

CHO-K1 cell membrane fractions expressing human recombinant M_2 or M_3 mAChRs were incubated with [^{3}H]revefenacin (18.5 Ci·$mmol^{-1}$), [^{3}H]glycopyrrolate (70 Ci·$mmol^{-1}$), for 1 hours at 37°C or [^{3}H]tiotropium (70 Ci·$mmol^{-1}$) for 4 hours at 37°C. Nonspecific binding was defined in the presence of 10 μmol L^{-1} atropine.

2.5 | Dissociation and association binding kinetics at human hM_2 and hM_3 mAChRs

Association and dissociation binding kinetic parameters for [^{3}H]revefenacin and other radiolabeled mAChR antagonists at hM_2 or hM_3 receptors were determined by filtration radioligand binding techniques. To determine dissociation rates and half-life ($t_{1/2}$), membranes prepared from cells expressing hM_2 or hM_3 receptors were incubated with tritium-labeled compounds, followed by the addition of 10 μmol L^{-1} atropine at 37°C. To determine the association rate, tritium-labeled compounds and membranes prepared from cells expressing hM_2 or hM_3 receptors were incubated together for varying lengths of time at 37°C prior to rapid filtration. Specific binding was determined during the association phase by measurement of binding in the presence or absence of 10 μmol L^{-1} atropine.

2.6 | Functional inhibition of human (hM_1, hM_3, hM_4) and chimpanzee (cM_5)-mAChR stimulated intracellular calcium mobilization

CHO-K1 (Chinese hamster ovary-K1) cell lines stably expressing hM_1, hM_3, M_4-G16α and cM_5 mAChR subtypes, respectively, were grown to near confluency in medium consisting of HAM's F-12 supplemented with 10% FBS and 250 μg mL^{-1} Geneticin. The chimpanzee M_5 receptor was utilized as a surrogate of the human M_5 due to intellectual property restrictions surrounding use of the latter in cell-based studies. Cells were gently washed and treated for 40 minutes at 37°C with the membrane permeable, calcium sensitive dye, FLUO-4AM and, following a wash, were then incubated with increasing concentrations of revefenacin for 20 minutes at 37°C. The cells were stimulated with oxotremorine at a concentration required to elicit 90% of the maximal response (EC_{90}). The change in fluorescence was measured using a FLIPR®-Tetra.

2.7 | Inhibition of agonist-stimulated [^{35}S]GTPγS binding at human hM_2 mAChRs

To measure antagonism of hM_2 mAChR activation, membranes prepared from CHOK1 cells expressing the hM_2 receptor were treated with various concentrations of revefenacin or other antagonists. Assays were conducted using 25 μL assay buffer containing [35S]GTPγS and GDP, 25 μL membrane, and 25 μL assay buffer were transferred to the 96-well microtiter plates. The final concentration of [35S]GTPγS was 0.4 nmol·L^{-1} and of GDP was 3 μmol·L^{-1} The membranes were subsequently treated with the mAChR agonist oxotremorine (EC_{90} value); for 1 hour to activate the receptors, enhancing $G\alpha_i/G\alpha_o$ protein binding to exogenous [^{35}S]GTPγS. The assay plate was then incubated at room temperature for 60 minutes prior to filtration over 1% bovine serum albumin-pretreated glass fiber GF/B filtermats using a PerkinElmer 96-well harvester. The plates were rinsed with ice-cold wash buffer and then air or vacuum dried. 40 μL of Microscint-20 scintillation liquid was added to each well, and each plate was sealed and radioactivity determined on a TopCount (PerkinElmer, San Jose, CA) scintillation counter. Bound [^{35}S]GTPγS was captured and analyzed as described above.

2.8 | Reversibility of antagonism at recombinant human hM_2 (w/Gqi5) and hM_3 mAChRs

CHO-K1 cells expressing hM_2 (w/Gqi5) or hM_3 receptors were grown to confluence at 37°C in a humidified incubator containing 5% CO2/95% O2. Cells expressing the hM_2 receptor were cultured in DMEM/F12 media, supplemented with 200 μg mL^{-1} G418 (geneticin), and 10% fetal calf serum. Cells containing hM_3 receptors were cultured in Alpha minimum essential medium (MEM) (Gibco, Green Island, NY) with nucleosides, L-glutamine, and 10% bovine calf serum.

Intracellular calcium mobilization was determined using a microtiter plate based calcium mobilization FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices, Sunnyvale CA,[11]) assay. On the day prior to assay, cells were plated with culture media in 96 well, black-wall, clear bottom plates (BD Biosciences) at a concentration of 40,000 cells per well and cultured at 37°C in a humidifier incubator with 5% CO2/95% air for 18-24 hours as described previously.[12]

Reversal of mAChR antagonism of calcium mobilization in CHO-K1 cells was determined by preincubation of cells with antagonist (0, 1.0, 10, 100 and 1000 nmol·L^{-1}) for 90 minutes. The cells were washed extensively over 180 minutes. ACh-induced concentration-response curves were then generated for each antagonist pretreatment condition. The potency of ACh for each pretreatment

condition was compared to the vehicle (DMSO) control to determine the magnitude of induced ACh potency shift post washout.

