Pharmacological Characterization of GSK573719 (Umeclidinium): A Novel, Long-Acting, Inhaled Antagonist of the Muscarinic Cholinergic Receptors for Treatment of Pulmonary Diseases


GlaxoSmithKline Pharmaceuticals, King of Prussia, Pennsylvania

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ABSTRACT

Activation of muscarinic subtype 3 (M3) muscarinic cholinergic receptors (mAChRs) increases airway tone, whereas its blockade improves lung function and quality of life in patients with pulmonary diseases. The present study evaluated the pharmacological properties of a novel mAChR antagonist, GSK573719 (4-[hydroxy(diphenyl)methyl]-1-{2-[(phenylmethyl)oxy]ethyl}-1-azoniabicyclo[2.2.2]octane; umeclidinium). The affinity (K_i) of GSK573719 for the cloned human M1–M5 mAChRs ranged from 0.05 to 0.16 nM. Dissociation of [3H]GSK573719 from the M3 mAChR was slower than that for the M2 mAChR (half-life (t_1/2) values: 82 and 9 minutes, respectively). In Chinese hamster ovary cells transfected with recombinant human M3 mAChRs, GSK573719 demonstrated picomolar potency (–log pA_2 = 33.9 pm) in an acetylcholine (Ach)-mediated Ca^2+ mobilization assay. Concentration-response curves indicate competitive antagonism with partial reversibility after drug washout. Using isolated human bronchial strips, GSK573719 was also potent and showed competitive antagonism (–log pA_2 = 316 pm) versus carbachol, and was slowly reversible in a concentration-dependent manner (1–100 nM). The time to 50% restoration of contraction at 10 nM was about 381 minutes (versus 413 minutes for tiotropium bromide). In mice, the ED_50 value was 0.02 μg/mouse intranasally. In conscious guinea pigs, intratracheal administration of GSK573719 dose dependently blocked Ach-induced bronchoconstriction with long duration of action, and was comparable to tiotropium; 2.5 μg elicited 50% bronchoprotection for >24 hours. Thus, GSK573719 is a potent anticholinergic agent that demonstrates slow functional reversibility at the human M3 mAChR and long duration of action in animal models. This pharmacological profile translated into a 24-hour duration of bronchodilation in vivo, which suggested umeclidinium will be a once-daily inhaled treatment of pulmonary diseases.

Introduction

Pulmonary diseases such as chronic obstructive pulmonary disease (COPD) remain a leading cause of disability and death while contributing to the global health burden. A primary manifestation of COPD is the inability of these patients to achieve sufficient oxygenation of extrapulmonary tissues. Oxygen transport from the atmosphere to the alveolar spaces for exchange to the bloodstream can be aided by dilating the airways using pharmacological agents. Some of these compounds are designed to block the activity of the muscarinic cholinergic receptors, in particular the muscarinic subtype 3 (M3) muscarinic cholinergic receptor (mAChR), which is highly expressed on the airway smooth muscle.

Maintenance of the caliber of the airway tree is supported by the parasympathetic nerve fibers carried within the vagus nerve. Parasympathetic ganglia are associated with the larger airways with postganglionic fibers innervating smaller-diameter bronchioli that are largely responsible for establishing resistance for airflow from the atmosphere to the gas exchange units (for review, refer to Canning and Fischer, 2001). Vagotomy or administration of atropine results in bronchodilation in both animals and humans, suggesting that the parasympathetic nerves are tonically active, and can provide a persistent obstruction (Canning and Undem, 1994). Neural output from both parasympathetic cholinergic and nonadrenergic, noncholinergic fibers affects airway smooth muscle contractile activity. Sympathetic fibers do not directly innervate these effector cells, even though β-adrenergic receptors are situated within their plasma membrane. Transmission of the parasympathetic neural impulse to the airway

ABBREVIATIONS: Ach, acetylcholine; Cch, carbachol; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CRC, concentration-response curve; DMSO, dimethylsulfoxide; FLIPR, fluorometric imaging plate reader; GSK573719, 4-[hydroxy(diphenyl)methyl]-1-2-[(phenylmethyl)oxy]ethyl)-1-azoniabicyclo[2.2.2]octane; M1–M5, muscarinic subtype 1–5; mAChR, muscarinic acetylcholine receptor; Mch, methacholine; MEM, minimum essential medium; ON t_1/2, time to 50% of maximal relaxation; OFF t_1/2, offset half-time of tension recovery; Penh, enhanced pause; t_1/2, half-life.
smooth muscle cells and consequent contraction occurs via the release of acetylcholine (Ach) and binding to the M3 mAChR. Of the five mAChRs, the M2 receptor is the most abundant on the airway smooth muscle cell, whereupon its action opposes β-adrenergic receptor–induced relaxation (Eglen et al., 1996; Coulson and Fryer, 2003). Modulation of the release of Ach is also influenced by M2 mAChRs located on the prejunctional fibers, where they exhibit an inhibitory influence. Activation of the M3 mAChR by Ach initiates a cascade of signaling events engaging G proteins (Gq), inositol triphosphate, and phosphoinositide phospholipase C with a consequent increase in intracellular calcium and enhancement of airway smooth muscle contraction (see for example Chilvers and Nahorski, 1990).

Bronchodilator therapy has become the mainstay for controlling symptoms brought about by enhanced parasympathetic nerve activity in patients with bronchoconstrictive pulmonary diseases such as COPD and asthma (Cazzola et al., 2012). β-Adrenergic receptor agonists and xanthines have been available for many years for symptom relief of these diseases. More recently, antimuscarinic cholinergic antagonists have been sought for the management of disease to maintain airway caliber for adequate gas exchange (Busch-Petersen and Lainé, 2011). To promote adherence to therapy, those agents with a long duration of action are more desirable, and to avoid systemic effects of these medicines, inhalation of the compound has benefitted these patients. This study was conducted to characterize the preclinical pharmacology of 4-[hydroxy(diphenyl)methyl]-1-[2-[(phenylmethyl)oxy]ethyl]-1-azoniabicyclo[2.2.2]octane (GSK573719; World Health Organization– and United States Adopted Name–approved term is umeclidinium), a potent mAChR antagonist that was designed to be used once daily via the inhaled route. Although this compound is a pan-active pharmacophore for each of the five muscarinic receptors, the data are, for the most part, derived from functional assay systems depicting M3 mAChR responses.