2.9 | Antagonism of acetylcholine-evoked contraction of rat isolated tracheal tissue: potency and offset time

Adult male Sprague-Dawley rats (200-250 g) were acclimated to their holding room for at least 1 week prior to any treatment. Rats were euthanized by CO_2 asphyxiation followed by thoracotomy. Trachea from each animal was rapidly excised, in <1 minutes following euthanasia, and placed in Krebs-Henseleit buffer (in mmol·L^{-1}: D-glucose, 10; $MgSO_4$, 1.6; $KHPO_4$, 1.2; KCl, 4.7; NaCl, 118; $NaHCO_3$, 24.9; $CaCl_2$, 2.5) containing indomethacin (1 μmol L^{-1}), aerated with 95% O2/5% CO2 gas mixture and maintained at 37°C. The trachea was sectioned into 5 mm rings and each ring was mounted in a tissue bath chamber filled with oxygenated Krebs-Henseleit buffer maintained at 37°C. The tissue was connected to a force transducer (Model 750TOBS, Danish Myo Technology, Ann Arbor, MI) for measurement of isometric tension. After 1 hour of equilibration at a resting tension of about 0.5 g, each tissue was repeatedly primed with ACh (0.1 mmol·L^{-1}) until a stable contractile response was achieved. Tissues were equilibrated for 2 hours with either vehicle or a predetermined concentration of test antagonist following which ACh was cumulatively added to the bath in half-log unit increments to generate a contractile concentration-response curve.

To assess the offset time of the test compound, tissues were primed with a submaximal concentration of ACh (EC_{80} = 3 μmol L^{-1}). Vehicle or a submaximal concentration (approximately IC_{90}) of test compound was added to each bath. After a 2 hours equilibration period, tissues were exposed to ACh (3 μmol L^{-1}) to determine the initial magnitude of antagonism of its contractile effect. This was followed by continuous perfusion of tissues with antagonist-free Krebs-Henseleit buffer at a rate of 2 mL·min^{-1} for up to 19 hours. Perfusion was stopped at predetermined time points to assess the recovery of the ACh contractile response.

2.10 | Inhibition of electrical-field-stimulated contraction of guinea pig isolated trachea: potency and offset time

Adult male Dunkin-Hartley guinea pigs (200-450 g) were euthanized by CO_2 asphyxiation followed by thoracotomy. The trachea was rapidly isolated, within 1 minute following euthanasia, and placed in Krebs buffer (in mmol·L^{-1}: D-glucose, 10; $MgSO_4$, 1.6; $KHPO_4$, 1.2; KCl, 4.7; NaCl, 118; $NaHCO_3$, 24.9; $CaCl_2$, 2.5) containing indomethacin, 10 μmol L^{-1}; choline, 1 μmol L^{-1} and guanethidine, 3 μmol L^{-1}, aerated with 95% O_2/5% CO_2 gas mixture and maintained at 37°C. Each tracheal ring was cut open and sutured using 4.0 silk thread. The tissue was positioned between 2 platinum electrodes and stimulated as follows: 1 msecond pulse duration, 10 pps pulse rate, 10 second train duration, 0.01 tps train rate, and 9 V (just maximal).

Concentration-response relationship was obtained by addition of test compound cumulatively in half log unit increments. At the end of the study, theophylline (2.2E-3 M) was added to induce maximum relaxation. Relaxation response from test compounds was normalized to theophylline.

For the washout study, a submaximal concentration (approximately IC_{90}) of test compound was added to the bath and allowed to equilibrate with the tissues. After the maximal inhibitory response was attained, the tissues were perfused (washed) with drug-free Krebs buffer (2 mL·min^{-1}) for 15-12 hours or until the inhibitory response was reversed completely. After 10 minutes of stopping perfusion, theophylline (2.2E 3M) was added to induce maximum relaxation.

2.11 | Antagonism of carbachol-evoked contraction of human isolated bronchial tissue: potency and offset time

Sections of bronchus were removed from fresh human lungs and cleaned of extraneous tissue. Bronchial strips of approximately 3-4 mm in width were prepared and placed into modified Krebs-Henseleit solution (in mmol·L^{-1}: NaCl, 113.0; KCl,4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0 and dextrose, 11.0) containing meclofenamic acid (1 μmol·L^{-1}) and equilibrated with 95% O_2/5% CO_2 and maintained at 37°C. Individual tissues were suspended via silk suture in either a superfusion chamber (superfusion studies) or a water-jacketed organ bath containing Krebs-Henseleit buffer (static studies) and connected to force transducers (Model TSD125C, BIOPAC Systems, Goleta, CA or Model FT03C, Grass Instruments, Austin, TX). Mechanical responses in both systems were recorded.

In static studies, paired tissues were exposed to either compound or vehicle for 120 minutes before cumulative concentration-response curves to CCh were generated. CCh-induced responses for each tissue were expressed as a percentage of histamine (1 mmol·L^{-1})-induced contraction obtained at the beginning of the experiment prior to addition of the antagonist.

In superfusion studies, the tissues were continuously superfused with Krebs-Henseleit solution at 2 mL·min^{-1} for the duration of the experiment. Stock solutions of agonist and antagonist were infused (0.02 mL·min^{-1}) via 22-gauge needle inserted into the superfusion tubing. The tissues were contracted with CCh (EC_{80} = 1 μmol L^{-1}) for the duration of the experiment. Upon reaching a sustained contraction isoproterenol (10 μmol·L^{-1}) was administered to maximally relax the tissue, and this change served as a reference. Isoproterenol exposure was halted and the carbachol-induced tension was allowed to recover. Test compounds were infused at a single concentration per tissue for a period of 6 hours until a sustained level of inhibition was attained. Infusion of compound was halted and the CCh-induced tension was allowed to recover for 10 hours. Antagonist-induced

inhibition of the CCh-induced contraction was expressed as a percent of the isoproterenol reference response.

2.12 | Off target activities

Revefenacin was evaluated in a panel of 81 receptors, enzymes, and channels at 1 $\mu mol \cdot L^{-1}$, a concentration that is >5000-fold over its affinity for the hM_3 receptor and the plasma exposure (C_{max}) at human efficacious doses of 88 and 175 μg.[13] The standard assays were performed at Cerep (Le Bois l'Evêque - B.P. 1 - 86600 Celle L'Evescault, France). Revefenacin was also tested separately in a concentration-response curve study in an H_1 radioligand binding assay and in a 5-HT4 agonist assay.