Materials and Methods

mAChR Binding. Studies were conducted using membranes prepared from Chinese hamster ovary (CHO) cells stably expressing the human M1–M5 mAChRs. The cloning, heterologous expression, and scale-up growth of CHO cells transfected with mAChRs were conducted to characterize the preclinical pharmacology of 4-[hydroxy(diphenyl)methyl]-1-[2-[(phenylmethyl)oxy]ethyl]-1-azoniabicyclo[2.2.2]octane (GSK573719; World Health Organization–and United States Adopted Name–approved term is umeclidinium), a potent mAChR antagonist that was designed to be used once daily via the inhaled route. Although this compound is a pan-active pharmacophore for each of the five muscarinic receptors, the data are, for the most part, derived from functional assay systems depicting M3 mAChR responses.

were collected for centrifugation (100,000 × 60 minutes at 4°C). Following resuspension of this pellet, aliquots were stored at −80°C until the day of experimentation. Protein concentration was quantified using the Bio-Rad protein assay (Hercules, CA).

Ligand binding assays with GSK573719 and [3H]-N-methyl scopolamine (0.5 nM) were performed using a scintillation proximity assay for M1, M2, and M3 mAChRs and a filtration assay for M4 and M5 mAChRs. For the scintillation proximity assay, membranes were incubated with wheat germ agglutinin beads in 50 mM HEPEs buffer, pH 7.4, at 4°C for 30 minutes and then with the radioligand in a 96-well OptiPlate (PerkinElmer, Waltham, MA) for 2 hours at room temperature in the presence of vehicle (1% dimethyloxide (DMSO)) or GSK573719 (0.01–300 nM). At the end of the incubation, the plates were centrifuged (Beckman CS-6R; Beckman Coulter, Brea, CA; for 5 minutes at 2000g), and radioactivity was counted (Top Counter, Model A9912; Packard, Meriden, CT). For the filtration assay, membranes (M4 and M5) were similarly incubated in HEPEs buffer containing the radioligand for 2 hours at room temperature in the presence of vehicle (1% DMSO) or GSK573719 (0.03–300 nM). Atropine was used as a reference agent. Reactions were terminated by rapid filtration (Brandel Cell Harvester; Brandel, Gaithersburg, MD) through GF/C filters (glass microfiber binder free 1.2 μm; Whatman, Kent, UK). Membranes were washed with ice-cold 50 mM HEPEs and transferred to scintillation vials. Radioactivity was counted in a Scintillation Counter (Beckman model LS6500). Reactions were terminated by rapid filtration as described earlier. Data were obtained from three independent experiments. Specific binding was determined by subtracting nonspecific binding (using 0.3 μM atropine) from total binding. The inhibition constant (K_i) for GSK573719 was calculated according to Cheng and Prusoff (1973), where K_i = IC_50/[L]/K_m + 1; K_m = 0.17, 0.28, 0.16, 0.07, and 0.2 nM for M1–M5 mAChRs, respectively, and [L] is the assay concentration of radioligand. Membranes containing M3 mAChRs were also incubated for 2 hours at room temperature with increasing concentrations of [3H]-N-methyl scopolamine (0.08–9.24 nM) in the presence or absence of GSK573719 (0.2–0.5 nM) in 50 mM HEPEs, pH 7.4. Nonspecific binding was determined using 10 μM atropine. The saturation data were converted to a scatchard plot for analysis.

For kinetic measurements of [3H]GSK573719 or [3H]tiotropium, membrane fragments from CHO cells stably expressing human M2 or M3 mAChRs were prepared as previously described (Slack et al., 2011). Fragments were placed in 96-well-plate wells maintained at 37°C in medium containing 50 mM HEPEs buffer, pH 7.4, with either [3H]GSK573719, [3H]tiotropium, or vehicle (2% DMSO) and 10 μM atropine for nonspecific binding and specific binding. After varying incubation periods, binding was terminated by rapid vacuum filtration (Brandel). The filtrate was collected on GF/B (glass microfiber binder free 1.0 μm, Whatman, Kent, UK) filter papers presoaked in 0.3% v/v polyethyleneimine, washed three times with ice-cold medium, and the filters were transferred into vials containing Ultima-Plu M (PerkinElmer, Beaconsfield, UK) and analyzed using a TriCarb 2900 TR LS counter (PerkinElmer). Specific binding of [3H]GSK573719 or [3H]tiotropium was not found on cells lacking muscarinic receptors (unpublished data). Saturation binding of [3H]GSK573719 (−0.01–2.4 nM) or [3H]tiotropium (−0.01–2.9 nM) to M2 or M3 mAChRs was determined after incubation for 24 hours. Association rates of [3H]GSK573719 (−0.02–0.43 nM) or [3H]tiotropium (−0.02–0.38 nM) to mAChRs were measured using incubation times up to 1 hour. Rates of dissociation of radioligand ([3H]GSK573719 ~0.1 nM or [3H]tiotropium ~0.2 nM) were determined after incubation of membranes for 1 hour, diluted (1:20) with medium containing 10 μM atropine and then incubated for varying times up to 24 hours prior to filtration. The equilibrium dissociation constant (K_d), total number of receptors (B_max), association rate (k_on), and dissociation rate (k_off) were calculated using commercially available software (Prism 5.0; GraphPad Software, San Diego, CA). A one-affinity site model was used to determine K_d and B_max values. Association binding data were globally fitted to an association kinetic model for k_on values, whereas dissociation
binding data were fitted to a one-dissociation model for $k_{\text{off}}$ values and estimates of dissociation half-lives ($t_{1/2}$). $K_D$ values were also calculated from $k_{\text{on}}$ and $k_{\text{off}}$ values using the equation $K_D = k_{\text{off}}/k_{\text{on}}$.

Potency measurements of GSK573719 for receptors or sites other than mAChRs were conducted by CEREPS Laboratories (Poitiers, France).