2.13 | Data analysis and statistics

Concentration-effect curves were analyzed through iterative curve fitting to a logistic equation using GraphPad Prism 5.0. Potency of antagonists was reported as Ki/IC_{50} or pKi/pIC_{50}. Apparent inhibition constants ($K_{I,App}$) for revefenacin were calculated from observed IC_{50} values according to Cheng and Prusoff.[14] In saturation binding studies binding studies, K_D was defined as the concentration of radioligand that resulted in 50% of the maximal specific binding signal (B_{Max}). In binding kinetic studies, values for the observed association rate (k_{obs}) from the association assay were calculated from "One phase exponential association" curve fit. The values for k_{on} were calculated from the equation: $k_{on} = (k_{obs}\text{-}k_{off})/L$, where L is the radioligand concentration. Agonist EC_{50} was defined as the concentration required to produce 50% of the maximum response. Antagonist affinity estimates (pA_2 or pK_B) were determined using either Schild analysis.[15] or Gaddum equation.[16] In reversibility studies, the offset time ($t_{1/2}$), following removal of the antagonist, was determined by measuring the time required for response to recover by 50%. Whenever appropriate, data were analyzed statistically using a unpaired t-test with $P < .05$ considered as significant.

3 | RESULTS

3.1 | Competition and saturation binding at human hM_1-M_5 mAChRs

[^{3}H]NMS specific binding was saturable and K_D values were determined to be 0.45, 0.55, 0.47, 0.28, and 1.23 $nmol \cdot L^{-1}$ for the hM_1, hM_2, hM_3, hM_4, and hM_5 receptors, respectively. Receptor densities were 2.7, 2.5, 2.4, 2.0, and 1-4 $pmol \cdot mg^{-1}$ protein, respectively. Due to slow binding kinetics for all test compounds, incubation times used for competition studies were carefully considered to avoid underestimations of the K_I.[17] Revefenacin and other mAChR antagonists inhibited [^{3}H]NMS specific binding in a concentration-dependent manner with full inhibition observed at concentrations above 10 $\mu mol \cdot L^{-1}$. Because inhibition curves shapes were consistent with competitive interactions, inhibition binding constants (K_I) were calculated from IC_{50} values according to methods described by Cheng and Prusoff.[14] The inhibition constants of revefenacin for each receptor subtype was hM_1 $K_I = 0.42$ $nmol \cdot L^{-1}$, hM_2 $K_I = 0.32$ $nmol \cdot L^{-1}$, hM_3 $K_I = 0.18$ $nmol \cdot L^{-1}$, hM_4 $K_I = 0.56$ $nmol \cdot L^{-1}$, hM_5 $K_I = 6.7$ $nmol \cdot L^{-1}$. Affinity measurements for other mAChR antagonists were similar to previously published results [18,19] and the negative logarithm values of the inhibition binding constants (pK_I) are summarized in Table 1. Comparing the affinities for the test compounds at the human M_3 receptor indicated a rank order of affinities of tiotropium ($pK_I = 10.71 \pm 0.12$) > revefenacin ($pK_I = 9.75 \pm 0.11$) > glycopyrrolate ($pK_I = 9.61 \pm 0.09$) > ipratropium ($pK_I = 8.97 \pm 0.15$).

The specific binding for [^{3}H]revefenacin at M_1-M_5 receptors was saturable and curve shapes were consistent with radioligand binding to a single receptor population in each membrane preparation. The dissociation affinity constant (K_D) values for [^{3}H]revefenacin determined with plasma membrane fractions from CHO-K1 cells expressing the receptors, were as follows: hM_1 $K_D = 1.0$ $nmol \cdot L^{-1}$; hM_2 $K_D = 0.61$ $nmol \cdot L^{-1}$; hM_3 $K_D = 0.39$ $nmol \cdot L^{-1}$; hM_4 $K_D = 0.46$ $nmol \cdot L^{-1}$; hM_5 $K_D = 4.2$ $nmol \cdot L^{-1}$; logarithm K_D values are in agreement with the calculated inhibition binding constants for each receptor.

3.2 | Binding kinetics at human hM_2 and hM_3 mAChRs

At concentrations of approximately 1 $nmol \cdot L^{-1}$, [^{3}H]revefenacin exhibited fast association with the hM_2 receptor ($k_{on} = 0.25 \pm 0.15$ $nM^{-1} \cdot min^{-1}$) and to the hM_3 receptor ($k_{on} = 0.092 \pm 0.029$ $nM^{-1} \cdot min^{-1}$). In atropine displacement assays, [^{3}H]revefenacin binding was reversible at the hM_2 or hM_3 receptors with dissociation rates (k_{off}) of 0.10 ± 0.00 min^{-1} for hM_2 receptor and 0.0085 ± 0.0004 min^{-1} for hM_3. The $t_{½}$ values for hM_2 receptor was 6.9 minutes which was significantly ($P < .05$) lower than 82 minutes for hM_3 (Table 2) at physiological temperature. Using the dissociation half-lives, revefenacin demonstrated kinetic selectivity for hM_3 receptors over hM_2 receptors (Figure 2), with a selectivity ratio of 12. The negative logarithm dissociation constant (Log K_D = Log k_{off}–Log k_{on}) estimated from association and dissociation rate parameters was estimated for hM_2 to be 9.40 and for hM_3 to be 10.03, which are similar to the pK_I values determined from competition radioligand binding assays. The rank order of dissociation $t_{1/2}$ values reported in Table 2 was tiotropium (230 minutes) > revefenacin (82 minutes) > glycopyrrolate (25 minutes).

3.3 | Functional competitive antagonism at human (hM_1, hM_2, hM_3, hM_4) and chimpanzee (cM_5) mAChRs

Revefenacin potently inhibited oxotremorine-stimulated [^{35}S]GTPγS binding at hM_2 receptors, with $pK_{I,App}$ (mean ± SD) of 9.77 ± 0.15, consistent with the radioligand binding affinity at hM_2. Revefenacin did not stimulate [^{35}S]GTPγS binding at hM_2 receptors.

In calcium mobilization studies designed to measure functional inhibition of agonist-stimulated mAChR activity, revefenacin was a potent functional antagonist at the hM_1, hM_3, hM_4-$G_{\alpha 16}$, or the

TABLE 1 Negative Logarithm Inhibition Binding Constants (pKi) for Revefenacin and Other Antagonists at M_1, M_2, M_3, M_4, and M_5 mAChRs

	hM_1 pK_I	hM_2 pK_I	hM_3 pK_I	hM_4 pK_I	hM_5 pK_I
Revefenacin	9.38 ± 0.03	9.52 ± 0.15	9.75 ± 0.11	9.26 ± 0.09	8.20 ± 0.18
Ipratropium	8.75 ± 0.30	9.03 ± 0.16	8.97 ± 0.15	8.87 ± 0.16	8.27 ± 0.15
Tiotropium	10.56 ± 0.18	10.47 ± 0.13	10.71 ± 0.12	10.69 ± 0.10	10.09 ± 0.08
Glycopyrrolate	9.56 ± 0.09	9.09 ± 0.10	9.61 ± 0.09	9.43 ± 0.09	9.05 ± 0.14

Inhibition binding constants for revefenacin and other antagonists at CHO-K1 cell membranes expressing M_1, M_2, M_3, M_4, M_5 mAChRs were determined using [^{3}H]NMS inhibition radioligand binding assays. Data are expressed as mean ± SD; *n* = at least 4.

TABLE 2 Association and dissociation binding kinetics for [^{3}H]revefenacin and other tritium-labeled antagonists at M_2 and M_3 mAChRs

	k_{on} ($nM^{-1}\cdot min^{-1}$)		k_{off} (min^{-1})		$t_{1/2}$ (min)		$t_{1/2}$ Ratio (M_3/M_2)
	M_2	M_3	M_2	M_3	M_2	M_3	
[^{3}H]Revefenacin	0.25 ± 0.15	0.092 ± 0.029	0.10 ± 0.00	0.0085 ± 0.0004	6.9	82	12
[^{3}H]Glycopyrrolate	0.18 ± 0.10	0.10 ± 0.03	0.16 ± 0.03	0.027 ± 0.006	4.2	25	6.0
[^{3}H]Tiotropium	1.07 ± 0.07	0.21 ± 0.00	0.020 ± 0.001	0.0030 ± 0.0002	35	230	6.6

To determine dissociation rates, membranes prepared from cells expressing hM_2 or hM_3 receptors were incubated with tritium-labeled compounds, followed by the addition of 10 μmol L^{-1} atropine at 37°C. Membranes were then filtered at various times after atropine addition. To determine the association rate, tritium-labeled compounds, and membranes prepared from cells expressing hM_2 or hM_3 receptors were incubated together for varying lengths of time at 37°C prior to rapid filtration. Data are reported as mean ± SD; *n* = 3-7.

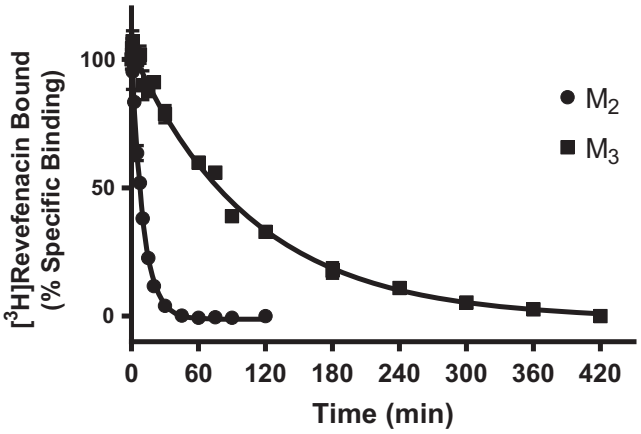

FIGURE 2 Dissociation of [^{3}H]revefenacin from human hM_2 or hM_3 mAChRs. To determine dissociation rates, membranes prepared from cells expressing hM_2 or hM_3 receptors were incubated with [^{3}H]revefenacin, followed by the addition of 10 μmol L^{-1} atropine at 37°C. Membranes were then filtered at various times after atropine addition. Dissociation of [^{3}H]revefenacin from M_2 or M_3 mAChRs was measured either over a 2 hours or 7 hours time course, respectively. Data are expressed as mean ± SEM; *n* = 3

chimpanzee M_5 (cM_5) receptor expressed in CHO-K1 cells. Owing to nonequilibrium conditions, antagonist potencies might be underestimated and apparent negative logarithm inhibition constants ($pK_{I,App}$) are reported. Revefenacin had the following inhibitory constants (mean ± SD) as summarized in Table 3: hM_1 $pK_{I,App}$ = 9.90 ± 0.17, hM_3 $pK_{I,App}$ = 10.02 ± 0.32, hM_4-$G_{\alpha 16}$ $pK_{I,App}$ = 10.95 ± 0.25, and cM_5 $pK_{I,App}$ = 9.44 ± 0.16. When tested alone in cells expressing mAChRs, revefenacin evoked no Ca^{2+} response, indicating that there was no detectable agonist or partial agonist activity (data not shown).

3.4 | Reversibility of antagonism at recombinant human hM_2 (w/Gqi5) and hM_3 mAChRs

Treatment of hM_3 and hM_2 cells with revefancin, at concentrations of 1-1000 nmol·L^{-1}, was associated with rightward shifts of the ACh-induced calcium mobilization concentration-response curve with concentration-related suppression of the maximum response. The pK_B estimate (mean ± SD) of revefenacin (at 1 nmol·L^{-1}) was 10.03 ± 0.08 and 10.54 ± 0.11 at hM_3 and hM_2 receptors, respectively.