**Calcium Mobilization in CHO Cells.** Functional antagonism of Ach-mediated calcium transients via human M1, M2, and M3 AchR was carried out using a microtiter plate-based fluorometric imaging plate reader (FLIPR) assay (Molecular Devices, Sunnyvale, CA) (Schroeder and Neagle, 1999). CHO cells were plated in 96-well plates (40,000 cells/well; Packard) for 24 hours and then the well plates were replaced with Eagle’s minimal essential medium containing Earl's salts, 1-glutamine, 0.1% bovine serum albumin (Sigma A-788; Sigma-Aldrich, St. Louis, MO), 4 μM Fluo-4-acetoxymethyl ester fluorescent indicator dye (Fluo-4 AM; Molecular Probes, Grand Island, NY), and 2.5 mM probenecid for 1 hour at 37°C. This medium was replaced with a similar one minus the dye and 0.1% gelatin instead of albumin. After 10 minutes, the cells were washed (3×) with Krebs–Ringer-Henseleit buffer: NaCl (120 mM), KCl (4.6 mM), K$_2$HPO$_4$ (1.0 mM), NaHCO$_3$ (25 mM), CaCl$_2$ (1.0 mM), MgCl$_2$ (1.1 mM), glucose (11 mM), HEPES (20 mM), pH 7.4, plus 0.1% gelatin and 2.5 mM probenecid. Cellular responses to Ach in the presence or absence of antagonist were monitored (FLIPR excitation = 488 nm, and emission = 516 nm). Data were acquired every second and analyzed using commercially available software (Prism 5.0, GraphPad Software). Potency and mode of receptor interaction were determined by classic Schild analysis (Schild, 1949) using calculations of $EC_{50}$ (half-maximal calcium response) or $pA_2$ values, where $pA_2 = \log (DR-1) – \log [B]$; $DR$ was the dose ratio defined as the ratio of equiactive concentration ($EC_{50}$) of agonist in the presence or absence of antagonist, and $[B]$ was the concentration of antagonist.

To evaluate the recovery of the Ach-mediated calcium responses in CHO cells containing M3 mAChRs after exposure to antagonist, the cells were either treated only with antagonist or exposed to the compound and then washed (3× in 30-minute intervals) with Krebs–Ringer-Henseleit to remove the compound. The latter group of cells was then exposed to Ach for determination of the residual inhibitory response to the agonist-mediated increase in intracellular calcium. The antagonist exposure period was 30 minutes in all cases, whereas the duration of clearance was 180 minutes; the exception was for tiotropium (90 minutes in most experiments and 180 minutes in one experiment, with similar outcomes).

**Isolated Airway Responses.** Human lungs from organ donors (five males, two females) were obtained from the National Disease Research Interchange (Philadelphia, PA). The human biologic samples were sourced ethically, and their research use was in accord with the terms of the informed consents. Sections of bronchus were either treated only with antagonist or exposed to the mAChR antagonists, referred to as ON $t_{1/2}$, and upon removal of drug, i.e., their ex vivo duration of effect or the offset half-time of tension recovery (OFF $t_{1/2}$). In additional experiments, the trachea was removed from male guinea pigs (450–650 g, Dunkin-Hartley; Charles River, Portage, MI). The epithelium was removed, and tracheal strips (2 cartilage rings) were prepared and similarly evaluated.

**Airway Mechanics.** Mice were pretreated intranasally (50 μl per mouse) with vehicle (0.9% saline) or GSK573719 at intervals (0.25–48 hours) prior to methacholine challenge, and placed into individual plethysmograph chambers (Buxco Electronics, Troy, NY). Fresh air was supplied by bias flow pumps to the chambers. After baseline respiratory [enhanced pause (Penh)] values were collected, the mice received methacholine (30 mg/ml or $EC_{50}$) by aerosol delivery (flow = 1.6 ml/min × 2 minutes; DeVilbiss model 5500D, Utrasonic Nebulizer; DeVilbiss, Somerset, PA). An average Penh was then calculated for 5 minutes. Penh = [(expiratory time / relaxation time) – 1] × (peak expiratory flow / peak inspiratory flow), and relaxation time is the amount of time required for 70% of the tidal volume to expire. In some cases, animals were treated on multiple, consecutive days as described in the figure legends. The data were expressed as the mean ± S.E.M. percent inhibition of Penh or (mean Penh value of vehicle treated group – Penh for each drug-treated animal) divided by (mean Penh value of vehicle treated group) × 100%. Data were analyzed using commercially available software (GraphPad Prism 3.0).

In guinea pigs (600–800 g), mAChR antagonists or vehicle (0.5% Tween; Sigma-Aldrich) were administered intratracheally by instillation (200 μl) under anesthesia (5% isoflurane). The animals were reanesthetized (ketamine 90 mg/kg, xylazine 15 mg/kg intramuscularly) after 4 hours for cannulation of the jugular vein, carotid artery, and trachea for drug delivery, blood pressure recording, and ventilation of the animal (pressure = 8 cm H$_2$O, 60 breaths/min; model 683; Harvard Apparatus, South Natick, MA). Succinylcholine was used to facilitate intubation and ventilation.
was administered (2.0 mg/kg i.v.) to paralyze the animal. Resistance and dynamic compliance were measured throughout the experiment (Palecek, 1969; Diamond and O’Donnell, 1977). Once the animal was stable, Ach (10–100 μg/kg i.v.) was administered. Alternate studies used whole-body plethysmography (BuxcoXA; Buxco Electronics) for determination of Penh as an indicator of airway obstruction (Hamelmann et al., 1997). In these studies, animals were exposed to an aerosol of Ach (3.5 mg/ml, flow 0.6 ml/min × 36 seconds followed by a 2-minute drying time; DeVilbiss Pulmosonic 5000D; DeVilbiss). Data (Penh) were collected for 10 minutes following the Ach exposure, and the area under the curve was calculated.

Statistical Analyses. The data are presented as means ± S.E.M. Statistical differences were analyzed using either analysis of variance or Student’s t test using commercially available software (GraphPad Prism), with a minimum level of significance established at P < 0.05.

Animals. All studies were conducted in accordance with the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Laboratory Animals, and were reviewed by the Institutional Animal Care and Use Committee at GlaxoSmithKline facilities where the work was performed. Age-matched male BALB/c mice (23–25 gm; Charles River Breeding Laboratories) and guinea pigs (Dunkin-Hartley; Charles River Breeding Laboratories) were allowed free access to food and water.

Chemicals. The mACHr antagonist GSK573719 (Fig. 2) was prepared as the crystalline bromine salt. This compound and other muscarinic agonists, e.g., ipratropium and tiotropium (for chemical structures, refer to Barnes 2001), were synthesized in the laboratories of GlaxoSmithKline. For radioligand binding kinetics, all chemicals were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK) unless otherwise stated. [3H]GSK573719 and [3H]tiotropium (specific activity 43 and 82 Ci/mM, respectively) were synthesized by Quotient Bioscience (Radiochemicals) Ltd. (Cardiff, UK). Scopolamine methyl chloride [(-)-(S)-3-hydroxy-2-phenylpropionic acid 1R,2R,4S,7S,8S,9-methyl-9-[methyl-3H]-3-aza-9-azatricyclo[3.3.1.02,6]nona-7-yl ester chloride], was purchased from PerkinElmer. CHO cells were purchased from American Type Culture Collection (Manassas, VA). Eagle’s MEM and Alpha MEM were purchased from GIBCO BRL (Grand Island, NY). All other reagents were purchased from commercial sources.

Results

In Vitro Receptor Binding Studies. The binding affinity values (Kᵢ) for GSK573719 obtained using membranes prepared from CHO cells stably expressing the individual five recombinant human mACHRs are displayed in Table 1. The values (nM) and corresponding negative log value pKᵢ) obtained under steady-state conditions using [3H]-N-methylscopolamine as the competing ligand are as follows: M1 = 159 (9.8), M2 = 151 (9.8), M3 = 62 (10.2), M4 = 50 (10.3), and M5 = 131 (9.9). Saturation binding of [3H]-N-methyl-scopolamine to the M3 receptor was shifted rightward in the presence of increasing concentrations of GSK573719 without a change in the maximum number of binding sites (B_max = 5.75 pmol/ml). The dissociation constant, K_D, increased with each incremental change of GSK573719.

Additional saturation, association, and dissociation binding studies were performed using [3H]GSK573719, and for comparison [3H]tiotropium, to determine receptor binding kinetics at the M2 and M3 mACHRs. The values (equilibrium dissociation constant (Kᵢ), total number of receptors (B_max) association rate (k_on), and dissociation rate (k_off)) are also shown in Table 1. The data indicate that association was rapid for both compounds at each receptor type. [3H]GSK573719 dissociates from the M3 mACHR more slowly, about 8-fold, than from the M2 mACHR (82 versus 9 minutes, respectively). This difference in dissociation between receptor subtype was also found for [3H]tiotropium, although the time to reach 50% receptor occupancy, t½, was longer than that for GSK573719. Of note is the finding that [3H]GSK573719 dissociates from the M2 mACHR more readily (about 4-fold) than does [3H]tiotropium. The affinity values, pKᵢ, for the M3 and M2 mACHRs in these saturation studies showed marginally wider separation for [3H]GSK573719 (10.5 versus 9.8) than for [3H]tiotropium (10.7 versus 10.3).

Evaluation of the selectivity of GSK573719 using a standardized panel of receptors and channels suggested that it is not likely to produce biologic effects unrelated to acetylcholine or mACHr activity. Affinity values (Kᵢ) for those proteins with the most avid binding are 69 and 220 nM for kᵢ and σ opioid receptors, respectively; 330 nM for L-type Ca²⁺ channel; 170 nM for site 2 Na⁺ channel; and 780 nM for dopamine transporter. In human embryonic kidney 293 cells, GSK573719...


**Table 1**

Receptor binding data

Data represent the mean ± S.E.M., where N = 3 for [3H-NMS]-based experiments and N = 4 for [3H]GSK573719 and [3H]tiotropium. Units for kinetic parameters are $k_{on} = M^{-1} \cdot \text{min}^{-1}$, $k_{off} = \text{min}^{-1}$, and $t_{1/2} = \text{minutes}$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK573719 versus [3H-NMS]</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.13 ± 0.003</td>
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<tr>
<td>pKᵢ (nM)</td>
<td>9.8</td>
<td>9.8</td>
<td>10.2</td>
<td>10.3</td>
<td>9.9</td>
</tr>
<tr>
<td>[3H]GSK573719</td>
<td>9.79 ± 0.08</td>
<td>10.5 ± 0.01</td>
<td>6.77 ± 4.5 × 10⁻⁶</td>
<td>0.0989 ± 0.0012</td>
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<tr>
<td>$k_{on}$</td>
<td>0.074 ± 0.004</td>
<td>0.0760 ± 0.0012</td>
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<tr>
<td>$k_{off}$</td>
<td>0.94 ± 0.5</td>
<td>82.2 ± 0.0012</td>
<td></td>
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<tr>
<td>$t_{1/2}$</td>
<td>39.2 ± 9.7</td>
<td>272.8 ± 27.6</td>
<td></td>
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<tr>
<td>[3H]Tiotropium</td>
<td>10.3 ± 0.08</td>
<td>10.7 ± 0.07</td>
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<tr>
<td>pKᵢ (nM)</td>
<td>1.26 ± 0.10 × 10⁻⁶</td>
<td>4.09 ± 5.55 × 10⁻⁸</td>
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<tr>
<td>$k_{on}$</td>
<td>0.94 ± 0.008</td>
<td>0.0026 ± 0.0003</td>
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<tr>
<td>$k_{off}$</td>
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[3H-NMS], (−)-3-hydroxy-2-phenylpropionic acid 1R,2S,4S,7S,8S-9-methyl-9-[methyl-3H]-3-oxa-9-azatricyclo[3.3.1.0²,4]non-7-yl ester chloride.

Inhibited the human ether-a-go-go-related gene channel tail current in a concentration-dependent manner ($IC_{50} = 9.4 \mu M$).

**In Vitro Functional Studies.** Functional pharmacological activity of GSK573719 was evaluated using two in vitro systems: first, by monitoring calcium flux in CHO cells transfected with the human recombinant mAChRs (and G-coupled proteins) and stimulated with Ach, and second, by measuring contractile responses in strips of airway isolated from human bronchus or guinea pig trachea.

In cells containing the human recombinant M3 mAChR, the CRC obtained with Ach was shifted rightward in the presence of GSK573719 (1–1000 nM) in a concentration-dependent manner (see Fig. 3). The maximal Ach response was minimally influenced by the presence of the antagonist. Potency as described by the pA₂ value was in the pM range for M1 mAChRs (9.6–10.6; see Table 2). Schild analysis indicated a slope of unity consistent with receptor antagonism of the competitive type for the M3 mAChR (slope = 0.96; see Fig. 3) as well as for the M1 and M2 mAChRs (slope = 0.83 and 0.93, respectively; unpublished data).