In reversibility studies at hM_3 receptors, the ACh concentration-response curve remained shifted by 6-, 7-, 16-, and 68-fold at revefenacin concentrations of 1 nmol·L^{-1}, 10 nmol·L^{-1}, 100 nmol·L^{-1}, and 1000 nmol·L^{-1}, respectively (Figure 3A) following 180 minutes of washout of the antagonist. In contrast, under similar experimental conditions at hM_2 receptors, revefenacin produced significantly ($P < .05$) smaller shifts with no meaningful shift after washout of concentrations of 1 or 10 nmol·L^{-1} and 4- and 13-fold shifts after washout of concentrations of 100 and 1000 nmol·L^{-1}, respectively (Figure 3B). Tiotropium was evaluated in similar studies, the results of which have been published previously.[12]

3.5 | Off target activities

Drug-related side effects are often the result of compound activities at undesired targets. To check for possible off-target effects, revefenacin was screened for activity in a panel of 81 receptors, enzymes, and channels at a concentration of 1 μmol L^{-1} (a concentration that was >5000-fold over its hM_3 K_I). Revefenacin

TABLE 3 Negative logarithm of apparent inhibition constants (pki_{app}) for revefenacin and other antagonists in calcium mobilization and [^{35}S]GTPγS binding assays

	Ca^{2+} Mobilization				[^{35}S]GTPγS Binding
	hM_1	hM_3	hM_4-$G_{\alpha16}$	cM_5	hM_2
Revefenacin	9.90 ± 0.17	10.02 ± 0.32	10.95 ± 0.25	9.44 ± 0.16	9.77 ± 0.15
Ipratropium	9.82 ± 0.23	10.37 ± 0.24	10.71 ± 0.27	9.72 ± 0.09	9.76 ± 0.25
Tiotropium	10.11 ± 0.22	10.54 ± 0.37	10.21 ± 0.10	9.98 ± 0.35	8.99 ± 0.27

Apparent inhibition constants for revefenacin and other antagonists were determined using agonist (ACh) stimulated calcium mobilization assays using CHO-K1 cells expressing hM_1, hM_3, hM_4-$G_{\alpha16}$, cM_5 mAChRs. Apparent inhibition constants for revefenacin and other antagonists were determined using agonist (ACh) stimulated [^{35}S]GTPγS binding assays using CHO-K1 cell membranes expressing hM_2 mAChRs. Data are reported as mean values ± SD; *n* = at least 3.

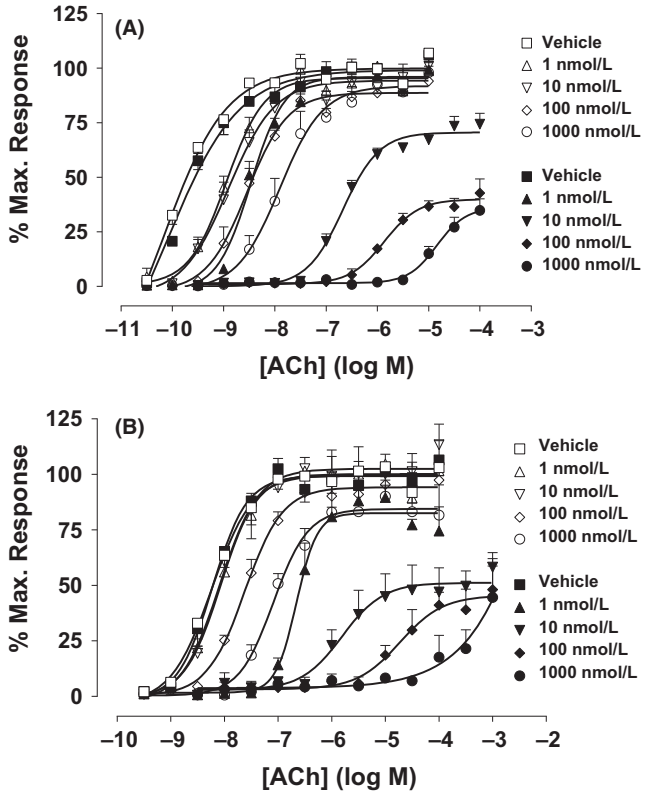

FIGURE 3 Reversibility profile of revefenacin at human hM_3 (A) and hM_2 (B) mAChRs as measured by ACh-induced calcium mobilization responses. Reponses to acetylcholine (ACh) were measured after preincubation of cells for 90 minutes with vehicle or revefenacin (1-1000 nmol·L^{-1}) in unwashed cells or washed (180 minutes) cells. Closed symbols denote values with no washout. Open symbols denote values post washout. Data are expressed as mean ± SEM; *n* = 3

exhibited <75% activity at a majority of the targets (Appendix: Table S1). In a radioligand binding assay for the closely related biogenic amine receptor histamine H_1, revefenacin exhibited 87% inhibition of radioligand binding relative to the control. The inhibition binding constant (K_I) was measured to be 498 nmol·L^{-1} at the H_1 receptor. Revefenacin was also found to be a weak (pEC_{50} = 6.59) partial agonist (intrinsic activity = 9%) at cloned human 5-HT4(C) receptors.

3.6 | Antagonism of ACh-evoked contraction in rat isolated tracheal tissue: potency and offset time

In rat isolated tracheal tissues, revefenacin (3-100 nmol·L^{-1}) produced concentration-dependent dextral shifts of the ACh contractile concentration-response curve with reduction (11%-35%) of the maximum response (Figure 4A). The pK_B estimate [mean ± SEM (*n*)] of revefenacin was 10.5 ± 0.1 (36). Similar effects were observed with tiotropium (pK_B estimate = 11.1 ± 0.2 (9)); whereas ipratropium behaved as a surmountable antagonist with pA_2 of 9.2 ± 0.1 (data not shown).

In studies to assess the antagonist recovery time in rat isolated trachea, tissues treated with IC_{90} concentrations of revefenacin (10 nmol·L^{-1}) and tiotropium (1 nmol·L^{-1}) showed approximately 70% and 40% recovery from the maximum inhibitory effect, respectively, after 17 hours of continuous tissue perfusion with drug-free buffer, whereas tissues exposed to ipratropium (10 nmol·L^{-1}) showed approximately 60% recovery of ACh contractile activity after only 4 hours of continuous perfusion (Figure 4B). The $t_{1/2}$ values were estimated to be 13.3 ± 3.1 hours for revefenacin, >17 hours for tiotropium which was significantly ($P < .05$) lower than that of ipratropium (1.6 ± 0.8 hours).

3.7 | Inhibition of EFS contraction of guinea pig isolated trachea: potency and offset time

Revefenacin (0.1-100 nmol·L^{-1}) (Figure 5A), tiotropium (0.1-100 nmol·L^{-1})), and atropine (0.1-100 nmol·L^{-1}) produced concentration-dependent inhibition of EFS contractions of the isolated guinea pig trachea with potencies ($pIC_{50's}$) of 8.1 ± 0.1, 8.7 ± 0.1, and 8.3 ± 0.03, respectively.

In antagonist washout studies using IC_{90} concentrations, the inhibitory effects of atropine (100 nmol·L^{-1}) were rapidly reversed ($t_{1/2}$ = 1.3 hours), whereas those of revefenacin (30 nmol·L^{-1}) and tiotropium (3 nmol·L^{-1}) persisted for significantly ($P < .05$) longer periods ($t_{1/2}$ = >16 and >20 hours, respectively) (Figure 5B).

3.8 | Antagonism of carbachol-evoked contraction of human isolated bronchial tissue: potency and offset time

In static tissue bath studies, revefenacin (1-1000 nmol·L^{-1}) caused dextral shifts of the CCh concentration-response curves in human

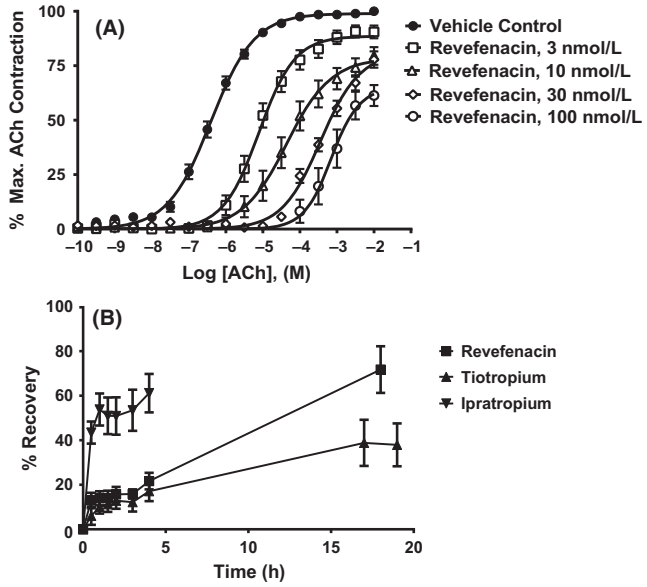

FIGURE 4 Antagonism of acetylcholine (ACh)-evoked contraction of rat isolated tracheal tissues. (A) Contractile responses to ACh were measured after tissues were incubated for 120 minutes with various concentrations of revefenacin (3-100 nmol·L^{-1}) (B) Reversibility of the effects at IC_{90} concentrations of revefenacin, tiotropium, and ipratropium, after 4-18 hours of washout of the antagonist. Data are expressed as mean ± SEM; n = 5-12 for "A" and 4-5 for "B"

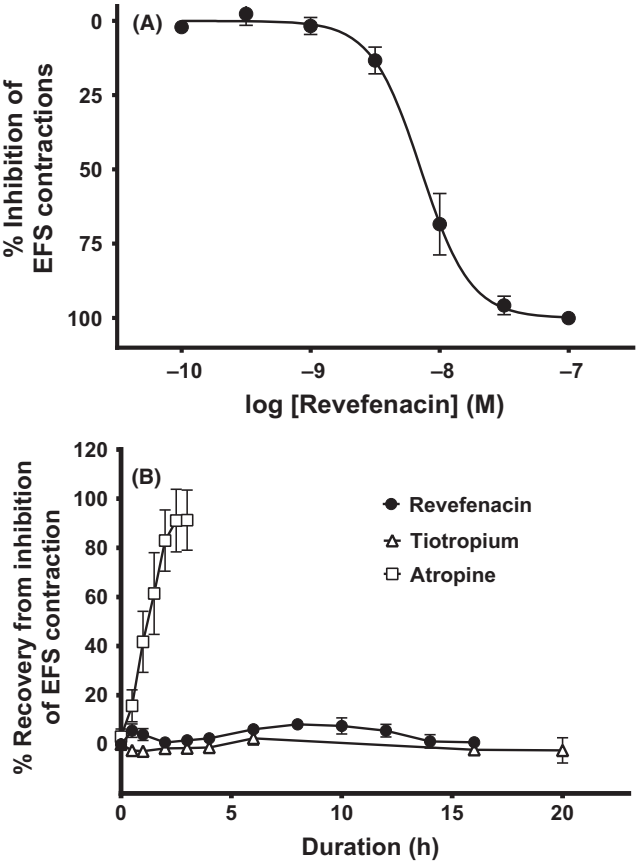

FIGURE 5 Inhibition of EFS contractions of guinea pig isolated tracheal tissues. (A) EFS contractile responses were measured after tissues were incubated for 120 minutes with various concentrations of revefenacin (0.1-100 nmol·L^{-1}) (B) Reversibility of the effects at IC_{90} concentrations of revefenacin, tiotropium, and atropine, after 3-20 hours of washout of the antagonist. Data are expressed as mean ± SEM; n = 4 for "A" and 3-4 for "B." EFS, electrical field stimulated