Additional cellular studies showed that the effects of GSK573719 on the M3 mAChR–mediated activity were slowly reversible. In Fig. 4, it can be seen that pretreatment of cells with GSK573719 (3.3–330 nM for 30 minutes) produced rightward displacement of the Ach CRC comparable to that obtained previously, and when followed by extensive washing for 180 minutes before Ach challenge, a residual concentration-dependent rightward shift of the Ach-induced CRC was still evident. Nonetheless, the amount of rightward shift was markedly less than that produced in the absence of washing. A different pattern emerged for both ipratropium and tiotropium. Ipratropium (0.1–1 μM) inhibited the human-induced calcium flux in a concentration-dependent manner; however, there was essentially no inhibition remaining after washing the cells. The initial exposure of cells to tiotropium (3.3–330 nM) produced concentration-dependent rightward shifts associated with progressive and marked suppression of the maximal Ach response. In the presence of the high concentration of tiotropium (330 nM), there was only a marginal calcium response stimulated by Ach. A rightward shift of the Ach CRC remained after clearance of the antagonist from the cells; however, the three concentrations of tiotropium resulted in the same magnitude of rightward movement of the CRC (relative to Ach alone), seemingly independent of the drug concentration applied initially to the cells. In addition, there was no loss of the maximal response after clearance. These data indicate that these three muscarinic receptor antagonists display independent patterns of pharmacological activity upon exposure and after clearance of the compounds.

![Fig. 3. Blockade of Ach-induced calcium mobilization by GSK573719.](image-url)
The pharmacological activity of GSK573719 was also determined using the more complex tissue matrix of isolated preparations of human airway (bronchial strips) in a conventional static tissue bath. In contrast to the studies described earlier using individual mAChRs expressed in CHO cells, the human bronchial tissue contains a distribution of muscarinic receptors, both M2 and M3 mAChRs, found in the normal airway (Coulson and Fryer, 2003), albeit at a given segment of the bronchial tree. Thus, any potential interplay of antagonist activity at the M2 mAChR or M3 mAChR would come to bear on the contractile event. These data were compared with those obtained for ipratropium, tiotropium, and atropine by using paired bronchial strips excised from the same donors (Fig. 5). Carbachol (Cch), an Ach mimetic, produced a cumulative concentration-dependent increase in tension development (referenced to that obtained with 1 mM histamine; the absolute increase in tension was 1.59 ± 0.14 g). The changes in contractile activity were concentration-dependently blocked by preincubation (120 minutes) of the tissues with GSK573719 (1–100 nM), thereby displacing the Cch-CRC to the right in a parallel manner. The potency value, i.e., pA2 = 9.5, indicated sub-nM affinity with only a modest suppression of the maximal response to Cch. Using only a single concentration of atropine (10 nM), a rightward shift of the Cch-CRC also occurred, and the magnitude was comparable to that obtained at the lowest concentration (1 nM) of GSK573719. Ipratropium (1–100 nM) also shifted the Cch-CRC to the right (pA2 = 9.2), but was without effect on the maximal Cch-induced contraction. In this case, the blockade achieved with 10 nM ipratropium was equivalent to that produced by 10 nM atropine. Tiotropium at the low concentration (0.1 nM) had negligible effects on the Cch-CRC, whereas contractile responses to the middle (1 nM) and high concentrations (10 nM) were similar, both markedly suppressing the maximal Cch-induced tension development by more than 60%.

These latter studies were extended using a modified system to determine the functional onset of antagonism and the recovery of tension development by Cch upon removal of GSK573719 by continuously superfusing the tissues with Cch. In this manner, the duration of action was estimated by extrapolation of the half-time to tension recovery (OFF t1/2) from a plot of tension developed versus time, and this was compared with ipratropium and tiotropium (see Fig. 6 for data using 10 nM) and atropine as an additional reference (unpublished data). The time to 50% of maximal relaxation (termed ON t1/2) for GSK573719 was concentration-dependent and decreased in value as the concentration of antagonist was increased. At 1 nM, the measured ON t1/2 (mean ± S.E.M.) was 102 ± 10 minutes (n = 3), whereas at 10 or 100 nM, it decreased to 43 ± 5 (n = 5) and 20 ± 2 (n = 3) minutes, respectively. Similarly, concentration-dependent ON t1/2 values were obtained for ipratropium [19 ± 1 (n = 3), 14 ± 3 (n = 5), and 6 ± 0.4 (n = 3) minutes, respectively] and tiotropium [24 ± 2 (n = 3), 10 ± 1 (n = 7), and 6 ± 0.1 (n = 3) minutes, respectively; 10 nM atropine = 20 minutes, n = 2, for paired tissues, historical reference = 9 ± 1 minutes, n = 17]. When these tissues were washed free of GSK573719, the OFF t1/2 (with confidence interval range) was also concentration-dependent. At 1 nM, the OFF t1/2 = 71 minutes (25–170 minutes; n = 3) and recovery was fully restored, similar to atropine. However, at the middle and high concentrations, tension recovery was more prolonged [OFF t1/2 = 381 minutes (241 to >600 minutes; n = 7) and > 600 minutes (n = 3)], and only returned at 10 hours to 67% (an average derived from those tissues recovering by 50% or more at 10 hours; some tissues did not reach this level) and 6%, respectively. By

### Table 2

<table>
<thead>
<tr>
<th>mAChR Subtype</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK573719</td>
<td>9.6</td>
<td>10.1</td>
<td>10.6</td>
</tr>
<tr>
<td>pA2 95% CI</td>
<td>9.5–9.7</td>
<td>10.0–10.2</td>
<td>10.5–10.8</td>
</tr>
</tbody>
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**Fig. 4.** Reversal of mAChR antagonism of calcium mobilization in CHO cells. CHO cells were preincubated for 30 minutes with several concentrations of mAChR antagonists (closed symbols as described in insets) and then cleared of drug by thorough washing of the cells (open symbols) prior to generation of Ach concentration-response curves. Cells exposed to ipratropium (A) or GSK573719 (B) were washed for a period of 180 minutes. For tiotropium, data were similar for 90 minutes of cell washing (C) and for 180 minutes (n = 1; unpublished data). For reference, some cells were treated with antagonists and Ach without washing. Studies were conducted independently and are shown here for comparison. The data represent the mean ± S.E.M. (n = 4).
contrast, contractile tension essentially recovered at 10 hours at all concentrations of ipratropium \(t_{1/2}\) (minutes) = 6 (4–9; \(n = 3\)), 63 (28–118; \(n = 5\)), and 224 (143–310; \(n = 3\)), respectively. Tiotropium was slow to recover contractile tension \(t_{1/2}\) (minutes) = 176 (62 to >600; \(n = 3\)), 434 (331 to >600; \(n = 7\)), and >600, respectively, and had negligible recovery of tension at 100 nM after 10 hours, about 1% (10 nM atropine \(t_{1/2}\) = 55 minutes, \(n = 2\); historical reference = 34 minutes, range = 23–49 minutes, \(n = 17\)). At the end of the protocol after clearance of Cch, histamine exposure elicited a robust contraction, indicating that the tissues were viable and responsive to nonmuscarinic mechanisms. When the ON \(t_{1/2}\) and OFF \(t_{1/2}\) values (at 10 nM) were measured in guinea pig tracheal strips, the average values \((n = 4)\) were as follows: GSK573719 = 34 and >600 minutes, ipratropium = 12 and 35 minutes, and tiotropium = 9 and >600 minutes, respectively.

**In Vivo Studies.** It is well established that administration of exogenous Ach (or mimetics) produces a robust bronchoconstriction that can be countered by administration of muscarinic antagonists. Pharmacodynamic activity of GSK573719 was evaluated in a murine model of methacholine (Mch)-induced bronchoconstriction using a standard plethysmograph (Penh served as a surrogate measure of changes in airway tone). When administered intranasally as a solution, GSK573719 blocked nebulized methacholine (30 mg/ml or ED\(_{80}\) value for maximal bronchoconstriction) in a dose-dependent manner (ED\(_{50}\) = 0.02 \(\mu\)g per mouse measured 5 hours after instillation). The inhibitory effect following a single administration was sustained for a prolonged period of time, e.g., at 0.05 \(\mu\)g per mouse, inhibition was about 50% or greater for up to 72 hours (data not shown). This inhibition continued, albeit at lower levels, for nearly 7 days. Comparable data were obtained for tiotropium at the same dose, although the maximal bronchodilatory effect occurred sooner (5 hours) than that of GSK573719 (24 hours post dose). When GSK573719 was given once daily to mice for 5 consecutive days (0.025 \(\mu\)g intranasally), the level of inhibition on the fifth day was modestly increased above that obtained after a single administration to the same mice (60 versus 35%, respectively). After the fifth day of dosing, the mice were rested for 5 additional days, allowing bronchomotor tone to return to baseline levels. On the sixth day, the mice received one last dose of antagonist and were once again challenged with Mch. The level of inhibition was essentially the same as that found on the first day of testing, indicating that tolerance was not evident with repeated intranasal delivery of GSK573719. By contrast, when GSK573719 was given orally (2.0 mg/kg) to mice at a dose 100 times the ED\(_{50}\) value (intranasal), there was no observable protection against an Mch challenge (monitored for 24 hours post dose; unpublished data).

In similar studies using guinea pigs rather than mice, intratracheal instillation of GSK573719 (0.25, 2.5, and 25 \(\mu\)g per guinea pig) dose dependently blocked the increase in Penh elicited by aerosolized Ach (Fig. 7A). Inhibitory effects were

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**Fig. 5.** Effects of mAChR antagonists on Cch-induced contraction of isolated strips of human bronchus. Segments of human bronchus were prepared as described in Materials and Methods and incubated with mAChR antagonists (A, ipratropium; B, GSK573719; C, tiotropium or vehicle) for 120 minutes prior to cumulative addition of carbachol in conventional tissue baths heated at 37°C. Atropine (10 nM) served as a reference. Values represent the mean ± S.E.M. \((n = 3–5\) tissues).}

**Fig. 6.** Recovery of tension development after clearance of mAChR antagonists from isolated strips of human bronchus. Strips of human bronchus were prepared as described in Materials and Methods and exposed to agents in accordance with the superfusion protocol shown in Fig. 1. All tissues were contracted with Cch (1 \(\mu\)M), and continued to receive the agonist until the end of the experiment. Afterward, tissues were evaluated for viability using histamine (1 \(\mu\)M). The data represent the mean ± S.E.M. \((n = 5–7\) and the line of best fit.
sustained for long periods of time, and the duration of the protection was more protracted with each increase in GSK573719. For example, the level of blockade corresponding to 50% inhibition was maintained for more than 2 days at 2.5 mg per guinea pig, and for more than 5 days at 25 μg per guinea pig. These increments were not, however, dose-proportional. When the inhibitory effects of GSK573719 were compared with those obtained with tiotropium (animals were given both compounds at 2.5 μg per guinea pig), the findings were comparable (Fig. 7B). Additional studies in which anesthetized Guinea pigs were instrumented for measurements of airway resistance and heart rate showed that GSK573719 (0.025, 0.25, and 2.5 μg) inhibited the Ach-induced dose-dependent increase in airway tone (Fig. 8A). At the highest dose of Ach (100 μg i.v.), significant inhibition, i.e., 74.4%, \( P < 0.05 \), was obtained 4 hours after instillation of 0.25 μg per guinea pig of GSK573719. Comparable data were obtained using tiotropium over the same dose range (Fig. 8B). Complete blockade resulted when the animals received the high dose of either agent. In these same animals, a dose-related decrease in heart rate occurred after each administration of Ach in vehicle and in the three drug-treated groups. Instillation of GSK573719 was not associated with a consistent dose-related blockade of the Ach-induced decrease in heart rate (unpublished data). For example, the average decrease in heart rate in the absence of antagonist was 148 ± 8 beats per minute after administration of 100 μg of Ach, and at the high dose of GSK573719 (2.5 μg), heart rate declined by 136 ± 14 beats per minute.

**Discussion**

The purpose of the present study was to characterize the pharmacological activity of a novel inhaled antagonist of the muscarinic cholinergic receptors: GSK573719, or umeclidinium. The major findings indicate that GSK573719 is a potent, competitive, pan-active mAChR antagonist with a long duration of action. Pharmacological activity was evaluated

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**Fig. 7.** Effect of intratracheal administration of GSK573719 on bronchoreactivity to Ach and comparison with tiotropium. Guinea pigs were anesthetized according to procedures described in Materials and Methods. Animals were administered varying doses of GSK573719 and challenged with an aerosol of Ach (3.5 mg/ml × 36 seconds) at time points over several days (A). In (B), a single dose of either GSK573719 or tiotropium was administered, and the animals were similarly monitored using plethysmography. The data represent the mean ± S.E.M. \(* P < 0.05\); \(** P < 0.01\); and \(*** P < 0.001\); (A) \(n = 6\); (B) \(n = 18\).