bronchus with concentration-related suppression (9%-70%) of the maximal carbachol responses (Figure 6A). Affinity values for revefenacin were not estimated given the high degree of suppression of the maximum response. We have previously shown that tiotropium, similar to REV, also behaves as a potent insurmountable antagonist, whereas ipratropium produces surmountable antagonism.[12] Concentrations of revefenacin (30 nmol·L^{-1}), ipratropium (10 nmol·L^{-1}), and tiotropium (10 nmol·L^{-1}) represent equieffective concentrations, producing approximately 30-fold shift of the carbachol concentration-effect curve, with respect to antagonism of carbachol contractile responses.

In superfusion washout studies, none of the tissues treated with revefenacin (10-100 nmol·L^{-1}) recovered to 50% of the 1 μmol L^{-1} CCh contraction ($t_{1/2}$ > 10 hours) following 10 hours of perfusion with antagonist-free buffer (Figure 6B). The $t_{1/2}$ for 1 and 10 nmol·L^{-1} tiotropium was 6.3 and >10 hours, respectively, and for 10 nmol·L^{-1} ipratropium, it was 2.9 hours. (Figure 6B). No statistics could be performed on this data because of the small number of donors.

4 | DISCUSSION

Revefenacin was designed to be a once-daily inhaled lung-selective LAMA and is being developed for COPD patients who require or prefer nebulized therapy. The present studies have shown that revefenacin is a high affinity competitive antagonist at human recombinant mAChRs with kinetic functional selectivity for M_3 over M_2 mAChRs and a potent, slowly reversible antagonist in rat, guinea pig, and human airway tissues expressing native mAChRs.

In a previous study, we demonstrated that inhaled revefenacin produced potent protection against ACh or MCh evoked bronchoconstriction in dogs and rats.[10] In this study, we investigated the antagonist potency and offset time in isolated tracheal tissues from rat in order to gain mechanistic insight into the long-acting bronchoprotective effects observed in rats in vivo. Revefenacin antagonized acetycholine-induced contraction of isolated rat tracheal tissues with a potency (pK_B = 10.5) that was marginally (4-fold) lower that of tiotropium (pK_B = 11.1) but greater (20-fold) than that of ipratropium (pA_2 = 9.2). The apparent insurmountable nature of the antagonism observed with revefenacin is unlikely to be due to a noncompetitive mode of interaction given the results from competition-binding studies discussed below. It is more likely that the pseudo-insurmountable behavior of revefenacin and tiotropium emanates from pseudo irreversible antagonism resulting from the failure to attain equilibrium conditions given the slow dissociation kinetics

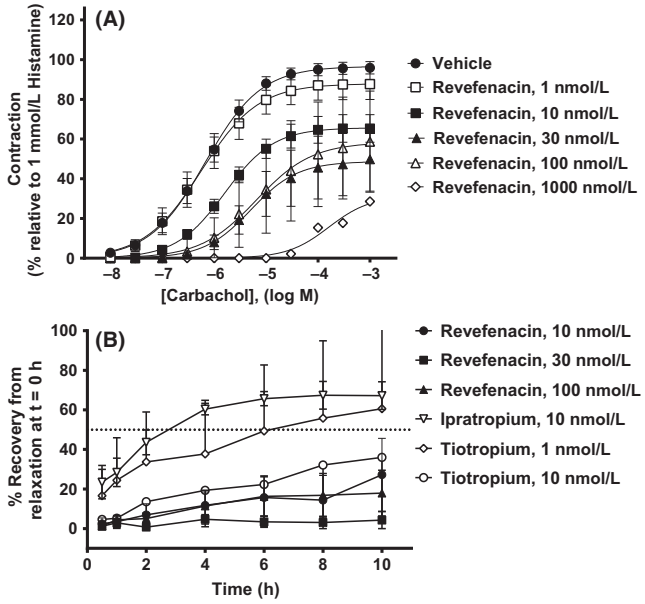

FIGURE 6 Antagonism of carbachol (CCh)-induced contraction of isolated bronchial strips from human donors. (A) Contractile responses to CCh were measured after tissues were incubated for 120 minutes with varying concentrations of revefenacin (1-1000 nmol·L^{-1}), n = 3: (B) Reversibility of effects of revefenacin, tiotropium, and ipratropium after 10 hours washout of the antagonist. Data are expressed as mean ± SEM; n = 6 (10 and 100 nmol·L^{-1}); n = 4 (1 and 30 nmol·L^{-1}); n = 2 (1000 nmol·L^{-1})

of both molecules at M_3 receptors (discussed below). The antagonist effects of revefenacin and tiotropium in rat isolated tracheal tissues persisted longer ($t_{1/2}$ of 13.3 and >17 hours, respectively) compared with the antagonist effects of ipratropium which reversed rapidly following removal of the antagonist from the bath buffer ($t_{1/2}$ = 1.6 hours). These results imply that the long-acting bronchoprotective effects of inhaled revefenacin and tiotropium in rats emanates, at least in part, from the intrinsically longer duration of antagonism of the two molecules in airway tissues expressing mAChRs although a pharmacokinetic contribution of increased lung-residence time of the molecules is a likely contributing factor as well. The slowly reversible antagonistic effects of revefenacin were also observed in guinea pig airway tissues indicating species-independent effects.