**Fig. 8.** Dose-dependent blockade of Ach-induced bronchoconstriction: comparison of GSK573719 (A) and tiotropium (B). Guinea pigs were treated similarly to those in Fig. 7 except that they were ventilated (pressure = 8 cm H2O at 60 breaths/min) and cannulated for heart rate and blood pressure recordings as well as for cumulative bolus injections of Ach. The data were analyzed using linear regression and represent the mean ± S.E.M. (\(n = 6\)). \(* P < 0.05\); \(** P < 0.01\); \(*** P < 0.001\).
using assays containing either the human recombinant mAChRs or endogenous ones resident in human airway and animal models. The animal models were not designed to simulate human pulmonary disease; rather, they address certain features of the disease process, i.e., mAChR-induced bronchoconstriction (Canning and Chou, 2008). In all cases, GSK573719 potently blocked agonist-mediated events in a manner consistent with a competitive type of antagonism.

The strong antagonist-receptor interaction of GSK573719 was favorably reflected in radioligand binding studies where affinity values were in the sub-nM range for the five human mAChRs. For example, the pKᵢ or pKᵢD values for the M3 mAChR were 10.2 and 10.5 using [³H]-N-methyl-scopolamine or [³H]GSK573719, respectively. The level of functional in vitro potency was obtained when the receptor system was coupled with its appropriate G-protein and intracellular molecular assembly to affect changes in intracellular calcium signaling measured in CHO cells and in isolated strips of human bronchus. In the former assay system, the potency described by the pA₂ value was also sub-nM (pA₂ = 10.6 for M3 mAChR). The parallel rightward shifts and modest change in the maximal response (at concentrations up to 10 nM) of the Ach-induced increase in intracellular calcium imparted by the presence of the antagonist indicated competitive antagonism. This was confirmed by Schild analysis, where the slope was essentially unity, e.g., M3 mAChR = 0.963. The temporal limits imposed by the FLIPR technology, i.e., the duration of the antagonist exposure (30 minutes) with the receptor was markedly greater than that for the agonist (peak Ach response occurs within 4–5 seconds in the absence of drug), may not have afforded an opportunity for equilibrium, especially at the higher concentrations of the antagonist. At the higher concentrations of GSK573719, there was partial suppression of the maximal response. The impact of incubation conditions and duration on pharmacological activity of long-acting muscarinic antagonists, such as tiotropium, has been brought out by previous investigations (Disse et al., 1993; Casarosa et al., 2009, 2010). These investigators suggested that the affinity and maximal mAChR-mediated effects of these agents may be underestimated should the incubation time be abbreviated within the chosen assay system.

A partial suppression of the maximal carbachol-induced contractile response, albeit only about 10–25%, also resulted when GSK573719 was exposed to strips of isolated human bronchus. In this case, suppression was independent of the amount added to the static tissue bath. Nonetheless, potency was consistent with that obtained in the other in vitro systems, with a calculated pA₂ value of 9.5. By comparison, the simple competitive muscarinic antagonist atropine was without effect on the maximal response. Ipratropium blocked tension development in a manner similar to that of atropine, and was equi-effective at 10 nM. Tiotropium at the lowest effective concentration used in these studies, i.e., 1 nM, reduced the maximal response to about 30% of that produced by carbachol alone, and this pattern deviated little at the higher concentration (10 nM). The latter effects likely represent an allostERIC modulation of the receptor conformation. These chemically distinct mAChR antagonists display quite varied responses, from competitive to noncompetitive, in complex human biologic systems. Taken together, the data suggest that the mode of antagonism provided by GSK573719 is competitive, although the possibility of insurmountable characteristics cannot be ruled out.

Dissociation of [³H]GSK573719 from the human recombinant M3 mAChR was slow. The time until 50% of [³H]GSK573719 remained bound to the receptor was about 82 minutes. Dissociation from the M3 mAChR occurred more slowly than from the M2 mAChR, i.e., half-life was 9 minutes. In our hands, [³H]tiotropium also dissociated slowly from the two receptors; however, the half-lives were longer lived, i.e., 273 versus 39 minutes. Both compounds avidly bind to each receptor with less than a log difference in binding affinities between the two receptors. Thus, potency per se was not the likely basis for differences in receptor dissociation. Consistent with the present findings, Casarosa et al. (2009) reported that tiotropium dissociates more slowly from the M3 mAChR than the M2 mAChR; however, the half-lives were 27 and 2.6 hours, respectively. Although both studies used human receptors stably expressed in CHO cells, methodological differences may have impacted the actual values (e.g., [³H]-N-methyl-scopolamine was used in competition kinetic studies to derive the values in the latter report rather than the more classic methods used in the present study). These authors also noted that residence time at the M3 mAChR was longer than that at the M2 mAChR for other antagonists, i.e., ipratropium, aclidinium, and glycopyrrolate. Within this group of compounds, the half-lives of ipratropium were the shortest. Recently, Sykes et al. (2012) suggested that “physiological” assay conditions, in particular the sodium ion concentration of the incubation medium, can markedly reduce M3 mAChR residence times by as much as 10-fold, e.g., the half-life of tiotropium decreased from 462 minutes in the absence of sodium to 46 minutes in the presence of sodium. Nonetheless, the trends for M3 versus M2 mAChR dissociation seem to be a characteristic that is common to this group of pharmacophores.