We also sought to translate the biological effects of revefenacin in rats and guinea pigs by characterizing the pharmacological effects of revefenacin at both human recombinant mAChRs and in isolated human airway tissues. In equilibrium competition radioligand binding studies, revefenacin, like tiotropium, and glycopyrrolate, demonstrated subnanomolar affinity for all five human mAChR subtypes, including M_2 (pK_I = 9.5) and M_3 (pK_I = 9.7) mAChRs, the two key receptors involved in airway smooth muscle tone and contraction.[3] The affinity estimates obtained for ipratropium, tiotropium, and glycopyrrolate were consistent with those reported previously.[18,19] The rank order of potency was tiotropium > revefenacin = glycopyrrolate > ipratropium. The high affinity of revefenacin for human mAChRs was replicated in equilibrium saturation binding studies that yielded a K_D range of 0.4-4 nmol·L^{-1} for [^{3}H]-revefenacin at the four subtypes. Revefenacin was also evaluated for potential off-target activity. When tested against a large panel of receptors, enzymes, and ion channels, revefenacin exhibited minimal activity at nonmuscarinic molecular targets. The potency of revefenacin at the histamine H1 receptor and 5-HT_4 receptor was >2700 fold and >1400-fold lower than its Ki at the hM_3 receptor implying these interactions are unlikely to have clinical significance at therapeutic doses.

In functional studies, using either calcium mobilization or [^{35}S]GTPγS binding as the endpoint, revefenacin, and other reference molecules behaved as functional muscarinic antagonists at all five subtypes including human M_2 and M_3 mAChRs. Revefenacin inhibited agonist-stimulated intracellular Ca^{2+} mobilization in M_3 receptor-expressing cells with $pK_{I,App}$ values closely matching radioligand binding receptor affinities. Notably, revefenacin did not stimulate a Ca^{2+} mobilization response when assayed in the absence of agonist, demonstrating the neutral antagonist properties of the molecule. Furthermore, in M_2 mAChR-expressing cells, revefenacin inhibited agonist-stimulated increases in [^{35}S]GTPγS binding, but did not stimulate [^{35}S]GTPγS binding in the absence of agonist.

The onset and duration of pharmacodynamics of LAMAs have been previously ascribed to slow dissociation kinetics at the M_3 receptor.[20,21] Kinetic studies, performed at physiological temperature (37°C), yielded [^{3}H]revefenacin, [^{3}H]tiotropium, and [^{3}H]glycopyrrolate dissociation $t_{1/2}$ values at the M_3 receptor that were 82, 230 and 25 minutes, respectively, and faster rates of dissociation from the M_2 receptor than the M_3 receptor. Comparing the ratio of half-lives of dissociation from the M_3 receptor versus the M_2 receptor, [^{3}H]revefenacin had the highest selectivity for the M_3 receptor (M_3: M_2 receptor half-life = 12) compared to the other antagonists (M_3: M_2 receptor half-life = 6.6 and 6.0, respectively). Although the kinetic behavior of ipratropium was not evaluated in this study, it has been reported to dissociate rapidly from both hM_2 and hM_3 receptors.[22]

Functional reversibility studies were conducted at human recombinant M_3 and M_2 receptors to determine whether the distinct off-rate kinetics of revefenacin at M_2 and M_3 receptors translated to differences in reversibility of functional antagonism. Revefenacin produced concentration-dependent antagonism of agonist-induced calcium mobilization responses at both M_2 and M_3 receptors. However, following 180 minutes of antagonist washout, the magnitude of rightward shift of the agonist curves was greater at M_3 receptors, as compared to M_2 receptors, consistent with more persistent antagonism of the former receptor.

In the human bronchus, revefenacin potently and insurmountably antagonized the carbachol-induced contractile responses. In superfusion drug washout studies, the antagonistic effects of ipratropium ($t_{1/2}$ = 2.9 hours) were rapidly reversible, whereas those of revefenacin and tiotropium persisted for several hours ($t_{1/2}$ of >10 hours at the highest concentration tested). These results imply that the slow functional reversibility of the antagonistic effects of revefenacin

observed in human recombinant M_3 mAChRs also translates to native mAChRs expressed in human airway tissues. It is interesting to note that despite having an hM_3 dissociation $t_{1/2}$ that is 2.8-fold shorter than that of tiotropium, the reversibility offset times in human bronchus of revefenacin was comparable to that of tiotropium suggesting that factors other than slow receptor off-rate also contribute to its duration at the tissue level.

In summary, the present studies illustrate that revefenacin produces potent and long-lasting antagonism of mAChR-mediated contractions of rat and guinea pig tracheal tissues. These data provide an explanation for the potent and long-acting bronchoprotective effects previously reported in rats after inhalation dosing. More importantly, the high affinity and selectivity of revefenacin for human recombinant mAChRs and its potent and long-lasting antagonism of mAChR-mediated contraction of human bronchial tissues provide confidence in the translational of preclinical findings to humans. Indeed, clinical Phase 2 dose-range studies have demonstrated sustained 24 hours bronchodilation in COPD patients following once-daily dosing.[7,9] Phase 3 studies with nebulized revefenacin are being conducted to confirm its long-acting bronchodilator effects and safety in COPD patients. Furthermore, recently reported Phase 3 efficacy studies [23,24] continue to support the safety and efficacy of revefenacin as a potential new therapeutic option for patients with COPD who may benefit from treatment with a once-daily, nebulized bronchodilator.

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DISCLOSURE

None declared.

AUTHOR CONTRIBUTION

Hegde, Pulido-Rios, Luttmann, Foley, Ji, Steinfeld, Lee, Mammen, and Jasper participated in research design. Pulido-Rios, Foley, Steinfeld, Lee, and Hunsberger conducted the experiments. Pulido-Rios, Foley, Steinfeld, Lee, and Hunsberger performed data analysis. Hegde, Pulido-Rios, Luttmann, Foley, Steinfeld, Lee, Mammen, and Jasper wrote or contributed to the writing of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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