Insight concerning the mechanism of the antagonist-mAChR interaction has been provided using ligand-bound crystal structures of M2 and M3 mAChRs (Haga et al., 2012; Kruse et al., 2012). Even though the five mAChR subtypes are separated into two major classes in accordance with their selectivity for G proteins—M1, M3, and M5 are associated with the Gq/11 family and M2 and M4 with the Gαo-type proteins (see, for example, Caulfield and Birdsall, 1998)—they show a high level of sequence homology and overall structural similarity in both intracellular and extracellular loops within the proteins (Kruse et al., 2012; Haga et al., 2012). Conservation of the orthosteric binding pocket also exists across M subtypes; however, structural divergence between M2 and M3 mAChR may lie in the three-dimensional architecture of the binding sites, and this feature may impact the different dissociation rates of the assorted antagonists (Kruse et al., 2012). Kruse et al. (2012) have suggested that in the case of tiotropium binds to and dissociates from the M3 mAChR, it pauses at an alternate, allosteric site in the extracellular vestibule. A portion of the extracellular loop (loop 2) nearest the binding pocket of M2 mAChR may be more mobile, and interacts with the thiophene ring of tiotropium, thereby promoting its egress from the orthosteric binding site to the extracellular vestibule and overcoming the largest energetic barrier of the binding and dissociation path. In the absence of additional crystallographic information for other M2 and M3 mAChR-antagonist complexes, the molecular mechanism(s)
of dissociation remain to be fully understood despite the recent progress in this area of drug design.

In the present study, slow receptor dissociation by GSK573719 is consistent with the persistent functional blockade observed in other in vitro assay systems. For example, extensive washing of intact CHO cells with antagonist-free media revealed that blockade of the Ach-induced increase in intracellular calcium was evident for both GSK573719 and tiotropium but not for ipratropium. Interestingly, any suppression of the maximal response that was observed prior to washing for either GSK573719 or tiotropium was not apparent during this “refractory” period. Moreover, for GSK573719, but not for tiotropium, the level of refractory antagonism was concentration-dependent, i.e., rightward displacement of the Ach CRC. To what extent (or proportion) these ligands remained bound to either the orthosteric or allosteric sites cannot be determined from the present experiments; however, they clearly indicate that the responses exhibited by ipratropium, GSK573719, and tiotropium during exposure and clearance are complex and provide a different array of receptor-ligand interactions.

Different recovery patterns by the antagonists were also borne out in the restoration of the carbachol-induced contraction of the isolated human bronchial strips after removing the compounds from the superfusion bathing media. In these studies, the temporal response after clearance of the low concentration (1 nM) of ipratropium or GSK573719 approximated that obtained upon clearance of atropine, in which case there was full restoration of tension development (for reference at 10 nM; unpublished data). At all concentrations (1–100 nM) of ipratropium, tension development returned during the washout period. On the other hand, removal of the middle (10 nM) and particularly the high (100 nM) concentrations of GSK573719 and tiotropium continued to suppress the tissue response to carbachol even after 10 hours of clearance. This prolonged duration of inhibition was also apparent for the latter two compounds when administered to either mice or guinea pigs.

Engagement of GSK573719 with its cognate receptors, mAChRs located within the airway, was responsible for its pharmacological activity in the animal models. Muscarinic receptors populate the airways of a variety of species, including humans, and are present on nerve terminals, airway smooth muscle, vascular endothelium, and submucosal glands (Cowson and Fryer, 2003). Although M2 mAChRs are located on prejunctional nerve terminals, and both M2 and M3 mAChRs are located on airway smooth muscle cells, it is the M3 mAChR that dominates the action of Ach upon release from parasympathetic nerves. Fisher et al. (2004) showed that, in mice deficient of the M2 mAChR, vagal stimulation or methacholine challenge produced enhanced bronchospastic activity, whereas in those lacking the M3 mAChR, bronchoconstriction was abolished. GSK573719 treatment prevented bronchoconstriction in both the murine and guinea pig models. Given the separation of potency, albeit a modest difference, and the kinetic differences in dissociation between the M2 and M3 mAChRs, it could be argued that the bronchoprotection obtained in animal models primarily resulted from blockade of the M3 mAChR. The potency value obtained in the mouse model, i.e., \( ED_{50} = 0.02 \, \mu g \) per mouse, is consistent with the levels of potency obtained in vitro using recombinant human receptors and samples of human airway. The protective effects are long lived after a single dose to animals and comparable to those obtained with tiotropium, yet GSK573719 does not appear to accumulate in the lung nor create drug-induced tolerance following several days of consecutive administration. The data underscore the view that very small amounts of GSK573719 are required to maintain a patent airway for gas exchange. These animal models simulate airway obstructive events but do not equate to the lung remodeling that takes place over decades in patients with pulmonary diseases such as COPD. Nonetheless, if there is a corollary in human disease for the bronchoprotection found in these animal models, GSK573719 should provide once daily control of Ach-driven enhancement of airway tone in the clinical setting.

Last, activity at the M2 mAChR represented by a blockade of the Ach-induced decline in heart rate measured in guinea pigs was not observed within the efficacious dose range used in the present investigation. It should be noted that diffusion of the compound from the lung parenchyma to the large plasma volume occupied by the systemic circulation dilutes the drug concentration severalfold, thereby diminishing any potential for M2 mAChR-mediated cardiac effects. Altered cardiac and pulmonary vagal tone is found in many pathophysiological conditions, including COPD. Clinical use of mAChR antagonists such as tiotropium is currently accepted as a treatment in this patient population. In a recent clinical study of patients with COPD, treatment with GSK573719 was well tolerated with no apparent treatment-related changes in vital signs, including pulse rate, while significant improvement in lung function (FEV\(_1\), forced expiratory volume in one second) at all doses was obtained (Decramer et al., 2013). The pharmacological findings of the present investigation using defined agonist-antagonist conditions would appear to have a corollary to those found in the clinical setting. Thus, these results have therapeutic relevance. GSK573719 may serve as a long-acting competitive antagonist providing bronchodilatory relief to patients with pulmonary diseases such as COPD.

**Authorship Contributions**

*Participated in research design:* Burman, DeHaas, Salmon, Lainé, Schmidt, Sarau, Hay, Foley, Slack, Barrett, Palovich, Buckley, Luttmann, Rumsey.

*Conducted experiments:* Burman, DeHaas, Schmidt, Foley, Slack, Barrett, Buckley, Luttmann, Kotzer, Webb.

*Contributed new reagents or analytic tools:* Lainé, Palovich, Luttmann.

*Performed data analysis:* Burman, DeHaas, Schmidt, Foley, Slack, Barrett, Buckley, Luttmann, Kotzer, Webb.

*Wrote or contributed to the writing of the manuscript:* Rumsey, Salmon, Palovich, Barrett, Slack, Luttmann, Webb.

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Address correspondence to: Dr. William L. Rumsey, 709 Swedeland Road,
King of Prussia, PA 19406-0935. E-mail: Bill.2.Rumsey@gsk.com